Thermophiles and Fouling Deposits in Milk Powder Plants

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To my loving wife Lisa, for
her help, support,
encouragement and
acceptance of long hours
and stressful times.

Abstract

Fouling deposits were suspected of playing a pivotal role in the thermophile contamination problem experienced in the dairy industry during milk powder manufacture. The objective of this work was to investigate thermophile growth and develop an understanding of how fouling deposits affect thermophile contamination in milk powder plants.

Pilot plant and laboratory scale studies were carried out investigating:

- The release of thermophiles from fouled and un-fouled surfaces;
- The survival of thermophiles in fouling during cleaning;
- The rate of re-contamination of thermal equipment after incomplete cleaning;
- and the adhesion of thermophiles to fouled and clean stainless steel.

Thermophile contamination from the pilot plant equipment was also modelled mathematically.

The bulk milk thermophile contamination from sanitised fouled and un-fouled surfaces was found to be not significantly different, showing that fouling deposits by themselves do not increase the steady state amount of bulk contamination and that the more important factor is the amount of surface area available for colonisation within the temperature growth range of the thermophiles.

Milk fouling layers provided much greater protection against cleaning than that of biofilms alone. Thermophiles that survive cleaning or greater initial thermophile concentrations in the raw milk were shown to reduce the plant production time available before concentrations of thermophiles in the bulk milk became excessive ($>1x10^6$ cfu.ml^{-1}).

Therefore, cleaning procedures in milk powder plants need to remove or destroy all traces of thermophiles to allow the maximum possible run length. It is similarly important to obtain raw milk with the lowest possible thermophile load before processing.

During adhesion studies, the number of thermophilic bacteria adhering to stainless steel surfaces increased with bulk cell concentration and increasing contact time for adhesion. The adhesion rate of thermophiles to whole milk fouling layers was found to be around ten times higher than the adhesion rate to stainless steel.

Steady state modelling provided a quick estimate of the level of bulk milk contamination that can be expected, however it was dependent on obtaining accurate measurements of the surface numbers. Since surface numbers were underestimated by approximately a decade using techniques that dislodged but did not enumerate loosely adhered cells, the model under predicted the bulk milk contamination.

Unsteady state modelling predicted the trends observed in the experimental data and provided reasonable estimates of the bulk contamination that can be expected over time from the pilot plant. Predictions from the model after changes in key parameters provide an insight to the magnitude of any reduction in contamination that can be made.

The results of this work have demonstrated that thermopile contamination during dairy processing can be minimised through:

- Re/design operating equipment to minimise the residence time of the product in the range of 40-70°C.
- Minimising the contact surface area of thermal equipment by use of alternative direct heating technologies.
- Minimising fouling by management of milk quality, optimising processing conditions, hygienic design of the plant equipment and ensuring the product mix is suited to the plant.
- Ensuring that the plant is thoroughly clean at the commencement of each run through attention to equipment design and optimisation of cleaning procedures.

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1. Introduction

In the dairy industry the manufacturing run times of milk powder plants are limited by the progressive build up of thermophilic bacteria and spores in the product stream over the course of the run. There is a gradual build up in microbial cell numbers in the product stream exiting a plant after it has been cleaned by clean in place (CIP) techniques. This gradual increase occurs up to a point at around 16-24 hours at which point the numbers in the product reach unacceptable levels and the plant has to shut down to be cleaned again. Owing to the large size of milk powder plants and the large quantities of milk powder being produced every hour, extensions in run times would provide economic benefits from increased plant utilisation and lowered chemical use. Therefore it is essential to control thermophiles in milk powder plants so that run times can be extended and product specifications met.

The residence time of milk within a milk powder manufacturing plant is of the order of minutes and thermophile numbers in the raw milk entering the plant are low. Therefore, bacteria merely growing in the liquid phase can not produce the large numbers of thermophiles exiting the plant in the product, as this would take several hours to achieve. Some sort of immobilisation of the bacteria against the plant contact surfaces must be occurring. This is most likely by attachment of bacteria to the numerous product contact surfaces within the plant, such as stainless steel, rubber gaskets or even fouling deposits. Once attached, these bacteria can replicate and contaminate the product stream, thus causing an increase in thermophile numbers. This bacterial contamination process is not limited to thermophiles in milk powder plants. It is also common for attached growth and release of bacteria to occur in other food processing systems and in water distribution networks.

Fouling deposits are suspected of playing a pivotal role in the thermophile contamination problem experienced in the dairy industry during milk powder manufacture. It is generally accepted in the dairy industry that fouling is linked to thermophile contamination, but until this work no specific study has looked at the interaction of fouling deposits with thermophile contamination and the precise nature of the interaction have been unknown.

1.1. Objectives

The broad objective of this thesis was to investigate thermophile growth and develop an understanding of how fouling deposits affect thermophile contamination in milk powder plants. The specific objectives were:

1. To determine if fouling increases the amount of thermophile contamination released from plant surfaces.

2. To determine whether fouling will enable thermophiles to survive on surfaces better during cleaning than on stainless steel alone.

3. To determine whether thermophiles remaining on the surface after CIP will re-contaminate the plant and reduce the plant availability by providing a faster rate of contamination.

4. To predict thermophile contamination of the bulk milk stream from surfaces through mathematical modelling.

5. To provide solutions to help alleviate the contamination problem.

2. Literature Review

This review examines the problem of thermophile growth in milk powder plants. The review covers issues of thermophiles in food products, bacterial relationships with surfaces, methods of study of attached bacteria, and the incidence of thermophiles in food processing plants.

2.1. Thermophiles in Food Products

Thermophilic bacteria and their endospores are known to occur in several food products and have been blamed for spoilage of a variety of food products. The control of thermophilic spoilage by various methods is of interest to the food industry for a variety of reasons including economic and safety.

2.1.1. Occurrence of thermophiles

Thermophilic bacteria and spores can be found in a wide variety of processed food products including canned food (Denny, 1981), sugar and flour (Denny, 1981), dairy products, such as milk (Koshy & Padmanaban, 1988; Koshy & Padmanaban, 1989; Koshy & Padmanaban, 1990a; Koshy & Padmanaban, 1990b; Rama Raju & Kiran Kumar, 1988), milk powder (Sharma *et al.*, 1978; Asperger, 1990) and cheese (Cosentino *et al.*, 1997). These thermophiles do not produce toxins and have no other ill health effects but can cause spoilage if present in sufficient quantities (Denny, 1981). If high numbers of thermophiles are present in products used as ingredients such as sugar, flour and milk powder this can cause high numbers of thermophiles in the product being produced. Thermophilic spores present can then prove difficult to kill in processing due to their heat resistance. Consequently, as they are difficult to kill, certain levels of thermophilic bacteria and spores are permitted in food products depending on their potential to spoil or their end use. Spores present in some products such as canned and dried food will not germinate if the food is handled properly. The spores may even die out if held under conditions at which they cannot germinate or outgrow (Denny, 1981). Tolerance of levels of thermophiles in products can be important in product quality.

This is because food of better quality (less thermal damage) can be produced if all thermophiles do not have to be destroyed (Denny, 1981)

Several types of thermophilic bacteria have been found in food products. The most common types reported in literature are listed below in Table 2.1 along with products that they have been commonly found associated with. The most likely original source of thermophiles is from soil, from which they can be isolated, and most food products can come into contact with soil at some time during harvesting or from contamination during processing.

Table 2.1. Common types of thermophiles found in food products.

Common thermophile types	**Food products most commonly found in**
Bacillus stearothermophilus, B. licheniformis, B. coagulans, B. subtilus	Dairy products (milk, milk powder, cheese) Canned food products Food ingredients (e.g. sugar, starch, spices, flour)
Desulfotomaculum nigrificans, Clostridium thermosaccharolyticum	Canned food products Food ingredients (e.g. sugar, starch, spices, flour)

2.1.2. Thermophilic Spoilage

Different types of thermophilic spoilage have been reported in the literature as the result of contamination by thermophilic bacteria. Most reports relate to spoilage of canned food products. Thermophilic spoilage in canning only accounts for a low proportion of spoiled cans (Davidson *et al.*, 1981; Pflug *et al.*, 1981). The types of thermophilic spoilage and the thermophiles that most commonly produce them are given below in Table 2.2.

An example of thermophilic spoilage not related to canning can be seen in the numbers of thermophilic and thermoduric bacteria in milk, which have been correlated to milk keeping quality. Higher numbers of thermophilic and thermoduric bacteria result in

reduced keeping quality of the milk (Koshy & Padmanaban, 1989; Koshy & Padmanaban, 1990a).

Table 2.2. Types of thermophilic spoilage and thermophiles that commonly produce them.

Thermophilic Spoilage Type	**Typical thermophilic bacteria producing spoilage type**
Anaerobic spoilage - H_2S not produced	*Clostridium thermosaccharolyticum* [A]
Anaerobic spoilage - H_2S produced	*Desulfotomaculum nigrificans* [B]
Aerobic spoilage - acid foods	*Bacillus coagulans* [C]
Aerobic spoilage - low acid foods	*Bacillus stearothermophilus* [D]

A – (Ashton, 1981); B – (Speck, 1981); C – (Thompson, 1981); D – (Ito, 1981).

2.1.3. Control

Several methods of control of thermophile numbers in food products have been discussed. One of these is adequate heat treatment of the product to kill as many thermophilic spores as possible. Hsieh *et al.* (1989) used ultra-high temperatures at 170-210 °C. Other approaches included changing the conditions faced by the spores to sensitise them to the heat process, such as acidity (Beelman *et al.*, 1989; Fernandez *et al.*, 1994), salinity (Periago *et al.*, 1998), and solids concentration (Behringer & Kessler, 1992).

Other methods include the use of effective sanitation, preventing steam leaks that may keep process equipment hot, rapid cooling of cans and using product ingredient specifications to inhibit growth (Denny, 1981). Further information on methods used to control thermophiles in milk powder production can be found in Section 2.4 of this literature review.

2.2. Bacterial Relationships with Surfaces.

2.2.1. Introduction – Bacterial adherence in food processing

Bacteria can attach to product contact surfaces in food processing environments (Hood & Zottola, 1995). These bacteria can then replicate on the surface with a ready supply of nutrients, detach from the surface and contaminate the product stream (Hood and Zottola, 1995). The adherent bacteria are normally described as a biofilm (Hood and Zottola, 1995), although the definition of a biofilm includes not only adherent bacteria but also any extracellular material produced at the surface and any material trapped within the resulting biofilm matrix (Characklis & Marshall, 1990). Therefore the term biofilm is probably often misused in food processing situations where only adherent bacteria are present. However, due to the ability of the adherent bacteria to contaminate the product stream, they may be as significant as a fully developed biofilm. The concept of bio-transfer potential has been introduced (Hood and Zottola, 1995) to more appropriately describe the ability of any adhered microorganisms to contaminate food products. Several reviews have been published in recent years on the significance of surface associated bacteria or biofilms in food processing (Pontefract, 1991; Notermans, 1991; Carpentier and Cerf, 1993; Zottola and Sasahara, 1994; Hood and Zottola, 1995; Bower *et al.*, 1996; Kumar and Anand, 1998; Mittelman, 1998), which shows the increased attention the topic has received in the last decade.

The sections below provide information on surface behaviour of attached bacteria and biofilms that may be applicable to attached thermophile growth in milk powder plants. Information on developments in mathematical modelling of biofilms relevant to the creation of models of thermophile growth in milk powder plants is also given.

2.2.2. Biofilm life cycle processes

Once bacteria have attached to a surface and a biofilm begins to form many different processes occur. Many of these apply to thick biofilms that develop over days or weeks rather than the adherent cells seen in the food industry that have only hours to develop. However some of these processes are relevant to bacterial adherence and its consequences in the food industry. The processes described below have been divided into the stages that occur as a biofilm completes its lifecycle, including colonisation,

growth, detachment, interactions and competition between different strains of bacteria, and succession.

2.2.2.1. Colonisation and Growth

Bacterial growth at surfaces has been observed to occur in two different forms. Either the bacteria are solitary in attachment or present as colonial groups on the surface (Lawrence *et al.*, 1995).

Bacteria that are solitary in attachment are first present as single adherent cells on the surface. These cells then replicate in a budding fashion, whereby the daughter cell is attached to the parent cell, which is adhered to the surface. The daughter cell can then be released into the bulk liquid. Examples of genera exhibiting this type of behaviour, are *Rhizobium*, *Caulobacter*, *Pedomicrobium* and *Hyphomicrobium* (Lawrence *et al.*, 1995).

The other more commonly observed form is the development of colonial groups on the surface. Once cells adhere to the surface, colonies of cells develop from these first cells after several division cycles. These eventually form bacterial microcolonies, which can then spread and converge, developing further into a biofilm covering the entire surface. Variations of this are possible whereby the daughter cells may spread along the surface away from parent cells or detach and reattach in new areas. Some bacteria also preferentially reattach to colonies on the surface rather than the surface itself, which creates more complex arrangements of cells (Lawrence *et al.*, 1995).

Biofilm accumulation normally takes the form of a sigmoidal curve consisting of an initial lag stage, an exponential accumulation and a steady state stage (Characklis and Marshall, 1990). Several processes occur in a biofilm system, which determine the net rate of accumulation. While bacterial growth and attachment increase the population within the biofilm, detachment of cells from the biofilm can also occur, reducing numbers. Also, products produced by the bacteria such as exopolysaccharides entrap cells and form the biofilm matrix. Other materials from the bulk liquid can also be entrained in the biofilm as it grows. Due to nutrient diffusion through the biofilm as its size increases, growth of cells within the biofilm becomes limited. Also, as the biofilm

grows any shear forces present increase which causes increased detachment of cells. These limiting factors cause the biofilm to attain a steady state thickness and a maximum population. The rate at which this occurs depends on the given situation. Factors such as nutrient supply, bacteria type and flow rate all may have an effect on how the biofilm progresses (Characklis and Marshall, 1990; Lawrence *et al.*, 1995).

2.2.2.2. Detachment

Detachment of biomass from biofilms back into the bulk liquid has many consequences. As well as being one of the factors determining the rate of biofilm accumulation, detachment also controls the migration and re-colonisation of biofilms (Lawrence *et al.*, 1995), as well as increasing the suspended biomass concentration in the bulk fluid. This last point is important in food processing and water distribution, as increased bacterial numbers contaminate the food or water and thus affect product quality.

Three different types of biofilm detachment have been recognised which are erosion, sloughing and abrasion. Erosion is the continuous loss of small portions of the biofilm through such forces as shear effects. Sloughing refers to rapid, massive loss of biofilm and is observed with thicker biofilms. Abrasion is the loss of biofilm due to collisions between particles as seen in fluidised bed reactors (Characklis and Marshall, 1990). As mentioned above, the term bio-transfer potential has been introduced with regard to food systems (Hood and Zottola, 1995). Bio-transfer potential describes the detachment or transfer of cells back into the bulk liquid from adherent cells as they multiply. It is useful in food systems as it can describe contamination from adherent cells that by themselves may not constitute a true biofilm but are still a threat to product quality and safety.

The rate of detachment has been shown to relate to the attachment rate and the growth rate for *Pseudomonas aeruginosa*. Escher (1986) reported that the detachment was linearly related to the attachment rate. Therefore the more cells that are attaching to the surface the more that will detach. (Peyton & Characklis, 1993) found that the detachment rate was directly related to the biofilm growth rate and that factors that limited growth rate also limited detachment rate.

2.2.2.3. Interaction, Competition and Succession

Interactions between bacteria in biofilms can be co-operative, with bacteria benefiting from the presence of others, or competitive, with the most dominant bacteria succeeding the other.

In certain situations some bacteria may need the presence of others to attach to a surface. The ability of *Listeria monocytogenes* to attach to and colonise a surface seems to be greatly enhanced by the presence of primary colonising bacteria such as *Pseudomonas fragi* (Sasahara & Zottola, 1993). Another co-operative relationship between bacterial species in biofilms is the production of vitamins or growth factors by one population that can be utilised by another (Characklis and Marshall, 1990; Lawrence *et al.*, 1995). An interesting difference between mixed population and mono-population biofilms is that mixed population biofilms can obtain a greater steady state thickness than mono-population biofilms in the same conditions (Characklis and Marshall, 1990). Another interaction that may occur between cells in biofilms is genetic exchange. The high population density may lead to good opportunities for genetic exchange in biofilms. This would provide a greater gene pool for biofilm bacteria to adapt and survive adverse conditions (Characklis and Marshall, 1990).

Competition between bacteria for resources such as growth substrates and attachment sites causes some strains present in biofilms to succeed others. The different growth rates of competing bacteria generally are used as an explanation of dominance of one strain over another, with the bacteria with the fastest growth rate dominating (Lawrence *et al.*, 1995). Motility could also be a reason for dominance in some situations. (Korber *et al.*, 1994) reported that enhanced cell transport, flow resistance and improved re-colonisation success led to the dominance of a motile strain of *Pseudomonas fluorescens* over a non motile strain, even though the growth rates of the two were identical. Therefore there can be several reasons why some bacterial strains may dominate others in biofilms.

2.2.3. Surface behaviour of attached bacteria and biofilms.

It is important to understand and know as much about biofilms and surface associated bacteria as possible to develop methods of controlling adherent bacteria in the food and

dairy industry. This section therefore covers the theoretical aspects of bacterial adhesion, the factors that may influence adhesion, and the characteristics of processes that occur in biofilms. As there is little information in the literature on thermophilic or even thermotolerant bacteria associated with surfaces, this section will cover important information concerning adherent bacteria and biofilms in general. This information can be applied later to thermophilic surface associated bacteria. Wherever possible however, examples from the literature regarding thermophilic or thermotolerant bacteria are used.

2.2.3.1. Proposed Mechanisms of Bacterial adhesion to surfaces

Several theories have been put forward but as yet the exact nature of the attachment process is unknown. The theories suggest that from two to five stages occur in the attachment process. In early studies the interaction between bacteria and solid surfaces was described as a time dependent process comprising of two stages, named reversible and irreversible adhesion, after the force required to remove the cells (Marshall *et al.*, 1971). The first stage is random contact of the bacterium with the solid surface, and firstly consists of transport of the bacterium to the surface from the bulk liquid followed by a weak association between the two components, where the bacterium can be easily removed from the surface by currents in the liquid medium, hence reversible. The second irreversible stage is subsequently reached as a result of the synthesis of adhesive polymers by the bacterium, cementing the bacterium in place. This basic model has been expanded over time to include further stages. For example, such stages, as cell transport to a wetted surface and adsorption of a conditioning film (Zottola & Sasahara, 1994). The events occurring between the substratum and the cell during attachment are described further by Busscher & Weerkamp (1987) and Zottola & Sasahara (1994). They used a three step model where different forces dominate at different cell to substratum distances. At greater than 50 nm Van der Waals forces predominate, from 10 to 20 nm both Van der Waals forces and electrostatic interactions predominate. To approach closer to the surface the cell has to overcome the interaction or repulsion barrier of these forces by some other specific interaction such as the presence of surface appendages to lower the projected surface area of the cell and hence reduce the repulsive force (Zottola & Sasahara, 1994).

How many of these stages are actually important for a given situation is not known. Electrostatic and hydrophobic interactions, the presence of external appendages on cells and polymer production are generally considered to contribute to adhesion. The influence of each of these on adhesion (covered in more detail below) is not fully understood and probably differs from case to case, depending on the bacterial type and the conditions faced.

Whether or not the potential for microorganisms to adhere can be predicted from the physiochemical properties of the surfaces concerned is another issue. Carpentier and Cerf (1993) suggest that it cannot be predicted. However, Meinders *et al.* (1995) concluded that initial bacterial adhesion could be explained in terms of overall physicochemical surface properties and that it is mediated by reversible, secondary minimum DLVO (Lifshitz-Van der Waals and electrostatic) interactions. Also, Bellon Fontaine *et al.* (1990) had success in predicting the adhesion of two fouling micro-organisms from dairy processing to various solid substrata using four different thermodynamic approaches. However the adhesion could not be accurately predicted in all cases.

2.2.3.2. Adhesion Factors

Several factors have been reported to have an effect on the attachment of bacteria to surfaces. These can be divided up into those factors dealing with cell properties, surface properties and the suspending fluid properties.

2.2.3.3. Cell Properties

Protein Interaction

The interaction of proteins on the cell surface is one factor that has been investigated for its effect on the attachment of cells to surfaces.

In Flint *et al.* (1997a) cell surface properties of 12 strains of thermophilic streptococci were examined. These cell surface properties were then correlated with the ability of the cell to attach to stainless steel surfaces. One of the properties examined was the effect of the cell surface protein on attachment to surfaces. The cell surface proteins of the 12 strains of thermophilic streptococci were removed, using either trypsin or sodium

sulphate. The attachment of the cells with proteins removed was then compared to the untreated cells. Treatment to remove the protein resulted in a 100-fold reduction in the number of cells adhering to the surface. This suggests that the surface proteins of the thermophilic streptococci are important in their attachment to stainless steel.

Bidle *et al.* (1993) tested the interaction of surface layer proteins with the attachment of bacteria, one of which was the thermophile *Bacillus coagulans*, to polystyrene substrata with different hydrophobicities. Some of the results found indicated that surface layers may play a role in bacterial adhesion to solid surfaces but a consistent correlation was not be found between surface layer adsorption and bacterial adhesion. This was thought to be due to the dependency of surface layer adhesiveness on chemical structure and environmental conditions. It was concluded that further evidence was required to determine the possible role of surface layers in bacterial adhesion.

The effect of cell surface proteins on attachment was also investigated by Herald and Zottola (1989). Free living cells were treated with compounds that either disrupted carbohydrates or proteins. The adherence to surfaces was then compared to adherence from untreated cells. It was found that treatments specific for disrupting proteins had little effect on the adherence of *Pseudomonas fragi* to stainless steel.

Parkar *et al.* (2001) found that spores and vegetative cells of thermophilic bacilli treated with protein denaturing agents (sodium dodecyl sulphate (SDS), and trypsin) showed decreased attachment to stainless steel surfaces. This indicates that these proteins were involved in adherence of the bacteria and spores to the surface.

Therefore cell surface proteins may play a role in adherence in some cases but not in others.

Cell Hydrophobicity

Another property that has been investigated for its effect on attachment of cells to surfaces is the cell surface hydrophobicity. There is no precise definition of hydrophobicity and there are several methods for determining cell surface hydrophobicity. The most common methods cited are bacterial adhesion to hydrocarbon

(BATH), hydrophobic interaction chromatography (HIC), and the salt aggregation test (SAT).

HIC uses aqueous suspensions of Sepharose beads covalently bound to hydrophobic moieties such as octal or phenyl groups. The beads are often packed into small columns and the cells are put on the column and eluted with a buffer solution. However, it is possible for the bacteria to be non-specifically trapped in the column. Alternatively, by mixing the beads with the cells and then separating the beads from the suspension, non-specific binding may be reduced (Hood and Zottola, 1995).

In the BATH method, a hydrocarbon such as hexadecane or xylene is mixed with a suspension of bacterial cells. Hydrophobic cells will adhere to the hydrocarbon and the decrease in absorbance of the bacterial suspension can be measured (Hood and Zottola, 1995). The hydrocarbons used may damage cells however and thus cause errors in measurements.

The SAT method is based on the theory that as hydrophobicity increases, the cells are more likely to precipitate out of solution at lower concentrations of salting-out agents. Bacteria are suspended in a dilute buffer solution and ammonium sulphate is added until aggregation occurs. High salt concentrations may cause damage to cell surface structure and therefore induce errors (Hood and Zottola, 1995).

Other methods that have been mentioned for use in determining cell hydrophobicity include adhesion to polystyrene, the use of molecular probes specific for hydrophobic surface components, determination of contact angles and two-phase partitioning (Hood and Zottola, 1995).

Due to the use of several different methods and in the absence of an accepted standard method it is possible that contradictory results may be obtained by various studies. This is particularly true since in many cases strong correlations between the different measurements have only been observed when the organisms were strongly hydrophobic or strongly hydrophilic (Mozes & Rouxhet, 1987; Sorongon *et al.*, 1991).

The hydrophobicity of the cell surface using the BATH test was another one of the properties examined in Flint *et al.* (1997a). No clear relationship between the degree of hydrophobicity and the attachment of thermophilic *streptococci* cells to stainless steel could be established. This suggests that cell hydrophobicity alone is not the determining factor in the number of cells attaching. However, it was found that all the thermophilic *streptococci* isolated from dairy plants were highly hydrophobic. On the other hand Van Der Mei *et al.* (1993) found that *streptococci* isolated from dairy pasteurisers were hydrophilic using a similar BATH method. This large variation between very similar adherent bacterial strains from the same type of environment shows that hydrophobicity may not be very important in determining adhering ability.

Gilbert *et al.* (1991) investigated the effect of hydrophobicity on the adherence of *Escherichia coli* and *Staphylococcus epidermidis* to glass using the HIC method. They found a strong correlation between hydrophobicity and adherence for *S. epidermidis* but not for *E. coli.* The methods they used were suspect however as they did not determine the non specific binding of cells in the column and they also used a removal technique for enumeration of bacteria at the surface which most probably would not have removed all bacteria.

Sorongon *et al.* (1991) found no correlation between the hydrophobicity of nine strains of swarming bacteria measured by three methods (BATH, HIC, and SAT) and their adhesion to glass surfaces. In Mafu *et al.* (1991) 22 strains of *Listeria monocytogenes* were tested for their hydrophobicity by SAT, HIC and contact angle measurements and were all found to be hydrophilic. The adherence of these bacteria could not be correlated to theoretical mechanisms based on the hydrophobicity and free energy of adhesion.

Parkar *et al.* (2001) also found no correlation between the surface hydrophobicity of vegetative cells and spores of thermophilic bacilli and the degree of attachment to stainless steel surfaces.

Therefore, there is no firm evidence in the literature that hydrophobicity is a strong predictive factor in the adherence of bacteria to surfaces. It could however still be involved as one of many factors effecting initial adherence. The measurement of the

hydrophobicity is a difficult task as it depends on a number of parameters. Hydrophobicity varies in the course of bacterial growth and with culture conditions. For example the hydrophobicity can decrease as the growth rate increases and mechanical and enzymatic treatments can alter the hydrophobicity of cells (Carpentier and Cerf, 1993). Because of such difficulties it is possible that hydrophobicity has an effect on adhesion but it has not been measured accurately or repeatedly.

Cell surface charge

The surface charge of cells is another factor that has been investigated for its influence on the initial attachment to surfaces. Bacterial cells generally carry a net negative charge on their cell wall with the magnitude varying between strains and depending on culturing conditions (Gilbert *et al.*. 1991). Two methods to measure the net surface charge that have been reported are electrophoretic mobility and electrostatic interaction chromatography (ESIC). ESIC is reported to be faster, simpler and more adequate when determining relative values for surface charge (Pedersen, 1980).

The cell surface charge was another property examined in Flint *et al.* (1997a). All the 12 thermophilic streptococci isolates possessed a net negative charge, but no relationship was found between the percentage of negatively charged cells and the numbers of cells adhering to the stainless steel. This suggests that cell surface charge alone is not the determining factor in the number of cells attaching.

Gilbert *et al.* (1991) have reported that the adherence of *E. coli* to glass surfaces has a strong inverse relationship with the negative charge on the cell surface. The same authors found no such relationship for *S. epidermidis* however.

With respect to the attachment to meat surfaces, Dickson and Koohmaraie (1989), have shown a relatively strong correlation between the net negative surface charge of several bacterial strains and the initial attachment to lean and fat meat tissue.

Therefore, though there is no strong evidence, the net charge of bacterial cells may have a role in the initial attachment of cells to surfaces even if it is only in some situations or in combination with other factors. As with hydrophobicity the charge is difficult to

measure as it can vary with culturing conditions as mentioned above. Thus the ability to determine whether a relationship exists or not is hampered.

External Polysaccharide Production

External polysaccharide (EPS) production of bacteria has also been investigated for its effect on attachment of bacteria to surfaces. The mechanisms of adhesion for bacteria to surfaces suggest that EPS plays an important role in the attachment process. Electron microscopy has been used to show the presence of EPS with attached cells in numerous reports. However, the literature regarding the relationship of EPS and initial attachment is inconclusive.

In Flint *et al.* (1997a) the effect of external polysaccharide production was yet another cell surface property that was examined in that article. It was found that the amount of EPS produced could not be directly related to the number of cells attaching to stainless steel. Parkar *et al.* (2001) also found no correlation between attachment to stainless steel of thermophilc bacilli and the amount of extracellular polysaccharide produced. This suggests that polysaccharide production alone is not the determining factor in the number of cells attaching. The work of Becker (1996) agrees with this, as it was shown that polysaccharide production is not always linked with the improvement of bacterial adhesion.

Support for EPS being involved in initial attachment is reported in Herald and Zottola (1989). They treated free living cells with compounds that either disrupted carbohydrates or proteins. The adherence to surfaces was then compared to adherence from untreated cells. Treatments that disrupted carbohydrates decreased attachment of *Pseudomonas fragi* to stainless steel.

Allison and Sutherland (1987) compared the adherence of a polysaccharide-producing wild strain of bacteria with that of a non-polysaccharide-producing mutant. They found that there was no difference in the attachment to glass between the two strains. However, it was noted that over time the polysaccharide-producing type formed microcolonies and the mutant remained as single attached cells. On eliminating glucose or Ca^{2+} from the medium the wild type did not produce polysaccharide. Again it was seen that although adherence was not affected, microcolony formation was prevented.

These indifferent results suggest that EPS production may not always be involved in the initial attachment of cells to surfaces and its main effect in some cases may be to assist in the colonisation of surfaces and not the attachment process itself.

Biosurfactant Production

Production of biosurfactants by bacteria is another factor that can influence the attachment of cells to surfaces. *Streptococcus thermophilus* is one bacterium that has been shown to produce biosurfactants and has been isolated from heat exchangers in dairy plants.

Busscher *et al.* (1990) found the adherence of *S. thermophilus* to surfaces was affected by its own production of biosurfactants. The deposition of the strain to a glass surface was initially fast, but after 80 minutes adhering cells detached and no new cells were found to adhere to the surface. This brought forward the hypothesis that the strain produced an anti-adhesive biosurfactant that would inhibit attachment. In further work, Busscher *et al.* (1994) investigated the biosurfactant production of eight strains *of S. thermophilus.* The strains were isolated from the downstream side of the regenerator section in pasteurisers in the dairy industry. The production of biosurfactants was investigated in water and also in a pH 7.0 buffer, with lactose, saccharose or glucose added. All strains were shown to produce biosurfactants in the buffer solution, while five strains produced biosurfactants in water. Also, most strains produced maximally when saccharose was added. It was suggested in this article that these biosurfactants could have a role as an anti-adhesive in the dairy industry. Here post pasteurisation contamination of product by bacteria adhering in biofilms is a major problem, particularly during long operating times, when the number of thermoresistant bacteria in the pasteurised milk gradually increases. Shorter run times and more frequent cleaning are strategies used to combat this. Busscher *et al.* (1996) isolated and purified biosurfactants from dairy isolates of *S. thermophilus.* Some of the compounds isolated were extremely surface active, reducing the surface tension to values around 30 $mJ.m^{-2}$ at a concentration of 10 $mg.ml^{-1}$. Also, an absorbed purified compound from one isolate inhibited the attachment of a different isolate by a factor of two. It was noted by the authors, that if these biosurfactants could be adsorbed to heat exchanger plates in pasteurisers and in doing so inhibit the attachment of other bacteria, then the compounds

would have major economic implications in the dairy industry, offering longer run times and reduced cleaning frequency. However, the likely high cost of the compounds and the necessity to re-treat the surfaces after they are cleaned may outweigh the advantages of being able to carry out longer production runs.

Spores

Attachment of spores to surfaces (as compared to vegetative cells) has also been investigated (Parkar *et al.*, 2001; (Flint *et al.*, 2001; Husmark & Ronner, 1992; Ronner *et al.*, 1990). These authors show that spores attach more readily to surfaces than vegetative cells, possibly facilitated by their relatively high hydrophobicity. Spores of *B. stearothermophilus* have low hyrdophobicity and attach to stainless steel surfaces much less efficiently than the spores of some other *Bacillus* species (Husmark and Rönner, 1992). Despite this Parkar *et al.* (2001), found that spores of thermophilic bacilli (such as *B. stearothermophilus* attached to stainless steel in greater numbers than vegetative cells of the same bacilli strains. The spores were also found to be more hydrophobic than the vegetative cells.

2.2.3.4. Attachment Surface Properties

Surface Polarisation

In Boulange Petermann *et al.* (1995) electrochemical techniques were used to investigate interactions between stainless steel surfaces and some biological materials. Two lactic bacteria were studied: *Leuconostoc mesenteroides* (encapsulated and biosurfactant (dextran) producing) and *Streptococcus thermophilus* (not encapsulated). When bacteria or dextran were added to a NaCl-containing electrolyte in contact with stainless steel, significant variations in the electrode potential were observed. It was found that a decrease in the electrode potential lowered the number of deposited bacteria. Their results suggested that the surface polarisation acts as an inhibitor of dextran (biosurfactant) adsorption, which would allow more cells to adhere due to the decreased biosurfactant level. Therefore a greater polarisation would facilitate greater bacterial adhesion.

Surface Roughness

The effect of surface roughness on the attachment of bacteria to surfaces has been investigated as rough surfaces may provide a more advantageous situation for adherence of bacteria. Boulange Petermann *et al.* (1997) studied the adhesion of *S. thermophilus* to stainless steel with different finishes, of varying roughness and topography. They could find no clear relationship existing between roughness or topographic parameters and the number of viable adhering bacteria. However, surface irregularities, such as roughness, crevices and pits have been shown to increase bacterial adhesion, by both increasing bacterial cell attachment and decreasing removal of attached cells by cleaning (Austin & Bergeron, 1995). Depressions and cracks in Buna-N and PTFE gaskets were regions where extensive biofilms were found. Austin and Bergeron (1995) advised that processing lines should be manufactured using smooth materials with as few depressions and crevices as possible, and that joining pipes by gaskets presents a surface irregularity that may lead to biofilm formation. In addition, increasing the surface micro-roughness may increase bacterial adhesion. In some cases cells were observed to attach to the flat regions around the crevices, while in others the bacteria were associated with the crevices (Lawrence *et al.*, 1995). This may indicate that irregularities on an otherwise smooth surface may cause an increase in bacterial transport and attachment to surface sites within that region rather than just in the crevices themselves.

However, Barnes *et al.* (1999) found that the difference in surface roughness between two stainless steel finishes was not great enough to affect bacterial attachment. The two finishes tested were a 2B and a no. 8 mirror finish, which had surfaces roughness (R_a) values of 0.412 μm and 0.035 μm respectively.

Several studies have investigated the effect of surface roughness on the ease of cleaning stainless steel surfaces with microbial contamination (Hoffman and Reuter, 1984; Leclercq-Perlat and Lalande, 1994; Steiner *et al.*, 2000; Frank and Chmielewski, 2001). Hoffman and Reuter (1984) found a linear relationship between roughness and residual spore counts of *Bacillus stearothermophilus* on cleaned stainless steel surfaces with a wide range of roughness values from 0.20 μm to 9.12 μm. Steiner *et al.* (2000) also found that physically roughened surfaces (sand blasted) had more residual contamination of *Bacillus stearothermophilus* spores after cleaning than standard

finishes. However, contamination differences between standard finishes with different roughness values were not statistically significant, indicating that only large changes in surface roughness affected cleanability. Frank and Chmielewski (2001) also investigated surface roughness and cleanability. They found that the number of surface defects present on a cleaned surface could be correlated to residual microbial contamination, but correlations with surface roughness were poor. Surfaces with different surface roughness values but with a similar number of surface defects had comparable residual contamination. They concluded that the absence of defects in the finish of stainless steel was more important than low surface roughness in obtaining an easily cleanable surface. This observation was also made earlier by Leclercq-Perlat and Lalande (1994) who found that surfaces with poor cleanability had many surface defects and that treatments that reduced the number of surface defects increased the hygienic qualities of surfaces in comparison to treatments that increased surface damage.

Therefore, surface roughness seems to contribute to the attachment of bacteria to surfaces. This may be due to increasing bacterial transport to the surface by changing the surface flow patterns or merely by providing a harbouring location for bacteria. However, the change in surface roughness required before adhesion to the surface is affected may need to be quite large. For example, variations of surface roughness provided by different stainless steel finishes may not be great enough to noticeably affect attachment. If this is the case then there would be no advantage in manufacturing food processing plants with more expensive smoother stainless steel to help control biofilm formation. However there is evidence to suggest that rough surfaces are not as cleanable as smooth, defect free surfaces. Therefore the stainless steel surface finish chosen for use in food processing environments should ideally be as smooth and defect free as financial and other constraints allow.

Substratum Hydrophobicity

Substratum surface hydrophobicity may be an important factor in the initial attachment of bacteria to surfaces. There is limited published work in this area, of what there is offers few details concerning surface preparation prior to experimentation, which is most influential on results, and quite often simply refers to the surface under study as either “hydrophobic” or “hydrophilic” (Carpentier and Cerf, 1993).

Boulange Petermann *et al.* (1997) investigated the surface hydrophobicity of stainless steel with different finishes and the adhesion of *S. thermophilus* to them before and after cleaning cycles. When cycles of bacterial adhesion were followed by chemical cleaning, it was found that the number of viable adhering bacteria decreased with surface hydrophobicity.

Pedersen (1990) used epi-fluorescence microscopy to quantify bacteria adhering to samples of hydrophilic stainless steel and hydrophobic PVC from a municipal drinking water system. There was no significant difference observed between the number of cells adhered to either surface.

The effect of surface hydrophobicity on attachment is not apparent from the literature and probably varies from case to case depending on the bacterial strain, the conditions involved and the methods used to prepare surfaces before testing.

Temperature

The temperature of the surface to which bacteria attach may be an important factor. Different bacterial phenotypes will grow in different areas of heat exchangers, depending on the temperature. For example, Langeveld *et al.* (1995) observed that in a heat exchanger where wall temperatures ranged from 22-49 °C, coliform bacteria were dominant, while in the regions where the wall temperature was 67-83 °C a *Thermus thermophilus* strain was dominant. However, this observation could be explained as a result of surface colonisation in the optimum temperature conditions rather than better initial adhesion to the surface.

Shea *et al.* (1991) showed that the marine bacterium *Deleya marina* showed optimum adherence on polystyrene at 25 °C, the organism optimum growth temperature. The attachment was less at 19 °C and less still at 37 °C. Klotz *et al.* (1989) observed that *Pseudomonas aeruginosa* adhered better to contact lenses at 37 °C than at 26 °C, with 37 °C again being the optimum temperature.

However Flint *et al.* (2001) found no relationship between temperature and adhesion when investigating the adhesion of the vegetative cells and spores of two strains of *B.*

stearothermophilus isolated from industrial milk powder plants over a temperature range of 20-55 °C.

Therefore it seems that although temperature may be a factor on the attachment of some bacteria, from the work available this doesn't appear to be the case for the thermophilic bacilli isolated from milk powder plants.

Type of Substratum

The adhesion of bacteria to different types of substratum has been reported. Adhesion to stainless steel is the most commonly studied as it widely encountered in industry. Adhesion to rubber has also been studied, (Lee Wong and Cerf, 1995; Austin and Bergeron, 1995; Criado *et al.*, 1994) as rubber gaskets are important sites for bacterial adhesion in food plants. Other materials such as glass, polystyrene and organic matter have also been studied for bacterial adhesion. Lee Wong and Cerf (1995) reported that the type of surface material affects the strength of adhesion.

The type of attachment surface also affects the degree of resistance to disinfectants. For example, the efficacy of disinfectants on biofilms developed on Buna-N rubber has been found to be much lower than on stainless steel. The residual antimicrobial activity on different surfaces also varies. Glass surfaces have been observed to retain higher residual antimicrobial activity than stainless steel after treatment with calcium hypochlorite or a quaternary ammonium compound (Lee Wong and Cerf, 1995).

Effect of Conditioning Films

The effect of the deposition of milk constituents on adhesion surfaces, as a conditioning film, has been investigated. In continuous flow and batch operations, a calcium phosphate pre-coating on stainless steel reduced adhesion of *S. thermophilus* by 50 % with pasteurised milk but had no clear effect when raw milk was used (Driessen *et al.*, 1984). Additionally the calcium phosphate coating was greater in the heating section of pasteuriser than in the downstream pasteurised milk section. Similar observations were also made by Bouman *et al.* (1982) who reported an increase in milk fouling deposits in the raw and heating sections of the pasteuriser over long operating times, whereas on the pasteurised side no detectable deposits were formed. Bacteria adhering to the plates of the heat exchanger adhered mainly to the pasteurised section where no or little

deposit was detected. Also, when the bacteria on the plates were viewed by electron microscope, the bacteria seemed to adhere directly to the metal surface without calcium phosphate acting as an intermediary.

Austin and Bergeron (1995) reported that a coating of milk solids on the inside surface of gaskets may have been a reason for the lack of bacterial adherence to the milk contact surfaces. This reasoning was supported by work that indicated that milk and individual milk components are capable of reducing bacterial adherence to stainless steel and Buna-N and that the inside surfaces of the gaskets were coated with milk solids (Helke *et al.*, 1993). Also, most of the bacterial adherence occurred on the surfaces of the gaskets that were not coated with a visible layer of milk solids. The areas of the stainless steel pipeline that formed the seal with Buna-N gaskets were also fouled with milk solids and lacked adherent bacterial cells (Austin and Bergeron, 1995).

Barnes *et al.* (1999) also found that milk proteins inhibit adhesion of bacteria. Stainless steel coupons were pre-treated with skim milk and the attachment to the surfaces of five kinds of bacteria was studied. Skim milk was found to reduce adhesion of all five strains. Individual milk proteins α-casein, β-casein, κ-casein and α-lactalbumin were also found to reduce adhesion of two of the five organisms. It was also found that adhered numbers were inversely proportional to the amount of skim milk protein present on the surface.

In agreement with this, Lee Wong and Cerf (1995) have reported that stainless steel surfaces pre-coated with milk or milk proteins such as casein and beta-lactoglobulin have been found to inhibit attachment of *Listeria monocytogenes* and *Salmonella typhimurium.* Contrarily, the same report also states that an increase in the attachment of several milk-associated microorganisms to stainless steel, rubber, and glass surfaces in the presence of whey proteins was observed. This discrepancy may indicate that whey proteins may not be a milk deposit that inhibits attachment, rather one that increases attachment.

Flint *et al.* (2001) investigated the attachment of *Bacillus stearothermophilus* to stainless steel coupons coated in a skim milk foulant created by denaturing skim milk on to the surface in an autoclave. It was found that the attachment of cells was increased

10-100 fold by the presence of the skim milk fouling. However, Parkar *et al.* (2001) found that coating stainless steel surfaces with skim milk proteins (undenatured) reduced attachment of both spores and vegetative cells of thermophilic bacilli.

As well as influencing bacterial attachment, milk protein and fat have reputed protective effects on certain microbes and also inactivate chemical sanitisers (Dunsmore, 1981; Mattila *et al.*, 1990). Criado *et al.* (1994) also states that milk components deposited on contact surfaces form residues, which serve to protect the associated bacteria from cleaning products and disinfectants, while at the same time, provide a source of nutrients which stimulate growth.

The effect of milk fouling deposit on the survival of *Listeria monocytogenes* was investigated as the presence of these components may allow improved survival over time of the attached bacteria (Wong, 1998). Under conditions where the numbers of viable bacteria on clean stainless steel and rubber surfaces died off after 3 to 10 days, the bacteria attached to the milk fouling actually grew and initially increased in numbers or held constant over the 10 days of measurement. Therefore, the milk fouling was acting as a nutrient supply for the bacteria.

Wirtanen *et al.* (1996) performed an experiment in which *Bacillus* biofilms attached to artificially created food soiling and biofilms on stainless steel were cleaned with an alkali and acid procedure in a test rig. It was found that for two of the three *Bacillus* species tested the bacteria remained attached to stainless steel better than to the soiled surface, so that the biofilm cells survived cleaning better than those on the soiling. This was due to the artificial soiling being easily removed from the surface (along with the attached cells) whereas the biofilm attached directly to the surface was much more difficult to remove. Therefore evaluation of any protective effect of the fouling material could not be made. Frank and Chmielewski (2001) also found that biofilm was more difficult to remove than dried on soiling.

Bredholt *et al.* (1999) incubated pre-soiled and clean stainless steel surfaces in cultures of several different bacteria for 4 days under continuous shaking. After incubation similar amounts of residual matter were present on both types of surface. Bacterial numbers detected were also similar (10^5-10^6 cfu.ml^{-1}) on both types of surface.

Therefore over this time period of 4 days any increased initial attachment that the soiling may have had did not have any effect on final numbers. After these surfaces had been exposed to low pressure cleaning a similar amount of residual material was left behind and similar numbers of bacteria were found on both types of surface. Therefore the presence of the pre-soiling had no protective effect over that provided within an established 4 day old biofilm.

Therefore, from the published work it appears that milk components deposited on the surface can inhibit or enhance the attachment of bacteria to surfaces depending on bacterial species and the nature of the milk protein. Bacteria associated with milk fouling deposits may also show improved survival of environmental and cleaning conditions in some situations.

2.2.3.5. Suspending Fluid Properties

Nutrient Limitation

Under conditions where nutrient concentrations are low, increased attachment to surfaces has been observed by many authors (Brown *et al.*, 1977; Kjelleberg and Hermansson, 1984; Gilbert *et al.*, 1991). The reason for enhanced attachment could be due to the available nutrients being concentrated at the surface rather than in the bulk liquid. Therefore, it would act as a survival technique. This would be different for bacteria in high nutrient environments, which would be saturated with nutrients and less likely to travel to the surface for nutrients (Zottola & Sasahara, 1994).

Another explanation for increased attachment under nutrient limited conditions is due to the starved state of the cells. Bacterial cells in their starved (or stationary) state are smaller and researchers have tried to relate the starved state of the cells to their hydrophobicity and surface charge. Kjelleberg and Hermansson (1984) showed that in some bacteria, but not others, low nutrient levels induced an increase in cell surface hydrophobicity and a greater number of irreversibly bound cells.

Therefore in nutrient limited conditions attachment of bacteria cells appears to be greater, however the reason for this is unclear.

Number of cells present

The number of cells present in the bulk fluid seems to have an effect on the number of cells attaching to surfaces. Both Notermans and Kampelmacher (1974) and Butler *et al.* (1979) have reported that the adherence of cells to surfaces is related to the number of cells present in the bulk fluid. Notermans and Kampelmacher (1974) found that the rate of adherence to chicken skin increased as the number of cells in the adherence media increased. Likewise, Butler *et al.* (1979) observed that the number of attached bacteria on beef, pork and lamb can be directly related to the number of bacterial cells in the adherence media. Escher (1986) showed that the adsorption rate for cells of *Pseudomonas aeruginosa* is linearly proportional to the cell concentration in the bulk fluid. Also, de Jong *et al.* (2002) states that the rate of adhesion of bacterial cells to a surface is linearly proportional to the cell concentration near the surface.

Therefore, it seems that higher numbers of cells in the bulk fluid results in a greater adhered population and greater adhesion rate to the surface.

Flow Regime / Shear stress

The adherence of bacteria to surfaces has been compared under different flow regimes of the bulk fluid. Authors use the fluid shear stress to compare attachment results. As the shear stress (or Reynolds number) increases lower adsorbed cell densities occur (Characklis & Marshall, 1990; Dickinson & Cooper, 1995; Duddridge *et al.*, 1982). A term that has been defined to help describe the adherence of bacteria under different conditions is the sticking efficiency. This is defined as the rate of cells adsorbing to the substratum divided by the flux of cells from the bulk liquid to the substratum, which can be calculated based on the transport mechanism involved (Characklis and Marshall, 1990). Thus, sticking efficiency describes the probability that a cell transported to the surface from the bulk liquid will adsorb. Characklis and Marshall (1990) using data from a variety of authors have compared adherence data from a variety of shear stress conditions. Their conclusion was that the calculated sticking efficiency was inversely proportional to the fluid shear stress. This indicates that under higher shear stress, fewer cells from those transported to the surface adhere to the surface. Also, Escher (1986) found that the net cell accumulation rate decreases with increasing shear stress, showing that not just the number of cells adhering initially, but also the rate at which they adhere over time is reduced under higher shear stress.

The structure of the biofilm that develops from attached cells also appears to be influenced by the shear forces at the biofilm surface. Van Loosdrecht *et al.* (1995) found that biofilms exposed to high shear stress developed into thin patchy biofilms, while under low shear the biofilm develops a loose structure with many pores and protrusions.

Therefore higher shear stress (or greater Reynolds numbers) can cause a reduction in the number and rate of cells adhering to a surface.

Recent work (Bakker *et al.*, 2002; Gomez-Suarez *et al.*, 2001) has shown that this effect of shear forces on adhesion has implications important to all studies where adhesion of bacteria to surfaces is measured. They report that forces as small as the passing of air-liquid interfaces over the surface can detach up to 80-90 % of bacteria adhered to the surface. These results were obtained using a parallel plate flow chamber so that adhered numbers could be measured without disturbing the surface. This has implications for methodologies where adhesion is measured by first removing the surface from the suspending medium and rinsing the surface. Gomez-Suarez *et al.* (2001) suggests that the results of such methods should be referred to as "bacterial retention" rather than "bacterial adhesion".

Electrolyte concentration

Van Hoogmoed *et al.* (1997) investigated the effect of electrolyte concentration on the adherence of three strains of *Streptococcus thermophilus* to stainless steel. The electrolyte concentration was varied by using buffers containing different concentrations of $CaCl_2$. Metallurgical microscopy was used to examine the adherence of the *S. thermophilus* dairy strains to the steel. Adhesion was measured *in situ*, in a parallel plate flow chamber that had a glass top plate and a stainless steel bottom plate. Initial deposition rates did not show systematic variation with amounts of $CaCl_2$ in the buffer. From this they concluded that electrolyte concentration, and therefore the electrostatic interactions related to the electrolyte concentration, only play a minor role in bacterial adhesion to stainless steel.

However, Barnes *et al.* (1999) found that increasing the ionic strength of the suspending medium increased attachment of *S. aureus*. With reverse osmosis (RO) purified water,

attachment was less than 1 % of that observed with quarter strength Ringer's solution. The individual components of Ringer's solution gave increasing attachment in proportion to their contribution to the overall ionic strength. Also, solutions of salts of divalent and monovalent cations at concentrations of 20 to 40 mM showed enhancement of attachment to clean stainless steel.

Therefore, the electrolyte concentration may have a significant effect on attachment in some cases. Due to this, it would be recommended to use constant electrolyte concentrations during investigations into attachment to avoid discrepancies in the results.

Raw/Pasteurised Medium

The effect of pasteurisation of the bulk liquid on attachment of cells to surfaces has been examined by Driessen *et al.* (1984). It was found that *S. thermophilus* initially adhered equally well to stainless steel in raw and pasteurised milk (initial adhesion time of one hour). However, in raw milk over long operating times (continuous feeding of milk over test plates for >1h) the number of bacteria on the stainless steel increased more slowly than with pasteurised milk. After 4 hours the count on the stainless steel was considerably less when using raw milk (7.0×10^5 cfu.cm^{-2}) than when using pasteurised milk (1.0×10^7 cfu.cm^{-2}). In the milk itself, up to 10^6 cells per ml were reached. The authors suggested that raw milk contains inhibiting compounds, for example thermolabile immunglobulins, that inhibit bacterial growth in raw milk. The proposed theory on this is that the bacteria initially attach to the wall with the initial adhering concentration being proportional to the number of bacteria in the milk. With raw milk, the inhibitors present inhibit the growth of the adhered bacteria and the increase of bacteria on the wall is slow compared to the pasteurised milk situation where there are no inhibitors present. Therefore, a higher count of cells is seen adhered to the surface with pasteurised milk. The findings in Rademacher *et al.* (1996) agree with this. They reported that milk that has had a thermal pre-treatment before pasteurisation causes an increased rate of growth of bacteria (*S. thermophilus* was studied) on plate surfaces in heat exchangers, and therefore an increase in the bacteria counts of the pasteurised milk.

Therefore, it seems that bacterial attachment to surfaces from raw milk is less than that from pasteurised milk. Consequently, there may be benefits in delaying the pasteurisation as long as possible when manufacturing dairy products such as milk powder, thereby minimising bacterial attachment to surfaces before pasteurisation.

Milk Components Present

Speers and Gilmour (1985) evaluated the effect of the presence of lactose, casein, milk fat, non casein protein, and whole milk in the bulk fluid on the attachment of bacteria to dairy plant surfaces. Bacterial adhesion was highly promoted by the presence of lactose and non-casein protein solutions but there was no increase in the numbers of bacteria attaching in the presence of whole milk.

This effect could again be due to inhibitory effects on the cells or possibly the influence of different conditioning films on the surface that are developed with the different components.

2.2.3.6. Resistance to Sanitation

An important property of adhered cells for the food industry is that once attached they are more resistant to sanitisers than free living planktonic cells. Several authors (Austin & Bergeron, 1995; Bower *et al.*, 1996; Flint *et al.*, 1997c; Hood & Zottola, 1995; Kumar & Anand, 1998) report that bacteria adhered to a surface have an increased resistance to sanitisers. This is important to the food industry as CIP operations in food processing plants may not remove or sanitise spoilage or pathogenic bacteria present in the plant after a processing run. These bacteria would then be able to contaminate the next run.

S. thermophilus has been shown to be more heat and chemical resistant than free living cells when attached to stainless steel (Flint *et al.*, 1997c). When attached and planktonic cells were exposed to temperatures of 60 °C and 65 °C the D-values for the attached cells were higher than those of the planktonic cells indicating that attached cells have an enhanced heat resistance. Similarly, the attached cells were more resistant to sanitisers than the planktonic cells. The planktonic cells were totally inactivated by 20 ppm of sodium hypochlorite or a type of quaternary ammonium compound (CTAB),

while attached cells survived 400 ppm sodium hypochlorite and 1000 ppm of CTAB. Sodium hypochlorite was found to be more effective against the attached cells. It was stated that this may be due to the different action each sanitiser has on microbial cells, and that sodium hypochlorite may be better at penetrating the protective mechanism that attachment provides, which has not yet been defined (Flint *et al.*, 1997c).

Carpentier *et al.* (1998) and Lee Wong and Cerf (1995) suggest that attached cells are more resistant to exposure to heat, antibiotics and disinfectants than are planktonic cells, but no mechanism was offered. Austin and Bergeron (1995) also reported that bacteria growing in a biofilm are more resistant to antibiotics and chemical sanitisers than corresponding planktonic bacteria. Similarly, Mattila *et al.* (1990) demonstrated that wild bacteria isolated from a milking line were susceptible to sanitisers if exposed as a cell suspension, but were resistant to the same reagents when they were attached to a surface. Frank and Koffi (1990) reported that *L. monocytogenes* adhered to a glass surface survived more than 10 times longer than free living cells when exposed to benzalkonium chloride (BAC), anionic acid sanitiser or heat (50 °C and 70 °C).

Some sanitisers, when used against attached bacteria instead of planktonic bacteria, show less reduction in effectiveness than others. Also, the sanitisers that are most effective against planktonic bacteria are not always the ones most effective against attached bacteria. Peracetic acid is more effective against attached cells than aldehydes, hydrogen peroxide or chlorine. However, hydrogen peroxide has the advantage that it sanitises and removes attached bacteria (Carpentier and Cerf, 1993). Quaternary ammonium compounds (QAC's) are generally not very effective against attached bacteria, as they do not penetrate layers of cells (Carpentier and Cerf, 1993). This is thought to be due to their hydrophilic nature which does not allow them to penetrate lipophilic surfaces like of the cell walls of gram-positive bacteria (Bower *et al.*, 1996). This is supported by the results of Flint *et al.* (1997c) mentioned above, where a QAC compound was less effective than sodium hypochlorite, and also Ronner and Lee Wong (1993) who found QAC compounds to be among the least effective on adhered bacteria.

The surface to which the bacteria adhere to also affects the resistance to sanitisers. Mafu *et al.* (1990) found that with cells attached to rubber, common sanitisers were needed at concentrations 5 to 10 times greater than were needed for stainless steel. Ronner and

Lee Wong (1993) found that bacteria adhered to Buna-N were more resistant than those on stainless steel. Krysinski *et al.* (1992) had similar results with polyester/polyurethane and polyester where attached bacteria on these two plastic surfaces were more resistant than those present on stainless steel. Therefore, it is possible that attached bacteria can be less resistant on stainless steel than they can be on surfaces such as rubber and plastics and that the surface adhered to may influence the resistance to sanitation. Also, surfaces such as rubber and plastics tend to wear more than stainless steel, providing a rougher surface, which could be more difficult to clean (Carpentier and Cerf, 1993).

As biofilms age the resistance to sanitisers of the cells within the biofilm increases (Carpentier & Cerf, 1993; Frank & Koffi, 1990; Lechevallier *et al.*, 1988; Lee & Frank, 1991).

The exact mechanism of how bacteria attached to surfaces become more resistant is unknown but several theories have been offered. Firstly, the resistance of the biofilm to sanitisers may be due to a protective effect conferred by exopolysaccharide (EPS) and other biofilm components. It is possible that the EPS acts as a diffusion or non-penetrable barrier for sanitisers (Carpentier and Cerf, 1993). However, Carpentier *et al.* (1998) states that diffusional resistance is not large enough to account for the increase in resistance to chemical sanitisers, especially across the thin biofilms or attached cells like those found in the food industry. Therefore, if this is the case the main protective effect of EPS may be through interaction and inactivation of sanitisers. Secondly, antimicrobial agents are more effective against actively growing cells (Morton *et al.*, 1998). Bacteria entrapped within biofilms may have reduced growth rates due to reductions in oxygen and nutrient transport, especially in thick biofilms. These bacteria could then be more resistant to sanitisers (Kumar & Anand, 1998; Morton *et al.*, 1998). Another mechanism put forward, and which may provide bacteria in biofilms an increased resistance to sanitisers, is through the production of antibiotic-degrading enzymes, such as β-lactamases. These enzymes may degrade and inactivate antibiotics as they permeate through the cell envelope to the target site. In biofilms these same enzymes could be produced and become trapped and concentrated within the biofilm matrix which in turn would provide greater protection from the sanitisers (Kumar and Anand, 1998). However, in reality, the enzymes present are unlikely to be able to act on many of the chemical structures that they are faced with. Another possible mechanism

for resistance to chemicals is through cell modifications on attachment. Carpentier *et al.* (1998) mention this and give an example where attached *E. coli* have a reduced amount of a surface protein (a porin) that permits passive diffusion of hydrophilic substances of less than 600 Daltons in size. This could explain the resistance of attached *E. coli* to certain antibiotics that would normally enter the cell through the porin.

Attached bacteria may be more resistant to sanitisers than planktonic cells but proper cleaning and sanitation should still be effective in reducing contamination. Stone and Zottola (1985) found that when cleaning and sanitising a milk pipeline using different recommended procedures, attached *P. fragi* were inactivated and biofilms were removed. In sub-optimal conditions this was not always the case because attached *P. fragi* were not always removed or inactivated. In food processing sanitation is preceded by cleaning. In most of the laboratory work carried out this cleaning stage was not involved. Due to this, organic matter is present in the laboratory studies that may not be present to the same extent in the food processing situation. This organic matter may provide the attached cells in the laboratory studies with increased resistance above that which would occur in a food processing situation, due to organic matter inactivating the sanitisers. Therefore, one must be careful extrapolating laboratory data to process plant situations. Krysinski *et al.* (1992) reported that, in general, chemical cleaners were more effective than sanitisers in eliminating *L. monocytogenes* biofilms on stainless steel and plastic surfaces. This is not surprising as the cleaning chemicals are designed to remove organic matter, while sanitisers have traditionally been designed to only kill cells.

Therefore, the observations made with regard to increased resistance emphasise that following cleaning and sanitising recommendations and not altering recommended chemical concentrations or reducing temperatures to economise is very important.

2.2.3.7. Control of Biofilms in the food industry

An effective cleaning and sanitation programme when included in the process from the beginning will inhibit accumulation of cells and biofilm formation, and keep bio-transfer potential to a minimum (Kumar and Amand, 1998). However, when the cleaning and sanitation regime is ineffective biofilm formation can increase, as bacteria are left behind after cleaning and are able to contaminate the product much faster than

would otherwise be possible. Developing such a programme and making it cost effective is a difficult task however (Kumar and Amand, 1998). Control strategies can focus either on the removal of the attached cells or preventing microbial adhesion.

Removal of attached cells generally relies on cleaning regimes used to clean food contact surfaces such as various CIP procedures or mechanical action. Some novel methods for removal of attached cells are the use of ice crystal formation, where cycles of freezing and thawing remove the films; the application of an electric field which allows chemicals to penetrate the biofilm matrix more effectively and kill cells (Bower *et al.*, 1996; Mittelman, 1998) and treatment with ultrasound (Kumar and Amand, 1998; Carpentier and Cerf, 1993). As mentioned above, an important factor in the cleaning of surfaces is the roughness and the presence of crevices and cracks. These can shield attached cells and organic matter from the cleaning procedure and care should be taken in design to ensure that cracks and dead areas in which organic matter could accumulate are avoided (Bower *et al.*, 1996).

Several strategies and methods have been reported to help prevent bacterial adhesion to surfaces on food contact surfaces. These include the use of super-high magnetic fields (Bower *et al.*, 1996), modifying surface hydrophobicity, applying biosurfactants (Busscher *et al.*, 1996), and applying antimicrobial agents such as chemical biocides and bacteriocins (e.g. Nisin, (Bower *et al.*, 1998)) to the surface. At present developing these techniques for large scale use in the food or dairy industry would be impractical however, largely due to cost. Also, methods such as applying chemicals to surfaces, eventually lose their effectiveness and need to be retreated, which adds to the cost. In the future cost effective methods may be developed but as yet this is not the case.

Therefore, at present, the best means of control of bacterial adherence in the food industry may reside in development of cost-effective cleaning regimes and the intelligent design of processing equipment to avoid difficult to clean and sanitise locations.

2.2.4. Mathematical modelling of biofilms

Mathematical models simulating the behaviour of biofilms have been used over the last 30 years. Models of different types and of different levels of complexity have been developed over the years depending on the purpose of the model.

2.2.4.1. History and Current Status

The earliest and simplest biofilm models described biofilms as steady state uniform films containing a single type of bacterium and controlled by one-dimensional mass transfer and biochemical reactions (Atkinson & Davies, 1974; Rittmann & Mccarty, 1980). Although basic, this type of model is still useful to describe simple biofilm processes. These models are generally focused on biofilm development and their consumption of nutrients for applications such as waste water treatment. Later models took into account differences in the biofilm at different depths and were able to represent multi-substrate and multi-species biofilms (Rauch *et al.*, 1999; Rittmann & Manem, 1992; Rittmann & Manem, 1992; Wanner & Gujer W. 1986; Wanner & Reichert, 1996; Wanner & Reichert, 1996). Also, one dimensional models which considered several factors important to their given situations were developed, such as models of biofilms in water distribution systems which consider phenomena such as hydraulic conditions, temperature, detachment, pH and inactivation (Bois *et al.*, 1997; Bois *et al.*, 1997; Dukan *et al.*, 1996; Piriou *et al.*, 1998; Piriou *et al.*, 1997; Piriou *et al.*, 1997; Stewart *et al.*, 1996).

These one-dimensional models are advanced descriptions of multi-parameter biofilm interactions but do not represent the different structures of biofilms that have been discovered, consisting of heterogeneities such as cell clusters surrounded by pores and channels connected to the bulk fluid (de Beer & Stoodley, 1995; de Beer *et al.*, 1994b; Lawrence *et al.*, 1995; Lewandowski *et al.*, 1995). Models focused on biofilm structure that provide two and three-dimensional descriptions of biofilms are some of the latest that have been developed (Hermanowicz, 1998; Hermanowicz, 1999; Noguera *et al.*, 1999b; Picioreanu *et al.*, 1998; Picioreanu *et al.*, 1999; Wimpenny & Colasanti, 1997). The basis for these models involves the use of a cellular automaton approach whereby small units or cells that may represent single cells or aggregates of cells make up a 2-D

or 3-D array which represents the biofilm. The state of each unit changes according to a set of rules that are set by the conditions and interactions in the biofilm (Hermanowicz, 1998). Picioreanu *et al.* (1998) and Picioreanu *et al.* (1999) combined this with a differential approach to link the cellular automaton model parameters to real values of parameters such as diffusivities and reaction rate constants. This lead to combined discrete-differential models that were more real than cellular automaton alone. Cellular automaton approaches have led to models which resemble the new heterogeneous structures that have been found consisting of voids and channels (Noguera *et al.*, 1999a). While these models may not be of much use in industrial applications, they are important in developing the scientific knowledge on biofilms.

In addition to these models that are generally concerned with biofilm growth and activity, models have also been developed that focus on killing biofilms (Dodds *et al.*, 2000; Stewart *et al.*, 1996). These models are useful in investigating biofilm resistance to inactivation by antimicrobial agents. The model of Dodds *et al.* (2000) incorporated different mechanisms of biofilm resistance. Model predictions were compared to actual experimental biofilm inactivation data and estimates on which resistance mechanisms were likely to be involved for different biofilms and different antimicrobials could be made.

2.2.4.2. Biofilm modelling in food processing

Biofilm models for predicting contamination of food products during processing from biofilms are relatively uncommon. Driessen and Bouman (1979) roughly calculated the expected concentration of thermoresistant streptococci in milk passing out of a heat exchanger using the observed bacterial population present on the surface within the heat exchanger where bacterial growth at the surface was expected to occur. The calculation was made by multiplying the number of bacterial generation times that occur per hour by the number of bacteria present on wall and dividing the result by the flow passing through the heat exchanger per hour i.e.:

$$N = \frac{D.A}{V.g} \qquad 2.1$$

Where:

N = bacterial concentration in the milk ($cfu.ml^{-1}$)

D = bacterial population at the heat exchange surface ($cfu.cm^{-2}$)

V = volumetric flow of milk ($ml.hr^{-1}$)

A = internal surface area of heat exchanger where bacterial growth occurs (cm^2)

g = generation time of bacteria (hr)

The calculated bacterial concentration in the milk of 0.7×10^6 $cfu.ml^{-1}$ approximately correlated with the observed bacterial count in the milk of 1×10^6 $cfu.ml^{-1}$.

Langeveld *et al.* (1995) took this calculation method and applied it to bacterial contamination of milk flowing through a tubular heat exchanger. The following equation was used:

$$N = \frac{D.\pi.d.l.(1.15)}{V.g} \qquad 2.2$$

Where:

d = internal pipe diameter (cm)

l = pipe length (cm)

1.15 = correction factor for bends in the heat exchanger piping

The calculated bacterial concentration was much higher than that actually measured (1×10^7 $cfu.ml^{-1}$ compared to 7×10^5 $cfu.ml^{-1}$). This over estimate was suspected to have been due to entrapment of bacteria within the milk fouling deposit found on some of the heat exchanger tubes. Surface bacterial populations were measured in such a way as to enumerate all bacteria present within the tube, therefore some of the bacteria measured would have been entrapped within the fouling deposit and would not have been contributing to the contamination of the milk.

The calculations above also use a linear approximation of the growth rate of bacteria generated at the surface after a given time by taking the number of times the population doubles in an hour (based on the generation time) and multiplying this figure by the initial surface population to get an estimate of the numbers released during that hour. Ideally this should be calculated using exponential growth kinetics, as this would more closely represent the growth of the bacteria at the surface over time.

An unsteady state model described by de Jong *et al.* (2002) for *Streptococcus thermophilus* in pasteurisers relates growth at the surface with numbers contaminating the product stream over time. Mass balances of bacteria at the surface and in the bulk liquid formed the basis of the model. The bacterial growth at the surface as a function of the operating time (t) for a plug flow reactor was defined using the following equation:

$$\frac{dn_w}{dt} = \mu_T . n_w . (1 - \beta) + k_a . c \qquad 2.3$$

Where:

n_w = bacterial wall coverage (cfu.m^{-2})

μ_T = bacterial growth rate at temperature T (s^{-1})

β = fraction of generated bacteria released into the bulk

k_a = adhesion constant (m.s^{-1})

c = local bulk bacterial concentration (cfu.m^{-3})

t = operating time (s)

The bulk concentration of bacteria as a function of position in a plug flow reactor was defined by:

$$\frac{dc}{dx} = \frac{\pi . d}{\phi} . (\beta . \mu_T . n_w - k_a . c) + \frac{\pi . d^2}{4\phi} (\mu_T - k_d) . c \qquad 2.4$$

Where:

ϕ = product flow rate (m^3.s^{-1})

k_d = destruction constant (s^{-1})

d = hydraulic diameter of reactor (m)

x = position in reactor (m)

For a tank reactor the bulk concentration is independent of the position and was defined by:

$$\frac{dc}{dt} = \frac{4\phi}{\pi . d^2 . L} . (c_{in} - c) + \frac{4}{d} (\beta . \mu_T . n_w - k_a . c) + c . (\mu_T - k_d) \qquad 2.5$$

Where:

L = Liquid level in the tank (m)

c_{in} = bulk bacterial concentration entering reactor (cfu.m^{-3})

These three equations were solved numerically in parallel as a function of operating time and position. The model parameters were fitted to experimental data by a computer program which provided an optimised solution through application of an advanced simplex method.

This model also takes into account the effect that temperature has on bacterial growth and inactivation. A Ratkowsky square root model was used to describe the growth rate and an Arrhenius relationship to describe the destruction of bacteria as a function of temperature. The model for growth rate was fitted against experimental data of observed growth rates at a range of temperatures.

Model predictions for bacterial contamination in the bulk fluid were close to those observed experimentally (Figure 2.3.1). However, much higher surface numbers were predicted by the model than were observed experimentally. Approximately 100 times more numbers were predicted by the model than were actually observed. The authors concluded that this discrepancy was due to inaccurate measurement of surface numbers, as the swab method used was suspected of providing underestimates of the surface numbers.

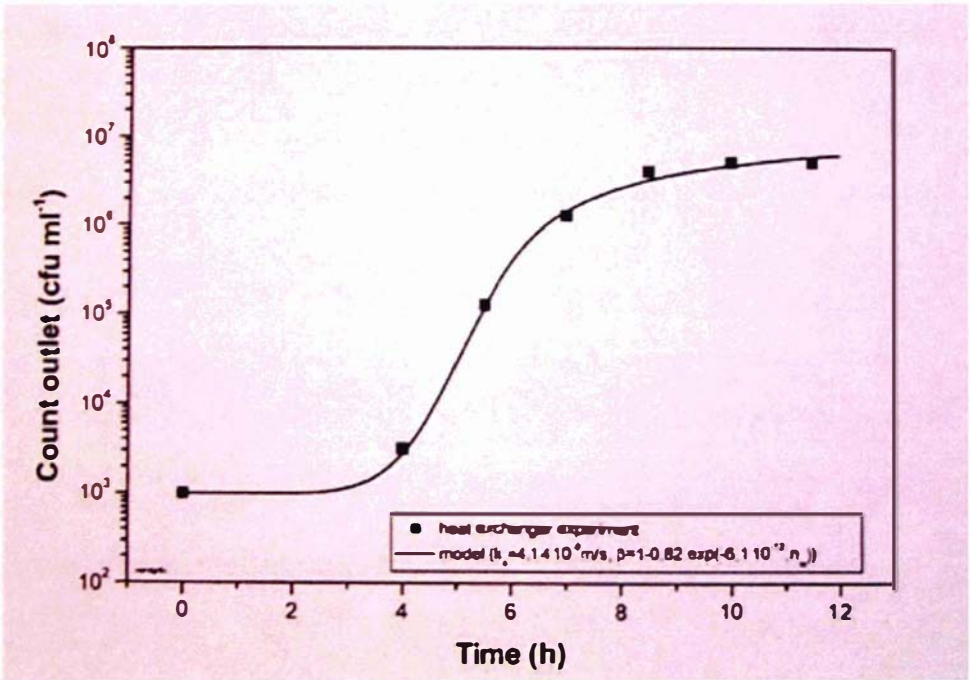


Figure 2.3.1. Experimental results and model predictions of de Jong *et al.* (2002) for the concentration of *S. thermophilus* at the outlet of a heat exchanger.

A simpler model was proposed by den Aantrekker *et al.* (2003) that performed adequately for *Staphylococcus aureus* biofilms on silicon tubing in laminar flow. No temperature dependant kinetics were incorporated and a power factor was used to describe the release of cells from the biofilm as the numbers of cells on the surface increased, rather than a seperate relationship controlling the release proportion as in de Jong. Surface predictions were close to what was measured experimentally as the experimental technique combined loosely, reversibly and irreversibly adhered cells in the estimate of total numbers at the surface.

Models such as this applied to thermophile contamination of the milk powder production process would be a useful aid in minimising such contamination through optimisation of the process based on model predictions. For example, the maximum operating time for processing equipment before thermophile contamination becomes a problem could be calculated based on the initial values of the important parameters in the model. Also, the most important process parameters could be identified by analysing the magnitude of the effect each parameter provides on the rate of increase in thermophile contamination. This knowledge could then be applied to determine the optimal conditions to minimise thermophile contamination.

In addition to models describing the contamination threat posed by biofilms in food processing environments, models that predict the cleaning of contaminated surfaces would also be a useful tool in minimising contamination. These models could help identify the important factors involved in cleaning and hence allow the cleaning process to be optimised by manipulation of these key factors. Lelievre *et al.* (2002) have recently developed a model describing the cleaning kinetics for pipes contaminated with *Bacillus cereus* spores. The model was based on an assumed process combining removal and deposition during cleaning and model predictions were confirmed experimentally. Predictions made by the model were then used to identify factors important to improved cleaning. For example, a significant effect on the effective removal rate constant by both the flow conditions during the soiling procedure and applied during cleaning was observed.

2.3. Methods of Detection, Study, and Enumeration of Bacteria on Surfaces.

2.3.1. Introduction

The problems that have arisen with large numbers of thermophiles in milk powder plants have come about due to their proliferation on the inner contact surfaces of the milk powder plants. When compiling the methods below this fact was taken into account. Hence, the following methods are largely for the detection, study, enumeration and identification of bacteria on surfaces. The methods that are not surface techniques were mentioned for their possible usefulness in detecting, studying, enumerating or identifying bacteria in the planktonic state to aid in the overall picture of contamination by thermophilic bacteria.

2.3.2. Generation of material for study

To study biofilms and bacteria attached to surfaces, methods of obtaining surface samples of biofilms or attached bacteria are needed. The type of sample depends on the environment of study and the observations that are needed. For applications relating to attached growth in engineering flow situations two different types of method dominate the literature.

The first of these is the use of removable test pieces that are placed inside equipment or pipe work. The Robbins device (Mccoy *et al.*, 1981) is a typical example of this technique. This is a multi-port sampling device that is placed in the pipe work to be investigated. Each port consists of a replaceable plug that sits level with the inside of the pipe work and that can be removed and replaced without draining the system. The plugs can then be studied for attached growth.

The second of these is the use of flow cells or flow chambers. In this technique the bulk fluid is passed over a glass slide in a flow system. Attachment of bacteria to this slide can then be viewed non-destructively microscopically over time without the need for removal of the slide. In this way the development of a biofilm can be viewed in place and microscopic biofilm events can be witnessed as they happen. A problem with this is

that the attachment surface, that is the slide, is limited to transparent materials such as glass. However, as mentioned above, recent work (Gomez-Suarez *et al.*, 2001; Bakker *et al.*, 2002) reports that forces as small as the passing of air-liquid interfaces over the attachment surface can detach up to 80-90 % of bacteria adhered to the surface. These results were obtained using a parallel plate flow chamber so that adhered numbers could be measured without disturbing the surface. This has implications for methodologies where adhesion is measured by first removing the surface from the suspending medium and rinsing the surface. Gomez-Suarez *et al.* (2001) suggest that the results of such methods should be referred to as "bacterial retention" rather than "bacterial adhesion".

2.3.3. Removal Methods

2.3.3.1. Introduction

Microbial cell removal methods are those that enumerate or detect bacteria on surfaces by removing them from the surface so that they are in a planktonic state and can be easily enumerated by traditional means such as plate counts and microscopic counts. The inability to remove all the bacteria from the surface is the main problem with these techniques. With the procedures such as rinsing, swabbing, shaking with beads, vortexing and sonication not all bacteria are removed from the surface and may lead to an underestimate of the absolute bacterial numbers present. Another problem with removal methods that require subsequent culture of the bacteria, such as with plate counts, is that cells that are respiring may not be cultured, due to damage from the removal process (Flint *et al.*, 1997c). Also, clumps of cells may not be broken up into individual cells by removal methods. These problems will also add to the underestimation of numbers that is involved with these methods. The surface material may also affect the results obtained by these sampling methods as the strength of adhesion of bacteria to different surfaces varies. Therefore, a sampling method may give very different results with different materials even if the same numbers of bacteria are present (Wong & Cerf, 1995). Another problem encountered with these methods is that results obtained may not be reproducible as slight variations in technique can alter the result found.

2.3.3.2. Swabbing

Swabbing can be used on any surface that can be reached with a swab and that is larger than a few square centimetres. Swabs are taken by streaking a known area of sampled surface with a wad of cotton wool, piece of sponge or similar. The swabs are then transferred to a recovery medium and agitated to remove the swab contents to the medium (usually peptone-saline). Aliquots of the recovery medium can then be taken and used to obtain the bacterial count (Lee Wong and Cerf, 1995).

Swabbing has been widely used in studying surface adherent bacteria. Driessen *et al.* (1984) swabbed *Streptococcus thermophilus* present on stainless steel from an area of $10cm^2$ with a cotton plug after which bacteria rinsed from the plug with 10ml of sterile peptone water. Bouman *et al.* (1982) used a similar method to sample bacteria from a swabbing area of 30 cm^2 on the walls of plates of a heat exchanger. In Langeveld *et al.* (1995) swabbing was used to measure the number of bacteria adhering inside tubes of a heat exchanger. The tubes were swabbed back and forth with a squeegee and the swab fluid cooled and the bacteria present enumerated. Swabbing was also used in Flint *et al.* (1997b) to measure the number of *Streptococcus thermophilus* cells attached to 10mm diameter stainless steel coupons that had been placed inside a milk pipeline located after a plate heat exchanger.

Swabbing has a disadvantage when it is being used for sampling of biofilms because it can only remove around 10 % or less of the cells adhered to the surface and therefore gives an underestimate of the actual numbers of cells present (Holah *et al.*, 1988; Flint *et al.*, 1997b). The swab also retains some of the bacteria that are removed from the surface, which adds to the underestimation. The use of soluble alginate swabs may eliminate this last problem as they dissolve and release all organisms collected on the swab (Lee Wong and Cerf, 1995).

Moore and Griffith (2002a) investigated the factors that influence the recovery of bacteria from surfaces using swabbing. It was found that up to around 90 % of the bacteria present on the surface could be removed from the surface, but overall only around 10 % of the bacteria initially present on the surface could be recovered and enumerated. This large difference was due to poor release of removed cells from the

swab, which was the main factor that limited effectiveness. They found that swab properties that helped in removal of cells from the surface, such as porosity, also hindered the release of cells into a suspending fluid. The best results were obtained using a cotton swab moistened with 3 % Tween solution to sample a wet surface. However this still only recovered 10 % of the actual surface population. In further work (Moore and Griffith, 2002b) swabs of contaminated surfaces were tested with non-microbial methods such as ATP detection and a method developed for protein detection (Pro-tect®, Biotrace). These results were compared to traditional bacterial counts from swabs of the same contaminated surfaces. It was found that the non-microbial and microbial methods did not correlate well. More of the surfaces tested failed using ATP and protein detection than with microbial detection. Also many surfaces that passed based on microbial assessment failed when tested with ATP and protein detection. This may have indicated that the surfaces were microbiologically clean but not chemically clean or that the microbial analysis failed to detect the presence of bacteria. Based on these results Moore and Griffith (2002b) recommended that non-microbial methods should be incorporated into industrial hygiene monitoring programs.

2.3.4. Microscopy

Because removal methods usually provide under estimations of surface numbers and are not always reliable for enumeration (Flint *et al.*, 1997b) microscopic methods provide an additional technique for obtaining information on the numbers of adhered bacteria present on a surface. Also, microscopic methods can be used not only for enumeration of the bacteria present, but also are capable of providing structural and other information on adhered cells and biofilms. Several different types of microscopy have been applied to the study of biofilms, some being more useful than others.

2.3.4.1. Types of Microscopy

Fluorescence microscopy

Fluorescence microscopy has been used widely in studies of attached bacterial growth. The technique utilises fluorescent stains or auto-fluorescent samples that emit fluorescence when excited by UV light. The image is then formed from this emitted light of longer wavelength in the visible range. Filters are required so that excitation of the specimen occurs at optimal wavelengths and so the emitted light can be detected and

harmful UV light blocked. Different excitation wavelengths of light are possible depending on the fluorescent dye used (the fluorochromes) to stain the object and the optimal excitation wavelength for that stained object. Likewise the wavelength of the emitted light varies depending on the object that is fluorescing. Different filter combinations are used to optimise the detection of the emitted light.

Many different types of fluorochromes are available depending on the type of material to be stained and what is to be visualised. The two most common traditional stains are acridine orange and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Kepner and Pratt, 1994). Acridine orange binds to both DNA and RNA with an excitation maximum wavelength of approximately 470 nm. It fluoresces red when bound to single stranded nucleic acids and green when bound to double stranded nucleic acids. DAPI is a DNA specific stain and fluoresces blue or bluish white (at or above 390 nm) when bound to DNA and excited at a wavelength of 365 nm (Kepner and Pratt, 1994). The concentration and duration of staining varies depending on the material to be stained. A typical staining time for acridine orange and DAPI would be 5 to 10 minutes with a concentration ranging from 0.001 to 1000 $mg.l^{-1}$. DAPI tends to be used at a lower concentration than acridine orange but for a longer time (Kepner and Pratt, 1994). The cell counts obtained by the two different stains seem to vary in some situations. Often acridine orange counts are significantly higher than those obtained with DAPI (Kepner and Pratt, 1994). The reason why some acridine orange stained cells are not seen with DAPI is unknown and researchers should be careful when choosing DAPI as a stain.

Several fluorescent molecular probes have been the developed over the last decade (Lawrence J.R. *et al.*, 1996). These are more specific in their binding than traditional fluorochromes (above) and have sharper wavelength excitation peaks allowing a better quality image and better ability to combine stains for different materials in the same sample without their excitations interfering (Lawrence *et al.*, 1996). These probes have mainly been developed for use in confocal scanning laser microscopy (CLSM) (below) but can be used in fluorescence microscopy also.

Epifluoresence microscopy has in the past been the most commonly used type of fluorescence microscopy system for studying bacteria on surfaces (Lawrence *et al.*, 1996; Lee Wong and Cerf, 1995). In this system the incident light passes through an

objective lens which functions as the condenser and the objective. Like dark-field microscopy, epifluoresence microscopy allows visualisation of bacteria on opaque surfaces.

Flint *et al.* (1997b,c) used epifluoresence microscopy to examine *Streptococcus thermophilus* cells attached to 10mm diameter stainless steel coupons that had been placed inside a milk pipeline located after a plate heat exchanger. The surface of the coupons were stained with 0.001 % w/v acridine orange and observed.

Epifluoresence microscopy was used in Bredholt *et al.* (1999) to enumerate bacteria on stainless steel surfaces soiled with a dairy based soil that had been applied and dried on to the surface. The surfaces were contaminated with bacteria by incubation for 4 days in cultures of different bacteria under continuous shaking. The surfaces were firstly stained with 5-cyano-2,3-ditolyltetrazolium chloride (CTC) at a concentration of 5 mM for 2 hours. Formaldehyde at 5 % strength was then used to fix the CTC stain before staining with DAPI at a concentration of 1 $\mu g.ml^{-1}$ for 3 minutes. The total number of cells and the number of living cells were counted from 120 fields for each sample.

Epifluoresence microscopy has been found to underestimate cell numbers when used to enumerate cells adhered to surfaces in biofilms (Flint *et al.* 1997b). This is because the cells tend to aggregate in three-dimensional arrangements (at concentrations $>10^6$ $cells.cm^{-2}$) and therefore it is difficult to visualise all the cells in one dimension as viewed from the microscope. Also, cells may be hidden by material on the surface or may be in crevices in the stainless steel and therefore be hard to see. Low concentrations of cells on the surface ($<10^4$ $cells.cm^{-2}$) may also be difficult to count accurately using this technique (Flint *et al.*, 1997b). Fluorescence microscopy also has the limitation that the bacteria seen on the surface can be either dead or alive. An alternative method for detection of bacteria in a biofilm to avoid this is the use of fluorochromes such as 5-cyano-2, 3-ditoyl tetrazolium chloride which enable respiring bacteria to be discriminated from dead bacteria (Caldwell *et al.*, 1992).

Confocal laser scanning microscopy (CLSM)

The use of confocal laser scanning microscopy (CLSM) has provided a new understanding of biofilm structure as heterogeneous structures consisting of open pores

and channels surrounding dense clusters of cells and extracellular polysaccharide (EPS) (de Beer & Stoodley, 1995; de Beer *et al.*, 1994b; Lawrence *et al.*, 1995; Lewandowski *et al.*, 1995). CLSM visualises optical 2D cross sections of the biofilm, and by capturing these images at different depths a computerised 3D image can be created. Imaging of fully hydrated specimens is possible in combination with fluorescence techniques, which allows 3D microscopic studies of biofilms, which are retained in a relatively unaltered state. Using the fluorescent probes, different components of biofilms can be stained and an image created showing the positioning of these constituents relative to each other in three dimensions. In combination with micro-injection of fluorescent dyes, flow effects in biofilms can also be examined using CLSM (de Beer & Stoodley, 1995; de Beer *et al.*, 1994b; de Beer *et al.*, 1997; Stoodley *et al.*, 1997).

The CLSM microscope is a combination of a conventional microscope with a laser light source and computerised digital imaging. In conventional light microscopy all light from the specimen is imaged directly and simultaneously. This results in images that lack clarity and fail to accurately represent 3D objects, due to stray light from around the specimen that is in focus interfering with image formation. With CLSM confocal pinholes at the laser (incident light) source and at the emitted light detector eliminate this interference by not allowing light other than that coming from the point of focus to reach the detector. This creates an optically thin section of around 0.2µm depending on lenses and the size of the confocal pinhole. The laser scans continuously across sections specified by the user, point by point, and line by line. Points on the specimen are then excited by the laser light and emit light which is digitally collected by the detector over the scan interval to build up the 2D image of the specimen. By building up a sequential series of these digital 2D images while focusing through the specimen in the third dimension, a 3D image can be constructed (Caldwell *et al.*, 1992).

There are some disadvantages with using CLSM with microbiological specimens however. CLSM is still essentially a light microscope and therefore has only relatively low magnification as compared to electron microscopes. Also laser microscopes tend to be more restrictive in the number of filter combinations available as compared to those used in epifluoresence microscopy. Another concern is that the laser takes time to scan a sample. To obtain a better image more time is required. Therefore construction of a 3D image consisting of many 2D images can be quite slow. Repeated scanning of the

sample can lead to fading of samples. This occurs from the laser passing through parts of the specimen that are not being imaged, both above and below the focal plane, subjecting them to repeated photo-bleaching. Another problem that may occur is caused by objects in the specimen that are opaque to the laser beam. These objects can cause shadows to appear in the focal plane. Therefore, it is important that the beam be intense enough to penetrate the specimen at the maximum depth being imaged. Lastly, the combination of slow scan rate and the lack of an out of focus image can make very thin specimens difficult to find. If CLSM is combined with an epifluoresence microscopy this can help in finding specimens, as the epifluoresence microscope can be used to focus on the specimen of interest before switching to CLSM to visulise a 2D optical section or 3D image (Caldwell *et al.*, 1992).

Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) has been used widely in the study and enumeration of bacteria attached to surfaces (Austin & Bergeron, 1995; Eighmy *et al.*, 1983; Marshall, 1996; Massol-Deya *et al.*, 1995; Stone & Zottola, 1985; Surman *et al.*, 1996; Zoltai *et al.*, 1981). Put simply, SEM is where an electron beam is scanned across the specimen's surface with the electrons reflected from the surface being detected and used to form the image of the specimen. Specimens are viewed in a vacuum so they need to be dried. The specimen is also given a thin (about 10 nm) metal coating commonly of gold or palladium. This coating provides a better image by promoting electron reflection and providing a homogenous source of secondary electrons. SEM can provide a high level of magnification, with atomic resolution, but only the surface of the specimen is visualised. Therefore it is useful for enumerating bacteria on surfaces or at the surface of a biofilm. Different cell morphologies can be seen also, allowing some identification ability of what bacterial types are present. Samples can be prepared in approximately 40 minutes, so it has the potential to be quite a fast method (Pontefract, 1991).

A disadvantage of SEM is that the specimen preparation for SEM can alter the real appearance of cells attached to the surface due to the drying that takes place, so its value for studying biofilm structure is questionable (Little *et al.*, 1991). For example, the nature of the EPS material changes when samples are prepared for SEM. This has been shown by comparison with other microscopic techniques that view the samples in a

hydrated condition. When viewed with SEM, EPS material can be seen as a matrix of fibrils connecting cells and substratum. When viewed with techniques that use hydrated specimens, such as environmental SEM, no fibrils are present, rather the cells were surrounded in a gelatinous film (Little *et al.*, 1991). The cells could be seen more clearly with SEM however as they were not covered with the EPS film. Therefore as a method for quantification and enumeration of bacteria on surfaces, SEM can be quite useful.

Transmission Electron Microscopy (TEM)

In transmission electron microscopy (TEM) the electrons are passed through the specimen, with the electrons passing through being used to obtain the image. As with SEM, specimens are viewed under vacuum so samples need to be dried. TEM requires very thin specimens of 5-50 nm thick. To achieve this samples are embedded in resin or plastic so that they can be sectioned using a microtome. Staining of the specimen is also carried out to achieve contrast between the different specimen constituents (Caldwell *et al.*, 1992).

TEM has been used in numerous biofilm studies as a method for observing cross sections of biofilms (Eighmy *et al.*, 1983; Austin and Bergeron, 1995). A high level of magnification is possible and an indication of the different types of bacteria present, their spatial distribution, potential for interactions and physical separation by the polymeric matrix can be provided (Marshall, 1996). Three-dimensional visualisation of biofilms is possible with TEM by taking serial sections of an embedded biofilm and producing a 3D reconstruction from this. The process is quite slow however as the production of serial sections requires days for embedding and dehydration and may involve numerous attempts before a useable set of sections is obtained. Images from this must then be digitised to reconstruct the original object (Caldwell *et al.*, 1992).

As with SEM the specimen preparation may alter the real appearance of the biofilm, as the drying process tends to collapse previously hydrated samples. For example, observation of TEM sections did not reveal the void spaces between bacterial masses in biofilms that were detected by optical sectioning with CLSM (Caldwell *et al.*, 1992). Therefore due to this effect, biofilm structures observed with TEM, as with those from SEM, are not necessarily true indications of structural relationships.

2.3.5. Impedance Microbiology

Impedance microbiology has been used in biofilm and attached bacteria studies to enumerate bacteria present on surfaces. The basic concepts and how these relate to and can be used for surface enumeration of adhered bacteria are given below.

Impedance microbiology is based on chemical changes that occur in a medium when bacteria grow due to metabolic activity. The rate of these changes is related to the numbers of bacteria are present. The changes are detected by passing an alternating current through the medium via electrodes and determining the resulting impedance of the medium over time (Firstenberg-Eden & Eden, 1984). Impedance consists of two components, conductance and capacitance, and the medium under measurement can be thought of as a series combination of capacitance and resistance. As bacteria in the medium grow conductance and capacitance both normally increase, which causes the impedance to decrease (Firstenberg-Eden & Eden, 1984). Conductance increases due to the metabolic activity of the bacteria producing new end products in the medium. Generally, uncharged or weakly charged substrates are broken down into highly charged end products. Examples of this are proteins to amino acids and carbohydrates to organic acids. These products increase the conductance of the medium (Firstenberg-Eden & Eden, 1984). Capacitance also increases with bacterial growth within the medium. At the interface of the electrode and the medium solution a separation of charges occurs, creating an electrical double layer that exhibits capacitance. Microbiological growth which generates smaller ionic species can decrease the thickness of this layer and increase the effective surface area available by increasing the concentration of ions close to the electrode. This then increases the capacitance of the medium solution (Firstenberg-Eden & Eden, 1984).

The resulting decrease in impedance due to increasing conductance and capacitance can then be related to the numbers of bacteria initially present in the medium. This is normally achieved by measuring the time it takes for the impedance to start to decrease, or more conveniently the time for the reciprocal impedance (admittance) to start to increase. The point of measurement is called the impedance detection time and is the point where a detectable acceleration in the impedance curve can be detected.

Detection time is dependent on several factors. Physical factors such as temperature, media, electrode type and microbial characteristics such as metabolism, generation times and microbial population numbers all effect the detection time (Firstenberg-Eden & Eden, 1984). Therefore to obtain useful information several factors need to be controlled when measuring impedance so that detection time can be related to the initial numbers present in the medium.

To relate the detection time to the initial numbers present, the system used to detect impedance changes must be calibrated so that for a given detection time the corresponding initial number of bacteria is known. This is normally done by using plate counts, where samples of bacteria of different concentrations are enumerated by both methods and a calibration curve relating initial numbers present (from the plate counts) to detection time, for a particular bacteria and under certain conditions, can be constructed.

A typical impedance microbiology system consists of several sample containers or ports and pairs of electrodes in each port where different samples can be measured for impedance simultaneously. The temperature of the ports is controlled and the impedance is normally followed using a computer monitoring system which can record changes and display a curve or trace of the impedance over time. Different systems also can vary in the type of impedance that is measured. Some systems measure conductance only while others measure conductance, capacitance or total impedance. The measured impedance depends on the frequency of the applied current. At low frequencies impedance is predominately affected by capacitance while at high frequencies it is mostly affected by conductance (Firstenberg-Eden & Eden, 1984). Depending on the bacteria and the media used in measuring the impedance changes, monitoring of conductance, capacitance or total impedance may provide the best observation of detection time. A good observation of detection time can be made from an impedance trace which shows an initial stable base line from which a rapid acceleration occurs after a certain length of time (the impedance detection time) after which the curve stabilises at the minimum impedance achievable for those conditions. In several situations, following the conductance changes alone provides the best trace, while in others capacitance or total impedance provides the best trace. This is due to the different

metabolites that are produced by different bacteria and the impedance of the media being used (Firstenberg-Eden & Eden, 1984).

An important point to note regarding impedance microbiology is that it measures metabolic changes rather than production of biomass as in other enumeration techniques, such as plate counts. Therefore, factors such as temperature, time and bacterial activity become critical parameters in the assay.

It is the opinion of the author that when dealing with adhered bacteria in impedance microbiology, calibration can involve quite a considerable assumption. If the calibration curve is constructed using liquid bacteria samples and plate counts as described above then the curve is calibrated for planktonic cells. When a sample with a certain number of adhered bacteria is put under the same conditions as that faced by the same number of planktonic bacteria it is not known whether the same detection time will be detected. This is because the activity of the planktonic bacteria may not be the same as that of the attached bacteria. If this type of calibration is used, then the assumption is made that the activities are the same. Therefore, measurements of attached bacterial numbers are in reported as the equivalent number of planktonic cells required to exhibit the same activity that was observed from the attached cells. If it were possible, then calibration of detection time through another surface enumeration technique such as epifluoresence, SEM or CLSM would avoid this assumption, as bacteria in an attached condition would be used to calibrate the method.

Also, there is a limitation with impedance microbiology when using mixed culture samples. In a sample with more than one type of bacteria present, how each of these bacteria will affect the impedance detection time is difficult to predict. This is especially so if the proportions of each type or the types that are present are not known (Firstenberg-Eden & Eden, 1984). Therefore, the accuracy of enumeration of samples taken from systems in which more than one type of bacterium is questionable. In this case the result could only be expressed as equivalence to the numbers of bacteria of the calibrated strain required to produce the observed activity.

Another potential problem that the author has identified with using impedance microbiology to enumerate adhered bacteria is due to contact of the bacteria with the

suspending medium. Even if the detection time is calibrated against a surface enumeration technique, it is not known whether all cells will interact equally with the suspending medium. If layering of cells or other materials such as EPS or other organic matter are present, where diffusion limitations will start to have an effect, the interaction with the medium is unlikely to be equal between cells. Therefore for these reasons, enumeration of bacteria in thick or protected films using impedance may be difficult. In these situations the impedance method would provide an estimate of the bacterial activity predominantly at the surface and would be applicable in situations where the interest is in the interaction of the bacteria at the surface with the suspending fluid. In this case, the numbers at the surface rather than total numbers would be more useful in assessing the impact of the bacteria.

One commercially available impedance-based system reported in literature and used for enumerating surface associated bacteria is the Malthus Conductance Growth Analyser (Flint *et al.*, 1997b) which uses the change in the conductance component of the electrical impedance. Since it only measures the conductance part of the impedance signal, not all bacteria in every situation can be enumerated with this system. As mentioned above, the capacitance part of the signal may change but not the conductance part and no change in impedance will be detected, despite bacterial activity being present.

The Malthus Conductance Growth Analyser was used in conjunction with epifluoresence microscopy and swabbing with plate counts to enumerate the number of bacteria in biofilms on the surface of 10mm stainless steel coupons by placing the coupons in the tubes of a Malthus Conductance Growth Analyser (Flint *et al.*, 1997b). The number of bacteria found by the Malthus technique was 1-2 $\log_{10}$ higher than that found with the other two methods. Some reasons given for this discrepancy were that swabbing only removes about 10 % of the bacteria from the biofilm, and that with epifluoresence microscopy the cells exist in three-dimensional aggregates and therefore it is difficult to visualise all the cells in one dimension. Another reason for the discrepancy may be associated with calibrating the impedance method, which assumes that attached cells behave in the same way as planktonic cells. If the attached cells grow faster for instance, then the method would over estimate the number of cells present, as the impedance detection time would be reduced.

Flint and Brooks (2001) used impedance to detect *Bacillus stearothermophilus* both in suspension and attached to stainless steel surfaces. The impedance device used for their measurements was a BacTrac 4000 microorganism growth analyser. Impedance changes were monitored at 55 °C. Eight different media were investigated, seven of which produced changes in the electrode impedance (E-value) and all of which provided negligible changes in the impedance of the culture medium (M-value). They found that tryptic soy broth as the medium provided a reliable method to enumerate *B. stearothermophilus.*

Bredholt *et al.* (1999) used a BacTrac 4100 instrument to measure bacterial activity on soiled surfaces (12x55 mm^2) contaminated with either monocultures or mixed cultures of bacteria. Comparisons were made based on the times when each sample reached the same E-value. No effort was made to convert these impedance detection times into estimates of actual numbers of bacteria present.

Lee Wong and Cerf (1995) described an impedance apparatus for measuring the number of bacteria adhering to industrial surfaces. The apparatus consisted of a cylinder closed at one end, but fastened tightly at its open end to the surface under study. A culture medium was introduced through an opening in the closed end and impedance was measured between two electrodes plunged into the culture medium. The impedance variation was converted to the number of bacteria using calibration curves.

Mosteller and Bishop (1993) also used impedance microbiology to enumerate surface adhered bacteria. They used a Bactometer® microbial monitoring system to enumerate bacteria adhering to pieces of rubber and Teflon gaskets. Separate trials with three different kinds of bacteria, *P. fluorescens, Y. enterocolitica* and *L. monocytogenes* were carried out. All three bacteria adhered to the gaskets. The authors commented on the fact that the impedance technique had an advantage in that it could measure reversibly as well as irreversibly adhered cells.

Coppola *et al.* (1988) used impedance to measure the number of thermophiles in UHT low-acid foods. The samples were incubated in a Bactometer® at 55 °C. Contaminated

foods containing approximately 10^4 cfu.g^{-1} resulted in detections within 8 hours, while foods with 10^1 cfu.g^{-1} resulted in detections within 24 hours, emphasising the relative speed of the impedance method as opposed to other methods requiring culture of the bacteria present.

Therefore in summary, impedance microbiology is useful in enumerating surface adhered bacteria, as it is a fast and relatively easy way to analyse samples of surface associated bacteria. However, there are issues with the calibration as it assumes planktonic behaviour of attached cells and also, there are diffusion limitations for embedded adhered cells meaning that all cells do not exhibit equal activity. When using this method for enumeration of adhered cells, care must be taken to ensure that the result obtained is meaningful.

2.4. Incidence of Thermophiles in Dairy Processing Plants

Thermophile growth and product contamination in food processing plants can occur in several of the unit operations present, with some facilities having a greater potential to support thermophile contamination than others. The nature of the contamination from these areas needs to be understood so that it can be controlled. The occurrence of thermophiles in different unit operations is covered below with many of the examples given taken from the dairy industry. This is because much of the available literature on thermophiles in food processing is centred on the dairy industry. Also, dairy industry examples are more relevant to the problem of thermophiles in milk powder plants.

2.4.1. Unit Operations

2.4.1.1. Prior to Plant

The total cell count and composition of micro-flora in raw milk varies during its production in farms, transportation and reception at the dairy factory (Unger & Babella, 1982). The number of bacteria in raw milk reaching the factory is important as a high population in the raw milk makes the production of quality dairy products with low counts of bacteria more difficult. Bacterial counts of the order of 1×10^{6} cfu.ml^{-1} have been shown to compromise final product quality in a range of different products (Muir *et al.*, 1986). Several studies on the microbial quality of raw milk supplied to factories have been carried out. The incidence of thermophilic bacteria in raw milk has also been reported, (Crielly *et al.*, 1994; Griffiths *et al.*, 1988; Hull *et al.*, 1992; Mahari & Gashe, 1990; Muir *et al.*, 1986; Roy, 1994; Unger & Babella, 1982) and is covered in more detail below.

Unger and Babella (1982) compared the bacterial counts at the farm and after delivery to the dairy. On farm milk was cooled to 4-5 °C, cold stored, prepared for transportation and tested. The milk was transported to the dairy plant in two uninsulated 2500 litre tankers made of fibreglass reinforced polyester. The transporting time was less than two hours and the temperature rise was less than 1 °C. The on farm thermophilic bacteria count was 0.5×10^{3} cfu.ml^{-1} and after delivery it was 0.8×10^{3} cfu.ml^{-1}. The slight increase

found was not of concern as the increase in other bacterial types was a lot higher. For example psychrotrophic bacterial counts increased by 21 times. The authors noted that the variations in bacterial counts are mainly dependent upon the hygienic conditions of the surfaces coming into contact with the milk.

Muir *et al.* (1986) found little increase in the counts of thermophilic bacteria after storage at the factory at 6-10 °C for up to three days (from 9 $cfu.ml^{-1}$ after one day to 17$cfu.ml^{-1}$ after three days). As noted by Unger and Babella (1982) the increase in psychrotrophic bacterial counts was much higher and more significant. This result is consistent with those of Griffiths *et al.* (1988). Here the storage temperature of the raw milk was at 2 °C and the milk was also stored for three days. They found that thermophile counts increased minimally from 3.2×10^{4} $cfu.ml^{-1}$ to 5.1×10^{4} $cfu.ml^{-1}$ after three days, compared to psychrotroph numbers which increased 100 fold.

Mahari and Gashe (1990) found that the thermophilic bacteria population in raw milk samples obtained from around Addis Ababa in Ethiopia made up 0.5 % of the total bacterial population, with psychrophilic bacteria making up 98.1 %. This indicates that the growth of thermophilic bacteria in the milk before reaching the factory is of little consequence compared to that of the psychrophilic bacteria.

Hull *et al.* (1992) states that farm milk becomes contaminated with high numbers of microorganisms from the use of low quality silage and from dirty and/or poorly maintained milking equipment and practices. Thermophilic organisms do not multiply appreciably in raw milk even at ambient temperatures, and thus a high thermophilic count in raw milk up to 24 hours old is reliable evidence of gross contamination from milking equipment or other sources. Hull *et al.* (1992) also reports on the sources and some typical numbers of different strains of thermophilic and thermoduric bacteria found in raw milk. The *Bacillus* count in raw milk rarely exceeds 5,000 $cfu.ml^{-1}$ and is influenced by seasonal factors such as barn housing of cattle, soil contamination of teats and water supplies. Also, *Bacillus* spore counts range from 10^{2}-10^{5} per teat depending on the environmental conditions. Clostridia spores are derived from poor quality silage and gain access to raw milk via dung and soil contamination of teats and milking equipment. Both aerobic and anaerobic spores gain access to raw milk supplies mainly

via contaminated teats. Other thermoduric bacteria listed as being found in milk by Hull *et al.* (1992) are mostly micrococci and result from poorly cleaned milking equipment and bulk farm milk tanks. The use of hot cleaning systems on farms can select for thermoduric and thermophilic organisms and these can multiply on poorly cleaned or maintained equipment, particularly rubber components.

Therefore, thermophilic contamination of raw milk does not contribute large numbers of bacteria to the contamination process in dairy processing plants. It is thus more likely to have a seeding effect, providing a source of bacteria that can replicate further downstream if suitable process conditions are met and then contaminate the product stream in greater numbers.

The incidence of thermophiles in the raw materials used for canning of food products has also been reported (Denny, 1981; Speck, 1981; Ito, 1981). Sugars and starch used in canning processes can contain high numbers of thermophilic spores and are difficult to kill due to their heat resistance. Thus the raw materials can be a major source of contamination in canned products. However, the numbers of thermophilic spores in raw materials, such as sugar and starch, used for canning today are low due to better control of thermophiles in the raw material processing (Denny, 1981). Therefore as with dairy processing, thermophile contamination problems in canning processes are the result of thermophile growth and contamination during heat treatments provided while processing (Denny, 1981).

2.4.1.2. Pre-heaters / Heat Exchangers/ Pasteurisers/ Separators

Heat treatment of natural products such as milk can provide an opportunity for the growth and multiplication of thermophiles if the optimum growth temperature of thermophiles is used. In the dairy industry the growth of thermophiles in heat exchangers and pre-heaters is often encountered and can result in increased thermophile counts in heat-treated milk over long operation times. This reduces the time that processes can be operated for as the thermophile numbers increase above that specified for the product, and the equipment needs to be cleaned. Several studies relating to this phenomenon have been carried out focusing on either thermophilic (such as *Bacillus stearothermophilus*) or thermoresistant bacteria (such as *Streptococcus thermophilus)*

(Becker, 1996; Bouman *et al.*, 1982; Busscher *et al.*, 1996; Driessen & Bouman, 1981; Lane, 1989; Langeveld *et al.*, 1995; Rademacher *et al.*, 1996; Refstrup, 1998; Refstrup, 2000).

Bouman *et al.* (1982) investigated the growth of *S. thermophilus* in a plate heat exchanger over operating times of 18 hours. The deposition of milk constituents in the heat exchanger over this time was also studied. A *S. thermophilus* strain was inoculated into the milk run through the heat exchanger at a concentration of 10^3 cfu.ml^{-1}. The bacterial adhesion on the pasteurised milk section of the heat exchanger was much greater than that on the raw milk side. This was shown by both the greater number of bacteria adhering to the plates on the pasteurised side of the regenerative section of the heat exchanger and the greater number of bacteria found in the milk after the pasteurised section, than the raw milk side. On the raw milk side only a slight increase in the number of bacteria in the milk was found, while on the pasteurised section after 8 hours a constant level of 5×10^6 cfu.ml^{-1} was reached in the milk. No bacteria were found adhering to the plates of the heat exchanger on the raw milk side of the regenerative section after 6 hours. After 12 hours on the same side the maximum numbers of *S. thermophilus* found were 10^4 cfu.cm^{-2} and were found in the area between 30 and 40 °C. On the pasteurised side, bacterial adhesion to the plates occurred more quickly than on the raw milk side. After 2 hours, adhesion was clearly observed and increased after 6 hours. After 12 hours the number of bacteria on the wall was about 10^6 cfu.cm^{-2}, on plates between 25 and 40 °C. The coverage of bacteria on the plates was also greater on the pasteurised side as about 2 % of the wall was covered compared to only 1.6×10^{-2} % on the raw side. Another observation that was noticed with bacterial adhesion was that the results were similar when using preheated skim milk to those obtained using raw milk. It was therefore concluded that the preheating treatment used or the fat content of the milk was unlikely to play a role in bacterial adhesion. An interesting observation can be made regarding the interaction of the contaminating bacteria and milk fouling deposits, as mentioned above in reference to conditioning films. This is that nearly all deposition of milk constituents was found on the raw milk side of the pasteuriser with hardly any deposition on the regenerative side, while most of the bacteria numbers at the surface were found on the regenerative section of the pasteuriser. This shows that the presence of milk fouling in association with

thermophilic bacteria is not always required to cause large levels of thermophilic contamination from pasteurisers.

Bacterial growth within a heat exchanger used for pasteurisation of milk was also studied in Driessen and Bouman (1981) where a model pasteuriser was used. Samples were taken periodically at five sampling points, and tested for total bacteria and heat-resistant streptococci. Growth of bacteria was detected after the pasteuriser had been in operation for greater than 13 hours and occurred in both the heating and cooling sections, but to a much greater extent in the cooling section, which agrees with the result found in Bouman *et al.* (1982) cited above. It was also found that both *Streptococcus lactis* and *S. thermophilus* grew in the heating section but only *S. thermophilus* in the cooling section. After 16 hours, bacterial counts on the pasteuriser walls reached greater than 10^6 cfu.cm^{-2} in the cooling section and about 10^4 cfu.cm^{-2} at less than 49 °C in the heating section. This also agrees with the results of Bouman *et al.* (1982) cited above. Also, the higher the initial count of *S. thermophilus* in the raw milk, the shorter was the operating time of the pasteuriser before the count in the pasteurised milk became too high and cleaning became necessary.

Lane (1988) found that milk separators were a key point where thermophile contamination could occur. By reducing the separation temperature from 55 °C to 40 °C, and increasing the cleaning frequency of separators low thermophilic spore counts for dried skim milk were obtained.

Becker (1996) investigated the growth of bacteria in cheese milk pasteurisers over operating times of up to 21 hours. The total bacterial numbers in the pasteurised milk increased slightly over the initial 8-9 hours of operation, then more rapidly over the remaining period of operation. Bacterial counts in excess of 10^6 cfu.ml^{-1} were reached in the pasteurised milk, far out numbering counts in the raw milk. This degree of bacterial contamination is consistent with that found by others cited above (Bouman *et al.*, 1982; Driessen and Bouman, 1981). The increase in the bacterial count in the pasteurised milk was not observed in the holding tube but was seen in samples from the cooling side of the regenerative section. This suggests that bacterial growth occurred on the plate walls of the regenerative section, which seeded the pasteurised milk. This also

agrees with what was found by Bouman *et al.* (1982) and Driessen and Bouman (1981) cited above. It was also found that the bacterial numbers in the pasteurised milk were influenced by raw milk bacterial quality. A lower total bacterial load in the raw milk entering the pasteuriser was shown to result in a lower total bacteria count in pasteurised milk over time, although some increase in numbers after 10-16 hours operation still generally occurred. Again, this agrees with what was found in other studies (Bouman *et al.*, 1982; Driessen and Bouman, 1981). In this study the use of a "mini-wash" procedure was used to try to control bacterial numbers during long runs. The mini-wash lasted for 20 minutes and was used after 10 hours of continuous operation. The mini-wash reduced the numbers of thermophilic and total bacteria in pasteurised milk and stopped the increase in bacteria at the point of the mini-wash. The numbers increased again however about 6 to 8 hours after the wash.

Rademacher *et al.* (1996) stated that growth of thermophilic micro-organisms preferentially occurs in the temperature range of 45-60 °C in the regeneration section of heat exchangers and contaminates the already pasteurised product. This then results in colony counts 10-100 times higher than counts of incoming milk over long operation times. Rademacher *et al.* (1996) also studied the effect of different parameters affecting increasing bacterial numbers in pasteurised milk. Heat exchangers pasteurising milk where operated for 10 hours, so in line with the results above, in most cases they only observed slight increases in bacteria numbers in the pasteurised milk leaving the heat exchanger over time. An operation time of 15 to 20 hours would have probably produced a greater increase in thermophilic bacterial counts. From the slight increases that were observed, it was concluded that parameters of regeneration efficiency, flow velocity in the gaps, and heating temperature may amplify the effect of increasing bacterial numbers in the milk but are not the initial cause of the problem. The main factors affecting increasing bacterial numbers in the milk were said to be the number of thermophiles in the milk prior to pasteurisation, as a greater number of thermophiles in the milk to be pasteurised results in a faster increase, and also whether or not the milk had been pre-pasteurised before pasteurisation, as this also results in a larger increase.

Langeveld *et al.* (1995) studied the adherence of five different strains of bacteria to the internal surface of a heat exchanger. The strains were a gram negative strain (growth range < 5-41 °C), a coliform type (< 5-44 °C), a *Streptococcus* strain (20-50 °C), an

aerobic spore former (*Bacillus stearothermophilus*, 40-68 °C) and a *Thermus thermophilus* strain (40-78 °C). Milk containing the bacterial strains under investigation was passed through a tubular heat exchanger for an operating time of 20 hours, in which the milk was heated to 80 °C, passing through the heat exchanger only once. During the experiment all five strains of bacteria adhered to the internal surface of the heat exchanger, the site being dependent on the wall temperature. The relationship between the density of bacteria on the surface and the concentration of bacteria in the product after passing that surface was also investigated. A model was derived to predict the number of bacteria in the bulk liquid from the density of bacteria on the wall. This model is based on the assumption that most newly grown cells are released into the product. The model predicted the number of bacteria in the bulk liquid reasonably well for those areas where there was little fouling of the tubes by the milk. In areas where there was fouling in the tubes the model over estimated the number of bacteria in the milk by over 10 fold in some cases. An explanation for this could be that many of the bacteria that grow on the surface when fouling is present are embedded in the fouling layer and newly grown cells cannot release into the pasteurised milk easily, therefore the model gives an over estimate.

Busscher *et al.* (1996) mentioned the use of biosurfactants as a retardant to colonisation of bacteria. It was stated that if these biosurfactants could be absorbed to heat exchanger plates in pasteurisers and in doing so inhibit the attachment of bacteria, the compounds would have major economic implications in the dairy industry, as longer run times and reduced cleaning frequency could be achieved.

Another investigation looked at the effectiveness of using direct steam injection to pasteurise or heat milk prior to evaporation (Refstrup, 1998). By using direct steam injection it was hoped that the growth of thermophilic organisms could be minimised as less area at the optimum temperature for thermophiles would be available for attachment. This could then enable operating times of at least 20 hours to be maintained without contamination from thermophilic organisms in the pasteurised or evaporated product. A different method covered in this study for controlling thermophile growth during pasteurisation or heating is to have two separate lines, where after a specified time (about 10 hours) the milk flow is changed over to the clean line, followed by CIP

of the other. This requires a sophisticated control system to avoid changes in the temperature. Both heating systems have been shown to result in significantly lower thermophilic counts even after 20 hours operation (Refstrup, 1998).

From these studies of increased bacterial contamination provided from pasteurisers, common findings can be identified.

- The growth of, and contamination from the thermophilic bacteria occurs mostly in the regeneration (or cooling) section of pasteurisers where the temperature range is normally 45-60 °C.
- The increase in contamination appears to occur more quickly if higher numbers of bacteria are present in the milk initially.

2.4.1.3. Evaporation

The evaporation of milk and whey involves long operation times and conditions in the equipment are suitable for the growth of thermophilic organisms. The large internal surface area provides good attachment and growth opportunities, and their concentration in the product may thus increase substantially (Langeveld *et al.*, 1990; Murphy *et al.*, 1999; Refstrup, 1998).

In Langeveld (1990) the bacterial growth in a four stage evaporator was investigated over an operating time of 34 hours. Observed concentrations in the product as a result of growth at the wall were as high as 10^6 cfu.ml^{-1} for gram negative and coliform bacteria and up to 3×10^7 cfu.ml^{-1} for streptococci and a non-spore forming thermophilic bacteria after 10 to 20 hours operating time. The gram negative bacteria, coliform bacteria and Streptococci were located in the greatest numbers after the preheater, which heated the milk to 45 °C. The non-spore forming thermophilic bacteria appeared in greatest numbers after the pasteuriser (10 seconds at 75 °C) and after the evaporator stages (70, 66, 60, and 45 °C). Numbers of thermophilic spore forming bacteria were lower than predicted as after 20 hours operating time they could hardly be detected or not at all, and the highest number detected was only of the order of 10^4 cfu.ml^{-1} after 30 hours in the last two stages of the evaporator. Langeveld (1990) states that it is known that these

bacteria may give rise to the formation of lactic acid at the end of a production run, but to do this, the bacteria must be present in the order of magnitude of 10^6 cfu.ml^{-1} in certain parts of the evaporator. The theory proposed was that spores of thermophilic sporeformers, such as *Bacillus calidolactis*, survive cleaning in residual product, in places that cannot be cleaned well or in insufficiently cleaned evaporators. During the run the bacteria can grow, increasing in numbers of vegetative cells and spores. If the surfaces on which the bacteria are growing are large enough, the lactic acid concentration may also increase after a certain production time, but the numbers of bacteria in the bulk liquid may not be excessively high (Langeveld, 1990).

Murphy *et al.* (1999) studied the survival and growth of *Bacillus stearothermophilus* and *Bacillus licheniformis* in a three effect evaporator during low heat skim milk powder manufacture. Substantial growth was shown to occur in the preheating stages prior to direct steam heating. A typical heat treatment (77 °C, 15 seconds) used in the manufacture of low heat powder did not inactivate the bacteria, which continued to grow in the heater. The importance of pre-heaters in influencing thermophile growth in the evaporator was demonstrated by the finding that the growth in the preheater stages was accompanied by growth in subsequent evaporator effects which significantly exceeded that observed when the final two preheaters were bypassed. The final evaporator effects in particular provided minimal to no additional thermophile contamination even though the temperature was favourable for thermophilic growth. This is thought to occur due to the growth being inhibited as the water activity is reduced through the evaporator. Also, a mid-run mini-clean procedure, incorporating 0.2 % hydrogen peroxide for decontaminating the evaporator was tested and proved useful in extending evaporator run times.

In contrast to the work on increasing numbers of bacteria in evaporators Reddy *et al.* (1975) studied the effect of vacuum evaporation on the destruction of thermophilic bacteria. They found that a decline in the numbers of *Bacillus stearothermophilus* and *Thermoactinomyces thalpophilus* over time occurred under vacuum evaporation. The run was only carried out for 5 hours though, so no contamination from colonisation of surfaces would have taken place and therefore no increase in numbers should have been observed.

2.4.1.4. Membranes

Thermophiles have also been shown to grow on membranes during hot membrane processes (Hull *et al.*, 1992; Lehmann, 1992b; Lehmann *et al.*, 1990). Two such processes are the hot ultrafiltration of milk in making cheddar cheese and the hot ultrafiltration of whey in concentrating whey proteins at 50-55 °C. Research has shown that milk concentrated 4-5 fold by ultrafiltration can have bacteria numbers increase by up to 10 fold during the ultrafiltration process (Lehmann *et al.*, 1990). This indicates that bacterial growth can occur in the ultrafiltration stage.

2.4.1.5. Drying

There is no literature available showing that thermophile counts increase during the powder drying process, apart from the increase per mass of product that occurs due to concentration effects as water is removed. However, counts of bacteria in the powder may decrease both during spray drying and storage.

Thompson *et al.* (1978) studied the survival of three selected bacterial strains including *Bacillus subtilis,* during spray drying and storage of milk powder and found reductions in numbers during both spray drying and storage. After spray drying, only 12 % to 22 % of *B. subtilis* initially in the milk concentrate survived the drying process and less than 0.5 % of less heat resistant bacteria such as *E. coli* survived drying. During storage of the powder for up to 36 weeks at 25 °C, *B. subtilis* numbers dropped steadily till after 36 weeks 32-40 % had died off. Over the same storage period 99.8-99.99 % of *E. coli* died off. Arun *et al.* (1978) also reported a decline in bacterial numbers in milk powder over time. They measured facultative and obligate thermophiles including *Bacillus coagulans*, *B. subtilis* and *B. stearothermophilus* and found that after storage for six months the thermophile counts were reduced by 32-67 % depending on the sample.

The use of methods to pasteurise powders with high counts of bacteria has also been studied. In Queguiner *et al.* (1989) a method for the pasteurisation of thermosensitive whey protein powder was studied. The whey protein powder was inoculated with 5×10^5 cfu.g^{-1} of *S. thermophilus* and continuously extruded in twin screw extruder to a moisture content of 4-5 % w/w. Reduction values of *S. thermophilus* of up to 10^5 fold

where obtained using barrel temperatures of 133-143 °C without any modification of protein solubility or gelling properties. This process could be of use in pasteurising powder products containing high levels of bacteria. Thermophilic spores may not be reduced to a great extent by this method however due to their high heat resistance.

2.4.1.6. Storage tanks

Another area in food processing operations that has been reported to cause growth and contamination of thermophiles is in tanks where warm or hot liquid is involved, such as in continuous cheese making (Hull *et al.*, 1992; Lehmann, 1992b; Lehmann *et al.*, 1990).

In continuous cheese making it has been shown that thermophile growth can occur in the retentate storage buffer tank (50 °C) and in the warm (30 °C) raw milk balance tank (Lehmann *et al.*, 1990). The retentate storage buffer tank is used to hold retentate (concentrated milk) exiting the ultrafiltration unit at around 50 °C prior to being moved on for starter and rennet inoculation at commencement of cheese making. Research has shown thermophilic bacteria can build up in this tank, particularly in the surface foam layer, which may be at a temperature slightly lower than 50 °C, therefore encouraging bacterial multiplication (Lehmann *et al.*, 1990). The warm raw milk balance tank holds milk at around 30 °C before being pasteurised. Milk at this temperature in the tank facilitates multiplication of thermophilic bacteria prior to the pasteurisation process (Lehmann, 1992b; Lehmann *et al.*, 1990).

2.4.1.7. Overall Plant

If the increase of thermophiles is viewed from an overall perspective in food plants, the contribution of each individual unit operation to thermophile counts in the final product can be seen. Literature on dairy product production processes where thermophile growth has been studied over the entire process exists. These processes are milk powder production (Griffiths *et al.*, 1988; Kwee *et al.*, 1986; Lane, 1989; Muir *et al.*, 1986), cheese making (Hull *et al.*, 1992; Lehmann, 1992b; Lehmann *et al.*, 1992a; Lehmann *et al.*, 1990) and liquid milk production (Mahari & Gashe, 1990).

In milk powder manufacture the level of thermophiles has been measured over the process. In Kwee *et al.* (1986) the number of thermophiles was measured before and after preheating, after concentration and drying. It was found that thermophile counts were reduced to negligible levels during preheating. Other steps did not show significant changes and the average number of thermophiles per gram was lower than in the raw milk. The experiment was only run for a few hours however so only little thermophile contamination from growth on surfaces would be expected in the milk and this was the case. This result was also found by Griffiths *et al.* (1988), who found that there were no substantial changes thermophilic counts during storage or processing during manufacture of skim milk powder with short operation times. The relationship between the bacterial quality of the raw milk used to make powders and the quality of the powder produced has also been studied (Griffiths *et al.*, 1988; Muir *et al.*, 1986). Both authors found that the bacterial quality of the raw milk does not significantly affect the quality of the powder produced. The experiments were again only run for a few hours however, which means no growth and recontamination by thermophiles on surfaces would be occurring.

In the cheese making process the growth of thermophilic bacteria has been studied (Lehmann, 1992b). Thermophilic bacteria numbers increased throughout the process during continuous production runs of cheddar cheese of 16-22 hours duration, reaching counts in the order of 10^6 cfu.g^{-1}. Counts of this order of magnitude have also been reported by others (Driessen *et al.*, 1984; Hup & Stadhouders, 1979; Lehmann *et al.*, 1990). The areas where thermophilic bacteria multiply were on the walls of the pasteuriser, in the ultrafiltration plant, the retentate storage buffer tank and in the pre-pasteuriser raw milk balance tanks (Lehmann, 1992). To minimise the opportunity for build up of thermophilic bacteria in the process it has been suggested to provide effective cleaning of all unit processes, by reducing foaming, and by cleaning holding tanks at frequent intervals of 3-4 hours, and giving pasteurisers a mini-wash after 8-10 hours continuous operation (Lehmann, 1992; Lehmann *et al.* 1992). It has been mentioned in several articles that high thermophilic counts in cheese cause adverse affects on the cheese quality (Hull *et al.*, 1992; Lehmann, 1992b). Effects such as late and early blowing, soft body defects and off flavours have all been reported so control of thermophiles is cheese production is important to ensure product quality.

Mahari *et al.* (1990) studied the sources of bacterial contamination in a liquid milk production process. Thermophilic bacteria survived the pasteurisation process and accounted for around 7.5 % of the total count in the pasteurised milk. It was also found that thermophilic bacteria that were isolated from the utensils holding the milk and from the plastic sheets used for bagging the pasteurised milk were contaminating the milk.

Karpinsky and Bradley (1988) investigated the cleanability of air-actuated butterfly valves in a pilot plant processing environment where contamination with *Bacillus stearothermophilus* spores occurred. The cleanability evaluation was carried out over a period of use designed to simulate 3, 6 and 12 months of use in an industrial manufacturing situation. It was found that the valves performed poorly in terms of cleanability and wear over all of the simulated time periods. All of the valves failed to be totally cleanable without maintenance within 12 months of simulated operation. Based on this finding the recommendation was made that butterfly valves should be installed in locations where they can be easily removed and that they should be completely disassembled daily for cleaning.

Lane (1988) studied the control of microorganisms in evaporation and spray drying processes. Thermophiles and mesophilic and thermophilic spores in dried milk from an industrial milk powder factory were monitored. Control of contamination was best achieved by avoiding holding the product at elevated temperatures (40-70 °C) and using an efficient cleaning system. Low mesophilic and thermophilic spore counts for dried skim milk were obtained by reducing the separation temperature from 55-40 °C and increasing the frequency of cleaning separators and evaporators. Thermophilic counts of dairy milk products were reduced by using a continuous fat remelt system for reprocessing recovered milk fat; vacreating the remelted fat without delay; and improving the cleaning-in-place (CIP) regime in the butter factory.

2.5. Milk Powder Manufacture

2.5.1. Milk Powder manufacturing process

A typical milk powder manufacturing process is represented and described below in Figure 2.5.1.

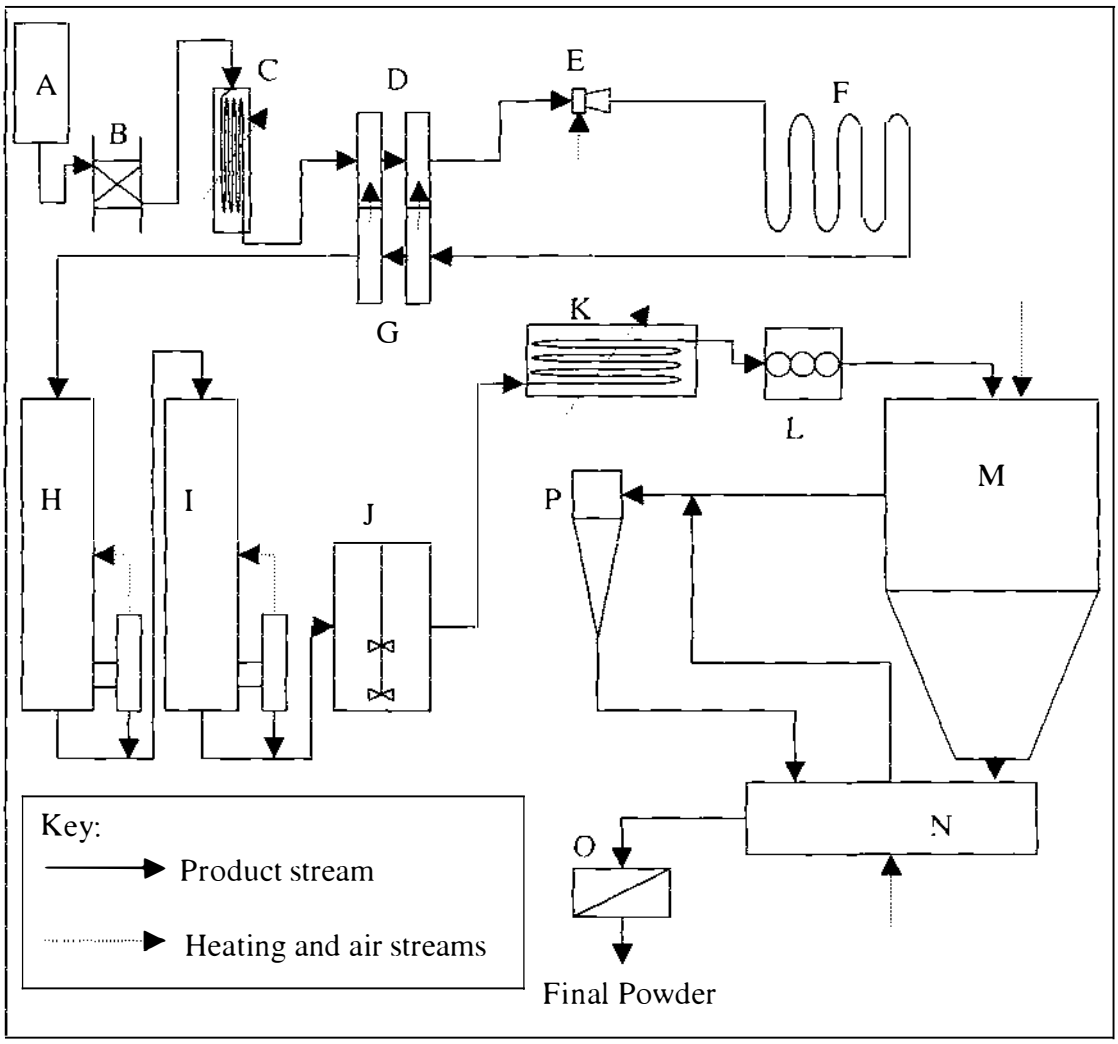


Figure 2.5.1a : Diagram of a typical milk powder manufacturing process.

Item	Description
A	Feed balance tank (milk temperature 10-14 °C). Prior to this milk may be separated, standardised and possibly thermalised or pasteurised which would involve exposure to heat treatments of 45-75 °C.
B	Plate heat exchanger used to heat milk to 50-55 °C.
C	Heat exchanger used to heat milk to 60-65 °C.
D	Direct contact heaters utilising heat from vapour removed from milk in flash vessels below to heat the milk up to 75-80 °C.
E	Direct steam injector using live steam to heat the milk to the desired temperature for the product being manufactured (e.g. typically a range of 90-100 °C for WMP).
F	Holding tubes used to maintain the temperature achieved for a set period of time depending on the product (e.g. typical range for WMP is 10 to 60 seconds).
G	Flash vessels used to quickly reduce the milk temperature by flashing the milk down to a lower pressure and hence temperature (around 75-80 °C).
H	First evaporation stage (effect) used concentrate the milk to around 35-40 % solids w/w) at a typical operating temperature of 60-70 °C.
I	Final evaporation stage (effect) producing concentrated milk at around 50 % solids w/w at a typical operating temperature of 50-60 °C.
J	Concentrated milk balance tank (45-55 °C). Gently agitated.
K	Heat exchanger used for heating the concentrated milk to 60 – 80 °C depending on the product.
L	Homogeniser used to homogenate the concentrated milk before spray drying.
M	Spray dryer used to dry the concentrated milk by atomising into small droplets and contacting with hot air at around 200 ± 20 °C.
N	Secondary dryer (vibrating fluidised bed) used to dry the powder produced in the spray dryer to the final desired moisture content of around 3 % w/w by contact with hot air at around 70-100 °C.
O	Powder sifter used to remove any large undesirable matter.
P	Cyclone used to separate any fine powder blown out of the spray dryer and secondary dryer and return it to the main product stream.

Figure 2.5.1b : Description of a typical milk powder manufacturing process.

2.5.2. Fouling and thermophiles in Milk Powder Manufacture

In the milk powder manufacturing process there are several locations where fouling deposits can form. Some of these locations overlap with regions where thermophile growth can occur. As mentioned earlier, fouling deposits are suspected of playing a pivotal role in the thermophile contamination. It is generally accepted in the dairy

industry that fouling is linked to thermophile contamination, but no specific study has looked at the interaction of fouling deposits with thermophile contamination and the precise nature of the interaction has been unknown.

In literature it has been found that fouling of milk constituents can develop at temperatures of 50-60 °C (Hegg *et al.*, 1985; Deplace *et al.*, 1994). In the New Zealand milk powder manufacture industry it is common knowledge that fouling is regularly observed in heat exchangers operating at temperatures as low as 50-55 °C. Therefore in the milk powder manufacture process fouling could occur from the early preheat stages throughout the evaporator and in concentrate heaters. Thermophilic Bacilli, such as *Bacillus stearothermophilus*, are capable of growing in temperatures of 50-65 °C (Flint *et al.*, 2001).

As mentioned above, Murphy *et al.* (1999) found that the majority of thermophile contamination occurs in the pre-heat sections of the milk powder plant. The final evaporator effects in particular provide minimal to no additional thermophile contamination even though the temperature is in the thermophilic growth region of 50-65 °C. This is thought to occur due to the growth being inhibited as the water activity is reduced through the evaporator.

Therefore there are several locations throughout the milk powder manufacturing process where both thermophiles and fouling deposits exist and hence interactions between the two could occur. In some of these regions thermophile growth is occurring while in others thermophiles may be present but not growing. Both of these regions are important however, as in both cases fouling deposits have the potential to become contaminated with thermophiles. Table 2.5.1 summarises the locations where thermophile and fouling interactions could occur.

Table 2.5.1. Summary of locations in milk powder manufacturing where fouling and thermophile interactions could occur, + indicates potential presence, - indicates likely absence.

Fouling and thermophile interaction summary							
	Separation/ Thermalisation / Pasteurisation (45 -75 °C)	Early preheat (50 – 80 °C)	High preheat (75 – 100 °C)	Early evaporation stages	Late evaporation stages	Concentrate storage (45 – 55 °C)	Concentrate heating (60-80 °C)
Thermophile growth	+	+	-	+	-/+ *	-/+ *	-/+ *
Thermophile presence	+	+	+	+	+	+	+
Fouling presence	+	+	+	+	+	-	+
Fouling and thermophile interactions possible	+	+	+	+	+	-	+

* Growth potential dependant on concentration and associated water activity of concentrated product.

2.6. Literature Summary

2.6.1. Summary of Literature

Literature has been discussed above covering issues of thermophiles in food products, bacterial relationships with surfaces, methods of study of attached bacteria, and the incidence of thermophiles in food processing plants.

Overall the literature shows that bacterial attachment to food processing equipment can occur and cause contamination problems in food products. This includes thermophilic bacterial contamination in milk powder processing with bacterial strains like those found in the New Zealand dairy industry such as *B. stearothermophilus.*

However, no literature details the role that fouling deposits play in the thermophilic contamination issue. As mentioned previously, thermophile problems have been traditionally linked to fouling problems in the New Zealand dairy industry. Langeveld (1990) touches on the issue by hypothesising that residual product left behind in evaporators after cleaning may provide contamination points for future manufacturing runs. However, further investigation is needed to define the role that fouling plays in thermophile contamination.

3. The Methods and Materials

This chapter covers the methods and materials used throughout the work. It is broken into sections to cover the different areas of work.

3.1. Pilot Plant (design, construction, description)

A milk pilot plant was designed and constructed to enable experimental runs to be conducted on a system that simulated industrial conditions. In the first stage of construction, this pilot plant was essentially equivalent to the pre-heat section of a milk powder plant where the milk is heat treated before entering the first evaporation effect. In the second stage, a small three effect evaporator was installed down stream of the pre-heat section. A spray drier is to be added on-line in the third stage. All milk contact surfaces in the pilot plant were constructed with 304 grade stainless steel with a number 4 finish. A photograph is shown in Figure 3.1.1 and a schematic drawing is shown in Figure 3.1.2. A detailed piping and instrumentation diagram is given in Appendix A, page A-1.

A team of six postgraduate students designed and built this plant, which was awarded the Food and Biosciences Supreme Award of Engineering Excellence of the New Zealand Institution of Professional Engineers for 2001. Each student was responsible for the design and construction of a particular section of the pilot plant. The pre-heating section received the most emphasis for the work reported here and is discussed in detail below. A more complete description of the other sections of the pilot plant is provided in Bennett (2000) and Croy (2000).

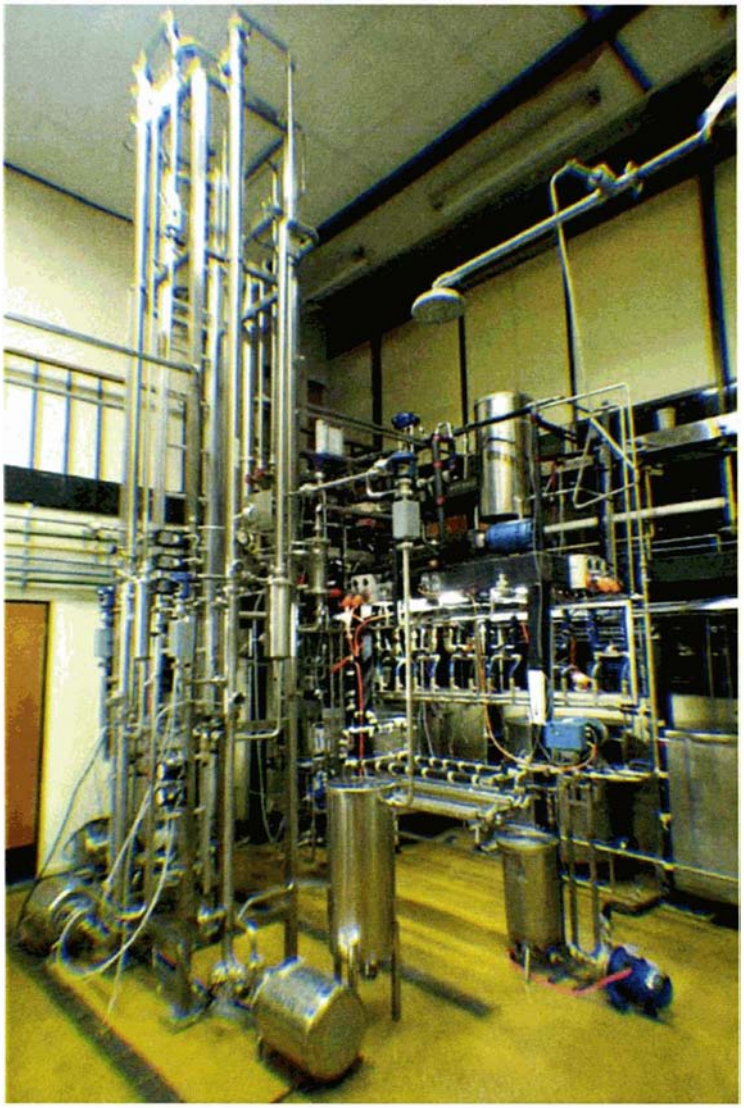
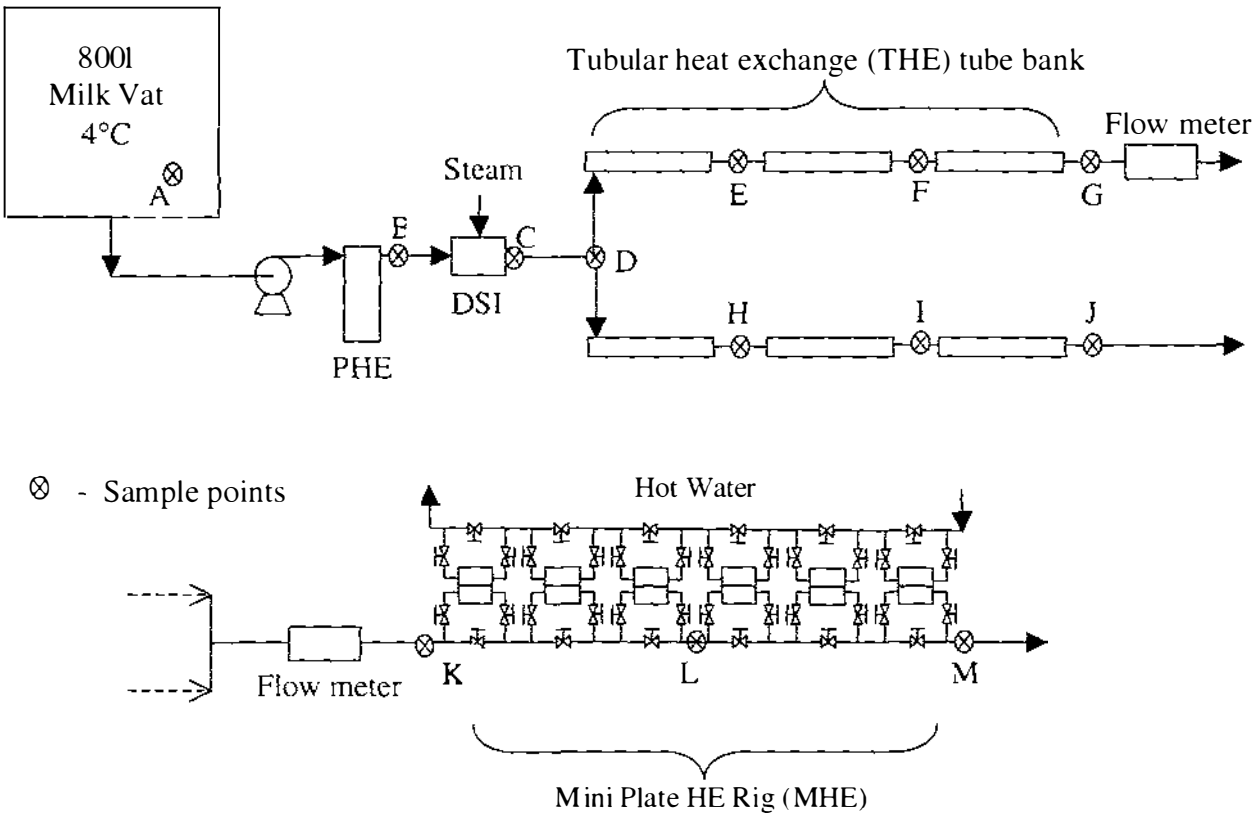


Figure 3.1.1. Photograph of the milk pilot plant showing preheating (on the right side of the photo) and evaporator (on the left side of the photo) sections.



Location of sample ports:

A – Milk vat

B – After PHE

C – After DSI

D – HE tube inlet

E – First pre-fouled HE tube

F – Second pre-fouled HE tube

G –Third pre-fouled HE tube

H – First Un-fouled HE tube

I – Second Un-fouled HE tube

J – Third Un-fouled HE tube

K – Inlet mini HE rig

L – Mid pont mini HE rig

M – Outlet mini HE rig

Figure 3.1.2. Diagram of typical the set up of the milk pilot plant preheating section showing locations of sampling points.

3.1.1. Pre-heat section (overview)

The pre-heat section consisted of several elements connected in a modular fashion to make the pilot plant as versatile as possible. Many different pre-heating scenarios were conducted in different experiments, both in this research project and others.

The typical layout of the pilot plant preheating section is shown in Figure 3.1.2 above. Milk previously standardised (3.3 % fat) and pasteurised was supplied to the pilot plant

from a local dairy factory (Fonterra, Longburn). The milk could be pumped with a centrifugal pump (Ebara, CDX70/05, 0.37kW, Keith R. Norling Ltd., Palmerston North, N.Z.) from the refrigerated 800 litre milk vat at a constant flow rate as low as 30 litres per hour. A small commercial plate heat exchanger (U265R, APV, Denmark) was then used to increase the temperature of the milk from the 4 °C vat storage temperature up to 30-70 °C depending on the experiment conducted. Direct steam injection (described below) could then be used to increase the temperature stepwise by up to 20-25 °C with a milk flow of 30 litres per hour.

The use of the plate heat exchanger (PHE) and direct steam injector (DSI) to heat the milk in stages was set up mainly to allow thermophile growth to be targeted in the area downstream of the DSI. This was achieved by maintaining the PHE temperature at 30-40 °C, which is below the typical thermophilic bacterial growth temperature range. Temperatures in the growth range of 50-65 °C are then only reached after the DSI. Also, during fouling experiments, the DSI enabled hot side temperatures in the PHE to be low enough so that minimal fouling occurred in the PHE. Thus fouling development was also targeted downstream of the DSI.

The next two elements of the pilot plant could be set up in any order. Either of the tubular heat exchange (THE) tube bank or the miniature plate heat exchanger (MHE) rig could come first. Figure 3.1.2 depicts the set up with the THE tube bank first. To maintain even flow through both sides of the THE tube bank, two paddle flow meters (Flow Sensor Dual Range, 256-225, RS Components Ltd., Auckland, N.Z.) were used to monitor the flow and adjustments were made on either side of the tube bank with hand valves. The MHE rig was designed so that each individual heat exchange unit could be separately isolated from the main flow. More detailed drawings of the process equipment can be found in the appendix.

3.1.2. Direct steam injection (DSI)

3.1.2.1. DSI description

Direct steam injection provides a near instantaneous heating of the milk stream by mixing steam directly into the milk flow thus providing little opportunity for thermophile contamination of the milk at this stage. This is because only a small amount of surface area is available in the DSI for thermophile colonisation as compared to the large surface area in heat exchangers. Thus the design targeted thermophile growth down stream of the DSI.

The DSI units were designed so that only one was required to heat the milk flow, however two DSI units were installed in parallel to allow continuous running in case one failed from fouling build up mid run. In practice, each unit lasted for approximately 10 hours before fouling build up prevented the unit from heating the milk effectively.

3.1.2.2. DSI design

The design of the unit was a simplified version of a design developed by the Fonterra Research Centre for a much larger pilot plant. Each unit consisted of a Teflon insert surrounded with a stainless steel outer tube. The insert was shaped so that the steam is injected as milk passes through a venturi. The steam fills an outer chamber surrounding the venturi and is injected into the milk via small holes through the Teflon. This is shown in Figures 3.1.3 and 3.1.4.

The design of the DSI unit required that the right amount of steam be injected into the milk stream to obtain the right temperature increase at the specified flow rate. This necessitated calculations to estimate the number and size of steam injection holes required.

First the energy input into milk required to give the likely maximum operating temperature increase, and the corresponding amount of steam needed were calculated. Then the cross section area of steam aperture required was calculated from equations found in literature (Vennard J.K. & Street R.L., 1976) based on the likely flow rate of

the milk and available pressure of the steam. An example calculation is given in Appendix B, pages B1-B4.

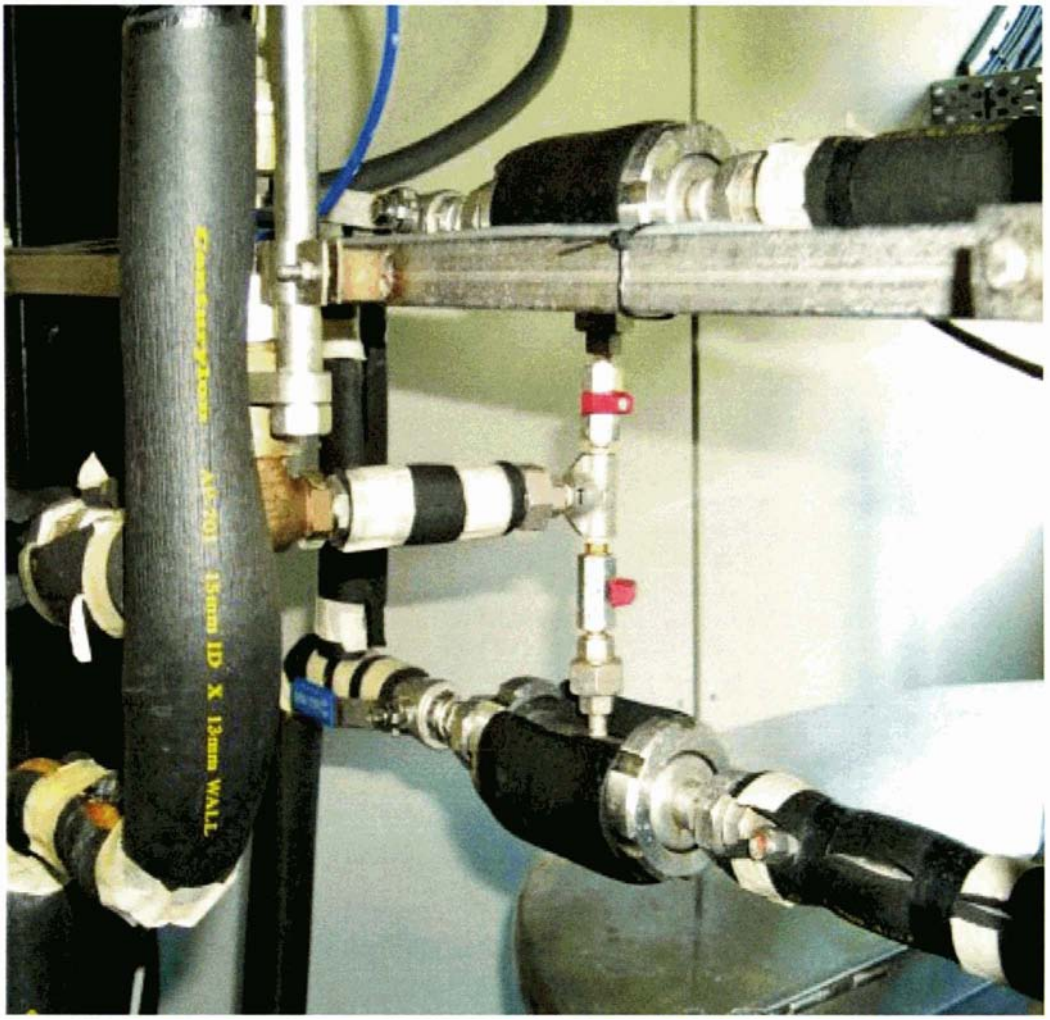


Figure 3.1.3. Photograph showing two of the DSI units installed in the pilot plant.

The correct number of steam holes to give the steam aperture area required can then be calculated depending on the size of the holes. Many smaller holes provide more even heating than a few larger ones. However, with milk, the minimum hole size is around 1 mm in diameter due to the tendency of holes to block with fouling which increases as the holes decrease in size. Initially a hole size of 0.75 mm was chosen, but during commissioning this was found to foul too rapidly so the hole size was increased to 1 mm. The milk line pressure needed to be at least 100 kPa below the available steam pressure of 300-400 kPa.a to avoid occasional back flow of the milk into the steam line due to unstable pressures. This phenomenon considerably increased fouling of the steam injection holes and in the steam line.

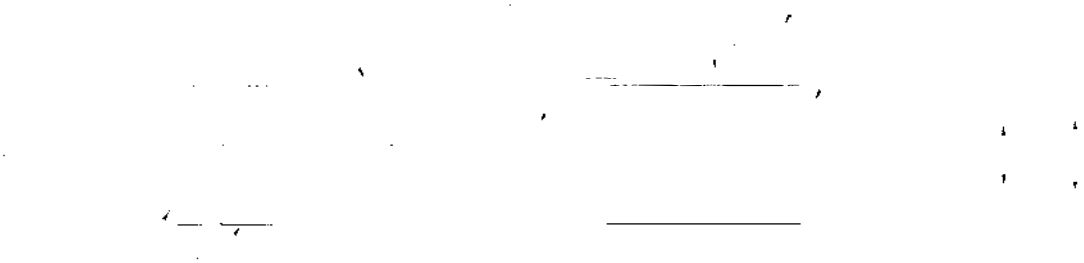


Figure 3.1.4. Diagram of a direct steam injection unit. A complete drawing is provided in Appendix A, page A-6.

3.1.3. Tubular heat exchanger (THE)

The tubular heat exchange (THE) rig was designed to provide surface area at a controlled temperature for colonisation of thermophiles so that the release of thermophiles into the bulk milk flow could be studied. Thermophile growth was targeted through temperature control both up stream of the THE as mentioned previously and also within the THE rig itself by maintaining a constant milk temperature at the optimum range for thermophile growth.

The THE rig was also used by other postgraduate students to study fouling so had to be versatile enough that fouling layers could be developed within the tubes and removed for examination.

The THE rig was arranged into two parallel banks of three tubes in series. This set up provided the ability for different initial surface conditions on each side of the rig or within each tube to study release into the milk stream. Comparison between the release of thermophiles with different surface conditions could then be made. For example one side of the THE rig could be pre-fouled before an experimental run, while the other could be clean and the release of thermophiles into the bulk milk stream from each side over time could be compared. The THE rig is shown in Figure 3.1.5 below. More detailed drawings of the THE given in Appendix A, pages A4 and A5.

Milk sample ports were located before the inlet to the THE and after each tube (as shown on Figure 3.1.2) to obtain the profile of thermophile release into the bulk milk stream during experiments. Temperature measurement devices were located at key positions on each side of the tube bank to allow control and monitoring of temperature profiles, as shown in the process and instrumentation diagram in Appendix A, page A4.

Figure 3.1.5. Photograph of tubular heat exchanger installed in the pilot plant.

The THE tubes were designed concentrically with three tubes inside one another. Milk flowed through the central chamber while the heating medium (hot water) could flow on either or both sides of the milk, providing heating on both the inner and outer surfaces if necessary. This is shown in Figure 3.1.6. Figure 3.1.7 shows the assembly of a single THE tube. The hot water was heated in a separate heating circuit, which is shown in the process and instrumentation diagrams in Appendix A, pages A1 and A4. The THE was constructed with 304 grade stainless steel with a number 4 finish (approximate relative surface roughness Ra = 1.0 μm).

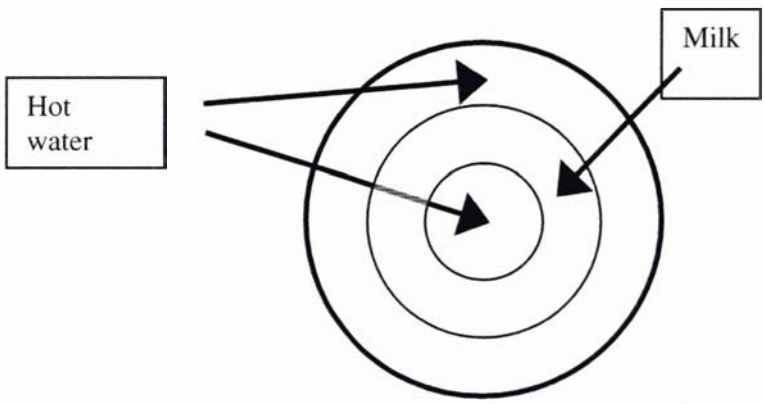


Figure 3.1.6. Cross section of tubular heat exchanger tube, showing the location of the milk in the central tube with hot water heating on both the inner and outer surfaces.

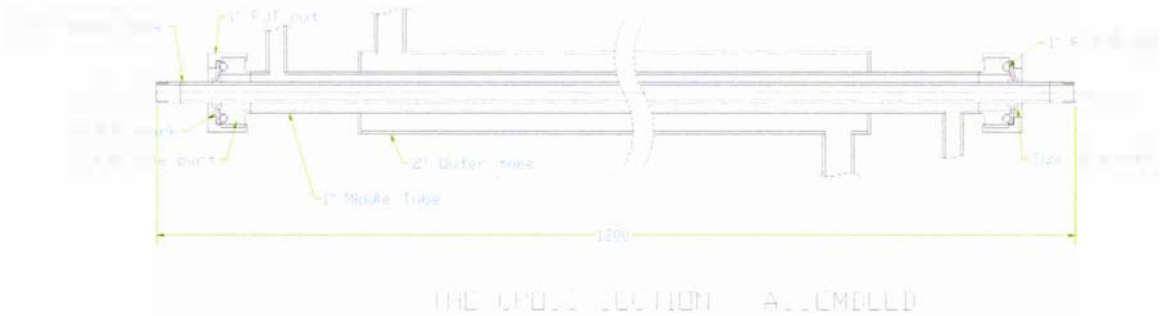


Figure 3.1.7. Drawing of the assembly of an individual heat exchange tube. A complete drawing is provided in Appendix A, page A-5.

The THE rig was especially designed for easy disassembly for examination of the milk contact surfaces after a run. This enabled visual and analytical study of the fouling and also allowed measurements of thermophile surfaces numbers.

In this design, individual tubes could also be isolated through a system of piping by-passes, without stopping the bulk milk flow. Any tube could therefore be removed and studied at any time during a run the run. This is shown in the piping and instrumentation diagram of the THE in Appendix A, page A4.

3.1.4. Mini plate heat exchangers (MHE)

The miniature plate heat exchanger (MHE) rig was used to study surface colonisation of thermophiles over time during experiments. It was also used to create and study the

development of fouling on surfaces that could be removed and manipulated for further study such as microscopy.

The rig consisted of six units in series, each one able to be independently isolated from the main flow, as depicted in Figure 3.1.2. A photograph of one MHE unit is shown below in Figure 3.1.8. More detail is provided on the MHE piping and instrumentation diagram in Appendix A, page A3.

Each unit provides approximately 10 cm^2 of heat exchange surface area where thermophile colonisation or fouling developed could be studied. Because the surface area of heating is quite small, the milk in the six consecutive MHE units were at the same temperature, within the sensitivity of the thermocouples used and were essentially replicates. Thin grade 304 stainless steel (0.08 mm, Ra 1.2 μm) was used as the plate within the units as it could be easily cut into sections for measurement of surface thermophile numbers. Rubber gaskets were used to seal the unit against foil plate. To provide extra strength and rigidity to the thin foil surface, the foil was placed on top of thicker stainless steel plate (0.6 mm). Thermal contact between the steel foils and plates were achieved by an adhesive layer of silicone heat transfer compound (Electrolube, HTS35SL, Spectron Electronics Ltd., Palmerston North, N.Z.). Hot water was used as the heating fluid which was circulated in its own heating circuit that also supplied the THE rig. The milk temperature in each unit was able to be monitored. Figure 3.1.9 shows the assembly and approximate dimensions of each MHE unit. Further detail on the MHE rig can be found in Bennett (2000).

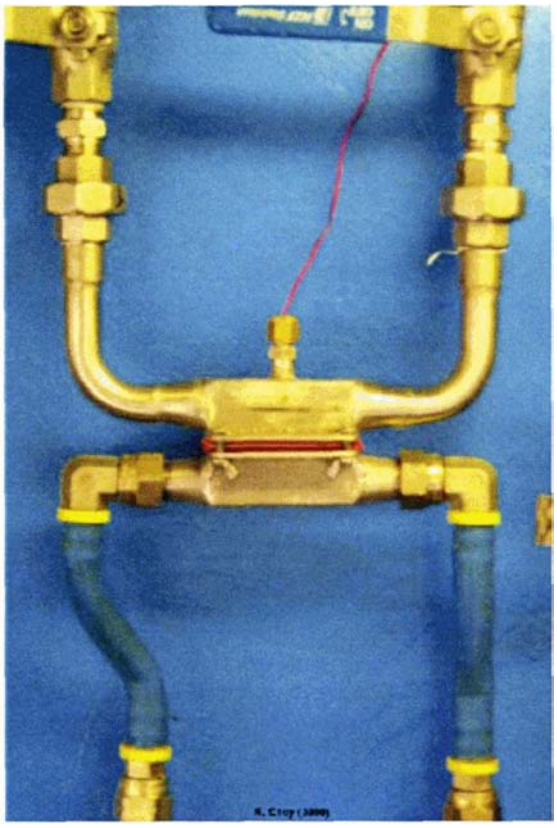


Figure 3.1.8. Photograph of a single MHE unit. Note the thermocouple wire entering from the top of the unit. Milk passes through the top half and hot water through the bottom half of the unit.

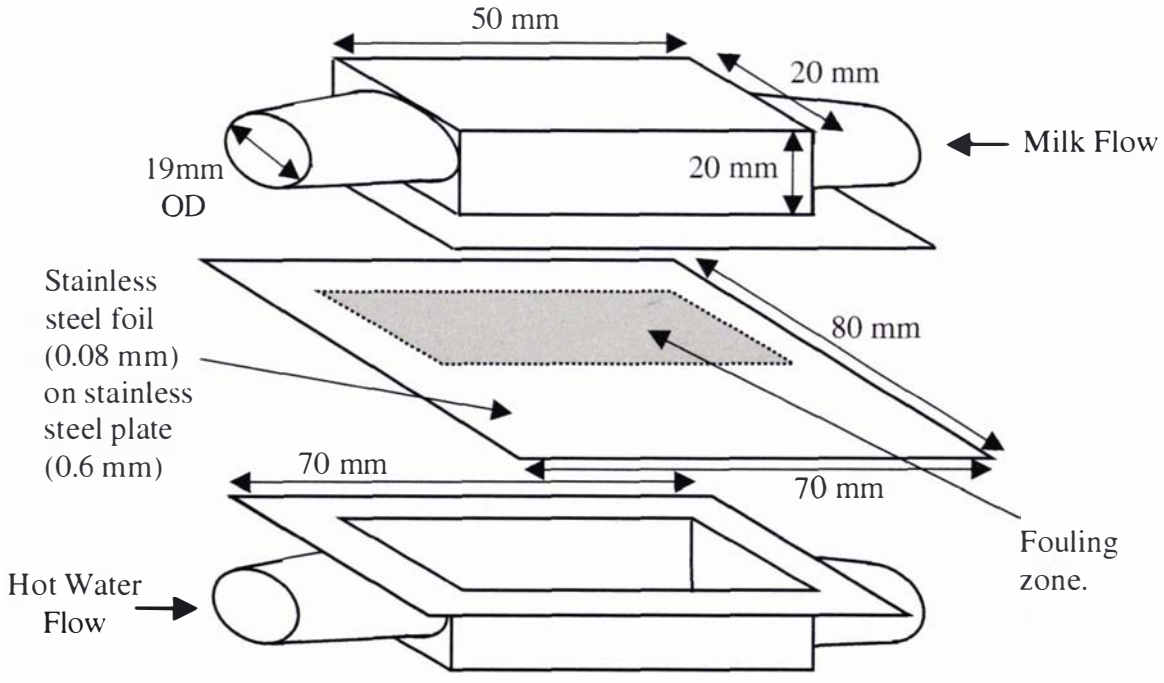


Figure 3.1.9. Diagram of MHE unit assembly showing approximate dimensions.

3.2. Acquisition of operational data

3.2.1. Computer interface

The data from sensors in the pilot plant was fed to a computer in the control room for monitoring and control of on-line measurements. A photograph of the control room is shown in Figure 3.2.1.

Most on-line sensors were wired back to a programmable logic computer (PLC) that consisted of an Allen Bradley SLC 5/03 processor and two racks each with 12 module bays. There were 2 analog current modules with 16 channels each, 2 counter modules of 4 channels each, 5 analog modules of 4 channels each, 2 relay output modules of 8 channels each, 3 thermocouple (millivolt) modules of 4 channels each and 4 RTD (resistance) input modules of 4 channels each. A total of 104 channels were accessible through approximately several hundred meters of wiring. Because of lack of space in the main PLC, some temperature sensors from the THE rig were fed to a separate portable PLC supplied with its own computer. This computer was networked to the main control computer so that the measurements could be recorded on-line with the rest of the plant sensors. Both computers were running the FIX 32 DMACS 7.0 (Intellution Inc., Industrial Interface Ltd., Auckland, N.Z.) as the user interface for monitoring, control and historical data collection. Historical data from each major experimental run conducted on the pilot plant is shown in Appendix D.

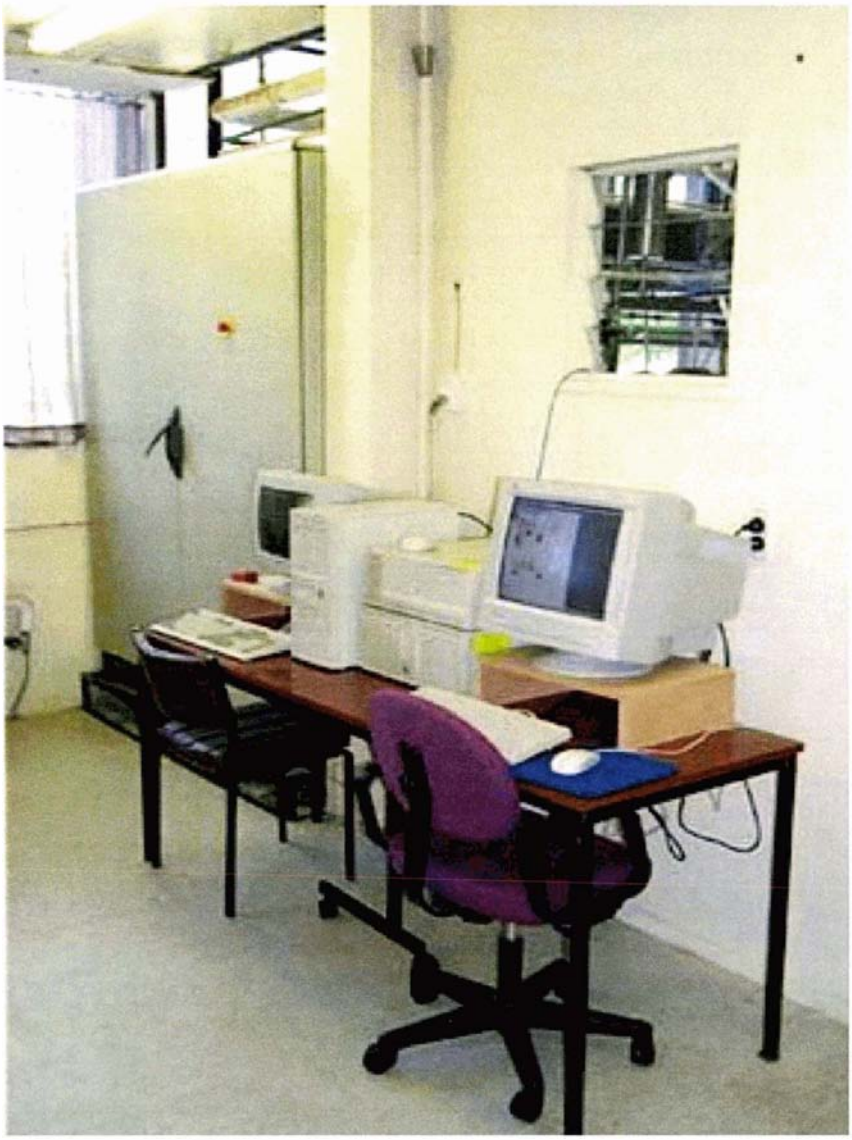


Figure 3.2.1. Photograph of control room

3.2.2. Temperature Measurement

A combination of T type thermocouples and resistance temperature devices (RTDs) were used to measure temperatures around the pilot plant. Thermocouples were used for the majority of temperature measurements, with RTDs only being used in the less crucial areas were measurement accuracy was less important such as the milk vat temperature. Initially RTDs were installed for all temperature measurements but during commissioning of the pilot plant it was found that these had a slow response time and hence could not accurately measure rapid changes in temperature.

These were calibrated by recording the temperature output from the sensors at 0 °C and 100 °C reference temperatures and producing a calibration equation for each sensor based on these measurements. The 0 °C and 100 °C reference temperatures were obtained by immersion in distilled ice/water slurry and boiling distilled water respectively. Measurements were recorded at these temperatures for 5 minutes each and the average value over the five-minute period taken as the recorded temperature for each reference temperature.

The calibration equation was assumed to form a linear relationship between the recorded and calibrated values:

$$\theta_c = a.\theta_r + b \qquad 3.1$$

Where:

θ_c = Calibrated temperature (°C)

θ_r = Recorded temperature (°C)

a = Gradient constant

b = Y – axis intercept constant

The gradient constant is given by:

$$a = 100/(\theta_{100} - \theta_0) \qquad 3.2$$

Y – axis intercept is given by:

$$b = -a.\ \theta_0 \qquad 3.3$$

Where:

θ_{100} = Temperature recorded at 100 °C reference temperature.

θ_c = Temperature recorded at 0 °C reference temperature.

These equation constants were entered into the computer interface to display the calibrated values on-screen, not the raw recorded values.

3.2.3. Flow rate

A combination of flow meters types were used for the measurement of the milk and cleaning fluids flow rates in the pilot plant.

Two paddle flow meters (Flow Sensor Dual Range, Model No. 256-225, RS Components Ltd., Auckland, N.Z.) were used to obtain even flow either side of the THE rig. These provided cost effective flow rate measurement. Each flow meter was calibrated by operating at a range of flows in which the flow meter was needed and recording the actual flow rate with a measuring cylinder and stopwatch. Collected data were recorded in an Excel spreadsheet and linear equations fitted to create calibration equations relating recorded flow rate to actual flow rate. As with the temperature calibration, the constants for these equations were entered into the computer interface so that the calibrated measurement was displayed on-line. The calibration plot for the two paddle flow meters are shown in Figure 3.2.2.

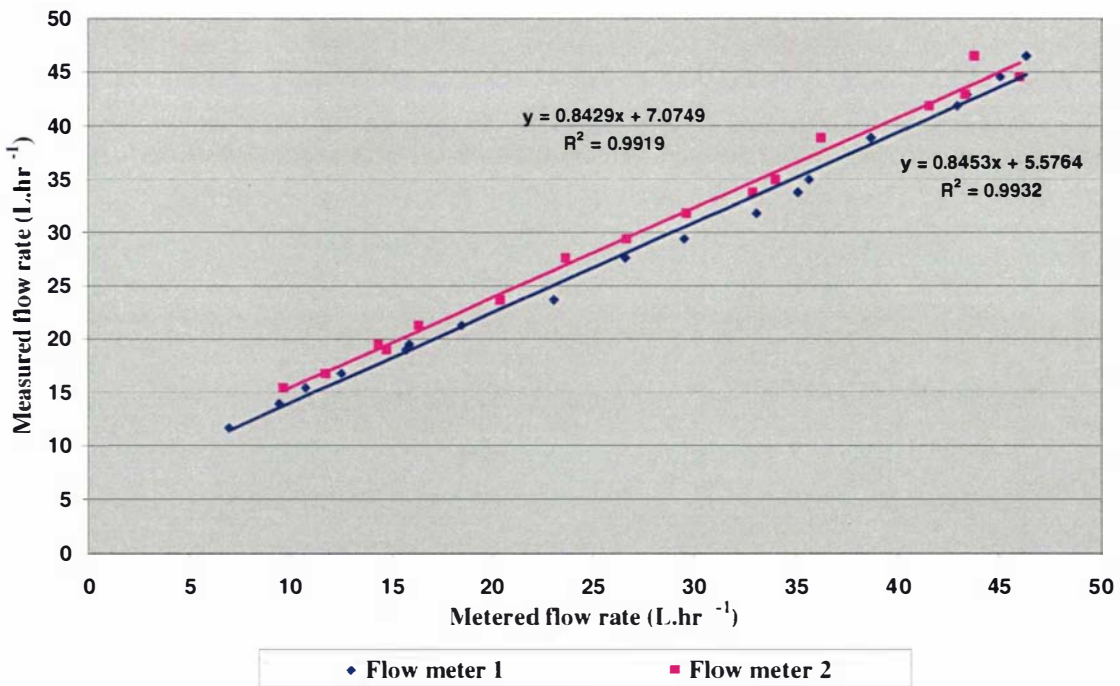


Figure 3.2.2. Calibration curve for the two paddle flow meters used to maintain even flow through either side of the THE rig.

The flow spilt between the two sides of the THE was balanced with the mean flow measurement of the flow down each side of the THE over the duration of an

experimental run being within ±0.3 $l.min^{-1}$ of an even flow split. For 95% of time the individual flow measurements down each side of the THE fell within ±2-3 $l.min^{-1}$ (depending on the experimental run) of the actual halved total flow (based on statistical analysis of experimental flow data).

Two magnetic flow meters were also used. One for the main milk flow rate in the pilot plant (Endress Hauser Picomag, 11 PM 16533, EMC Industrial Instrumentation, Auckland, N.Z.) and the other when higher flows were being used such as when cleaning the plant by clean in place (CIP) (Endress Hauser Promag, 3FT25 – AA1AA11A2113, EMC Industrial Instrumentation, Auckland, N.Z.). These flow meters were calibrated in the same fashion as for the paddle flow meters however only marginal adjustment was necessary.

3.2.4. Pressure

On-line measurement of pressure in the pilot plant was used for steam and milk line pressures. These were monitored mainly to obtain good performance from the DSI units, as when the milk line pressure approached the steam pressure, the performance of the DSI dropped. Measurement was made using 0 – 50 PSIG pressure sensors (Data Instruments XPRO Pressure Transmitter, 9907202, EMC Industrial Instrumentation, Auckland, N.Z.). Calibration was made against a manual pressure gauge over a range of different pressures.

3.3. Fouling methodology

3.3.1. Preparation

Fresh foil MHE sections were cleaned in a 1 % (w/v) caustic soda solution at 50 °C, then rinsed thoroughly in distilled water before being autoclaved at 121 °C for 15 minutes. All surfaces used in the experiments (both the removable THE inner tubes and the MHE plates) were removed and cleaned with 1 % (w/v) caustic soda at 55 °C for 30 minutes, then cleaned further in nitric acid (0.5 % w/v) at 55 °C for 10 minutes. As the MHE plates were used to determine surface bacterial activity they were further autoclaved again at 121 °C for 15 minutes before use. The remaining pre-heat sections of the pilot plant were cleaned as described in Section 3.4 below.

In experimental runs 1 to 5 (see Section 3.6) comparing the behaviour of clean and pre-fouled surfaces, the pre-fouled and originally clean tubes and plates were sanitized prior to the experiments in 200 ppm hydrogen peroxide at 25 °C. The surfaces were then rinsed until no hydrogen peroxide could be detected on the surface using peroxide test strips (Peroxide Test, Merck, Germany) which had a minimum detection of 1 $mg.l^{-1}$. Small samples of fouling were also taken after sanitising and tested for thermophile activity using the impedance technique. No thermophile activity was found on any of the sanitised fouled surfaces.

3.3.2. Operation

Pre-fouling of surfaces was carried out in the pilot plant generally for four hours with milk flowing at 30-45 $l.hr^{-1}$ on a once through system. The tubular heat exchanger inner tubes were fouled with the hot surface at 95 °C and the milk inlet temperature to the THE at 65 °C (outlet temperature 85 °C). The MHE plates were fouled with the hot surface at 95 °C and the milk temperature at 65 °C.

At the beginning of each run water was first pumped through the pilot plant until flow rates and temperatures became stable, then the system was switched over to milk supplied from the vat. Similar practice is found in the New Zealand dairy industry. Air was released from the milk through the heating process and found to collect in a number

of locations around the plant. This air was removed where possible by sterile syringes through rubber septa using the same system as for taking milk samples mentioned later in Section 3.5.1. Any variations from this basic methodology in a particular experimental run are described later in Section 3.6.

This fouling methodology produced fouling structures with a cratered appearance as seen in Figure 3.6.4 below. This fouling structure is likely to have formed due to the development of bubbles where the milk contacts the hot surface. These bubbles would have formed due to the high temperature difference between the hot surface and the milk and the laminar flow regime in the heat exchangers. This fouling structure is likely to have a rougher surface than fouling developed under industrial conditions with a lower temperature difference and much higher flow rate, as bubbles will be less likely to form. Even though this is not desirable as the results from this work aim to be representative of industrial situations, the fouling deposits could not be developed over long runs on the pilot plant due to cost of milk required to operate the plant for this length of time. This may have resulted in fouled surfaces capable of harbouring more bacteria at the surface than industrial fouling deposits due to the rougher surface nature.

3.3.3. Recording of fouling structures

To record fouling structures a digital camera was used (Kodak DC 290, Eastman Kodak Company 1999, New York, U.S.).

Some magnified images where also taken with a fluorescence microscope (Leitz Ortholux II) with an H2 incident light excitation filter block (Ernst Leitz Wetzlar, GmbH, Wetzlar, Germany). An Acridine orange stain was used with a magnification of 40x. Staining procedure was as per epi-fluorescence procedure in Section 3.5.7 below.

3.4. Pilot Plant Clean in Place (CIP)

3.4.1. CIP Procedure

The pilot plant was cleaned using a five cycle clean in place (CIP) procedure. This consisted of a hot water flush, caustic, hot water rinse, acid and final hot water rinse. Hot water for the CIP was heated to 55°C using direct steam injection. The caustic cycle used caustic soda at 55°C with a concentration of 1 % w/v and consisted of a 5 minute flush (once through) followed by a 15 minute circulation period. The acid cycle used 0.5 % w/v nitric acid at 55°C, circulating for 10 minutes. During all cycles of the CIP both the CIP pump and milk pump (refer to Figure A.1 in Appendix A) were run at their maximum sustainable speed of 50 Hz. This provided a flow rate of 0.6 $l.s^{-1}$, which produced turbulent cleaning conditions (Re = 24000) in the 15.8 mm inner diameter pipes that were used in the majority of the pilot plant milk transport lines. More detail on the CIP equipment and CIP optimisation can be found in Croy (2000).

3.5. Microbiological Techniques

3.5.1. Milk sampling

Milk samples were drawn aseptically in duplicate from the pilot plant with sterile 30 ml syringes (Terumo, Terumo Corporation, Tokyo, Japan) and 20 gauge (0.9 x 25 mm) needles (Precision glide, Becton Dickinson Medical Pte. Ltd., Singapore) through ports containing rubber septa (Septa 77, Chromatography Research Supplies, Inc., USA). The ports holding these septa were quarter inch stainless steel tube sockets (Swagelock, SS-400-6-4W, Auckland Valve and Fitting Co. Ltd.). These allowed easy removal of each septum for replacement at the start of new experimental runs.

3.5.2. Bulk milk thermophile counts

A modified form of the Fonterra Research Centre (FRC) method for assessing thermophilic spores (NZMP, 2002a) and vegetative cells (NZMP, 2002b) was used . The modification consisted in the use of spread plates, where the sample is spread over the surface of prepared agar, rather than pour plates, where the sample is mixed with molten agar. This was necessary because such a large number of plates were required (up to 2000 in some experimental runs) that not enough agar could be kept molten during the experiments. Also, as the spread plating technique could be carried out much faster than pour plating, the milk samples collected at each sampling time could be processed in the time available between sampling, avoiding the creation of a backlog of samples during the experimental runs. To differentiate between spores and vegetative cells, samples were exposed to a heat treatment of 100 °C for 30 minutes prior to dilution. Plates were incubated at 55 °C for 48 hours inside plastic bags, which provided humid conditions so that plates did not dry out. The base agar used was standard methods agar (BBL, Becton Dickinson & Co., Cockeysville, USA). Agar concentrations used for thermophile counts were as follows:

Table 3.5.1. Agar composition used for thermophile counts.

	$g.l^{-1}$
Standard Methods Agar	23.5
Skim Milk powder	1.0
Soluble starch	2.0

Thermophile spore counts were performed using the same agar, with the exception that 2.5 ml Bromocresol purple solution (4 % w/v) were added per litre.

For dilution of samples, buffered peptone water (Merck, Darmstadt, Germany) was used at a concentration of 5 $g.l^{-1}$.

This method of sampling and enumeration of thermophiles and thermophilic spores in the bulk milk produced duplicate counts that never differed by more than a factor of 2. The greatest amount of variation in measurement of thermophile numbers from duplicate samples taken from the pilot plant was from sample variation rather than actual test method reproducibility. Duplicate determinations of the same sample were more repeatable than duplicate sample determinations (repeatability typically 10%). This indicates that the variations in the measurements from duplicate samples are due to actual variation in the thermophile load in the bulk milk.

3.5.3. Isolate

The isolate of *Bacillus stearothermophilus* used in this study (identified as B12 Cm) was from a stock culture held in culture collection (Biofilm Research Unit, Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, N.Z.) originally isolated from a milk powder manufacturing plant and provided to the Biofilm Research Unit by Professor Hugh Morgan of the Thermophile Research Unit, University of Waikato. The culture was maintained at –80 °C using Microbank Cyrobeads® (Pro-Lab Diagnostics, Austin, Texas, USA). Random amplified polymorphic DNA (RAPD) analysis, carried out at the Thermophile Research Unit, University of Waikato, was used to identify the isolate (Ronimus *et al.*, 1997; Parker, *et al.* 1997). This isolate was chosen as it was one of the most commonly encountered thermophilic bacilli isolated from milk powder plants and milk powder around New Zealand. It was therefore considered to be representative of the thermophiles causing contamination problems within New Zealand.

Actively growing cultures used in this study were prepared by placing one bead from the stock culture into 10 ml of tryptic soy broth (TSB) (Difco, Becton Dickinson & Co., Sparks, USA) then incubating this culture overnight (15 hours) at 55 °C. One millilitre

of this culture was then used to inoculate 10 ml of fresh TSB. After 5-8 hours at 55 °C actively growing cultures with cell concentrations in the range of 10^8 cfu.ml^{-1} could be expected. For situations where cultures with specific cell concentrations were required, growth was monitored by taking regular direct microscope counts of samples from the growing culture. To obtain a culture of a required cell concentration, dilutions were made in peptone water (5 g.l^{-1}). To assess the viable cell concentration accurately, a thermophilic plate count was carried out (as in Section 3.5.2 above) on the culture before dilution. Viable cell concentrations in the diluted cultures were calculated by applying the dilution factor to the viable cell concentration of the initial culture.

3.5.4. Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) was used to obtain direct microscopic counts of thermophile colonies on surfaces and also to study the distribution of thermophiles on surfaces.

3.5.4.1. Method development

As no previous CLSM method for observing bacteria on milk fouled surfaces was found in the literature, a method was developed.

Several different types of stain were investigated. A DNA stain was thought to be the best option due to the relatively long time that samples can be kept after staining, as the DNA stains are much more permanent, since they bind to the nucleic acids of the cells. However, some stains, such as the esterase substrate Calcein AM (Molecular Probes Inc., Eugene, Oregon, USA), are converted into fluorescent molecules by intracellular enzymes. These stains are initially able to penetrate the cell, but once inside the cell they are converted by cell enzymes such as esterase into a fluorescent state that is unable to pass out of the cell. These stains would be more specific than DNA stains, as only the cells are stained and therefore there is very little background fluorescence. However, these stains can leak back out of the cells over a matter of hours, which was unacceptable in this situation as the time required to observe and count the colonies in all the samples was up to one week after the samples were stained. As a result, a DNA stain was chosen.

The greatest problem encountered with DNA stains was with background fluorescence, where the fouling material would irreversibly take up the stain and would then appear as a bright background on the CLSM images. This background then made it difficult in some situations to identify bacteria present on the surface of the fouling. This was not a problem where the surfaces where free of fouling, such as on clean stainless steel surfaces.

The first DNA stain trialed was Acridine orange (BDH Chemicals Ltd, Palmerston North, N.Z.) at a concentration of 0.01 % (w/v) suspended in 0.05M pH 7.5 Tris buffer (United States Biochemical Corp., Cleveland, Ohio, USA). However, the problem with the background fluorescence was very apparent because this material stained many substances other than DNA. Therefore, a more specific stain, SYTO 13 (Molecular Probes Inc., Eugene, Oregon, USA) was trialed. This had a high specificity to DNA and RNA and showed increased green fluorescence on binding to nucleic acids, but was rather expensive.

Different concentrations of the stain (1 μM, 5 μM, 10 μM and 20 μM) were investigated, based on the suppliers suggested concentration range. A concentration of 10 μM was found to be the best compromise between sample fluorescence and the amount of dye used.

With this stain less background fluorescence occurred, but it was still a problem. Only groups of bacteria were bright enough to be seen; single adherent cells were lost in the background fluorescence. For MHE plate surface samples taken from the pilot plant this was less of a problem, as bacteria could be seen present in groups. However, it did cause difficulties when trying to observe single adherent cells, such as when adhesion studies were conducted. To overcome this shortcoming, a method was developed where single adherent cells were allowed to grow to form small micro colonies around 10 μm in diameter. These were then visible when carrying out the CLSM surface counts.

This method was carried out by immersing the fouled sample in sterile peptone water (5 $g.l^{-1}$) under quiescent conditions for a period of 12 hours at 55 °C. During method

development, surfaces were left in the peptone water for varying durations up to 48 hours and no significant increase in the number of colonies was observed between 12 and 24 hours. After this period the fouling material started to break up, so the minimum incubation time possible to see results was used (i.e. 12 hours). Thermophile counts on the bulk peptone showed no thermophile contamination after 24 hours, indicating that the thermophiles were not able to proliferate in the peptone solution alone. Therefore the method was assumed to be suitable for enumerating adhered thermophiles.

An example image from the CLSM is shown in Figure 3.5.1 (a). However, even using this technique, thermophile colonies were difficult to differentiate on fouling deposits that were not fully developed, that is, when the fouling was present as number of small islands. Some of these fouling deposits closely resembled thermophile colonies and added to the experimental uncertainty. An example CLSM image of this condition is shown in Figure 3.5.1 (b). Despite the experimental uncertainty with the CLSM method it does have the advantage that it allows examination of the distribution of thermophiles on the surface.

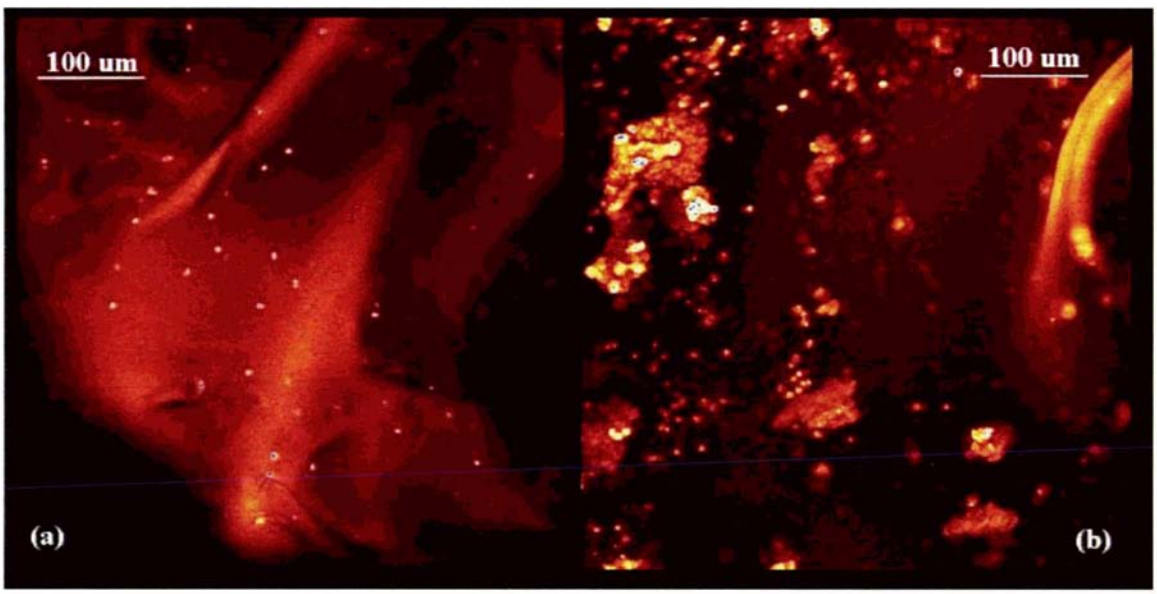


Figure 3.5.1. CLSM images of thermophilic bacterial colonies on a fouling layer stained with SYTO 13 at 200x magnification. Bacterial colonies appear as the bright dots, while the fouling layer causes the background fluorescence. Higher areas of the fouling layer appear brighter than the lower regions. In (b) a poorly developed fouling layer is shown, where some of the deposit resembles thermophilic colonies, adding uncertainty to the counts.

3.5.4.2. CLSM technique

The staining technique for the direct counts using CLSM is outlined below:

1. Each MHE plate surface sample was cut into ~1cm square pieces and placed in a sterile test tube.
2. The sample was rinsed twice in sterile filtered Tris buffer (0.05 M pH 7.5) made up from Tris and Tris HCL (USB – United States Biomedical Corp., Cleveland, Ohio). Buffer was filtered using a 0.2 µm Sartorius filter (Medic Corporation, Lower Hutt, N.Z.).
3. The sample was then stained for 10 minutes in 10 µM SYTO 13 green fluorescent nucleic acid stain, made up in sterile filtered Tris buffer (0.05 M pH 7.5).
4. The sample was rinsed again five times in the sterile filtered Tris buffer.
5. The sample was removed from the test tube with flamed tweezers and affixed to a glass microscope slide with epoxy resin (Araldite, Selleys, Auckland, N.Z.).
6. The sample was left to air dry for 30 minutes at room temperature before being placed in storage at -18 °C until observation.

A Leica TCS 4D Confocal Laser Scanning Micrsocope with an Argon/Krypton mixed gas laser was used (Leica Microsystems, Mannheim, Germany). Filter sets used for SYTO 13 were an excitation of 488 nm with a RSP510 nm beamsplitter then a LP515 nm barrier filter to collect emission wavelengths 515 nm and above.

An objective of 10x magnification was used on the microscope. Fields of view to count the surface bacterial population were selected randomly and their position on the microscope stage scale recorded. Random selections were made by using two randomly generated numbers from a hand held calculator as a fraction the length and width of the sample as measured by the increments on the microscope stage scale (first number generated used for width and the second for the length). Once the field of view had been chosen a depth scan between the highest and lowest points on the field of view (up to the maximum possible 166 µm) was completed at 3x zoom. This produced a digital image of 300x magnification of the sample surface.

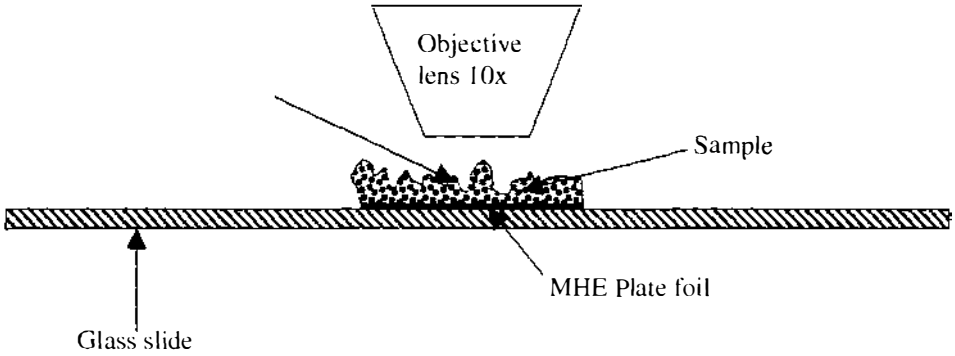


Figure 3.5.2. Diagram of the set up of the sample in relation to the CLSM objective lens.

To obtain measurements of the numbers of adhered cells the bacterial numbers in 8 randomly selected fields were counted for each sample and averaged. The number of fields selected was limited to eight, due to time and financial constraints. As the maximum depth that the CLSM can achieve for one scan is 166 μm, some images did not show the entire area of fouling in the selected field. This was because the fouling layer topography sometimes provided a situation where the depth of the fouling layer that was visible within each field was greater than 166 μm. The fouled area above and below the visible 166 μm was therefore not present in depth scan image generated by the CLSM. In this situation, instead of taking the fouled area as equal to the area of the field of view, the area cut out of the image (blank areas on the images above or below the 166 μm depth scan range) was subtracted from the total field area. This allowed the surface colony numbers adhered per unit area of fouled surface to be calculated as the measurement was then based on the area of the depth scan image where fouling surface was visible. The fouled area was calculated by multiplying the fraction of field of the depth scan image where fouled surface was visible by the total field area. Also, with fields where the depth range for a single depth scan was greater than 166 μm, the range of fouling that the CLSM could span was randomly selected to start from either the top or the bottom of the visible image so that samples were not biased towards either the higher or lower areas of fouling.

Images were saved and at a later time colonies of thermophiles on the surface were counted and a surface count calculated of colonies per square centimetre of fouled

stainless steel surface. These images can be found on the enclosed compact disc. Refer to the index of the compact disc in Appendix G for the location of the images

3.5.5. Swabbing

Swabs were taken from some surfaces that could not be tested for bacterial surface numbers via other methods, for example THE tubes and other pipe surfaces. Swabs were moistened with thiosulphate diluent (Merck, Germany) rubbed vigorously over the surface to be tested of 5 cm^2 and then suspended in peptone water (5 $g.l^{-1}$) (Merck, Darmstadt, Germany) by vigorous shaking. A thermophile count was then carried out (as per method described in Section 3.5.2) on the peptone water. Thermophile counts on some swabs were also made using impedance microbiology by placing the swab in the detection cell. Swabbing was not used to a great extent in the thesis, as it is well established that underestimates of about 10 times the surface population as measured by other techniques are obtained (Flint *et al.*, 1997b).

3.5.6. Impedance microbiology

The thermophilic bacterial activity of the MHE surfaces was estimated by impedance microbiology, using a MiniTrac 4000 impedance monitor (SyLab, MBH, Purkersdorf, Austria). This method has been shown to be reliable for enumeration of *Bacillus stearothermophilus* (Flint S.H. & Brooks J.D., 2001). Tryptic soy broth (TSB) (Difco, Becton Dickinson & Co., Sparks, USA) was used as the growth medium and was held at an incubation temperature of 55 °C. The time taken to reach a threshold level of impedance (set at 5 % of the electrode 'E' value) was measured and called the impedance detection time (IDT). The more bacterial activity present, the shorter the IDT was. The number of active cells was determined from a calibration curve created with serial dilutions of planktonic cells enumerated by plating onto milk plate count agar and incubating at 55 °C for 48 hours as per the plate count method described above in Section 3.5.2. The calibration curve for the impedance monitor as used in the experiment is shown as Figure 3.5.3. To avoid bacterial contamination from the surface of the MHE that was exposed to the heating water flow, that side of the MHE foil was swabbed with ethyl alcohol before being cut into smaller pieces and inserted into the impedance monitor.

Each piece of fouled or un-fouled stainless steel foil surface tested by impedance had around 0.5 cm^2 of surface area. However, the surface area of each sample was slightly different due to uneven cutting of the fouling layer when the foil was cut into pieces. Therefore, each test piece had to be measured individually so that the number of thermophiles per square centimetre of surface area could be accurately calculated.

The impedance technique assumes that the activity of surface adhered and planktonic cells are the same. In reality this may not be true and would thus introduce an error into the results. However this method is more sensitive than the microscopic methods.

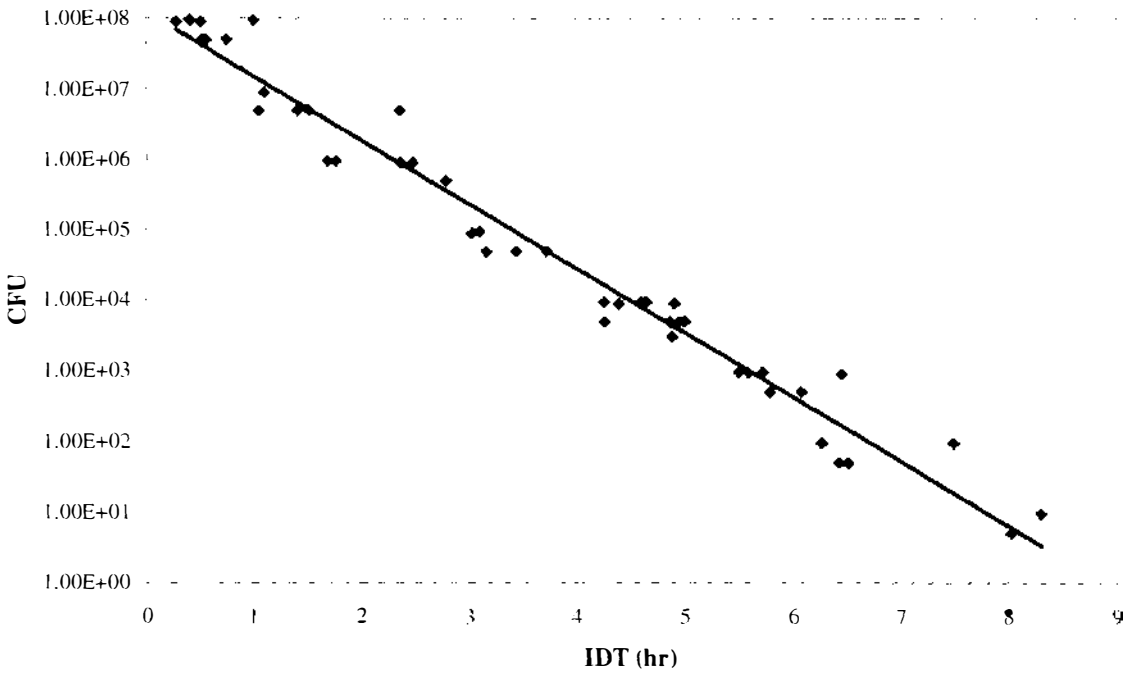


Figure 3.5.3. Calibration curve for planktonic *Bacillus stearothermophilus* C_m *(B12)* on the MiniTrac 4000 impedance monitor using TSB as the growth medium. Graph shows the number of colony forming units (CFU) measured in the samples by plate counts versus the impedance detection times (IDT) of the samples.

The number of viable spores was also determined using impedance, by first subjecting the surface samples to a heat treatment of 100 °C for 30 minutes before measuring the IDT to differentiate between spores and vegetative cells. This heat treatment was carried out by immersing the surface sample to be tested in a test tube filled with 10 ml of water placed in a water bath at 100 °C. A separate test tube with a temperature monitor immersed in 10 ml of water was used for the control. When the temperature in this tube

reached 98 °C the 30 minute time interval was started. After the heat treatment the samples were rapidly cooled in another water bath at 15-18 °C.

In the writer's view, this measurement only accounts for the activity of the bacteria present at the surface, which is the parameter of relevance in the study of contamination to the milk stream. A total count of bacteria present in the fouling layer requires dissolution of the foulant without affecting the bacterial activity. Such a count was not relevant to the problem at hand and was therefore not attempted.

The major source of variation in multiple determinations of the same sample when measuring numbers of thermophilic bacteria on the test surfaces seemed to originate from test repeatability of the impedance detection equipment rather than from variation in the sample itself. Multiple measurements of the same homogenous planktonic sample gave similar variation as multiple measurements of an individual surface sample (i.e. measurement of surface numbers on multiple pieces cut from a surface sample) where the actual variation in surface numbers over the surface was unknown. Variation between multiple impedance detection time determinations was typically between ±0.2 hours.

3.5.7. Epi – fluorescence microscopy

The other microscope method used for study and enumeration of surface adhered cells was direct epi-fluorescence microscopy (DEM) based on the method of Flint (1998). As with CLSM, DEM also involved DNA staining so that bacterial cells would fluoresce and direct counts of bacteria adhered to the surface could be made. The stain used was acridine orange (BDH Chemicals Ltd, Palmerston North, N.Z.) at a concentration of 0.01 % (w/v) suspended in Tris buffer (0.05 M pH 7.5) as for the SYTO 13 stain used in CLSM. Before the stain was used it was filtered through a 0.2 µm Sartorius filter (Medic Corporation, Lower Hutt, N.Z.).

The staining procedure was similar to that for CLSM, with each surface sample cut into approximately 1 cm square pieces. These were then rinsed in the sterile filtered Tris buffer. As above, the buffer was filtered using a 0.2 µm Sartorius filter (Medic Corporation, Lower Hutt, N.Z.). After this, samples were stained for 10 minutes in the

prepared stain before being rinsed again in sterile filtered Tris buffer with 5 charges of 5 ml.

Samples were then affixed to glass microscope slide with epoxy resin (Araldite, Selleys, Auckland, N.Z.) and left to air dry before storing at -18 °C to await observation.

Prepared samples were observed at 500x magnification under ultra violet (UV) light using a Leitz Ortholux II microscope with an H2 incident light excitation filter block (Ernst Leitz Wetzlar, GmbH, Wetzlar, Germany) and photographed using PJC 1600 film (Kodak, Rochester, New York, USA).

3.5.8. Random amplified polymorphic DNA (RAPD) analysis

Random amplified polymorphic DNA (RAPD) analysis, carried out at the Thermophile Research Unit, University of Waikato, was used to identify cultures isolated from the pilot plant (Ronimus *et al.*, 1997; Parker, *et al.* 1997).

3.6. Experimental procedures

3.6.1. Thermophile contamination experiments

Experiments in the pilot plant to investigate thermophile growth demanded long runs (up to 24 hours) which required at least two extra staff to operate the plant in shifts. Sampling and micro work occupied at least one person fully for the entire experiment duration plus 3-4 hours after the run had finished. Approximately 4-5 hours of preparation were also needed immediately before the start of each run. Therefore, these runs required one person to be awake for around 31-33 hours at a time.

The planning and preparation for each run of such complexity took around 2 months. This was necessary to organise people and equipment required. These experiments required nearly 200 milk samples to be tested, which in turn required up to 2000 agar plates. For example, a 24 hour experiment with sampling every 4 hours would have seven different sampling times. At each sampling time 26 milk samples were taken at 13 different locations in duplicate, thus a total of 182 milk samples were analysed. Each sample was diluted across a range of four fold dilutions for both thermophiles and thermophile spores after heat treatment. This required 1456 agar plates, excluding controls and a contingency for mistakes and contaminated plates of approximately 20 %, giving an overall number of 1750 plates to prepare. In practice the number of plates became smaller with experience, especially in the later runs, as not all milk samples required the full four dilutions mentioned above. However, some samples such as those for determining spores had more than four due to the unpredictable nature of the sporulation. The number of dilutions required for each sample was pre-determined so that enough plates could be made and so that plates could be pre-labeled with a unique numbering system to save time during the experiment. An example of the dilutions required for experimental Run 5 is shown in Appendix B, page B-5. As with agar plates, several hundred dilution bottles also had to be prepared before the experiment.

Below is a detailed description of each major run performed.

3.6.1.1. Run 1

Bulk contamination from a pre-fouled stainless steel surface was compared to that from a clean stainless steel surface. At this stage in the construction of the THE rig only one tube per side had been installed. On one side of the THE rig, the inner tubes were pre-fouled but the outer tubes were not pre-fouled. On the other side of the THE rig, the tubes were not pre-fouled at all. This provided pre-fouling on approximately one third of the milk contact surface area within the pre-fouled side of the THE rig. The targeted hot side surface temperature in the THE rig was 60 °C and the targeted milk inlet temperature was 55 °C. In this temperature range additional fouling was not expected to occur. Bulk milk contamination was measured by taking duplicate milk samples every 2-3 hours from the sample points D, E, H shown in Figure 3.1.2 over a 9 hour period. Photographs of the inner tubes before and after the experimental run were taken and are shown in Appendix C, page C-1. Data logged from the pilot plant during the run is given in Appendix D, page D-1.

3.6.1.2. Run 2

This was essentially a repeat of the first experiment but with more THE tubes installed, more thorough sampling and for a longer duration (15 hours opposed to 9 hours). Bulk contamination from a pre-fouled stainless steel surface was compared to that from a clean stainless steel surface. On one side of the THE, the inner tubes were pre-fouled but the outer tubes were not pre-fouled (Figure 3.1.2, H, I, J). On the other side of the THE (Figure 3.1.2, E, F, G) the tubes were not pre-fouled at all. This provided pre-fouling on approximately one third of the milk contact surface area within the pre-fouled side of the THE. The targeted hot side surface temperature in the THE was 60 °C and the targeted milk inlet temperature was at 55 °C. In this temperature range additional fouling was again not expected to occur. Bulk milk contamination was measured by taking duplicate milk samples every 2.5 hours from the sample points A-J shown in Figure 3.1.2. over a 15 hours period. Photographs of the inner tubes before and after the experimental run were taken and are shown in Appendix C, pages C-2 to C-5. Data logged from the pilot plant during the run is given in Appendix D, page D-2.

3.6.1.3. Run 3

The third experiment differed from the second in that it was longer, at 20 hours, and that the targeted temperature of the hot side was 85 °C, instead of 60 °C. This different temperature profile was used in an effort to see how contamination may change with a developing fouling layer. Cold water was also passed on the outside of the THE tubes to help maintain the milk temperature in the THE close to 55 °C. Surface numbers on pre-fouled and originally clean surfaces were also measured from the MHE plates six times throughout the run. The pre-fouled and originally clean surfaces were present on the same plate with half of the surface pre-fouled and the other half clean. Photographs of the inner tubes and of the MHE plate surfaces before and after the experimental run were taken and are shown in Appendix C, page C-6 to C-9. Data logged from the pilot plant during the run is given in Appendix D, page D-3 to D-4.

3.6.1.4. Run 4

Both the inner and outer tubes of the pre-fouled side of the THE were pre-fouled. This provided pre-fouling on all of the milk contact surface area on that side of the THE, so that the bulk contamination from a completely pre-fouled surface could be compared to an un-fouled surface. This experiment was carried out as differences in the bulk contamination from the first two experiments were difficult to gauge, so more fouled surface was provided. The temperature profile across the THE and MHE was as for the first run. Surface numbers on pre-fouled and originally clean surfaces were also measured from the MHE plates six times throughout the run. This run was carried out for 24 hours. Photographs of the inner tubes and of the MHE plate surfaces before and after the experimental run were taken and are shown in Appendix C, page C-10 to C-12. Data logged from the pilot plant during the run is given in Appendix D, page D-5 to D-9.

3.6.1.5. Run 5

This 24 hour run was used to investigate the re-contamination of the pilot plant when residual contamination from the preceding run is left behind after cleaning. The first inner tube on one side of the THE rig was fouled and contaminated with thermophiles during a preliminary run on a separate test rig and then inserted back into one side of the THE rig for Run 5. The details of this run are found below in Section 3.6.3.2.

The MHE rig was used down stream of the experiment for 24 hours so that surface numbers on pre-fouled and originally clean surfaces could be measured. This run was a repeat of the surface measurements made in the fourth experiment because in Run 4 both the pre-fouled and originally clean MHE surfaces had a similar amount of fouling present due to the surfaces fouling on initiation of milk flow through the MHE. In Run 5, the pre-fouled MHE surface had much more fouling than the originally clean MHE surface so a clear comparison could be made.

Photographs of the inner tubes and of the MHE plate surfaces before and after the experimental run were taken and are shown in Appendix C, page C-13 to C-14. Data logged from the pilot plant during the run is given in Appendix D, page D-10 to D-12.

3.6.2. Survival during cleaning

In this study the rates of thermophile survival during cleaning of fouled and un-fouled surfaces were compared to determine whether milk fouling can provide extra protection for surface associated thermophiles.

Fouling was first created in the MHE fouling rig set up with foil plates as described earlier in Section 3.1.4. This allowed the production of six similar samples of fouling layer per run. The milk temperature in the MHE was kept at 65 °C and the hot water side at 95 °C to develop fouling by the surface control mechanism.

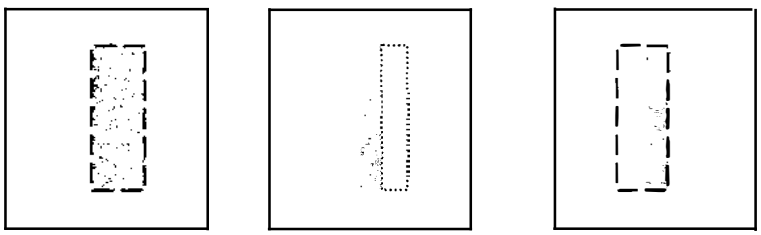
In the dairy industry, cleaning-in-place (CIP) is conducted at the end of each production run. While most of the plant surface, especially the straight length of pipes, is cleaned completely, there are hard-to-reach areas where the CIP is not completely effective. Samples retrieved from these areas when the plant is shut down annually for maintenance exhibit a typical layered structure. Fouling with this layered appearance is commonly encountered in New Zealand milk powder plants. The layers indicate that subsequent runs tend to deposit fouling on top of incompletely removed foulant. To simulate this situation, the fouling layers were produced by performing two runs in the pilot plant each of 4 hours duration. These run lengths were chosen for practical reasons to both minimise costs and to allow operation without requiring separate labour shifts.

However, because the runs were so short and the temperature too high for rapid thermophile growth, the surfaces had to be inoculated with thermophile cultures in between fouling runs to simulate the levels found at the end of industrial runs which last typically for 18 hours.

The cleaned stainless steel surfaces were first inoculated by immersing overnight (15 hours) in autoclaved milk at 55 °C that had been inoculated with *B. stearothermophilus* to a level of around 1 x 10^6 cfu.ml^{-1}. Surface numbers were not measured at this point as the enumeration methods were destructive.

The center 10cm^2 portion of each stainless steel surface was then fouled in milk flowing at 30 litres per hour for four hours as described previously. The surfaces were then inoculated again overnight, before being fouled again in the same location the following day using the same conditions, and then inoculated further overnight a third time.

The inoculated surfaces were then returned to the MHE so that the surface area inside the MHE exposed to the milk flow was half fouled and half un-fouled. This was achieved by changing the portion of the stainless steel surface that was exposed to the milk flow within the MHE. Each stainless steel surface was shifted sideways in the MHE so that only half of the fouling created previously was exposed to the milk flow. The other half of the surface now exposed to milk was previously hidden under the MHE seal (Figure 3.6.1.).



Fouled surface showing original position of Miniature HE

Remove half of fouling layer

New position of plate in Miniature HE.

Figure 3.6.1. Diagram showing procedure for changing the surface area inside the MHE exposed to the milk flow as half fouled and half un-fouled by changing the portion of the stainless steel surface exposed to the milk flow within the MHE.

The MHE rig was then operated with both the milk and the hot side at 55 °C, for 8 hours to develop an active bacterial layer under dynamic conditions on the fouled and un-fouled portions of each surface before the cleaning experiment. When both the milk and hot water side are held at 55 °C, no further fouling develops but the thermophiles continue to attach to the surface and grow at their optimum temperature. In this way in each MHE, the fouled and un-fouled areas were subject to the same growth conditions. A time period of 8 hours was chosen, as in other work on this experimental rig the thermophile surface population reached steady state after this time (Section 4.1.5). This process differs from that of the overnight treatments as the overnight inoculations were carried out in a closed system where the bacterial numbers will peak and then start to decline over time. Under dynamic conditions the surface numbers increase to a steady state maximum level.

3.6.2.1. Cleaning

The fouled and un-fouled stainless steel surfaces were then placed in a 2 % (w/v) solution of caustic soda at 65 °C for 10 s, 30 s, 1 min, 2 min and 15 min without agitation. A second trial was also conducted where the immersion times were 1 min, 2 min, 5 min, 10 min and 20 min. Quiescent immersion was chosen to simulate conditions in hard-to-clean areas in the plant such as dead spots and re-circulation regions. The surfaces were then rinsed thoroughly in sterile distilled water to remove caustic soda remaining on the surface. Fouling that had swelled and absorbed the caustic was removed when the surfaces were rinsed therefore little caustic should have remained at the surface.

The thermophile activity of the surfaces after cleaning was obtained by impedance microbiology as described previously in Section 3.5.5 to assess thermophile and thermophile spore numbers.

Confocal Laser Scanning Microscopy (CLSM) was used to determine if surface associated thermophiles were removed during cleaning as described in Section 3.5.3. This allowed the linkage of changes in activity of the surfaces with either removal or death of the thermophiles.

Impedance detection times were used to calculate the number colony forming units (cfu) per square centimetre of surface area. The surface area of each test piece was measured individually because the test pieces were slightly different due to uneven cutting.

For the first trial duplicate impedance samples were used for each determination of thermophile and spore surface numbers. In a second trial triplicate impedance samples were used and the standard error and 95 % confidence intervals on the mean were calculated.

3.6.3. Re-contamination after cleaning

Two different types of lab scale experiment were conducted to study the process of contamination of a plant during a production run after cleaning.

3.6.3.1. Lab scale – transport mechanism study

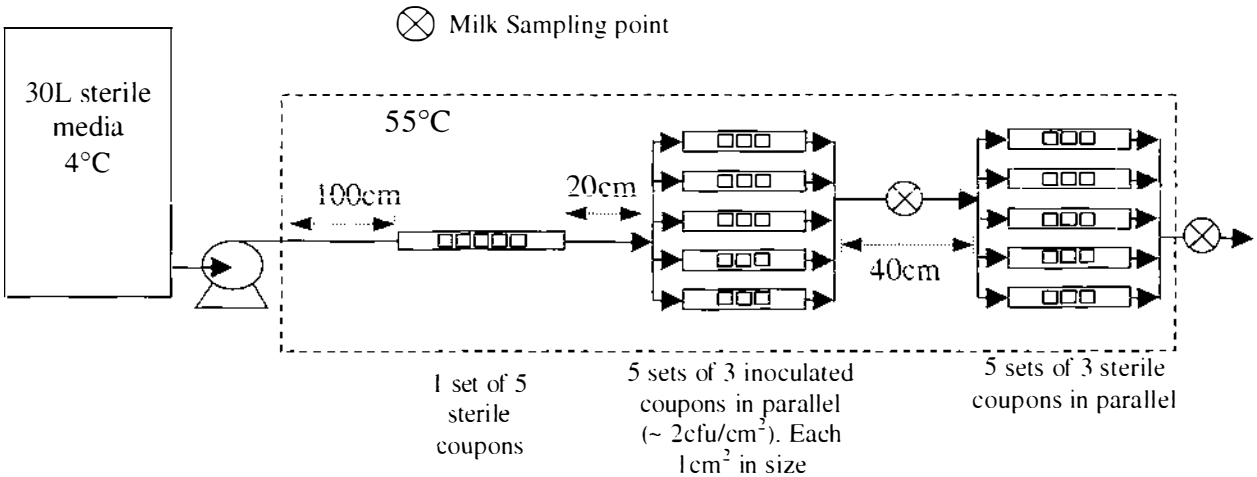
This lab scale experiment was used to identify the dominant transport mechanism of bacteria through the plant. Studies were conducted under flow (convection forces present) and static conditions.

The study with flow present utilised a bench top lab scale rig shown in Figure 3.6.2. The rig was constructed of flexible Masterflex tubing (Cole Parmer Instrument Co., Vernon Hills, Illinois) of different sizes. Coupons were inserted into 7.9 mm ID tubing (96400 – 18). These were connected with 4.8 mm ID tubing (96400 – 15) and 3.1 mm ID tubing (96400 –16) as shown in Figure 3.6.2. To help obtain even flow through the 5 parallel sets of coupons, 1.6 mm ID tubing (96400 – 14) was inserted inside the 4.5 mm ID tubing to add flow resistance. The approximate tube lengths between the sections of the rig are shown on Figure 3.6.2.

A combination of clean sterile (cleaned in 1 % 50 °C caustic for 20 minutes and autoclaved at 121 °C for 15 minutes) and inoculated (inoculated by exposure to an active bacterial culture of *B. stearothermophilus* containing $5x10^6$ cfu.ml^{-1} for 2 minutes) coupons were used. The inoculation left approximately 1.7 ± 0.5 cfu.cm^{-2} thermophiles on the surface of the coupons. This number was estimated by inoculating 18 coupons and testing three for surface numbers using impedance, while using the

other 15 in the experiment (5 parallel sets of 3 coupons) and assuming a surface population equal to the average of the three tested coupons. Five sterile coupons were placed upstream of the inoculated coupons to test for spread of contamination against the direction of flow. Sterile coupons were also placed down stream of the inoculated coupons to test for spread of contamination in the direction of flow (5 parallel sets of 3 coupons).

The experiment was run for 15 hours and used 30 litres of TSB (Difco, Becton Dickinson & Co., Sparks, USA). Samples of coupons from the two different groups of parallel coupon sets (the inoculated coupons and the coupons downstream of the inoculated coupons) were taken every three hours and surface numbers were measured using impedance (Section 3.5.5). These coupon samples were taken by removing one of the 5 parallel coupon sets from each group. This provided three coupons for measurement of surface numbers from each group (triplicate determination). The thermophile count in the broth was also measured every three hours prior to removing the coupons (using thermophile plate count technique mentioned in Section 3.5.1). The first 5 initially sterile coupons, located upstream of the inoculated coupons, were all removed after 15 hours. To remove a set of coupons the tubing was clamped off so that the rest of the rig remained closed during sampling.



Detail of tube connections:

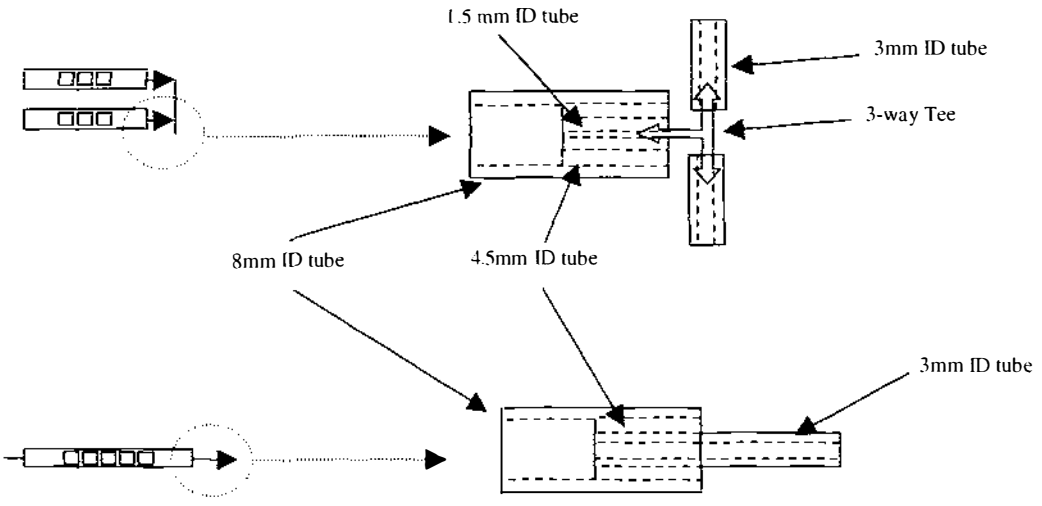


Figure 3.6.2. Bench scale convection experiment rig.

The peristaltic feed pump (7016, Masterflex, Barant, Barrington, IL, USA) was calibrated so that the flow rate could be adjusted quickly. The flow was adjusted each time a parallel set of coupons was removed to keep the flow rate past the coupons in the parallel sets constant. The initial total flow rate was 55.5 $ml.min^{-1}$, then as the number of parallel sets of samples decreased, the flow was decreased accordingly, to 44.4, 33.3, 22.2, then 11.1 $ml.min^{-1}$ for the last 3 hours. Keeping the flow in each parallel section constant in this way gave a flow velocity of approximately 0.36 $cm.s^{-1}$ past the coupons in each parallel section of tube.

A temperature of 55 °C was maintained in the tubing by immersing it in a water bath at 55 °C. Broth was pumped into the rig at 4 °C and the one metre length of tubing that was immersed the water bath before any coupons were reached, provided enough time for the broth to warm to the water bath temperature.

The experiments carried out under static conditions were used to determine the distance that the thermophiles could travel without convection forces present. This distance could then be compared to the speed of transport possible with flow present.

Static experiments used a 1.2 m long piece of Masterflex tubing (Norprene, 6404 – 18, Cole Parmer Instrument Co., Vernon Hills, Illinois) filled with autoclaved milk (autoclaved at 121 °C for 15 minutes). This milk filled tube was then inoculated at one

end with a 1 cm^2 stainless steel coupon (316 stainless steel with 2B surface finish) previously immersed in active thermophile culture of *B. stearothermophilus* (2 $x10^7$ $cfu.ml^{-1}$ for 10 minutes). Before inoculation coupons were cleaned in 1 % w/w caustic soda at 50 °C before being rinsed and autoclaved at 121 °C for 15 minutes. The inoculation left 6 $x10^3 \pm 3$ $x10^3$ $cfu.cm^{-2}$ thermophiles on the surface of the coupon. This number was estimated by inoculating four coupons and testing three for surface numbers using impedance, while using the fourth in the experiment and assuming it had a surface population equal to the average of the other three coupons. Once the coupon was inserted, the rig was left at 55 °C with no liquid movement for 5 hours. After this time 15 cm sections were clamped off at a time, starting from initially sterile end of tube. The milk contained in these sections was then tested for the presence of thermophiles by conducting a plate count on 1 ml (undiluted) of the milk from each section. A second trial was also conducted in the same way except that the tube length was shortened to 0.8 m and 10 cm sections were clamped off at a time to obtain a better estimate of the distance that the thermophiles could travel. The estimated surface thermophile population of the coupon used to inoculate the second trial was slightly higher at $8x10^3 \pm 4$ $x10^3$ $cfu.cm^2$.

3.6.3.2. Pilot Plant Experimental Run (Run 5)

This experiment (Run 5) was performed on the pre-heat section of a milk powder pilot plant to investigate the downstream contamination of the plant from an upstream thermophile source.

The tubular heat exchanger (THE) bank was used to study the thermophilic bacterial contamination of the heated milk stream. The milk temperature was held at 55 °C throughout the THE. An initially contaminated tube was inserted at the start of one side of the THE. Comparisons between the milk contamination from each side could then be made. A diagram of the equipment is shown in Figure 3.6.3.

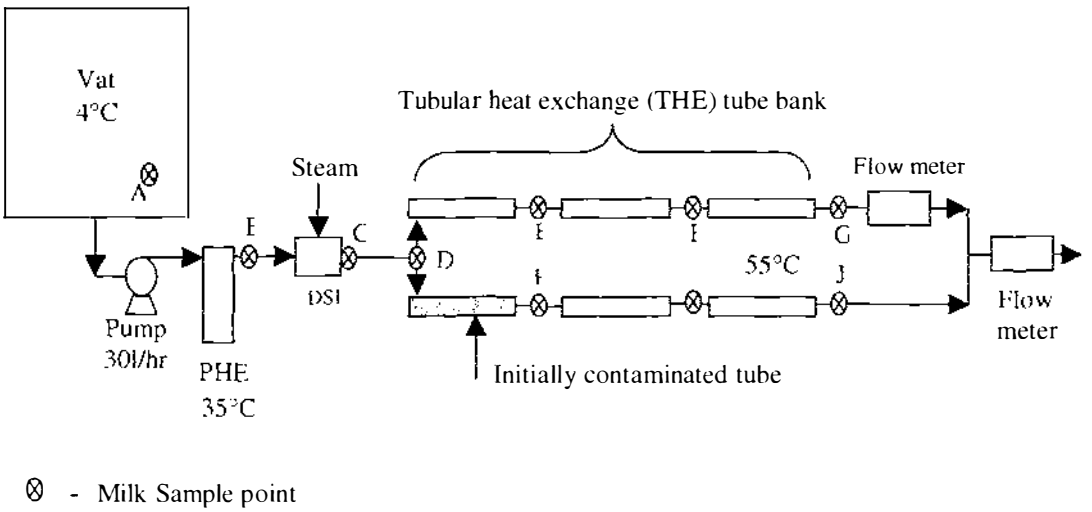


Figure 3.6.3. Diagram of pilot plant equipment used in the pilot plant recontamination experiment.

The initially contaminated tube was prepared by pre-fouling the surface of an inner tube of the tubular heat exchanger with a milk-fouling layer around 1-2 mm thick. Pre-fouling was carried out with a bulk milk inlet temperature of 65 °C and a hot side temperature of 95 °C for a period of four hours. A photograph of the fouling on the pre-fouled inner tube is shown in Figure 3.6.4. To inoculate the surface, the tube was then left overnight (10 hours) in a rig circulating 55 °C autoclaved milk that had been inoculated with the selected isolate of *B. stearothermophilus* used in the rest of the work. A peristaltic pump (7018, Masterflex, Barant, Barrington, IL, USA) was used to provide a flow rate of 250 $l.hr^{-1}$. A diagram of this rig is shown in Figure 3.6.5. Inoculation of the surface in this way provided $4x10^4 \pm 2x10^4$ $cfu.cm^2$ on the tube surface. This measurement was obtained by scraping 3 small 1 cm^2 portions of the fouling and measuring the surface numbers via the impedance technique.

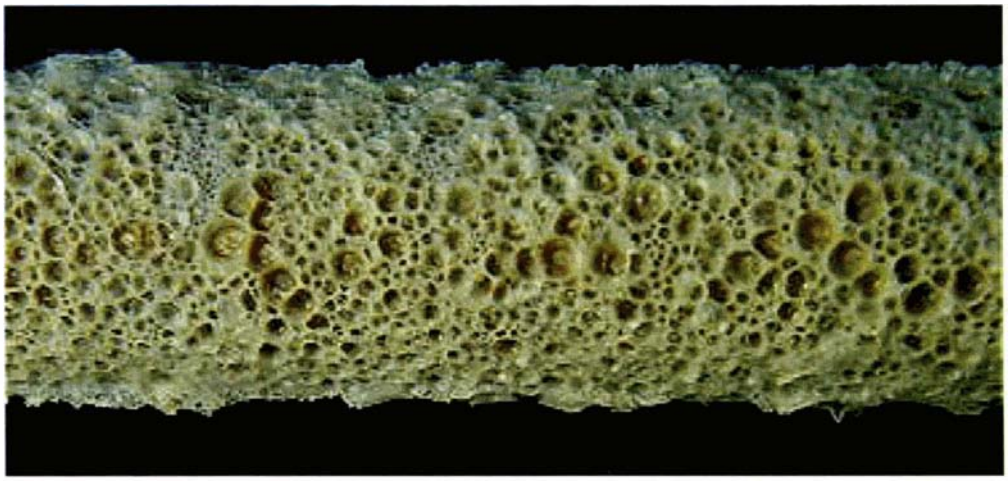


Figure 3.6.4. Photograph of pre-fouled inner tube after four hours of fouling with the milk inlet temperature at 65 °C and the hot side temperature at 95 °C.

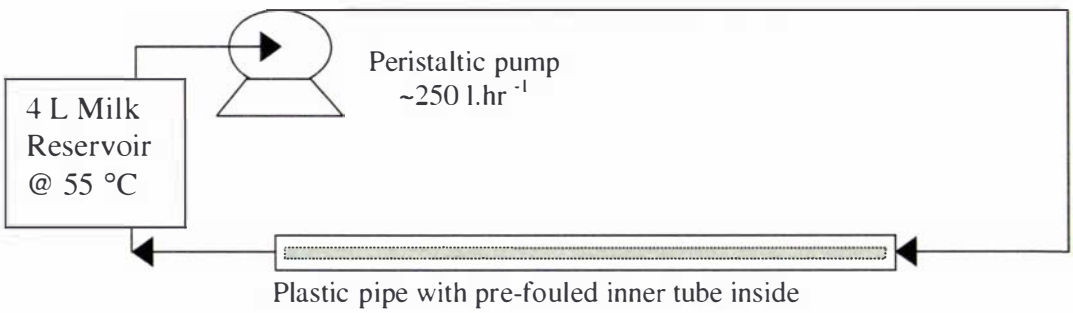


Figure 3.6.5. Rig for inoculation of the contaminated pre-fouled inner tube.

Milk samples were taken from 10 different points of the pilot plant every 4 hours to assess thermophilic vegetative cell and thermophilic spore counts over 24 hours. These points are labeled A to J as shown on Figure 3.1.2.

Surface thermophile numbers in the THE at the end of the experimental run were assessed by scraping small 1 cm^2 sections of the fouling layer and testing these using the impedance method.

The effect of varying the initial inlet bulk concentration of thermophiles on the bulk contamination over time was also investigated. This was carried out by comparing the bulk contamination from different pilot plant runs that had different initial thermophile concentrations in the milk and where the THE was initially clean.

3.6.4. Adhesion investigations

3.6.4.1. Adhesion

The detailed theory behind the adhesion study is given later in Section 4.4. Adhesion experiments measuring the numbers of cells (n_w) adhering to the surface with varying bulk concentration (C_b) and varying adhesion time (t_a) were conducted to get estimates of the adhesion constant (k_a).

$$k_a = \frac{(\Delta n_w / \Delta t_a)}{C_b} \qquad 3.4$$

Where n_w is the measured number of adhered cells (cfu.cm^{-2}), t_a is the adhesion time (s), k_a is the adhesion rate constant (cm.s^{-1}) and C_b is the concentration of bacteria in the bulk liquid (cfu.ml^{-1}).

In this study the adhesion of *Bacillus stearothermophilus* to whole milk foulant, skim milk foulant and to stainless steel was investigated under quiescent conditions with varying bulk cell concentrations and varying adhesion times to estimate the rate of adhesion of thermophiles to surfaces within milk powder pilot plants.

3.6.4.2. Method

Whole milk and skim milk fouling layers were created on stainless steel foil surfaces in the milk powder pilot plant using the MHE rig (Figures 3.6.6 and 3.6.7). The clean sterile surfaces were fouled for 4 hours in milk flowing at 30 litres per hour. The milk was heated firstly by the plate heat exchanger (PHE) to 50 °C then by direct steam injection (DSI) to 65 °C at the mini plate heat exchanger (MHE) rig inlet. Hot water at 95 °C heated the hot side to develop fouling on the plate surfaces.

As well as measuring adhesion to whole milk and skim milk fouling layers that had developed for four hours, six whole milk fouling layers were created with fouling durations of 1, 2, 3, 4, 5 and 6 hours. In this way the adhesion to fouling layers of increasing age could be measured. Photographs of the different types of fouling structures created are shown in Figure 3.6.8.

To measure adhesion to stainless steel the same clean and sterile stainless steel foil used in the creation of fouling layers was used.

Actively growing cultures of *B. stearothermophilus* grown at 55 °C in Tryptic Soy Broth (Difco, Becton Dickinson and Co., Sparks, USA) were used for adhesion studies. Bulk cell numbers were measured using a direct cell count on the broth and cultures were used in adhesion studies once the bulk concentration reached 10^8 cfu.ml^{-1}, near the end of the exponential growth phase. Viable cell numbers were measured with a vegetative plate count on the culture following the method mentioned previously in Section 3.5.2 to determine thermophile numbers from milk samples.

Cultures were diluted in peptone water (Merck, Darmstadt, Germany) to obtain different concentrations of bulk cells for adhesion studies. The bulk cell concentration of these diluted cultures was estimated from the dilution ratio, assuming perfect dilution. A range of bulk cell concentrations was tested from 10^2-10^7 cfu.ml^{-1}.

The adhesion test was carried out in quiescent conditions by adding 5 ml of diluted culture to a test tube containing a small piece of surface with a measured surface area of approximately 0.5 cm^2. Adhesion to the stainless steel on the clean side of fouled samples was avoided by covering the un-fouled side with adhesive tape during immersion in the culture. The tape was then removed before measuring the adhered number of cells. This was important when measuring the adhesion using impedance microbiology (see below) to ensure that only bacterial activity on the fouled side was measured.

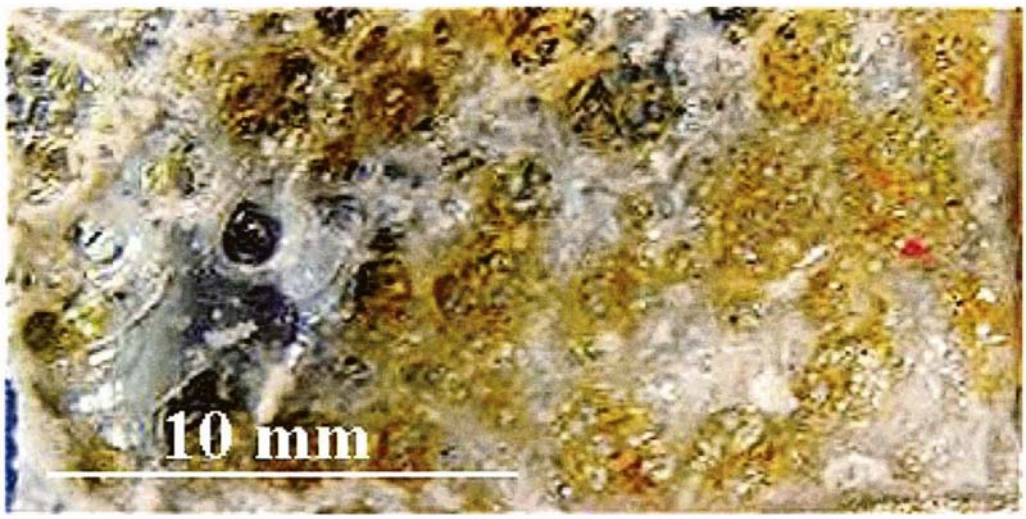


Figure 3.6.6. Structure of skim milk fouling layer used in adhesion studies.

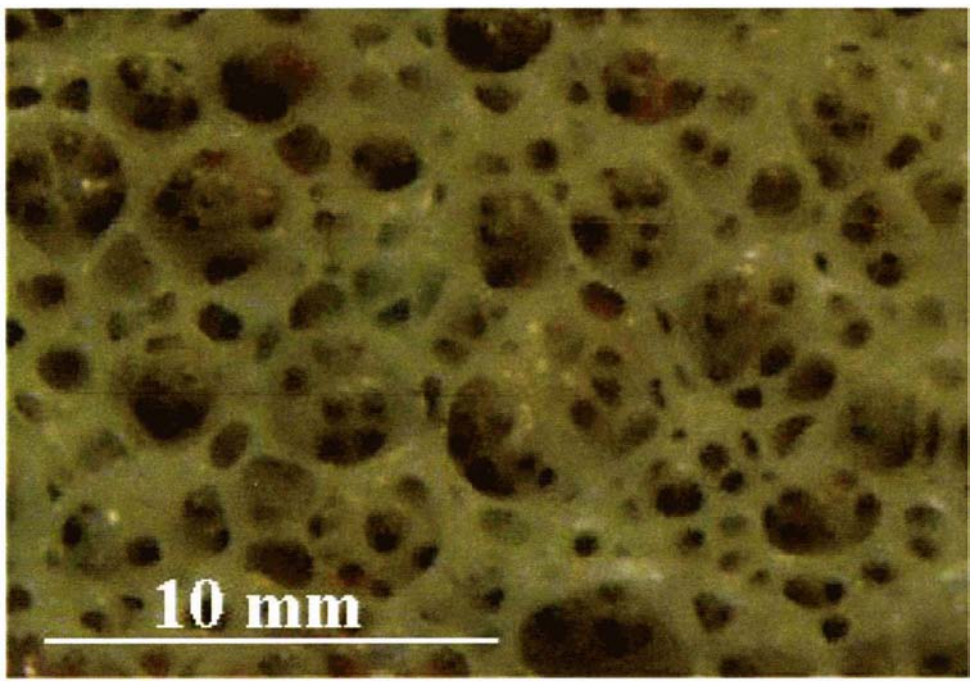


Figure 3.6.7. Structure of whole milk fouling layer used in adhesion studies.

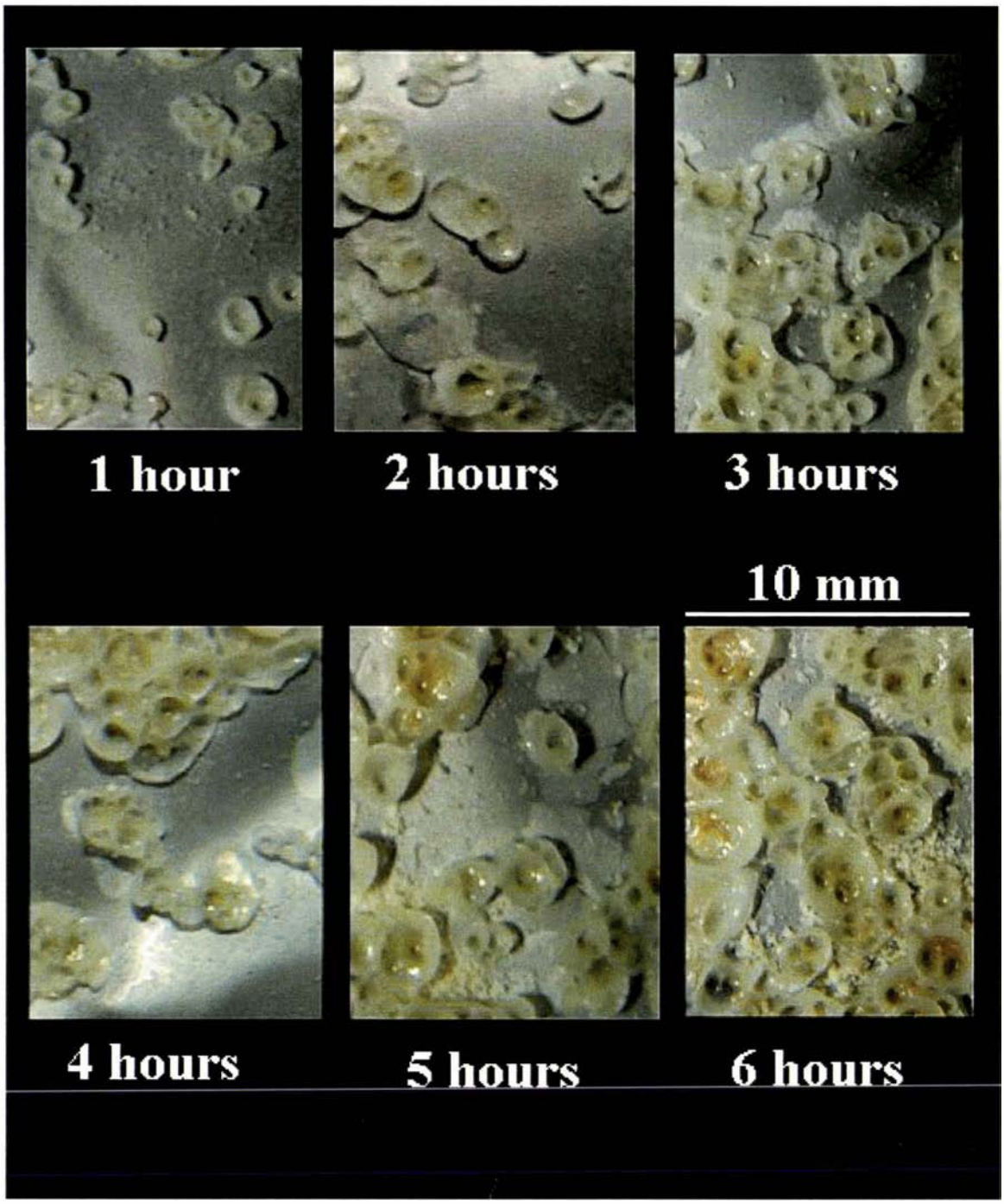


Figure 3.6.8. Structures of whole milk fouling layers used in adhesion studies where the amount of fouling was varied based on the fouling duration.

After each sample had been immersed in the culture for the given adhesion time, the sample was rinsed thoroughly in a test tube with five rinses of sterile distilled water. Three different methods were used to measure the numbers of cells adhering to the surfaces. These were confocal laser scanning microscopy (CLSM), epi-fluorescence

microscopy and impedance microbiology. These have been previously described in more detail in Section 3.5.

The surface samples intended for microscopy were all given a 20 minute adhesion time. This method examined the adhesion to different types of surfaces, including the skim milk and whole milk fouling surfaces obtained after 4 hours, the surfaces with different amounts of fouling, and to the clean un-fouled stainless steel.

Samples assessed by impedance microbiology were subjected to a range of adhesion times from 10 seconds to 20 minutes. This methodology was used to test adhesion to whole milk fouling layers with a constant amount of fouling and to clean stainless steel. Four trials were conducted, two with a constant adhesion time of 20 minutes and varying bulk cell concentrations, and two with constant bulk cell concentration but varying adhesion times of 10 s, 30 s, 1 min, 2.5 min, 5 min, and 10 min. The conditions used for each type of sample are outlined in Table 3.6.1.

The fouling layers created were not sterile so the residual numbers of thermophiles on the fouling layers were measured before any adhesion. This residual surface population was taken into account by subtracting the average of the control measurements from the measurements obtained after adhesion and combining errors of the two.

An overview of the surfaces, bulk cell concentrations, adhesion times and measurement methodologies used is shown in Table 3.6.1.

Table 3.6.1. The range of samples, adhesion times, bulk cell concentrations and measurement methods used in the adhesion studies.

Surface	**Adhesion times tested**	**Bulk cell concentrations tested (cfu.ml^{-1})**	**Measurement method used**
Skim milk foulant	20 mins	10^3-10^7	CLSM
Whole milk foulant (varying amount)	20 mins	10^7	CLSM
Whole milk foulant (constant amount - trial 1)	20 mins	10^3-10^7	CLSM
Whole milk foulant (constant amount - trial 2)	10 secs – 20 mins	10^2-10^7	Impedance microbiology
Stainless steel (trial 1)	20 mins	10^5-10^7	Epi-fluorescence microscopy
Stainless steel (trial 2)	10 secs – 20 mins	10^2-10^7	Impedance microbiology

3.7. Data Processing

3.7.1. Plate counts

Plate count data were entered into Excel (97 version, Microsoft Corporation) spreadsheets for analysis. This spreadsheet was in the form of a template, where numbers counted per dilution could be entered and the bulk numbers in $cfu.ml^{-1}$ calculated. Statistical analysis was also carried out in Excel such as regression. Excel was also used to plot all graphs.

3.7.2. CLSM

Images of the depth scan of each field were opened in Paint Shop Pro 5 (version 5.00, Jasc Software Inc.) and the colonies per field counted manually either on screen or via a print out if many were present. These data were entered into Excel, where surface populations were calculated and statistical analysis for confidence intervals on counts carried out.

The "CONFIDENCE" function in Excel was used to calculate 95 % confidence intervals on the mean. This function uses the following formula:

$$x \pm 1.96.\sqrt{\frac{\sigma}{\sqrt{n}}} \qquad 3.5$$

Where, χ is the mean of the samples, σ is the population standard deviation and n is the sample size.

3.7.3. Plant data

Historical plant data were downloaded in form of *.csv files. These were opened in Excel for further analysis and plotting.

3.7.4. Impedance

Impedance detection times were entered into Excel and were used to calculate the surface populations per square centimeter of surface area. If triplicate (or greater) determinations were available the standard error and 95 % confidence intervals on the mean were calculated using Excel as for CLSM data.

3.8. Thermophile modelling

Steady state and un-steady state thermophile contamination models were developed.

3.8.1. Steady state

A mathematical equation was derived from first principles to describe the release of thermophiles into milk from pipe surfaces. Experimental data were compared to model predictions. This is covered further in Section 4.5.

3.8.2. Unsteady state

MATLAB Version 5.2 (The Mathworks Inc. Natick, MA) was used to solve set of ordinary differential equations (ode45 solver) describing growth and release of thermophiles from the plant pipe surfaces into the bulk milk stream. Experimental data were compared to model predictions, including attachment rate. This model is covered further in Section 4.5. Matlab files and example model input and output can be found in Appendix E, pages E-1 to E-6.

4. Results and Discussion

This chapter is organised into sections discussing the different elements of the overall work as follows:

1. bulk milk contamination and surface numbers in pilot plant studies,
2. thermophile survival during cleaning,
3. re-contamination of the pilot plant from residual thermophiles after cleaning,
4. adhesion of thermophiles to surfaces and
5. modelling of thermophile contamination within the pilot plant

4.1. Bulk Milk Contamination and surface numbers

This section covers the release of thermophilic bacteria and spores into the bulk milk stream of the pilot plant and the growth of thermophilic bacteria on surfaces within the pilot plant. The contamination by thermophiles and spores that are released from surfaces into the bulk milk stream is discussed along with the growth of the thermophiles at the surface.

4.1.1. Typical thermophile contamination profile in pilot plant

Experimental runs on the pilot plant showed that the thermophile contamination of the bulk milk stream followed a typical profile. In Figure 4.1.1, the numbers of thermophiles present at different locations in the pilot plant are plotted against time. The numbers of thermophiles in the milk stream entering the plant (at the vat) are relatively low, in this case less than 300 cfu.ml^{-1}. Contamination by thermophiles begins only after the plate heat exchanger (PHE) where the milk temperature reaches 40 °C. In this pilot plant, the numbers of thermophiles exiting in the milk stream reach steady state concentrations of the order of 10^6 to 10^7 cfu.ml^{-1} after approximately 8-12 hours of operation. Since the typical residence time of milk in the pilot plant is less than 10 minutes, the planktonic growth in the milk stream cannot provide the amount of contamination observed in this time scale. Therefore the contamination must come from thermophiles attached to, growing on and then releasing from product contact surfaces within the plant.

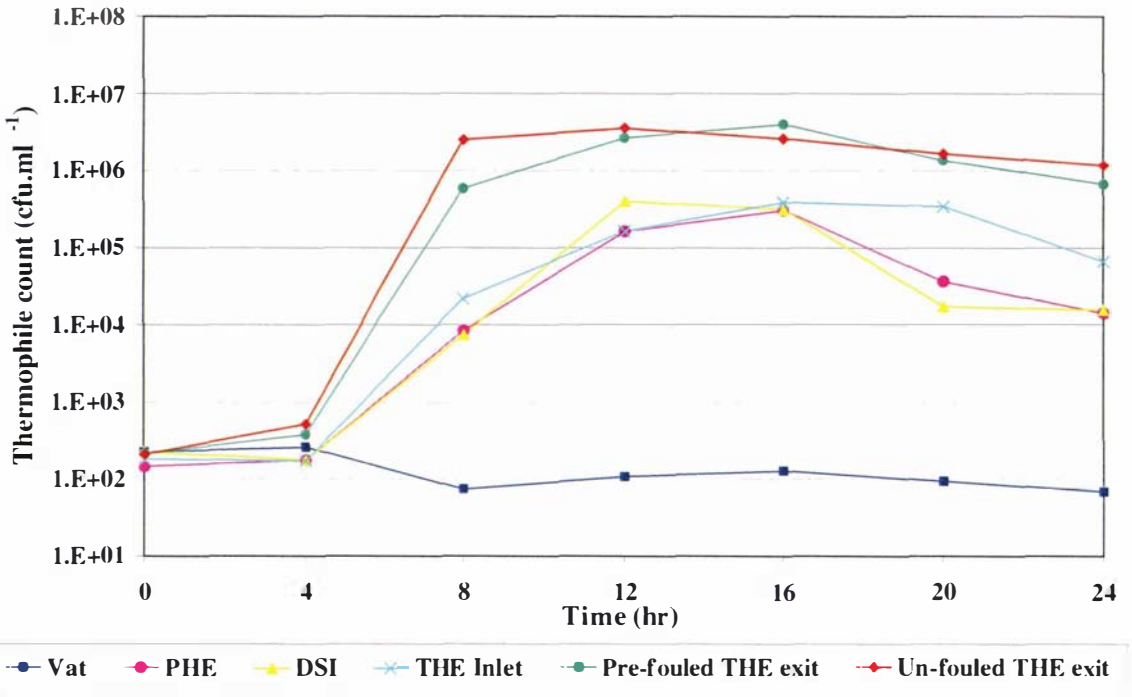


Figure 4.1.1. Thermophile bulk contamination at selected locations across pilot plant over time during Run 4. The pre-fouled and un-fouled THE were installed in parallel. Typical temperatures at each position were: Vat 4 °C, PHE 40 °C and other positions 55 °C.

The spore contamination across the pilot plant in the bulk milk also followed a typical profile, as shown in Figure 4.1.2, where the level of spores is now plotted at selected positions over time. Elevated numbers of spores were not observed in the bulk milk stream until after 12-16 hours of operation. The steady state contamination concentration was also lower than for vegetative cells at 10^4-10^5 cfu.ml^{-1}. However, the number of spores entering the plant from the vat are less than 10 per ml. Therefore this again shows that attached growth must be involved to produce this amount of contamination in the short resisdence time available.

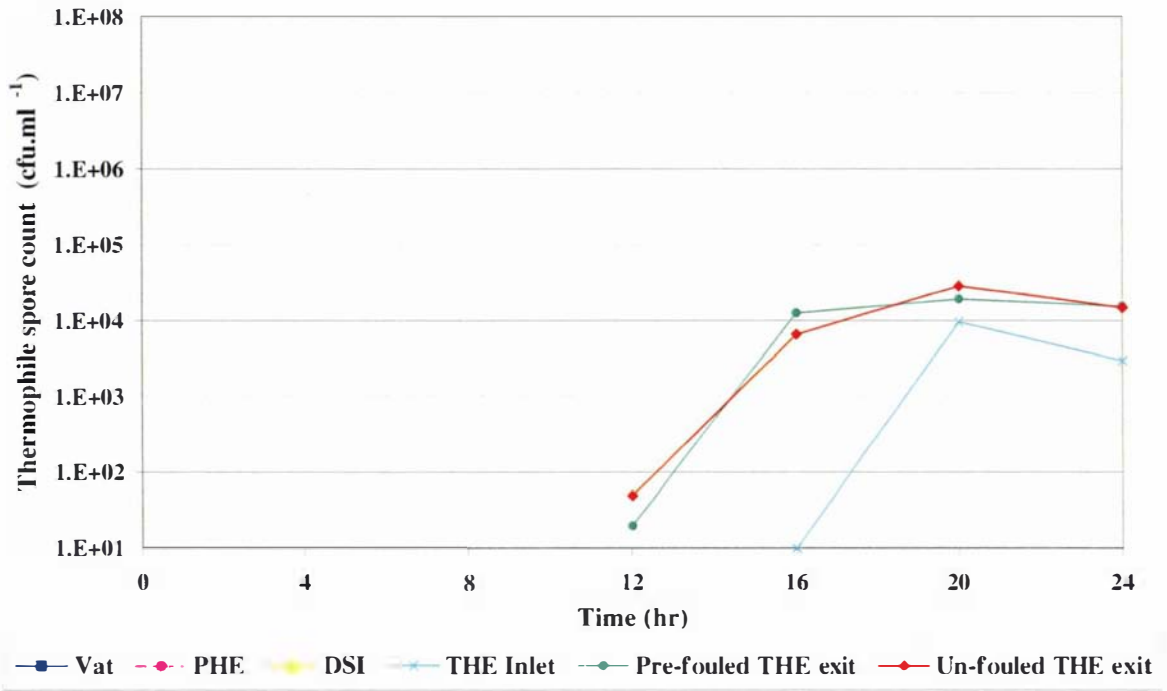


Figure 4.1.2. Thermophilic spore bulk contamination at selected locations across pilot plant over time during Run 4. The pre-fouled and un-fouled THE were installed in parallel.

4.1.2. Location of Thermophile bulk contamination

The contamination of the bulk milk flow from the inoculated thermophile strain was successfully targeted downstream of the DSI through temperature control in the experiments. Figure 4.1.1 shows typically that the microbial population in the bulk milk after the THE rig was one to two orders of magnitude greater than that after the PHE. This result validated the design of the plant, which sought to encourage the attached thermophile growth in specific locations that could be dismantled for observation. Data from the samples taken after 20 hours of operation already shown in Figure 4.1.1 are re-plotted differently below in Figure 4.1.3 to illustrate the distribution along the path of milk flow. This form of data representation clearly shows that the contamination is greatest in the THE rig. The high thermophile count at the THE inlet is due to attached growth in the 1 metre long pipe connecting the DSI to the THE. This was considered unavoidable and the connecting pipe length was minimised at 1 metre to reduce the contamination entering the THE rig.

Figure 4.1.3 indicates that some contamination occurs from the PHE but this contamination actually came from a different strain of thermophile than the target *Bacillus stearothermophilus* (type Cm) strain used to inoculate the vat. This strain could be enumerated by plate counts separately from the target strain, due to the distinct spreading colony morphology that it formed and was identified by RAPD as *Bacillus licheniformis* (type F/G). *B. licheniformis* (type F/G) was found to be the naturally dominant thermophile in the pasteurised milk used in all the pilot plant experiments and was initially present at concentrations of 1-100 cfu.ml^{-1}. As it was found to contaminate the PHE, which is at a lower temperature than the THE (35 to 40 °C compared to 55 °C), it seems better suited than the *B. stearothermophilus* strain to these lower temperature regions. No increase in contamination from the inoculated *B. stearothermophilus* strain was observed until after the DSI as shown in Figure 4.1.3. However, numbers of this strain in the low temperature areas of the pilot plant were difficult to quantify from plate counts, as the *B. licheniformis* numbers were much more numerous (around three orders of magnitude greater). Likewise *B. licheniformis* numbers were difficult to quantify in the higher temperature areas when the *B. stearothermophilus* strain was dominant. The occurrence of growth from two different strains of thermophile in the pilot plant also shows that it is possible for more than one strain of thermophile to be found in elevated numbers after the preheat section of a milk powder plant. The different temperature zones provided in a pre-heat section of a production plant could provide locations where different strains could dominate, just as observed on the pilot plant. However, the proportions of the thermophiles found in the powder may not line up with what is released from the pre-heat section, as this depends on the relative ability of the strains to survive further downstream processing.

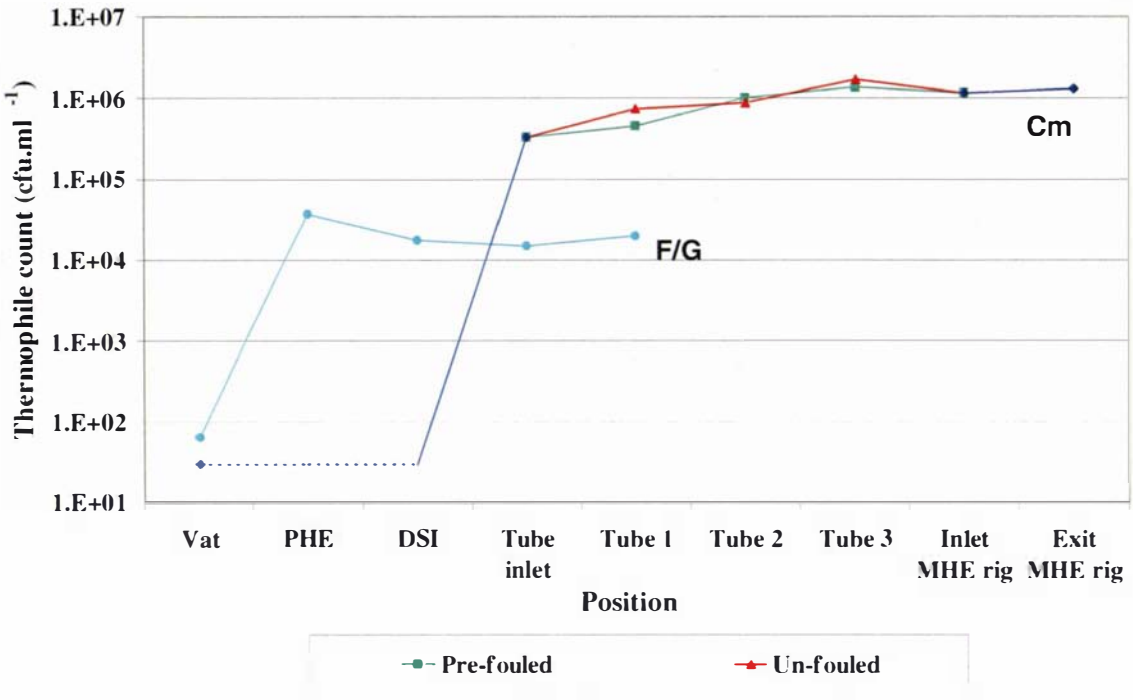


Figure 4.1.3. Increase in thermophile contamination in the bulk milk as it passes through the pilot plant after 20 hours of operation (Run 4). Two different strains of thermophile were found to contaminate the plant, the inoculated *Bacillus stearothermophilus* (type Cm) strain and a naturally occurring *Bacillus licheniformis* (type F/G) strain.

4.1.3. Bulk contamination and contact surface area.

Down stream of the DSI in the main thermophile contamination region of the pilot plant, the bulk milk contamination was proportional to the amount of wetted surface contact area that the milk passes, as shown in Figure 4.1.4 and Figure 4.1.5. The wetted surface contact area is defined as the amount of surface area of stainless steel pipe that is in contact with the milk as it flows past. Figure 4.1.4 shows the bulk contamination along the THE in Run 4, with bulk contamination values from the end of each tube plotted against the wetted milk contact surface area (1200 cm^2 per tube) for both the pre-fouled and un-fouled tube sets. This trend for bulk contamination to increase linearly with wetted milk contact surface area was observed in all the runs performed in this work. The data from Run 2 is shown in Figure 4.1.5

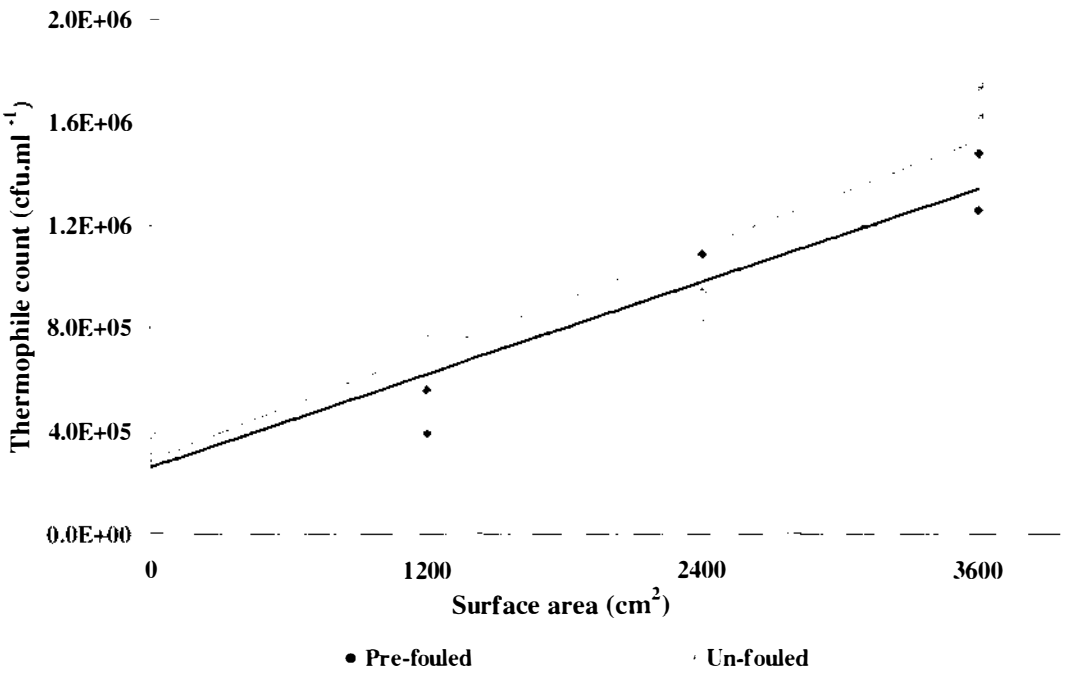


Figure 4.1.4. Thermophile bulk contamination along the THE at 20 hours during Run 4. Bulk contamination values from the end of each tube are plotted against surface area as each tube provides 1200cm^2 of wetted milk contact surface area.

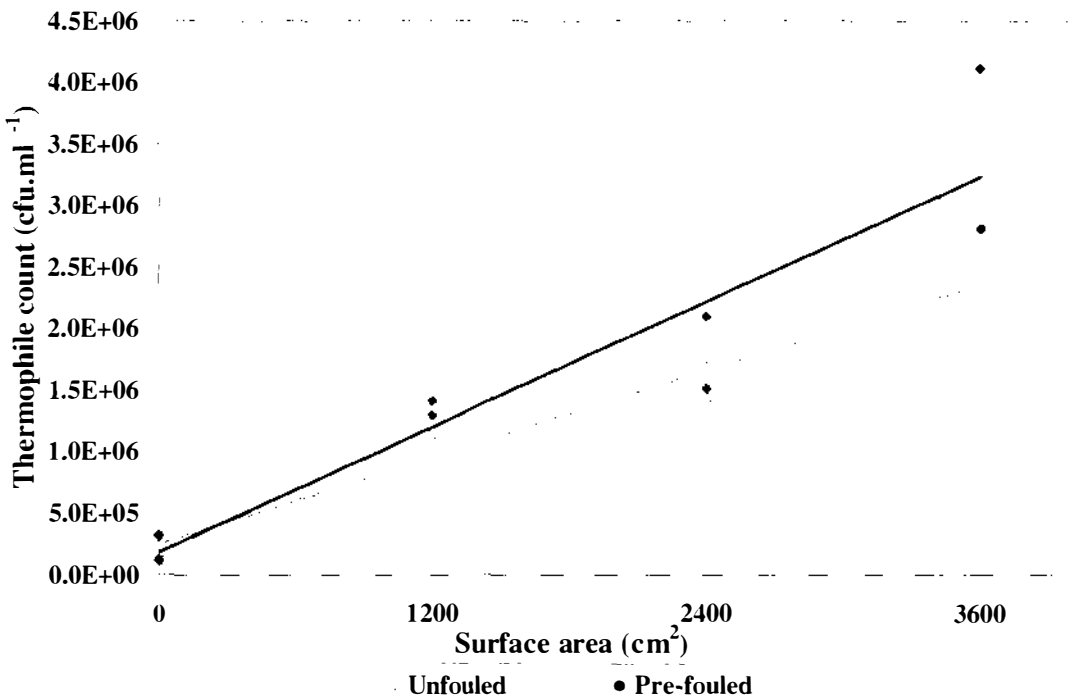


Figure 4.1.5. Thermophile bulk contamination along the THE at 12.5 hours during Run 2.

The number of spores released also increases linearly with surface area as shown in Figure 4.1.6 and Figure 4.1.7.

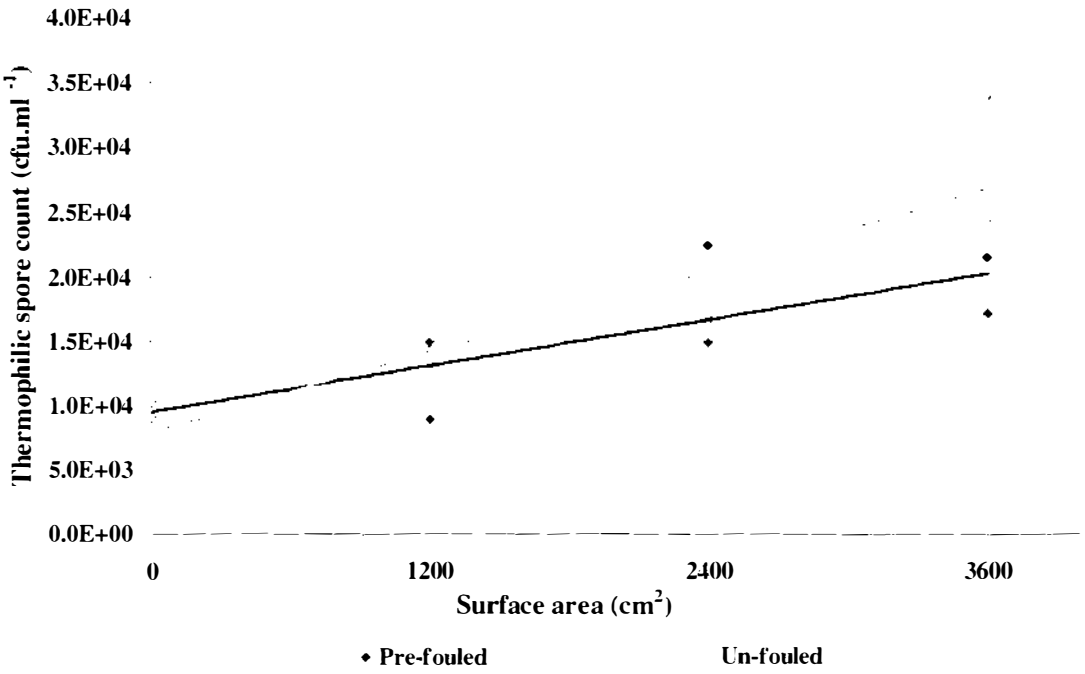


Figure 4.1.6. Thermophilic spore bulk contamination along THE at 20 hours during Run 4.

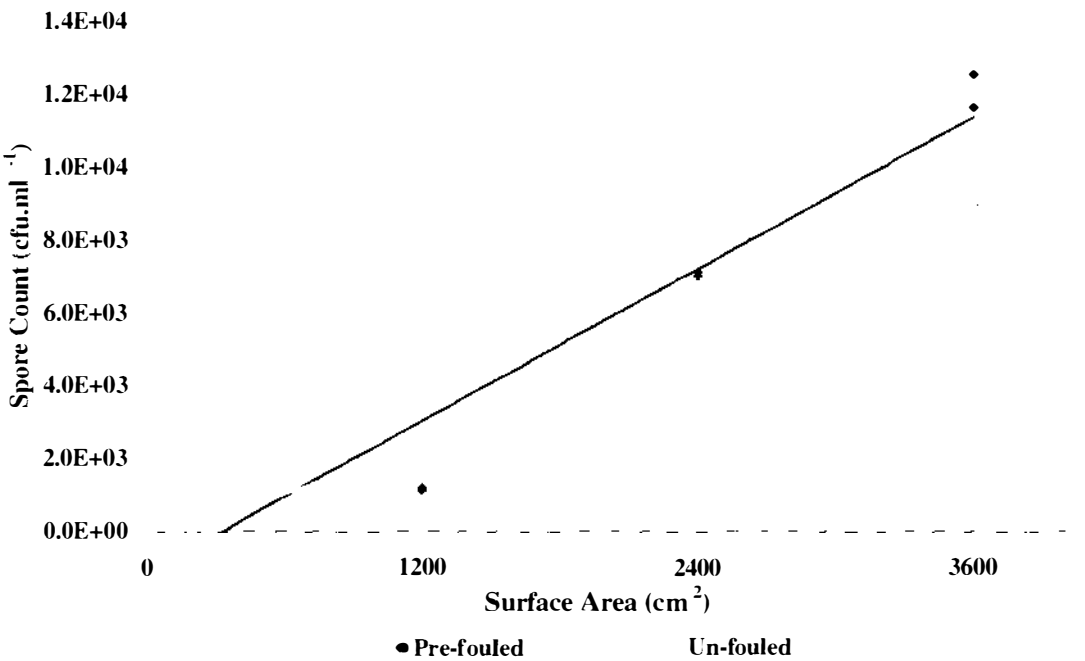


Figure 4.1.7. Thermophilic spore bulk contamination along THE at 12.5 hours during Run 2.

Since thermophilic bacterial contamination of the bulk milk stream is proportional to the surface contact area in the thermophilic bacteria growth temperature zone, it is important to design milk powder plants with a minimum amount of surface contact area available in this region. This then should provide the maximum possible production length before thermophilic bacteria or spores in the milk stream reach specification limits.

4.1.4. Contamination rate from clean and fouled surfaces.

A series of experiments were performed to elucidate the interaction between fouling and thermophile contamination. In the first 4 runs, flowing milk was heated in a clean THE and in a pre-fouled but sanitized THE arranged in parallel to use the same batches of milk. Within experimental error, thermophile contamination of the bulk milk arising from colonised pre-fouled and un-fouled stainless steel surfaces was similar at steady state in all the experimental runs where it was compared (Runs 1- 4). This similarity in the contamination levels can be seen in the data from the two tube banks presented above in Figure 4.1.4 for Run 4 and Figure 4.1.5 for Run 2.

The amount of thermophile contamination released into the bulk milk per unit area of wetted contact surface in the THE is shown in Figure 4.1.8 for Run 2. In Run 2 only the inner tubes were pre-fouled. This graph was generated by calculating through regression the slope of curves similar to that presented in Figure 4.1.5 for samples at 12.5 hours. The data are shown in Appendix B, pages B-6 to B-7. As shown in Figure 4.1.8, the 95 % confidence intervals for the thermophile release over time from both tube sets overlap, indicating that the values are not significantly different.

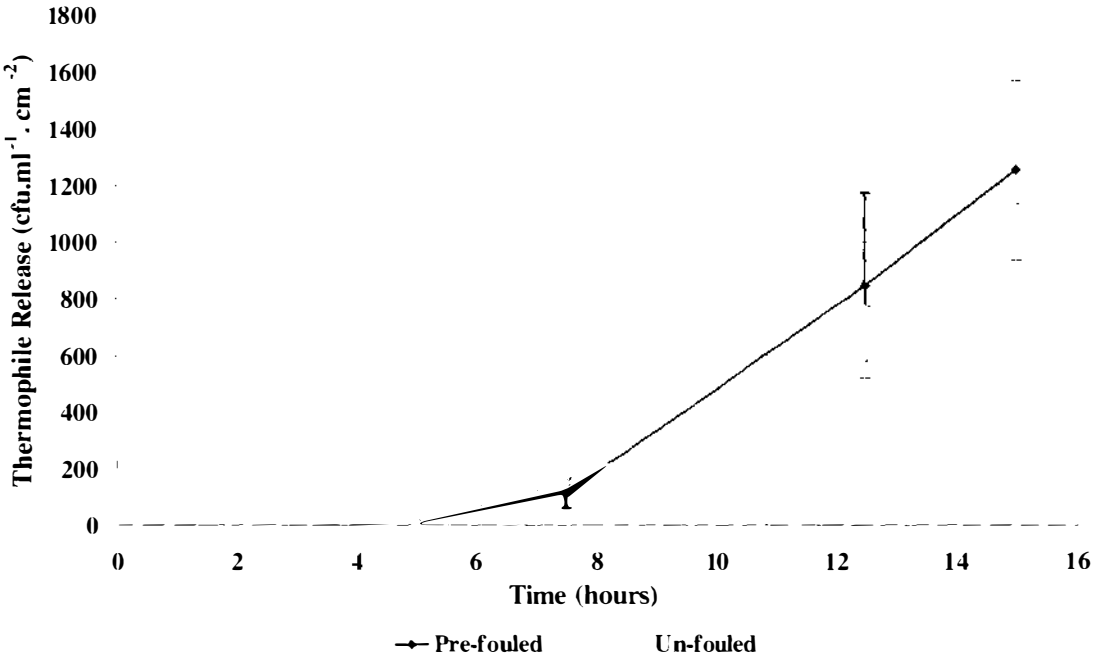


Figure 4.1.8. Thermophiles released into the bulk milk from the THE per unit wetted surface contact area over time for Run 2. These values are calculated from the slope of graphs such as in Figure 4.1.5. Errors bars show 95 % confidence intervals in the prediction of the slope through regression.

As for Run 2, the thermophile release along the THE per unit area of wetted contact surface for Run 4 was calculated and is shown in Figure 4.1.9. In Run 4 both the outer surface of the inner tube and the inner surface of the outer tube were pre-fouled. Figure 4.1.9 shows again that the 95 % confidence intervals for the thermophile release from 12 to 24 hours from both tube sets overlap, indicating that the values are not significantly different.

However, the contamination from the pre-fouled tube in Run 4 appears to lag behind the contamination from the un-fouled tube. After eight hours operation in Run 4, as shown on Figure 4.1.9, the thermophile release from the pre-fouled THE tube set was significantly lower than that of the un-fouled tube set (160 ± 20 cfu.ml^{-1}.cm^{-2}, compared to 730 ± 80 cfu.ml^{-1}.cm^{-2}). A possible explanation for this phenomenon is that pre-fouled surface may initially absorb more thermophiles than the un-fouled surface and therefore release fewer than the un-fouled surface. In effect it would be acting as a thermophile sponge, collecting more thermophiles but releasing less. This theory is supported by the assessment of surface numbers below (Figure 4.1.12) which shows a

faster accumulation of thermophiles at the pre-fouled surface than the un-fouled surface. Also in other work, the rate of attachment to fouled surfaces has been measured at ten times that to un-fouled stainless steel (Section 4.4).

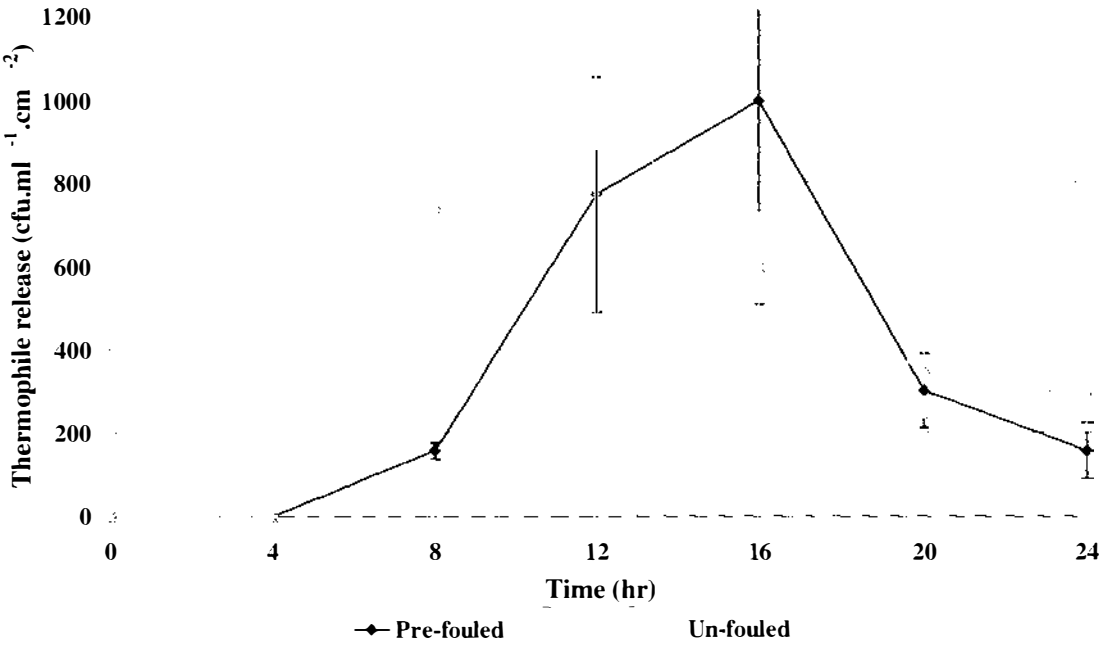


Figure 4.1.9. Thermophiles released into the bulk milk from the THE per unit wetted surface contact area over time for Run 4. These values are calculated from the slope of graphs such as Figure 4.1.4. Error bars show 95 % confidence intervals in the prediction of the slope through regression.

An interesting pattern of thermophile release was also observed during Run 4 (Figure 4.1.9). The release peaks at around 1000 cfu.ml^{-1}.cm^{-2} after 12 to 16 hours then drops to below 400 cfu.ml^{-1}.cm^{-2} when measured after 20 and 24 hours. This pattern was not observed in other runs and may be representative of the cyclic nature of biofilm growth that has been reported by other authors (Parkar *et al.*, 2003).

Spore contamination from the two THE tube sets was not significantly different throughout the experimental runs. Figure 4.1.10 and Figure 4.1.11 show the spore release per unit wetted contact surface area during Run 2 and Run 4 respectively. The spore release is also around 100 times less than the thermophile release. Release of spores ranged from 2-6 cfu.ml^{-1}.cm^{-2} compared to 200-1200 cfu.ml^{-1}.cm^{-2} for vegetative thermophile release.

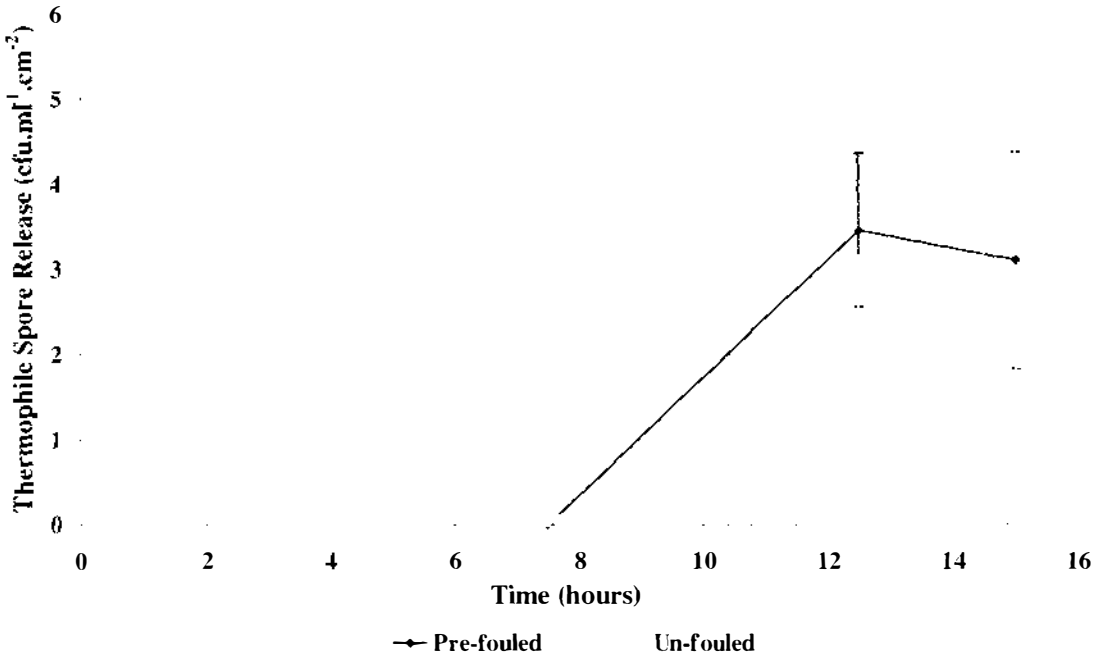


Figure 4.1.10. Thermophilic spores released to the bulk milk from the THE per unit wetted surface contact area over time for Run 2. These values are calculated from the slope of graphs such as Figure 4.1.6. Error bars show 95 % confidence intervals in the prediction of the slope through regression.

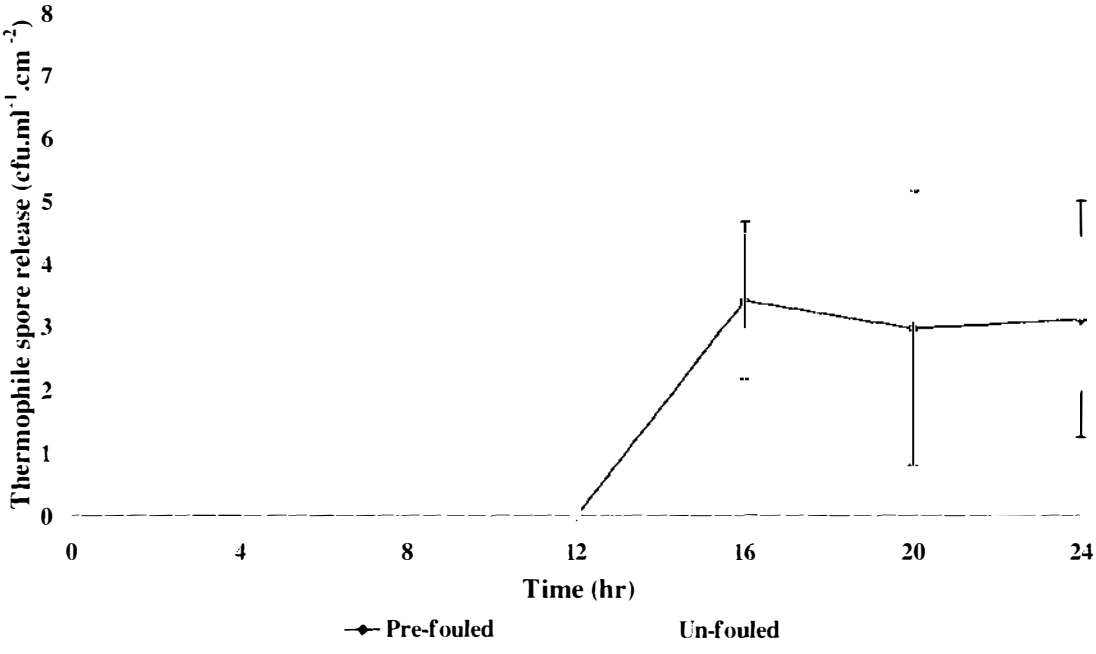


Figure 4.1.11. Thermophilic spores released to the bulk milk from the THE per unit wetted surface contact area over time for Run 4. These values are calculated from the slope of graphs such as Figure 4.1.7. Error bars show 95 % confidence intervals in the prediction of the slope through regression.

4.1.5. Surface populations of thermophiles

4.1.5.1. Surface numbers on fouled and un-fouled surfaces

From Run 5 the surface numbers of thermophilic bacteria measured by impedance on the fouling layer were found to be 10-100 times greater than on the un-fouled stainless steel (shown on Figure 4.1.12). Also, a higher proportion of spores per bacterial population were found on the fouling layer than on the stainless steel (shown in Figure 4.1.13). These numbers measured on the fouling layer are likely to be on surface or in locations where they can access the surface. The reader is reminded that in Run 5 (and all other runs except Run 3) both the heat exchanger surface and the milk stream were kept at 55 °C to encourage thermophile growth on pre fouled and clean surfaces and at the same time avoid further fouling that occurs significantly only above 65 °C. The thermophiles would not be expected to be trapped within the fouling layer as the fouling layer is not growing during the experiment and bacteria can attach only to the fouling surface.

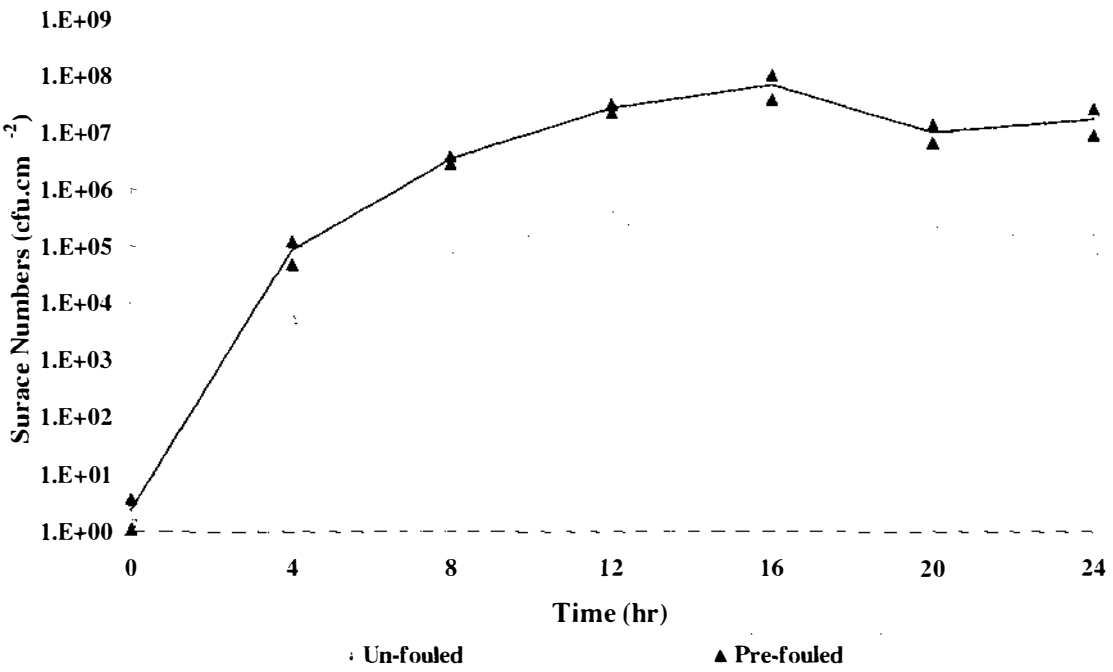


Figure 4.1.12. Numbers of vegetative thermophilic bacteria at the surface of the pre-fouled and un-fouled stainless steel surfaces (MHE) during Run 5, as measured by impedance microbiology.

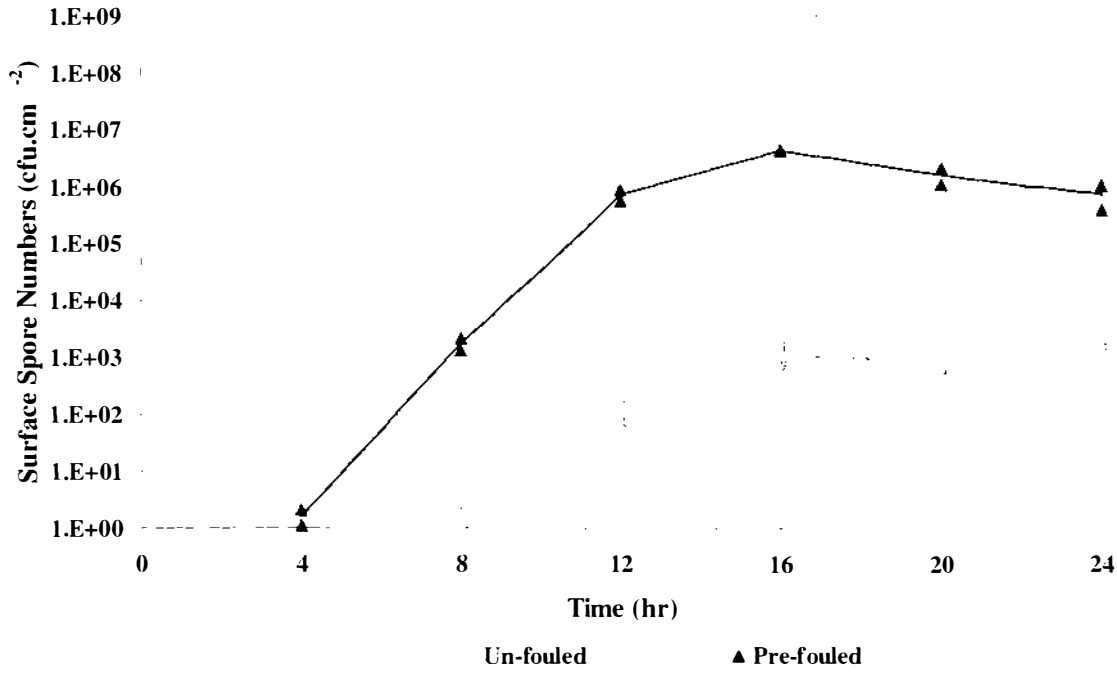


Figure 4.1.13. Numbers of thermophilic spores at the surface of the pre-fouled and un-fouled stainless steel surfaces (MHE) during Run 5 as measured by impedance microbiology.

Since the fouling layers are rugged, the total surface area in contact with the milk (available for colonisation) is increased over that of the original flat clean stainless steel surface. This structure can be seen in Figure 4.1.14. We have found that the bacteria tend to gather in sheltered areas, as shown in the CLSM image given as Figure 4.1.15. In this figure the bright spots indicate a high population density of bacteria, lighter coloured areas are fouling material elevated from the stainless steel wall (closer to the CLSM objective lens) and the dark areas are sunken towards the wall. Thus it is proposed that the topography of the surface and the accompanying hydrodynamic conditions affect the distribution of bacteria.

Langeveld *et al.* (1995) also found 10-100 times more bacteria in milk fouling deposits than observed on un-fouled stainless steel surfaces in a tubular milk heat exchanger run for 20 hours. However, in their work the hot surface of the heat exchanger was high enough that fouling developed over the 20 hour run (>70 °C). As the bacteria investigated (*Thermus thermophilus*) was capable of growth up to 78 °C, fouling and thermophile growth occurred together. Therefore this resulted in bacteria being embedded within the fouling deposit as the amount of fouling increased.

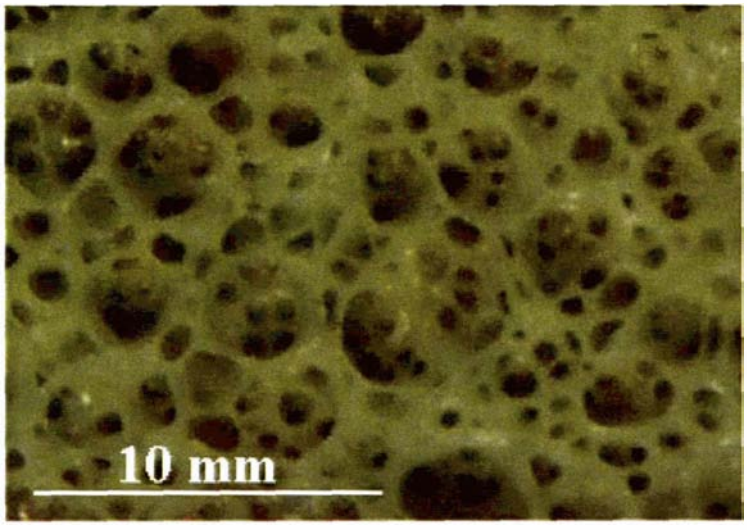


Figure 4.1.14: Typical fouling structure seen on the stainless steel showing the rough topography of the surface.

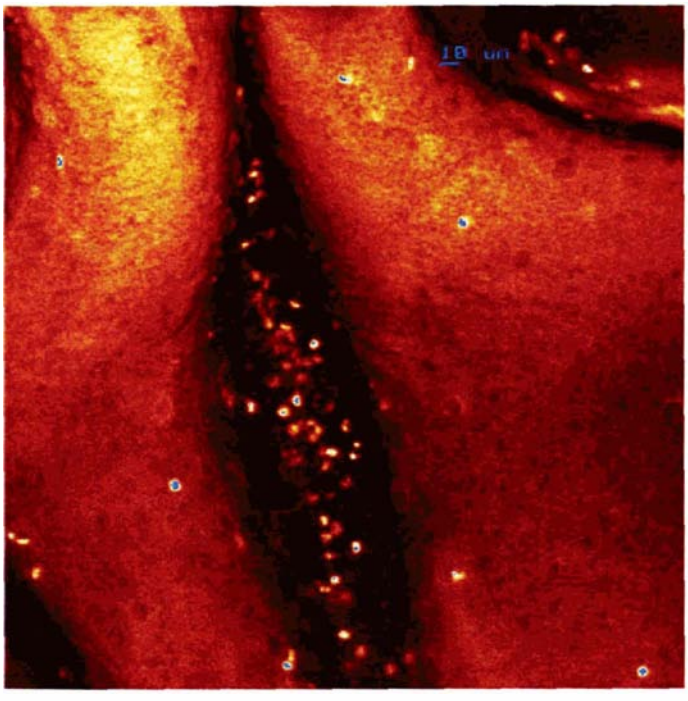


Figure 4.1.15. Confocal Laser Scanning Microscope (CLSM) image of bacteria on a milk fouling layer. This is a magnified view (300x – note 10μm scale bar) of how the structure shown in Figure 4.1.14 appears under CLSM.

4.1.5.2. Increased surface temperature

The thermophile numbers on the pre-fouled surface were still greater than on the originally clean surface at a higher surface temperature of 85 °C (Run 3) as shown below in Figure 4.1.16. The error in the measured values is quite large, as shown by the

95 % confidence intervals on the mean. However, the measurements of pre-fouled and initially clean surface numbers are significantly different at each point in time, except at 12.5 hours where the error bars overlap. Observation of thermophile colonies by CLSM on the originally clean surface also corresponded with the first development of the fouling layer, which occurs at around 7.5 hours (Figure 4.1.17). This effect provided approximately an eight hour lag in the thermophile surface numbers between the two surfaces.

There are two possible explanations to this situation:

1. The fouling may be able to insulate the bacteria from the hot surface (85 °C) and allow the thermophiles to grow in cooler conditions near to the milk temperature, which in this case is 55 °C. However, during industrial milk powder production this scenario where the surface is significantly hotter than the milk is unlikely to occur, as heat transfer equipment is operated with a temperature driving force of only a few degrees to reduce fouling and improve the efficiency of hot utility utilisation. Therefore, this insulating effect would be unlikely to increase the surface area that can be colonised in a production plant.
2. The rugged topography allows better capture of thermophiles at the wall as soon as the fouling deposit develops.

Langeveld *et al.* (1995) also found fouling deposits that formed in a milk heat exchanger over 20 hours at surface temperatures higher than that at which bacterial growth would have been expected (>78 °C for *Thermus thermophilus*) contained high numbers of bacteria (up to 9.6 $x10^{8}$ $cfu.cm^{-2}$). The temperature of the bulk milk stream however was low enough that bacterial growth could have occurred (<77 °C). Therefore this could again be the result of an insulating effect from the fouling deposit or a function of capture and accumulation of bacteria at the surface by the fouling deposit. However as bulk milk concentrations were low ($6x10^{5}$ $cfu.ml^{-1}$ after 20 hours) it is more likely that this high number of bacteria at the surface has resulted from surface growth rather than attachment. If a typical adhesion rate constant of $4x10^{-6}$ $cm.s^{-1}$ is assumed (see Section 4.4.2 below) and even if the bulk milk concentration of $6x10^{5}$ $cfu.ml^{-1}$ was present for the entire 20 hours, an attached surface population of only $2x10^{5}$ $cfu.cm^{-2}$ would be expected. This is much less than the observed 9.6 $x10^{8}$ $cfu.cm^{-2}$, therefore

growth must be occurring at the surface even though the surface temperature is higher than the growth range of the bacteria, indicating that the fouling is likely to have an insulating effect.

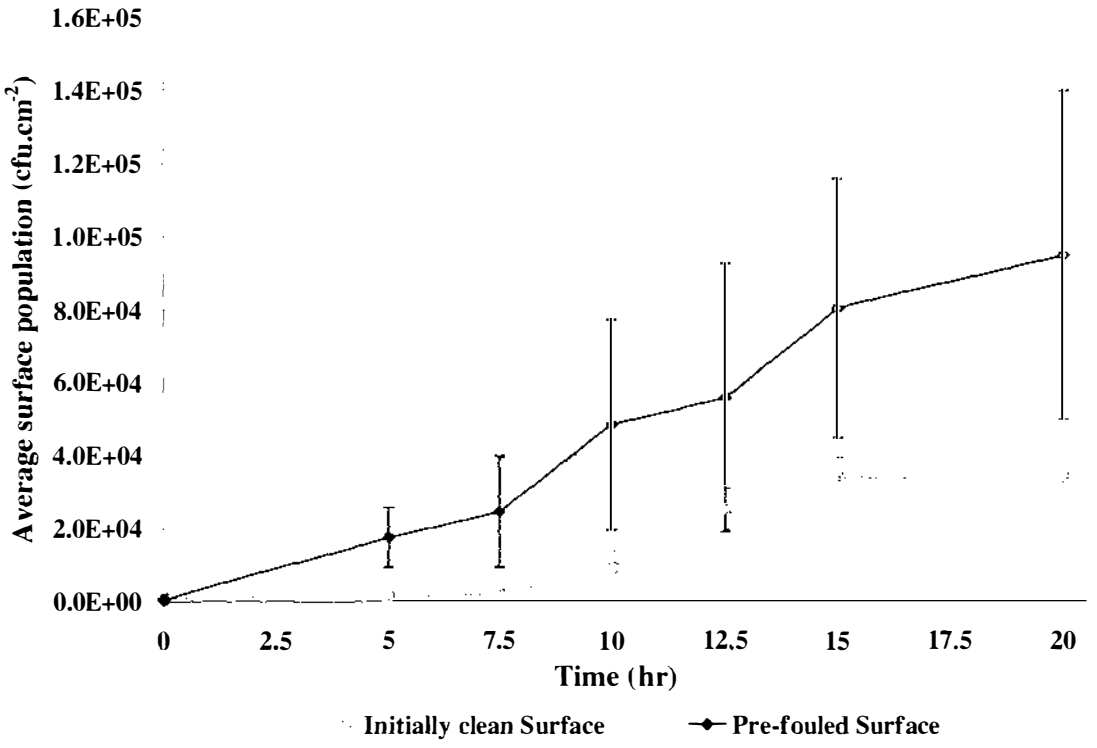


Figure 4.1.16. The development of thermophilic bacterial populations over time on the pre-fouled and initially clean stainless steel surfaces (MHE) during Run 3 as measured by CLSM where the surface temperature was 85 °C and the bulk milk was 55 °C. Error bars represent 95 % confidence intervals on the mean.

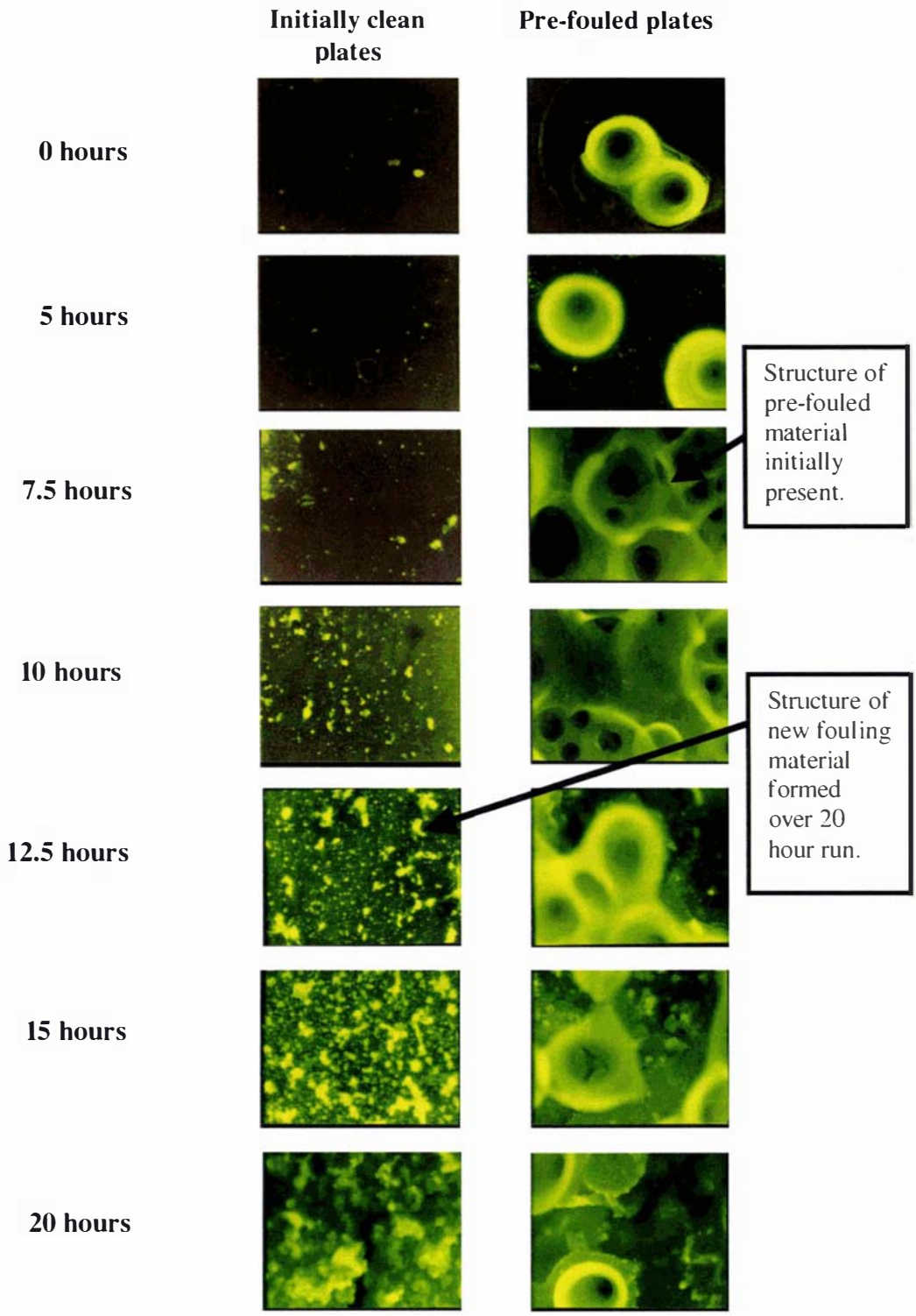


Figure 4.1.17. UV photomicrographs of the fouling structures present on the mini HE plates over time in Run 3 (40x magnification). Shows that fouling started to build up on the initially clean surfaces after 7.5 hours from the start of the run. Also, it can be seen that the pre-fouling and new fouling formed over the 20 hour run have different structures.

4.1.6. Further discussion of bulk contamination

It has been observed in this work that there are much higher thermophile numbers present on the fouling than on the stainless steel surfaces, yet the bulk contamination is similar. A possible explanation is proposed here. The thermophiles in low areas of fouling or in crevices are likely to have more difficulty being released into the bulk flow than bacteria at higher locations or on stainless steel, due to the sheltering effect that the topography of the fouling layer would provide to bacteria in low areas. Therefore, a smaller proportion of these thermophiles will be released into the bulk flow. This would cause numbers to build up within the fouling layer faster than would occur on stainless steel where the thermophiles can be released into the bulk flow more easily. As colonization progresses, eventually a steady state point seems to be reached as the surface numbers plateau. At this point the production of cells at the surface would be equal to the release of cells into the bulk flow. Therefore, to make it possible for the steady state release from the fouled and un-fouled surfaces to be similar, the number of bacteria generated from the two surfaces must be the same. Since there are more bacteria on the fouled surface than the stainless steel surface the average generation time of the bacterial population on the fouled surface must be longer at steady state than on the stainless steel. This could be due to pollution or nutrient limitation effects in the harder to access locations of the fouling layer. Thermophiles in the easier to access (that is higher and less sheltered) regions are likely to have similar generation times to the bacteria on stainless steel. But if nutrient limitation and pollution effects are great in the harder to access regions, then growth in these areas will be low. The higher proportion of spores per bacterial population on the fouling layer than on stainless steel may be one indicator that there is greater stress on the bacteria on the fouling layer. To prove this explanation would require measurements of concentration gradients across the depth of the fouling using micro-probes for example. Within the resources and time available in this PhD programme that work could not be performed and is left to future workers. However, the observation is firm and backed by many runs: although thermophile numbers present on the fouling layer are higher than on stainless steel, more will not necessarily be released into the bulk flow.

Langeveld *et al.* (1995) also made a similar observation regarding bacterial release from fouled and un-fouled heat exchanger surfaces. Much higher bacterial populations were

found on surfaces within the heat exchanger when fouling was present (10-100 times higher numbers than on un-fouled stainless steel). Despite this, higher measured numbers of bacteria were released into the bulk milk from the un-fouled surfaces than from the fouled surfaces. However, in the work of Langeveld *et al.* (1995) the heat exchanger tubes where significant fouling was present were also run at a higher temperature (75-83 °C) that would have been less favourable for bacteria growth (*Thermus thermophilus*) than the temperature of surfaces where significant fouling did not develop (<75 °C). This temperature difference would have affected the observation of fewer bacteria being released from the fouled surfaces, as slower bacterial growth would be expected to occur on the hotter surfaces and therefore fewer bacteria would be released from these surfaces.

Therefore the importance of fouling deposits in thermophilic bacterial contamination is not to increase the steady state release of bacteria into the bulk milk, but to provide areas where thermophiles can be protected from cleaning and act as seeding points for future runs as shown in Sections 4.2 and 4.3.

There is also another link between fouling and thermophile contamination. Since fouling acts as a thermal insulator it decreases the heat transfer coefficient. Therefore, to achieve the same level of heating throughout a production run the heat exchanger must be oversized to account for the development of fouling. Results from Section 4.13 indicate that the thermophilic contamination in the bulk stream will occur faster as a consequence of the increased surface area of the heat exchanger. Therefore minimization of fouling is beneficial.

4.2. *Thermophile Survival during Cleaning*

This section covers the ability of thermophiles to survive during a cleaning process. The rate of thermophile survival during cleaning of fouled and un-fouled surfaces is compared to determine whether milk fouling can provide extra protection for surface associated thermophiles.

4.2.1. Stages of cleaning

The response of fouling layers to the cleaning process can be seen in Figure 4.2.1. There is an initial swelling of the layer when it first encounters the caustic solution and then a gradual removal of the fouling layer. The caustic cleaning treatment was not harsh enough to remove all the fouling present on the fouled surfaces after 20 minutes, which was the longest treatment tested. The un-fouled surfaces did not change in visual appearance during the cleaning treatments given. These un-fouled surfaces were further examined using CLSM as described below.

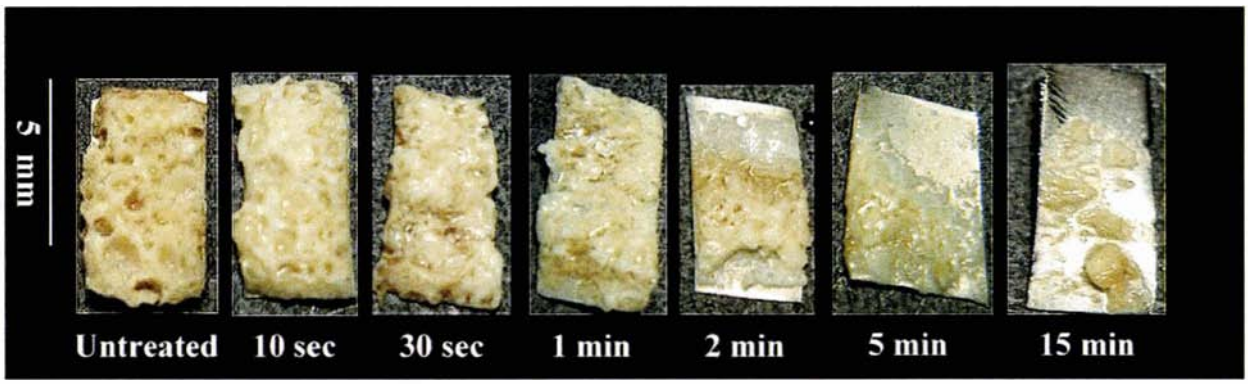


Figure 4.2.1. Amount of fouling remaining on fouled surfaces after caustic cleaning treatments (65 °C, 2 %) of increasing durations, from no treatment to 15 minutes (trial 1). The 10 and 30 second treatments show the initial swelling of the fouling layer. Samples exposed to cleaning from one minute to 15 minutes show gradual removal of the fouling layer.

4.2.2. Thermophile survival during cleaning

Thermophile surface activity measurements showed that the initial population on the fouled surface was around two orders of magnitude greater than that found on the un-fouled surface (10^8 cfu.cm^{-2} compared to 10^6 cfu.cm^{-2}). As the duration of the cleaning treatment increased, the population of bacteria on the un-fouled surface decreased, with no surface activity found on the un-fouled surfaces treated for 15 and 20 minutes. The population present on the fouled surface decreased with exposure to the caustic cleaning treatments. However, the population did not show a continuous decline as was seen on the un-fouled surface. Variations in surface numbers from 10^2 cfu.cm^{-2} to 10^5 cfu.cm^{-2} were obtained, as shown below in Figure 4.2.2.

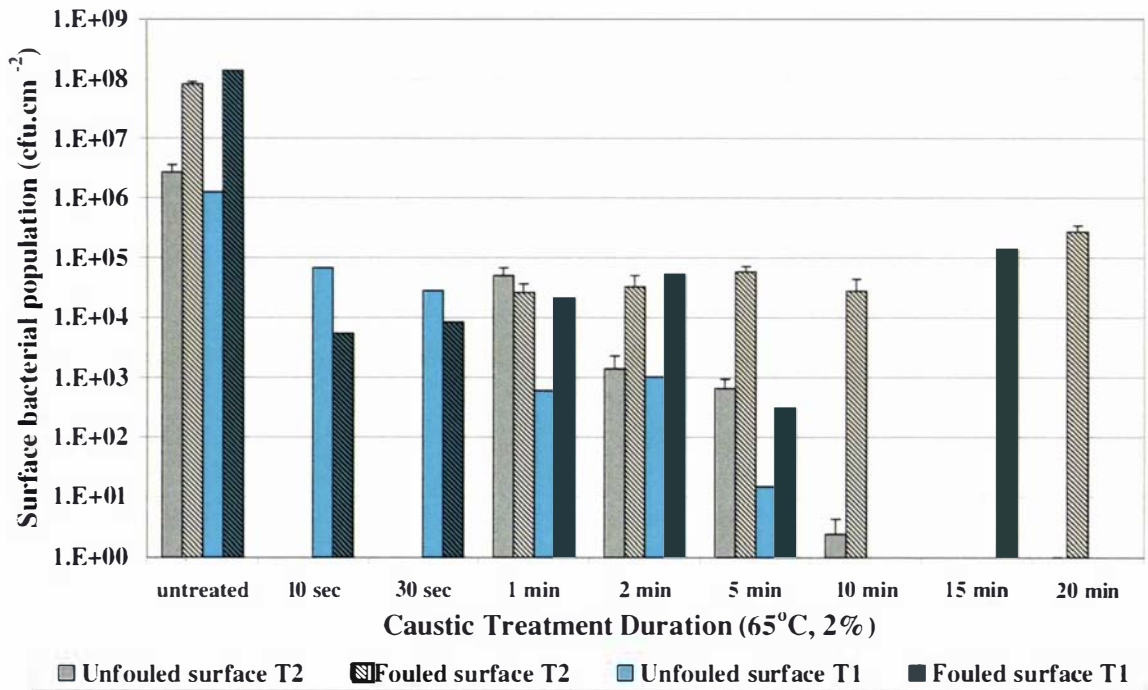


Figure 4.2.2. Surface bacterial population of the fouled and un-fouled surfaces before and after 2 % caustic cleaning treatments from 10 seconds to 20 minutes at 65 °C with no agitation. Population calculated from surface activity measured by impedance microbiology. Error bars show 95 % confidence intervals on the mean (triplicate samples). Data from trial one (T1) and trial two (T2) are shown.

The number of spores remaining active after cleaning showed a trend similar to the vegetative cells, with activity equivalent to 10^1 to 10^3 cfu.cm^{-2} found on the fouled surfaces even after 20 minutes of caustic treatment. The activity attributed to spores on the un-fouled surfaces ceased after caustic treatment of two minutes and longer (Figure

4.2.3). This result was unexpected, as spores would have been expected to have a higher chemical resistance than the bacterial cells. A possible explanation for the apparent higher chemical resistance of the bacterial cells as opposed to the spores is that the chemically treated spores may be more susceptible to the heating process used in the spore count to differentiate between spores and vegetative cells.

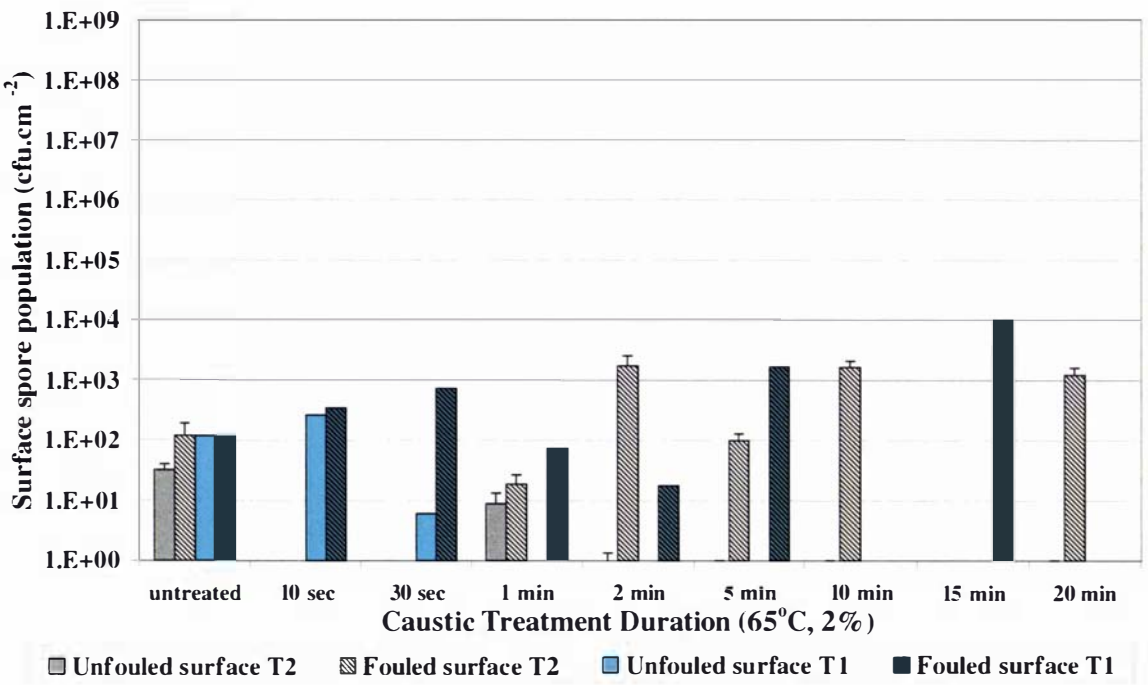
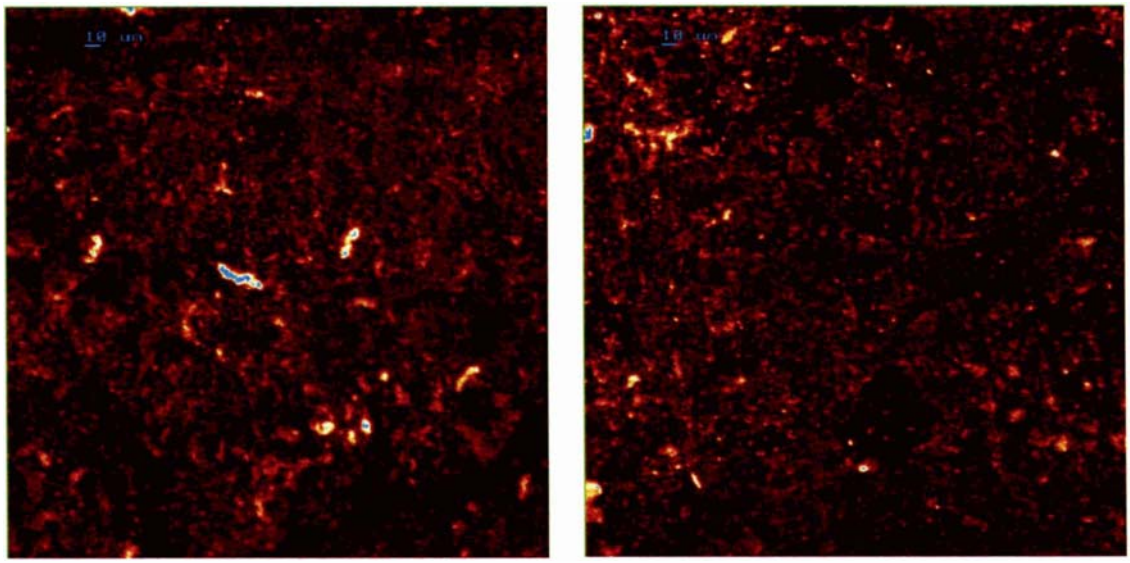


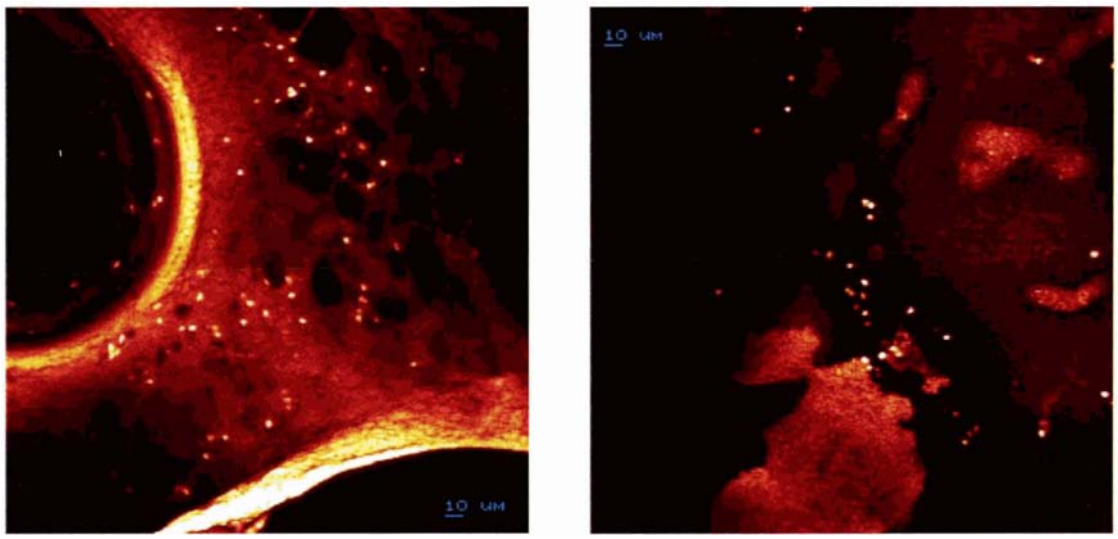
Figure 4.2.3. Surface spore population of the fouled and un-fouled surfaces before and after 2 % caustic cleaning treatments from 10 seconds to 15 minutes at 65 °C with no agitation. Population calculated from surface activity measured by impedance microbiology. Samples were given a 100 °C heat treatment for 30 minutes prior to impedance measurement to differentiate between vegetative cells and spores. Error bars show 95 % confidence intervals (triplicate samples). Data from trial one (T1) and trial two (T2) is shown.

Confocal laser scanning microscopy showed that intact thermophilic biofilm structure remained on the microbially inactive un-fouled surfaces. Therefore the inactivity seems to be due to death of the thermophiles rather than their removal (Figure 4.2.4). Thermophilic colonies could also be made out on the fouled surfaces before and after cleaning (Figure 4.2.5).



Before cleaning treatment | After 15min cleaning treatment

Figure 4.2.4. Confocal laser scanning microscopy image (300x magnification – note scale bar of 10 μm) of un-fouled surfaces before and after 15 minutes of cleaning treatment. As can be seen, the biofilm structure is relatively unchanged, indicating that reduced surface thermophile activity is due to cell death rather than removal.



Before cleaning treatment | After 15min cleaning treatment

Figure 4.2.5. Confocal laser scanning microscopy image (300x magnification – note scale bar of 10 μm) of fouled surfaces before and after 15 minutes of cleaning treatment.

4.2.3. Further Discussion

It has been widely published that bacterial cells attached to surfaces have a higher resistance to chemical and heat treatments than planktonic cells (Bower *et al.*, 1996; Flint *et al.*, 1997c; Hood & Zottola, 1995; Kumar & Anand, 1998). The work reported here suggests that the presence of a milk fouling layer provides further protection against chemicals. Other authors have also reported similar observations, that milk fouling build-up such as protein and fat can have protective effects and also inactivate chemical sanitisers (Dunsmore, 1981; Mattila, *et al.*, 1990).

During milk powder production runs thermophiles are continuously attaching and detaching from the plant surfaces. Simultaneously, milk solids are continuously deposited. Thus necessarily, some bacteria will be embedded in the fouling matrix found at the end of the run. The observed results indicate that the caustic solution is unable to deactivate or remove the bacteria entrapped in the foulant as long as some of it remains. As foulant is removed by the caustic solution, the bacteria present at the newly exposed surface become active and can recontaminate the next production run.

An acid cleaning step was not used in this work as a clear difference was seen between the survival of bacteria in fouled and on un-fouled surfaces after cleaning with the caustic stage alone. The acid cleaning stage is used to remove mineral fouling deposits and also has some bactericidal effect. There was sill residual milk fouling remaining after the caustic cleaning stage and an acid stage would not have removed this fouling. The acid stage may have provided some decrease in numbers surviving after cleaning but the difference between fouled and un-fouled would have remained.

At the beginning of a production run, the numbers of bacteria present in the milk are relatively low and so are the numbers at the surface, however the bacterial numbers in the milk and on the surface increase with time (shown previously in Section 4.1). Thus the distribution of bacteria across the depth of the each fouling layer is not expected to be even but should increase with distance from the wall. When several discrete layers are created on top of one another as in this study, the distribution is complex and the activity found at different depths of cleaning appears randomly varied as shown in Figures 4.2.2 and 4.2.3.

Wirtanen *et al.* (1996) performed an experiment in which *Bacillus* biofilms attached to artificially created food fouling layers and biofilms on stainless steel were cleaned with an alkali and acid procedure in a test rig. It was found that for two of the three *Bacillus* species tested the bacteria remained attached to stainless steel better than to the fouling layers, so that the biofilm cells survived better than those on the fouling layer. This may be considered to be in conflict with the findings of this study. However the fouling layers in their study were artificial with the fouling created by twice spraying stainless steel surfaces with either sour full milk or Campden soil with a two hour drying between applications. Therefore the properties such as the adherence of the sprayed deposits to the stainless steel surface, their strength or internal cohesion and subsequent resistance to CIP chemicals would differ from those of a real fouling layer as was used in this study. The study of Wirtanen *et al.* (1996) is more representative of the situation when milk is spilled onto the surface of heating equipment and then contaminated with bacteria. In addition, their bacteria were only located at the surface of their fouling, not distributed throughout the depth of fouling as would occur in a real processing situation. So once the colonised fouling used by Wirtanen *et al.* (1996) was removed, so would be the attached bacteria as they were present only on the surface of the fouling.

The present study simulated the hard to clean areas in the plant, as it is likely that cleaning procedures in the dairy industry are effective in the high flow areas (Parkar *et al.*, 2004) and that it is the hard to clean areas that are the problem as far as recontamination is concerned. That is, areas of low or slow flow (including partially blocked evaporator tubes and plate heat exchangers) where fouling may remain after cleaning. In these trials minimal agitation was used, whereas Wirtanen *et al.* (1996) used a high flow rate in their cleaning rig, which would simulate an ideal industrial situation.

Frank and Chmielewski (2001) also report a similar observation to Wirtanen *et al.* (1996) that biofilm is more difficult to remove than dried on milk based soil. Although Frank and Chmielewski used a soil with microbes (*B. stearothermophilus* spores) throughout the adhered deposit (unlike Wirtanen), a turbulent cleaning regime (1.28% NaOH, 66 °C) was still used, therefore their results would again be similar to what would be observed in an ideal industrial cleaning situation. Masurovsky *et al.*, (1959) also comments that milk soil is readily removed by chemical and physical cleaning

procedures, so bacteria embedded in the milk soil are also removed. This again would only apply to those situations within the food processing equipment were adequate mechanical action could be incorporated to remove the soil. However, it is important to ensure that the cleaning regime used is not only effective at removing fouling, but also at removing or inactivating attached cells.

Several other studies have also investigated the effect of different cleaning regimes on surface associated *Bacillus* bacteria and spores (Hoffmann and Reuter, 1984; Leclercq-Perlat and Lalande, 1994; Bredholt *et al.*, 1999; Steiner *et al.*, 2000; Lelievre *et al.*, 2002; Parkar *et al.*, 2004). In general these agree with the work reported here that the presence of residue soil does hinder inactivation of the bacteria present on the surface.

In cleaning investigations reported by Bredholt *et al.* (1999) it was found that a weak alkaline cleaning agent left more residual soil material and higher numbers of viable bacteria behind than a strong alkaline cleaner, but the use of a sanitiser after cleaning helped to reduce the viable count to a level similar to that found on surfaces cleaned with the strong alkaline cleaner alone. This result also supports the finding from the work in this thesis that residual soil remaining on the surface after cleaning is also likely to harbour bacteria. It also shows that if the cleaning procedure is ineffective at removing all traces of fouling deposits, sanitisers can be used to reduce the potential for re-contamination of the plant on start up.

Lelievre *et al.* (2002) investigated the cleaning of adhered *B. cereus* spores on stainless steel surfaces with 0.5 % sodium hydroxide at 60 °C. They found that the caustic had no killing effect on the spores and that the observed reduction in spore activity on the surfaces was due to spore removal. They also found that the removal was poor under low shear. Therefore, the higher numbers of spores found in the presence of fouling relative to stainless steel in the work reported in this thesis is likely to have been due to a reduced rate of removal of spores during cleaning rather than protection from any antimicrobial activity of the cleaning solution.

Steiner *et al.* (2000) reported few residual *B. stearothermophilus* spores remaining on stainless surfaces with a variety of surface finishes after a turbulent cleaning regime with a chlorinated alkaline cleaner at 66 °C. The spores were heat fixed to the stainless

steel surfaces before cleaning, however no data of spore levels before cleaning were given, making it difficult to determine the amount of reduction in surface numbers due to cleaning. However the work did show that the surface associated spores did not survive cleaning well when adhered to the surface without the presence of any soil or fouling material.

Parkar *et al.* (2004) found that biofilms of *B. stearothermophilus* adhered to 316 stainless steel coupons with a 2B finish (with no milk fouling residue present) were successfully inactivated with a caustic treatment (1% NaOH, 60°C, 30 min) followed by an acid treatment (0.9% HNO_3, 60°C, 30 min).

In conclusion, this work demonstrates that milk fouling layers can protect thermophiles against cleaning better than a biofilm alone. This could allow them to survive better during cleaning of dairy equipment, especially in hard to clean locations where fouling remains after cleaning. Therefore it is important when cleaning dairy equipment to remove all traces of fouling to minimise the numbers of thermophiles in the plant after cleaning, so that, in turn, the amount of recontamination in the plant in the following production run is kept to a minimum.

4.3. Re-contamination

Any thermophiles that survive cleaning after a run may re-contaminate the plant at the start of the next run. This recontamination may then shorten the lag time for thermophile contamination within the plant and reduce the time available for production. The lag time of the bulk contamination is important, as once the lag time is over and bulk contamination starts to increase, thermophile bulk contamination quickly becomes excessive (Section 4.1). This section covers work where thermophiles were purposely left in the pre-heat section of the milk powder pilot plant to investigate the effect on thermophile contamination down stream in the bulk milk flow during a processing run.

4.3.1. Effect of initially contaminated tube

The surface thermophile population of the initially contaminated pre-fouled inner tube inserted on one side of the THE at the beginning of the run (Run 5) was measured as $4x10^{4} \pm 2x10^{4}$ cfu.cm^{-2}. This would have provided a large bacterial population to contaminate the down stream sections of that side of the THE.

Figures 4.3.1, 4.3.2 and 4.3.3 below show the bulk contamination after each tube on both sides of the THE after 4 and 8 hours of operation. The contamination (or thermophile release) was found to be proportional to the surface area that the milk contacts within the growth temperature region as mentioned above in Section 4.1.2.

The bulk contamination after 4 hours, plotted in Figure 4.3.1, shows a large amount of contamination being released from the initially contaminated tube (>2 x10^{6} cfu.ml^{-1}). Also, the two tubes down stream of the tube are releasing significantly more contamination than the tubes on the initially clean tube bank per wetted contact surface area (600 ± 200 cfu.ml^{-1}.cm^{-2} compared to 80 ± 20 cfu.ml^{-1}.cm^{-2}). After 8 and 12 hours (Figure 4.3.2 and Figure 4.3.3) the tubes downstream of the initially contaminated tube are still providing significantly more contamination than the tubes on the initially clean side (1700 ± 300 cfu.ml^{-1}.cm^{-2} compared to 800 ± 200 cfu.ml^{-1}.cm^{-2} after 8 hours and 1900 ± 300 cfu.ml^{-1}.cm^{-2} compared to 1100 ± 200 cfu.ml^{-1}.cm^{-2} after 12 hours). It is not

until after 16 hours (Figure 4.3.4) that the contamination measured from the two tube banks is not significantly different (2500 ± 800 cfu.ml^{-1}.cm^{-2} compared to 2300 ± 700 cfu.ml^{-1}.cm^{-2}).

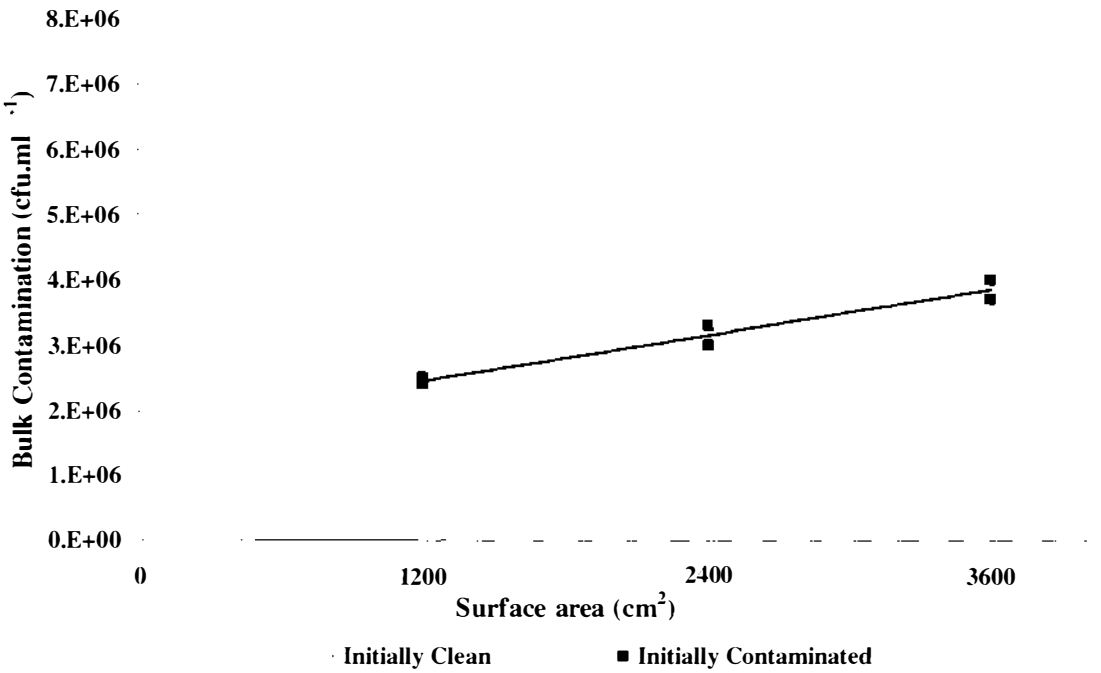


Figure 4.3.1. Bulk contamination across each side of the THE tube bank after 4 hours of operation against surface contact area (Run 5).

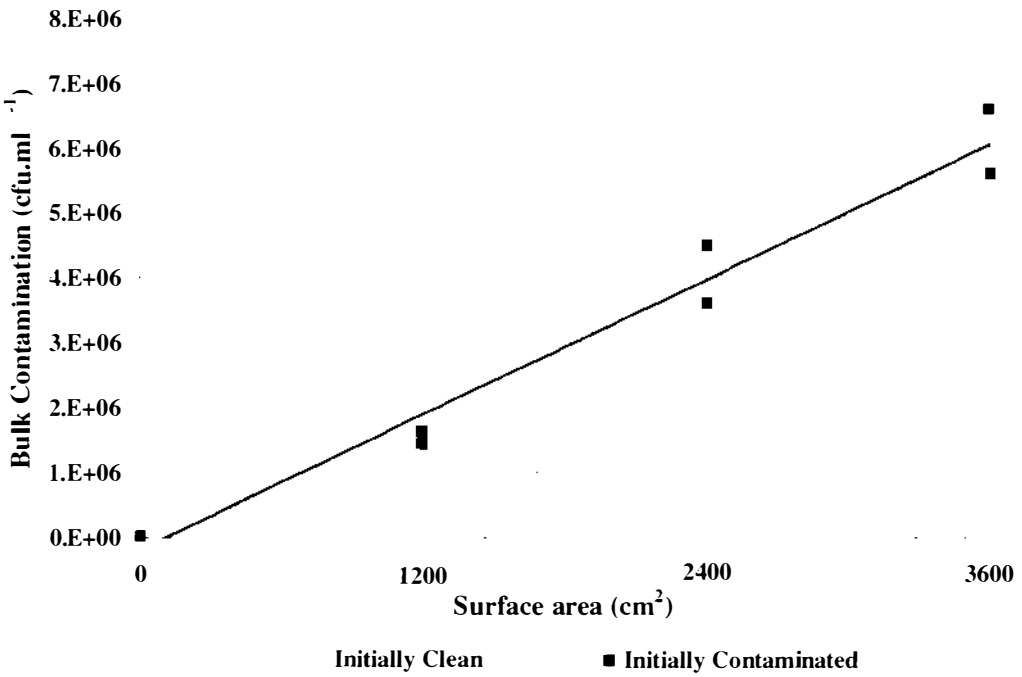


Figure 4.3.2. Bulk contamination across each side of the THE tube bank after 8 hours of operation showing the thermophile release as surface contact area increases (Run 5).

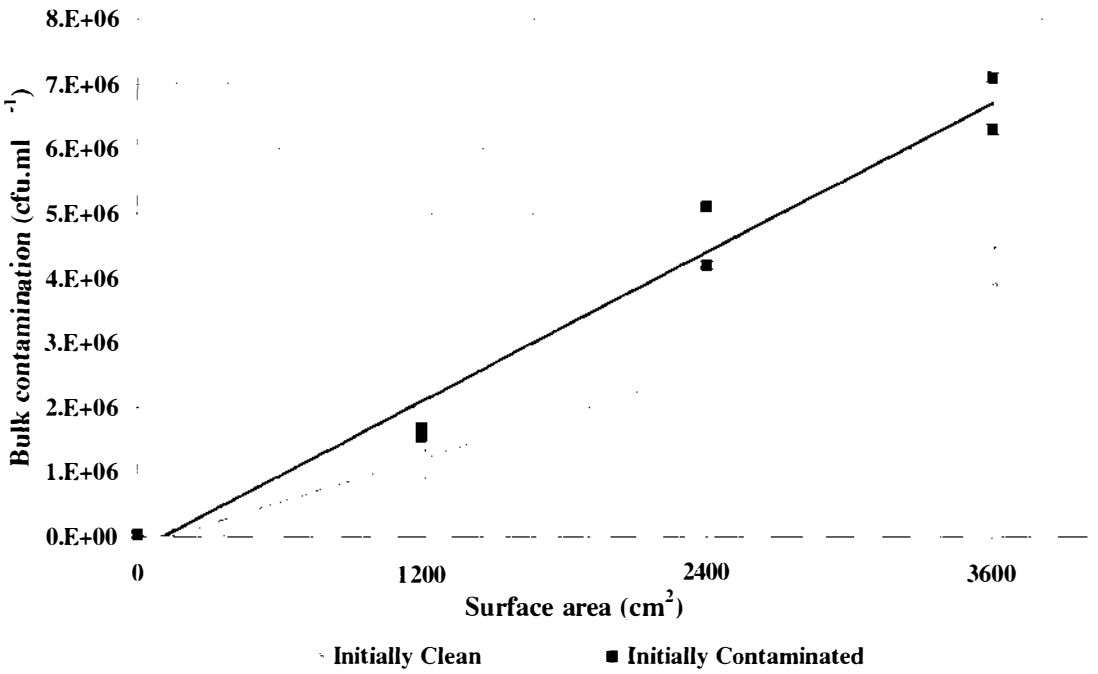


Figure 4.3.3. Bulk contamination across each side of the THE tube bank after 12 hours of operation showing the thermophile release as surface contact area increases (Run 5).

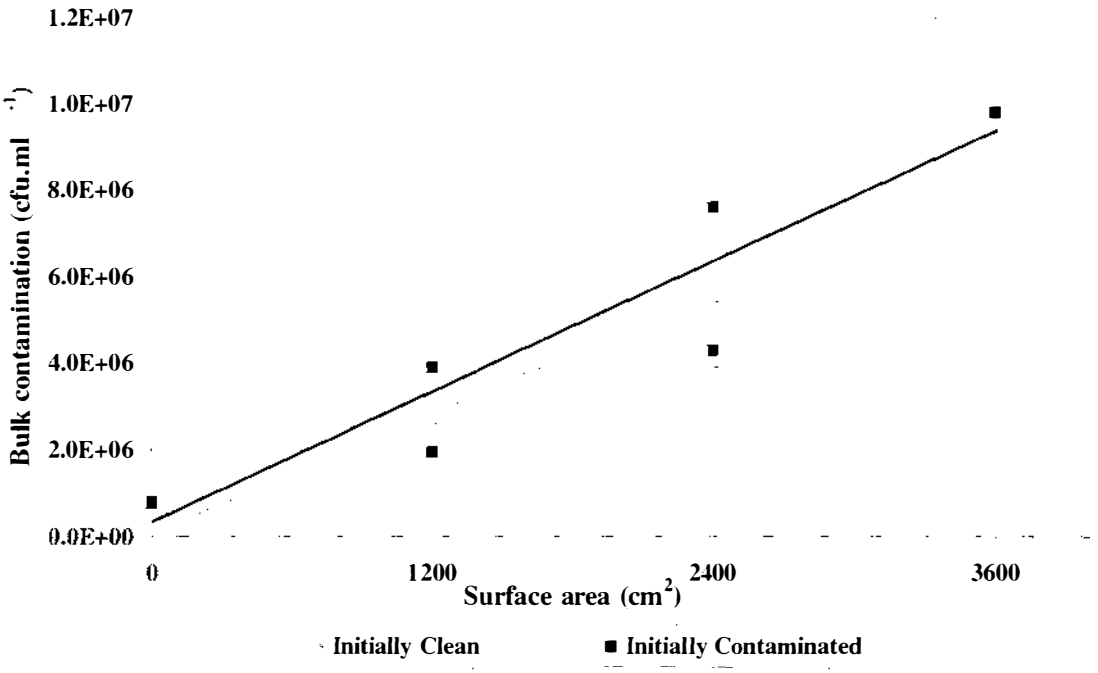


Figure 4.3.4. Bulk contamination across each side of the THE tube bank after 16 hours of operation showing the thermophile release as surface contact area increases (Run 5).

These data can also be represented as in Section 4.1.3, where the slope of graphs such as 4.3.1, 4.3.2, 4.3.3 and 4.3.4 are plotted against time. This plot then shows the release of bacteria per unit wetted milk contact surface area against time. The data from this run

(Run 5) are shown in this way in Figure 4.3.5. These data are also given in Appendix B, page B-7. With the data in this form, the difference in the release from each side can be more easily seen.

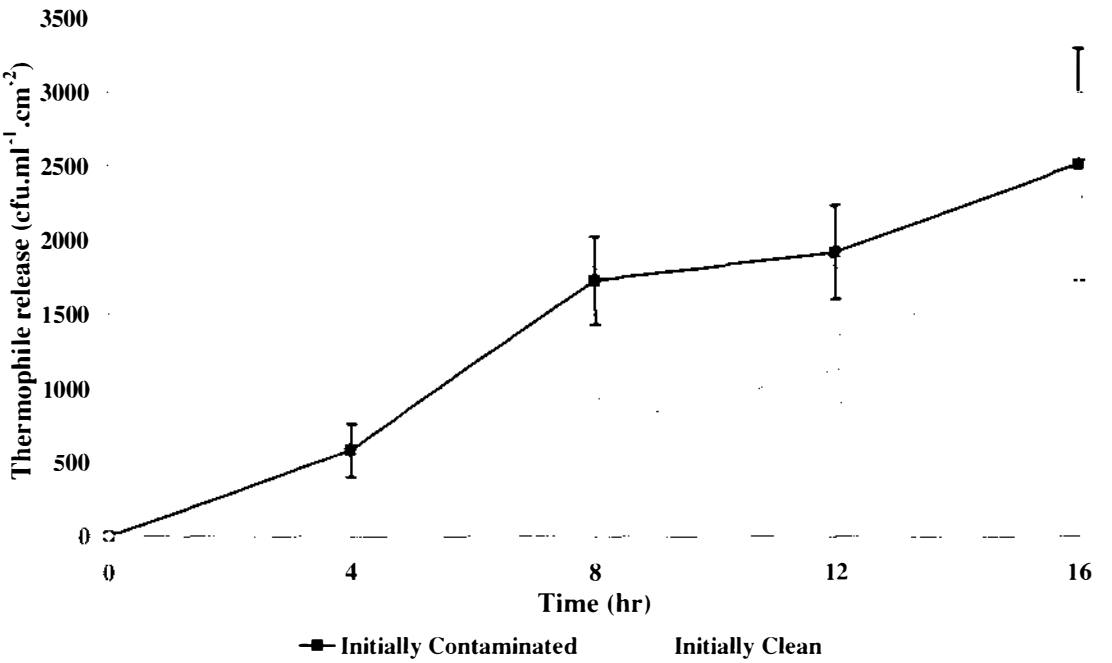


Figure 4.3.5. Thermophiles released into the bulk milk from the THE per unit wetted surface contact area over time for Run 5. These values are calculated from the slope of graphs such as Figure 4.3.1 and 4.3.2. Errors bars show 95 % confidence intervals in the prediction of the slope through regression.

Figure 4.3.6 shows the bulk contamination from each THE side over time up to 16 hours. As can be seen, the time taken for the bulk contamination to become excessive (greater than 1×10^6 cfu.ml^{-1}) is reduced on the initially contaminated side. The initially contaminated side exceeds 10^6 cfu.ml^{-1} after 4 hours of operation, while the initially clean side contamination is not measured as greater than 10^6 cfu.ml^{-1} until after 8 hours. Therefore, in terms of run length before thermophile contamination was excessive, the initially contaminated side run length would be only around half of that of the initially clean side run length (4 hours as opposed to 8 hours). This shows that the run length can be shortened dramatically if active thermophiles are left on the surfaces within the plant.

Another interesting observation that can be seen from the above results is that the contamination from the initially contaminated tube seems to reach a maximum at around $2 - 3 \times 10^6$ cfu.ml^{-1}. This indicates that there is a maximum contamination

potential that each THE tube can provide. This would also hold true for industrial dairy processing equipment, however by the time this is reached the amount of contamination in the product stream is likely to be unacceptable due to the amount of surface area available for colonisation at temperatures suited to thermophile growth. If the amount of surface area available for thermophile growth was small enough however, this maximum amount of contamination may be small enough that it is still acceptable in terms of product quality. This would then allow longer manufacturing runs as the product contamination would reach and maintain an acceptable maximum amount despite long operating times.

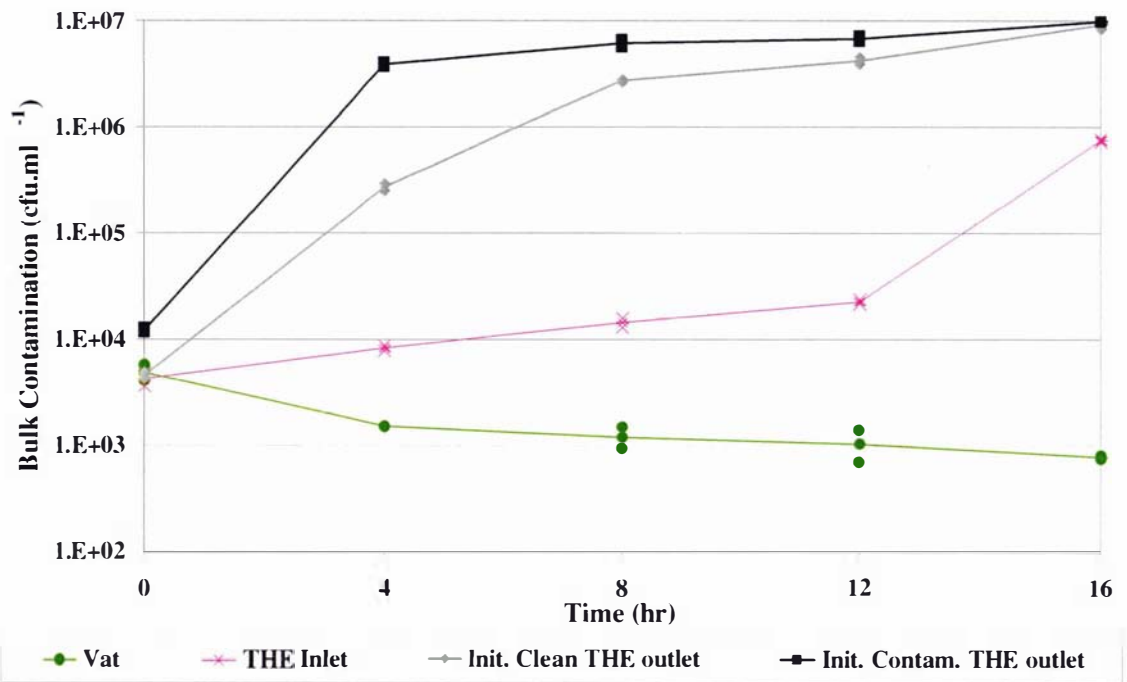


Figure 4.3.6. Graph of thermophile bulk contamination over time at the vat, THE inlet and THE outlet up to 16 hours (Run 5).

4.3.2. Effect of inlet thermophile concentration

Figure 4.3.7 compares the bulk contamination over time for runs where the THE was initially clean and different initial concentrations of thermophiles were present in the milk. It shows that as the initial bulk concentration increased the lag time of growth was reduced. The two runs with low levels of thermophiles marked as runs 2 and 4 had lag times of 1-4 hours. The other two runs (1 and 5) appeared to have no lag time, as the bulk concentration increased quickly right from the start of the runs. Also, it shows that

the time for the bulk contamination to reach steady state was reduced as the initial bulk concentration increased. The run with the highest initial bulk concentration of $3x10^4$ cfu.ml^{-1} (Run 1) took only 4 hours for the bulk contamination to be measured above 10^6 cfu.ml^{-1}. However, the run with the lowest bulk concentration of 10 cfu.ml^{-1} (Run 2) took 10 hours before the bulk concentration was recorded above 10^6 cfu.ml^{-1}.

Therefore, higher bulk concentrations of thermophiles at the start of the run or active thermophiles left on the surfaces within the plant can reduce both the lag time in contamination and the time available for production before thermophile bulk concentrations in the milk become excessive (>10^6 cfu.ml^{-1}).

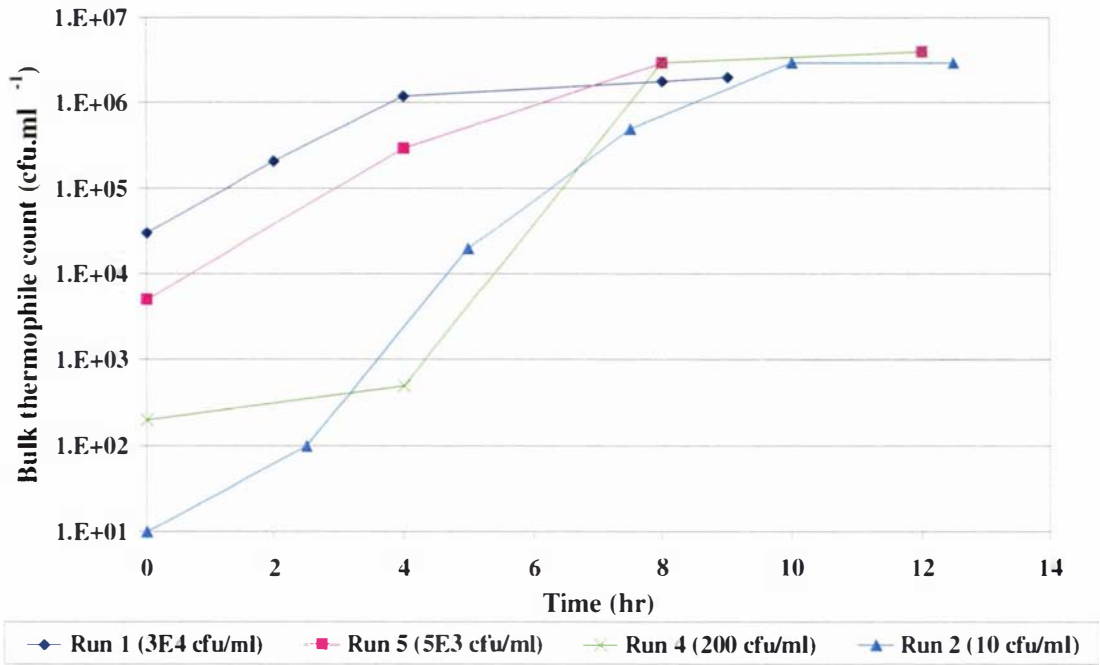


Figure 4.3.7. Graph comparing bulk concentration over time at the clean THE outlet between runs with different initial thermophile concentrations in the milk.

4.3.3. Dominant transport mechanism

The dominant mechanism for transport of thermophiles from one location to another within a plant was shown on the lab scale equipment to be convection rather than motility or spreading of the biofilm across the surface. Under laminar flow of initially sterile media (autoclaved milk) at a velocity of 1300 cm.hr^{-1} it was shown that thermophiles could grow and be released into the bulk milk downstream of an initially contaminated surface with an initial surface population of 2 cfu.cm^{-2} (Figure 4.3.8).

These released thermophiles were observed to populate initially sterile surfaces located 40 cm downstream of the initial surface population to a level of 500 cfu.cm $^{-2}$ after 15 hours (Figure 4.3.9). No contamination was observed on surfaces 20 cm up stream of the initially contaminated surfaces after 15 hours, showing minimal spread of thermophiles along the surface (<1.3 cm.hr $^{-1}$). Under static conditions the rate of transport of bacteria along a horizontal tube filled with initially sterile autoclaved milk was measured between 6 and 8 cm.hr $^{-1}$. As this is much slower than the rate of transport in the flow of milk (1300 cm.hr^{-1}) the dominant rate of transport is by convection with the bulk milk under flowing conditions.

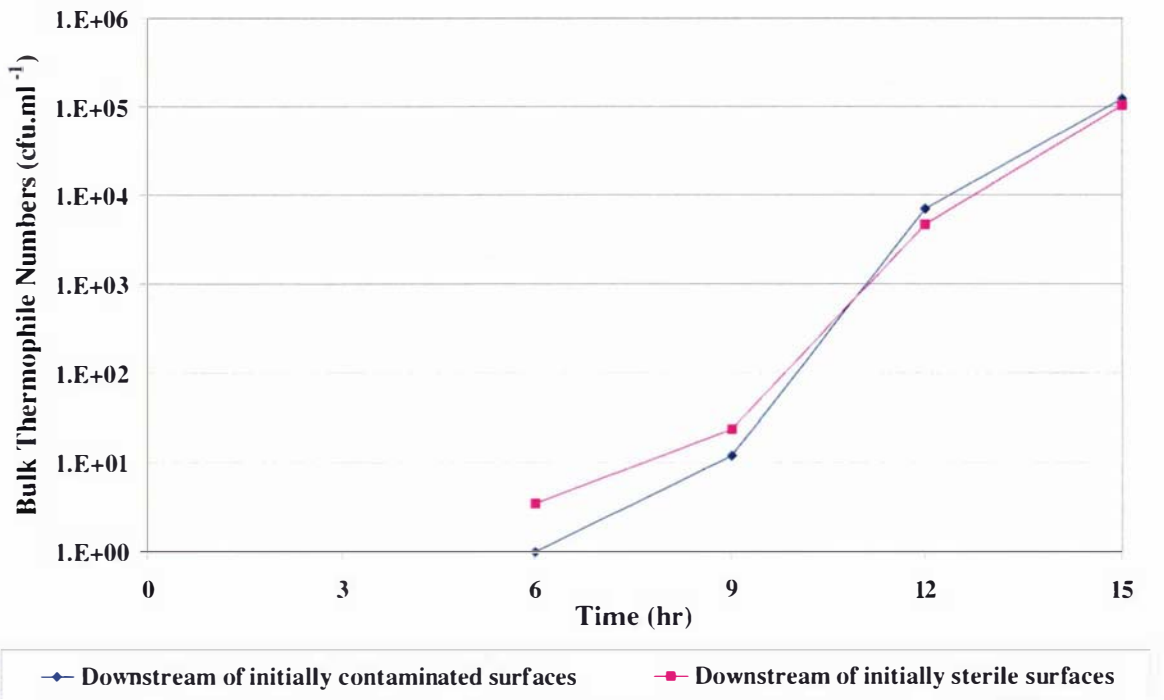


Figure 4.3.8. Bulk thermophile numbers in the lab scale rig at milk sample points down stream of initially contaminated surfaces and downstream of initially sterile coupons over 15 hours operation.

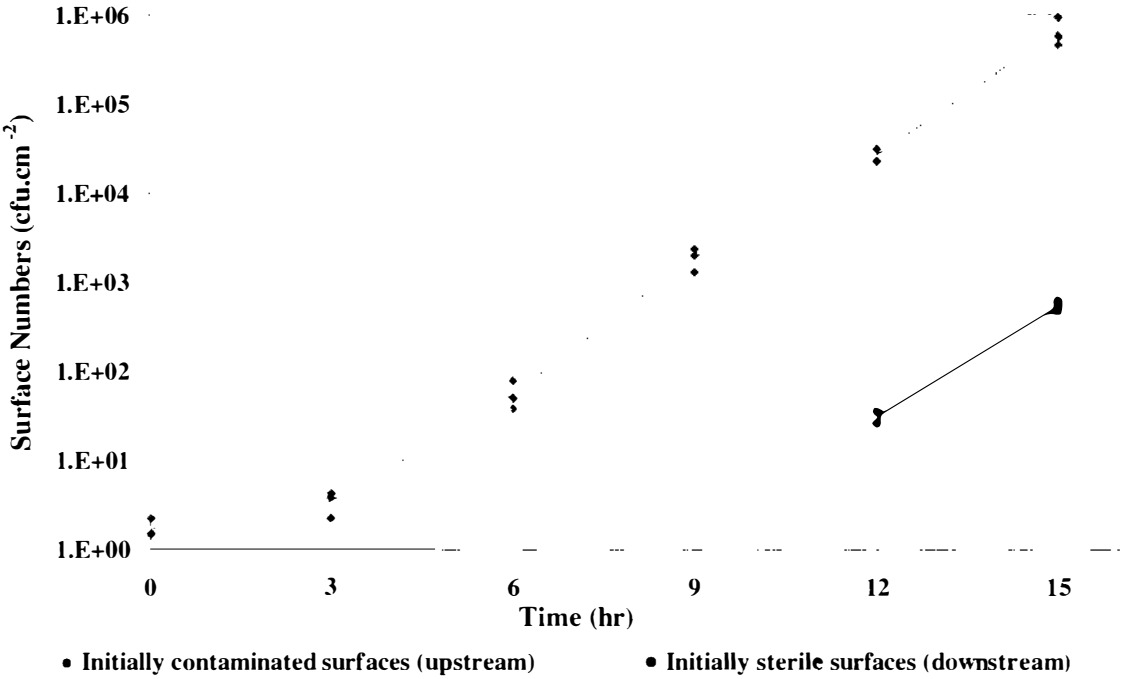


Figure 4.3.9. Surface populations on initially contaminated (upstream) and initially sterile (downstream) surfaces in the lab scale rig over 15 hours.

4.3.4. Further Discussion

This study shows that thermophiles surviving after cleaning or greater initial thermophile concentrations in the milk can reduce the plant production time available before bulk thermophile concentrations are excessive ($>10^6$ cfu.ml^{-1}). These thermophiles that re-contaminate the plant are transported from their original locations via convection in the bulk flow to down stream areas of the plant. It is thus crucial to ensure that cleaning procedures in milk powder plants remove or destroy all traces of thermophiles and that as few as possible thermophiles are present in the milk, especially at the start of the run to allow the maximum possible run length.

4.4. Adhesion

In the study of thermophile contamination, it is important to know the adhesion rate of thermophiles to different surfaces within the plant, so that the subsequent growth and bulk contamination can be modelled. The number of bacteria adhered to a surface depends upon the transport of bacteria to the surface and the adhesion reaction with the surface. In de Jong *et al.* (2002) it was suggested that the adhesion rate of bacteria to the surface is a first order function of the bulk bacteria concentration near the surface as given in the equation below.

$$r_a = k_a \times C_s \qquad 4.1$$

Where r_a is the rate of adhesion ($cfu.cm^{-2}.s^{-1}$), k_a is the adhesion rate constant ($cm.s^{-1}$) and C_s is the concentration of bacteria near the surface ($cfu.ml^{-1}$). Bacteria are quantified as colony forming units (cfu). The transport of bacteria to the surface is dependent upon the rate of transport through the boundary layer (de Jong *et al.,* 2002).

$$r_d = \frac{D(C_b - C_s)}{h} \qquad 4.2$$

Where r_d is the rate of transport through the boundary layer ($cfu.cm^{-2}.s^{-1}$), D is the transport coefficient ($cm^2.s^{-1}$), C_b is the concentration of bacteria in the bulk liquid ($cfu.ml^{-1}$) and h is the thickness of the diffusion boundary layer (cm). If the rate of adhesion is smaller than the rate of transport then the concentration near the surface (C_s) will not be significantly depleted and C_s=C_b. Then the adhesion rate constant, k_a, can be calculated from the equation:

$$k_a = \frac{r_a}{C_b} \qquad 4.3$$

Experiments measuring the numbers of adhered cells (n_w) over time with varying bulk concentration and varying adhesion time (t_a) can therefore be conducted to get estimates of the adhesion rate constant k_a.

$$k_a = \frac{(\Delta n_w . \Delta t_a)}{C_b} \qquad 4.4$$

Where n_w is the measured number of adhered cells (cfu.cm^{-2}) and t_a is the adhesion time (s).

In this study, the adhesion of *Bacillus stearothermophilus* to whole milk and skim milk foulant and to stainless steel was investigated under quiescent conditions with varying bulk cell concentrations and varying adhesion times to estimate the rate of adhesion of thermophiles to different types of surfaces within milk powder plants.

4.4.1. CLSM examination of adhesion to different surfaces

CLSM examination of the fouled surfaces did not show significant differences between the numbers adhering to skim milk and whole milk fouling. This is because with low levels of fouling it is very difficult to differentiate between the thermophile colonies and the small islands of fouling, even with the use of DNA stains like the SYTO 13 stain used in this work. This was mentioned previously in Chapter 3. The two sets of data are compared in Figure 4.4.1. When counting the number of thermophiles present on the surface of a fouling layer variation of up to 10 times between the counts of the fields selected was found. This large variation is reflected in the large 95 % confidence intervals for the data presented in Figure 4.4.1, which shows the average numbers of thermophiles adhered to skim milk and whole milk fouling layers when exposed to varying bulk milk concentrations. The control surfaces for the whole milk and skim milk samples were measured as $8.4 \times 10^3 \pm 5.5 \times 10^3$ cfu.cm^{-2} and $3.0 \times 10^3 \pm 2.1 \times 10^3$cfu.cm^{-2} respectively. The control values were obtained by measuring the thermophile population on the fouled surfaces before adhesion tests. These control values have been taken into account on the data in Figure 4.4.1, by subtraction of the control values from each total count. To identify trends with better confidence, many more than eight fields should have been counted per sample. As can be seen on Figure 4.4.1 the 95 % confidence intervals between the two sets of data overlap, indicating that the differences are not significant at the 95 % level. Figure 4.4.1 also shows no significant increase in numbers adhered to the fouling layers with increasing bulk cell

concentration. However, the more reliable data from impedance microbiology shown below did show an increase.

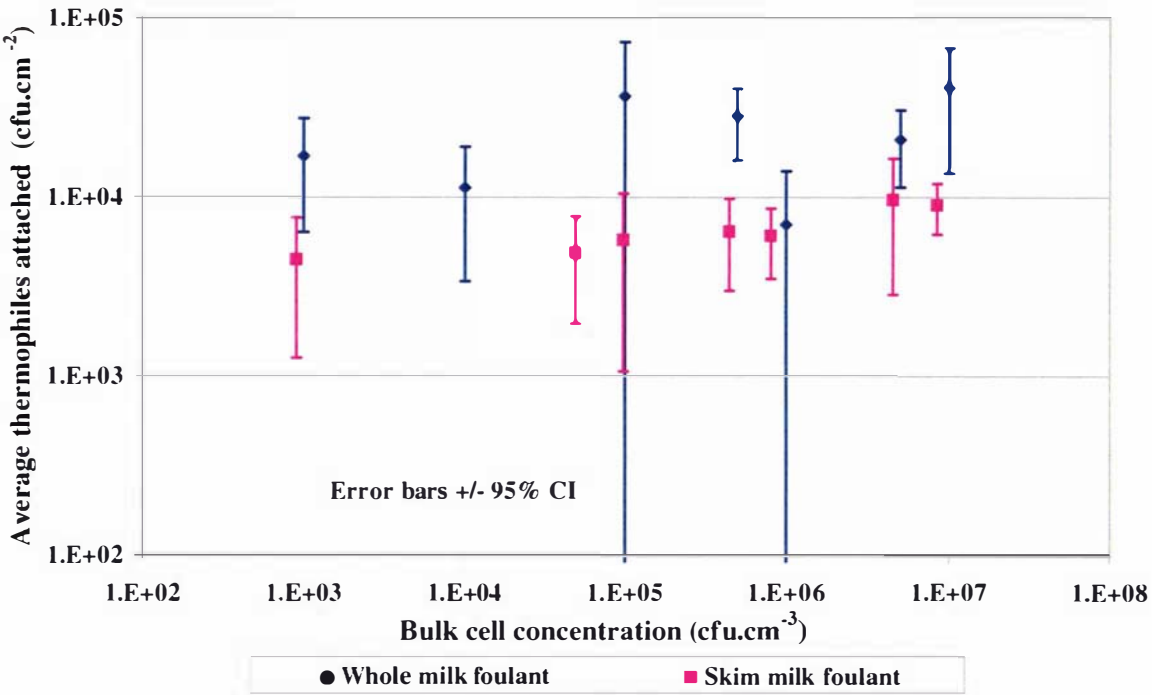


Figure 4.4.1. Numbers of thermophiles adhered to whole milk and skim milk fouling layers at varying bulk cell concentrations. Error bars show the 95 % confidence interval on the mean. Numbers assessed using CLSM.

The number of thermophiles adhered to the surface did seem to increase with the increasing duration of the fouling process, indicating an increase in adhered numbers with an increasing amount of fouling with a constant amount of time for adhesion. As shown in Figure 4.4.2, the confidence intervals are again large but the numbers adhered for the six hour sample are significantly greater at the 95 % confidence interval than the samples of one and two hours fouling duration. This trend is most likely indicative of an increased surface area for adhesion on the samples with a greater amount of fouling. To confirm this trend further experiments with larger differences in the amount of fouling present should be carried out. The surface population on the control samples was 900 ± 600 cfu.cm^{-2} and has been taken into account in the data shown, by subtracting the control values from the measured total counts.

Unlike microscopic measurements on the fouling layers, epi-fluorescence microscopy of un-fouled stainless steel surfaces did reveal an increase in adhered numbers with increasing bulk cell concentration. However the results were variable, with a difference

in adhered numbers of up to 100 times between duplicate trials as shown in Figure 4.4.3 below. Again, a greater number of fields selected when counting may have helped to reduce this variability.

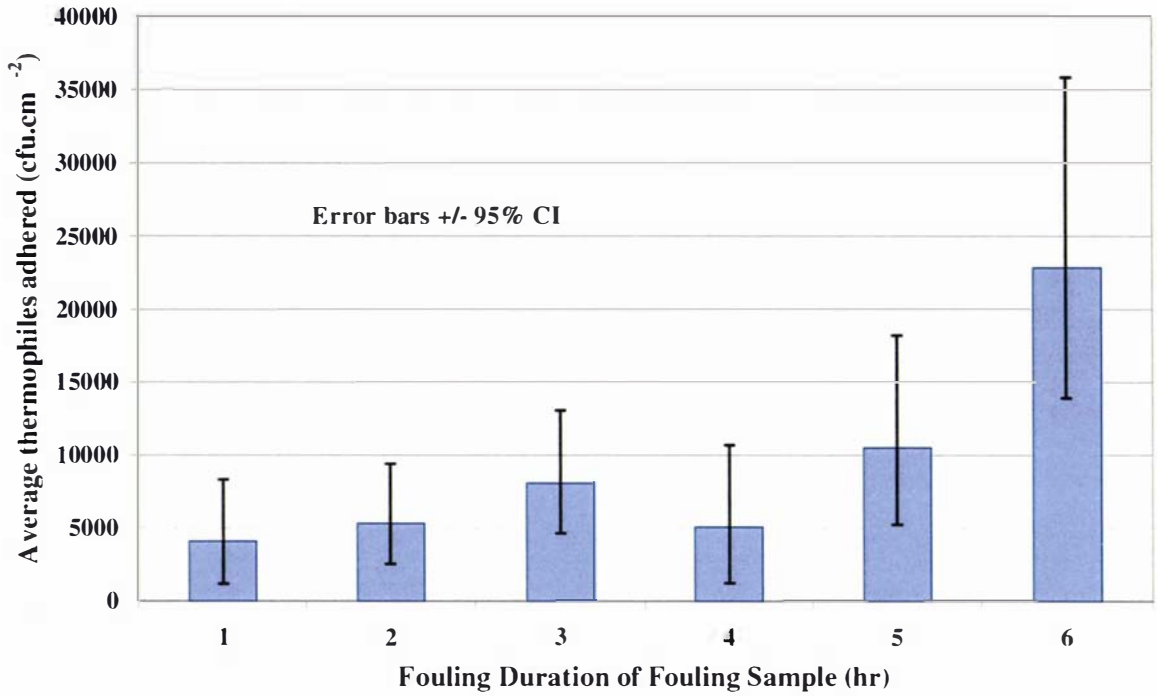


Figure 4.4.2. Average thermophile numbers adhered to whole milk fouling layers with increasing run duration. Error bars show the 95 % confidence interval on the mean. Numbers assessed using CLSM.

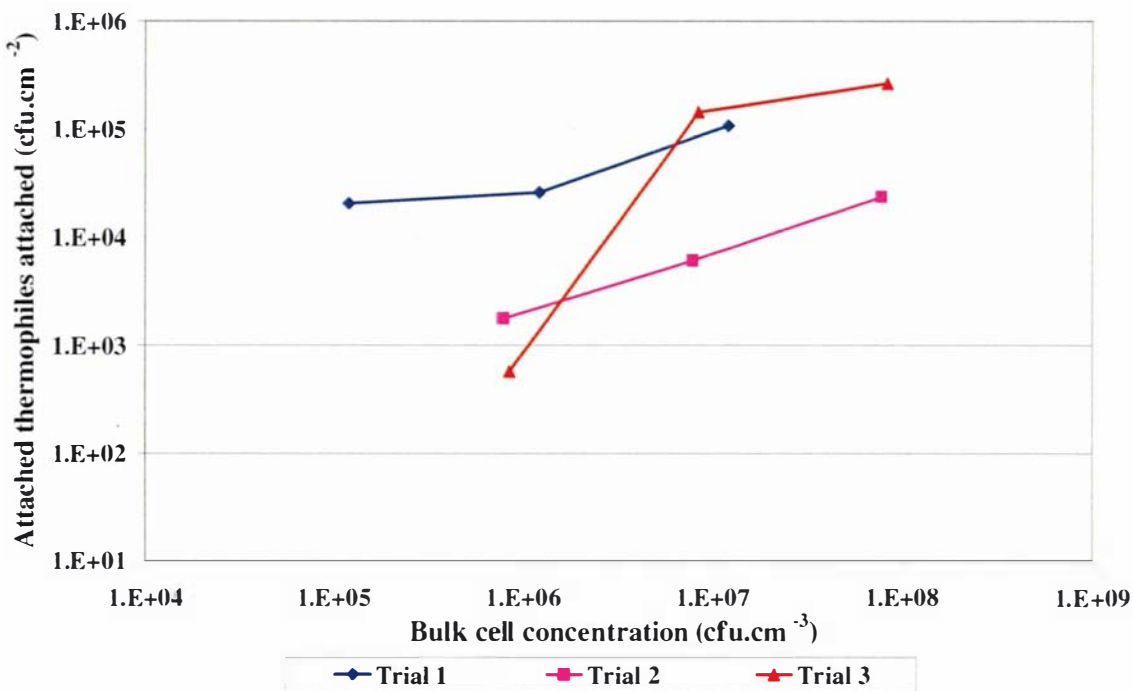


Figure 4.4.3. Numbers of thermophiles adhered to un-fouled stainless steel at varying bulk cell concentrations. Numbers were assessed using epi-fluorescence microscopy.

4.4.2. Examination of adhesion using impedance microbiology

Data from the impedance measurement technique did not have the same variability as the microscopic data. Duplicate measurements using impedance were within 50 % of each other for both fouling layer and stainless steel duplicates. Results were also reproducible between different trials conducted. These improved data allowed the observation of trends. Unlike the microscope measurements, impedance microbiology did detect an increase in numbers of adhered cells with increasing bulk cell concentration with both fouling and stainless steel surfaces. Numbers of adhered cells also increased with increasing adhesion time.

The data for both fouled surfaces and stainless steel surfaces were plotted to test for linearity and verify previous assumption of a first order relationship between adhesion rate and bulk concentration given in Equation 4.1 (de Jong *et al.*, 2002). Equation 4.4 indicated that a plot of numbers adhered (Δn_w) versus the product of the adhesion time and the bulk cell concentration ($\Delta t_a * C_b$) should be a straight line passing through the origin with the gradient equal to the adhesion rate constant (k_a). The data from all four trials using the impedance method are plotted in this form on Figure 4.4.4. The residual surface population (the initial population of thermophiles on the surface before inoculation) on the control samples of the fouled surfaces was measured as 47±22 cfu.cm^{-2} and was taken into account by subtraction from the raw data before plotting. As can be seen, both sets of data fall roughly on a straight line, indicating first order adhesion with bulk concentration. From linear regression, at the 95 % level of confidence the adhesion rate constant for the whole milk foulant has a value of $4x10^{-6} \pm 1x10^{-6}$ cm.s^{-1} and for stainless steel a value of $5x10^{-7} \pm 0.75x10^{-7}$ cm.s^{-1}. This indicates that the adhesion rate of thermophiles to the whole milk fouling layers is around ten times higher than the adhesion rate to clean stainless steel. Comparable values can be found in literature, den Aantrekker *et al.* (2003) obtained a value between $6.0x10^{-7}$ cm.s^{-1} and $2.1x10^{-6}$ cm.s^{-1} for *Staphylococcus aureus* adhesion to silicon rubber surfaces and de Jong *et al.* (2002), a value of $2x10^{-6}$ cm.s^{-1} for attachment of *Streptococcus thermophilus* to a rotating steel disk in laminar flow. We should note that den Aantrekker *et al.* used a technique that recovered all the bacteria in the biofilm, while de Jong *et al.* rinsed their disk, incubated and counted the colonies formed. Thus our

results are more comparable to de Jong's even though the organisms were different. However all three sources of adhesion constants are of similar magnitude.

Flint *et al.* (2001) conducted adhesion studies comparing adhesion to stainless steel and to a milk fouling deposit created by exposing stainless steel coupons to skim milk in an autoclave. They found that the presence of the milk fouling increased the number of cells adhering to the surfaces by 10 to 100 times. The nature of these fouling deposits is much different to the fouling deposits created on the pilot plant heat exchange rigs, however despite this a similar result was obtained.

The fouling layers have a greater surface area of contact with milk available for adhesion than the stainless steel surfaces, due to the rough nature of the fouling layer surface as seen previously in Figure 4.1.14. Although difficult to quantify without a full topographic analysis of the fouling samples, the increase in surface area available is unlikely to be ten times that of the base stainless steel sample, so this cannot account for all of the increase observed. Other unidentified factors must also be involved. For example the thermophiles may have a higher adhesion affinity to fouling than to stainless steel, but a full attempt to identify these issues could not be made within the time frame available in this project.

The present findings will nonetheless be useful in the modelling of thermophile contamination in milk powder plants. In the early stages of colonisation, the dominant factor increasing thermophile numbers on the surface is adhesion to the surface rather than growth at the surface. Therefore, in these early stages, the thermophile surface numbers present on fouled surfaces will be expected to be greater than on clean stainless steel, since the rate of adhesion to fouled surfaces is higher than that for clean stainless steel.

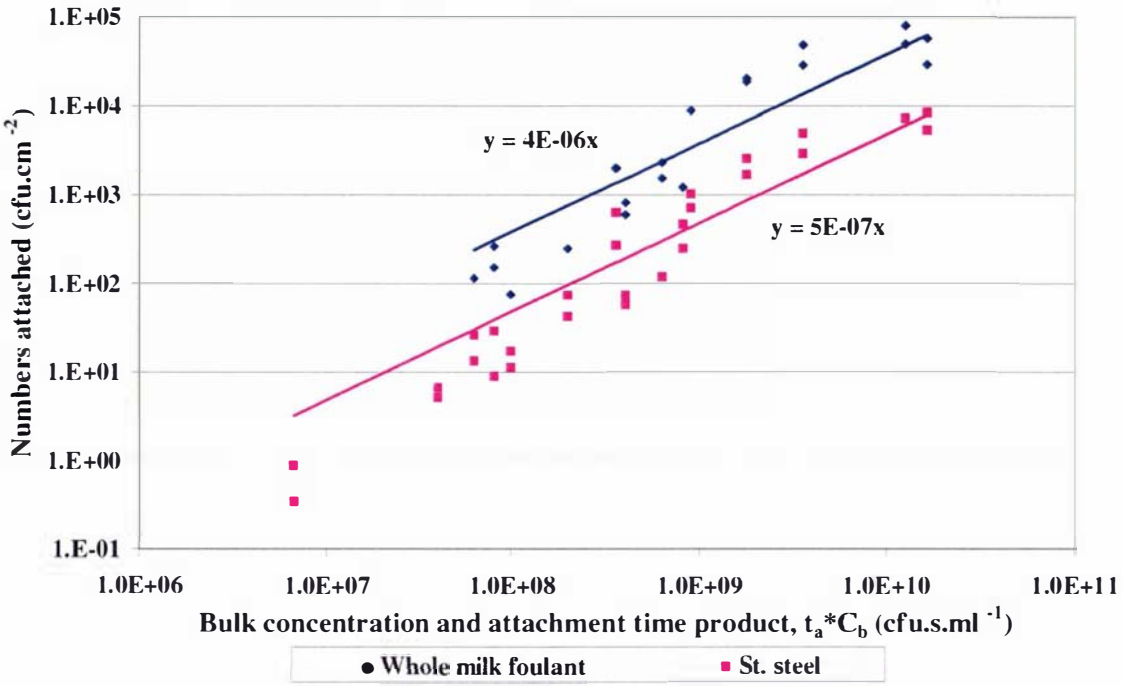


Figure 4.4.4. Adhesion data measured by impedance for whole milk foulant and stainless steel. Surface numbers assessed with impedance microbiology.

4.4.3. Further Discussion

Microscopic measurement of the surface counts gave results with large variation that made it difficult to determine trends in the data. No statistically significant increase in adhered thermophile numbers with increasing bulk cell concentration and no difference between numbers adhered to skim milk foulant and whole milk foulant was found with confidence using CLSM. However, numbers adhered did seem to increase with an increasing amount of foulant present, possibly at least partly due to an increase in the available surface area for adhesion.

Impedance microbiology provided data with less variation and it was found that thermophile numbers attaching to the surface increased with bulk cell concentration and increased with increasing adhesion time up to 20 minutes. It was possible from these data to estimate the adhesion rate constant for whole milk foulant and stainless steel. The whole milk foulant adhesion rate constant was found to be $4x10^{-6} \pm 1x10^{-6}$ $cm.s^{-1}$ and for stainless steel to be $5x10^{-7} \pm 0.75x10^{-7}$ $cm.s^{-1}$. Therefore the adhesion rate of thermophiles to the whole milk fouling layers would be around ten times higher than the adhesion rate to the stainless steel.

The adhesion rate constants found here are used in modelling thermophile contamination from surfaces in Section 4.5 below.

4.5. *Modelling of Thermophile Contamination.*

In this work, steady state and unsteady state models were developed to predict the growth and release of thermophilic bacteria into the bulk milk stream over time from a pilot scale tubular heat exchanger for milk. Initially a simple steady state model was developed to predict the maximum bacterial concentration in the bulk milk stream that could be expected from the heat exchanger. The purpose of the unsteady state model was to predict the effect that different scenarios would have on the bulk milk thermophile contamination over time within the heat exchanger.

4.5.1. Steady state contamination model theory

The steady state release of vegetative cells can be estimated with a simple model. The model assumes that at the end of the run the build up of bacteria adhered at the wall has ceased and all bacteria produced are released into the milk stream. Therefore, the model is more suited for dealing with established biofilm surfaces rather than growing biofilms. This model is similar to that of Langeveld *et al.* (1995) covered earlier (Section 2.2.4.2), however exponential growth kinetics have been incorporated from first principles rather than using the linear approximation of the growth rate used by Langeveld *et al.* (1995).

Let p be the concentration of cells at the surface at a given point in time (cfu.cm^{-2}). The exponential rate of growth is given by:

$$\frac{dp}{dt} = \mu.p \qquad 4.5$$

Where μ is the growth rate constant (s^{-1}). The population at a later time t (s) is obtained by integration:

$$\int_{p_0}^{p} \frac{dp}{p} = \int_{t_0}^{t} \mu.dt \qquad 4.6$$

$$p_t = p.e^{\mu t} \qquad 4.7$$

Our flow system is characteristerised by an average residence time, τ (s), through the THE tubes which is equal to the length, L (cm), divided by the flow velocity, v (cm.s^{-1}):

$$t = \tau = \frac{L}{v} \qquad 4.8$$

The increase in cells on the surface with time, Δp (cfu.cm^{-2}), is given by:

$$\Delta p = p_t - p \qquad 4.9$$

Substituting for p_t from Equation 4.7:

$$\Delta p = p(e^{\mu t} - 1) \qquad 4.10$$

Substituting for τ from Equation 4.8:

$$\Delta p = p(e^{\mu \frac{L}{v}} - 1) \qquad 4.11$$

The growth rate constant, μ (s^{-1}), can be evaluated by rearranging Equation 4.7:

$$k = -\frac{\ln\left(\frac{p_t}{p}\right)}{t} \qquad 4.12$$

Defining the generation time, g (s), as the time required for p_t to double p:

$$\mu = \frac{\ln 2}{g} \qquad 4.13$$

As it is assumed all cells produced at steady state are released into the bulk milk stream, the release of vegetative cells over the THE per square centmetre (cfu.cm^{-2}) is then given by:

$$\Delta p = p(\exp\left(\frac{\ln 2.L}{g.v}\right) - 1) \qquad 4.14$$

Which can be reduced to:

$$\Delta p = p(2^{\frac{L}{g.v}} - 1) \qquad 4.15$$

The increase in concentration of bacteria as the milk passes through the THE tube, ΔC, (cfu.ml^{-1}.tube^{-1}) is given by:

$$\Delta C = \Delta p.\frac{A}{V} = \Delta p.\frac{2.\pi.L(R_2 + R_1)}{\pi.L(R_2^2 - R_1^2)} = \frac{2.\Delta p}{(R_2 - R_1)} \qquad 4.16$$

Where R_1 and R_2 are the outer radius of the inner pipe and the inner radius of the outer pipe respectively (cm), A is the surface area in contact with the milk (cm^2) and V is the volume of milk that has passed through the THE in time τ (ml):

$$A = 2.\pi.L(R_2 + R_1) \qquad 4.17$$

$$V = \pi.L(R_2^2 - R_1^2) \qquad 4.18$$

Then:

$$\Delta C = \frac{2.\Delta p}{(R_2 - R_1)} \qquad 4.19$$

And thus:

$$\Delta C = \frac{2.p(2^{\left(\frac{t_2}{3.32}\right)} - 1)}{(R_2 - R_1)} \qquad 4.20$$

4.5.2. Steady state contamination model predictions

The predictions of the steady state model are shown in Table 4.5.1 below. As can be seen the model under-predicts the bulk bacterial release into the bulk milk stream. This model relies on accurate measurement of the surface bacterial populations and this is likely to be the biggest source of error in the model predictions. It is difficult to obtain an accurate value with the methods available for the bacterial population, as measuring the population involves disturbing the surface. It has been shown experimentally (Gomez-Suarez *et al.*, 2001; Bakker *et al.*, 2002) that disturbances as small as a passing liquid-air interface can remove large proportions (up to 80-90 %) of the bacterial surface population, depending on the surface properties of the bacterium and the substrate. Therefore the methods used here for obtaining surface population measurements by removing the surface from the liquid stream are likely to under estimate surface numbers, even if the numbers remaining on the surface after removal from the liquid stream can be measured accurately. The difference between predicted and actual contamination levels in Table 4.5.1 suggest that the numbers of bacteria on the surface have been underestimated by approximately one order of magnitude.

The steady state equation used by Langeveld *et al.* (1995) (Section 2.2.4.2) also under-predicted the actual measured concentration of bacteria found in the bulk milk stream released from un-fouled stainless steel tubes. Predicted bacterial concentrations were roughly half of the measured values. These predictions used swabbing to enumerate the population of bacteria on the tube surfaces and therefore are also subject to the same issues of underestimation mentioned above. Langeveld *et al.* (1995) also found that using estimates of the surface population on fouled surfaces, based on counts of the

bacteria from within the entire fouling layer, over-predicted the measured bulk milk stream contamination. This indicated that not all of the bacteria in the fouling layer were able to contribute to the release of bacteria into the bulk milk stream. This was not an unusual result as many of the bacteria were embedded within their fouling layers.

If the fouled surface numbers shown previously (Section 4.1.5) are used to predict the amount of bacteria released from the THE tubes the steady state model presented above also over predicts the actual release of thermophiles into the bulk milk stream (Table 4.5.1). Therefore this indicates that not all of the bacteria associated with the fouling layer have equal ability to contribute to the numbers in the bulk milk stream.

A generation time of 1000 seconds (16.7 minutes) is used in these calculations. This value was chosen as it provides a reasonable fit to the experimental data in both the steady state and unsteady state modelling (see below), and is within the range of generation times measured by other authors for similar thermophilic Bacilli in milk of 15 to 25 minutes (Basappa *et al.*, 1974; Flint *et al.*, 2001). The other model parameter used in the calculations were a THE tube length of 110 cm, middle THE tube inner radius (R_2) of 2.36 cm, inner THE tube outer radius (R_1) 1.27 cm and a flow rate of 15 $l.hr^{-1}$ per THE tube.

Table 4.5.1. Predicted and actual release of bacteria from THE tubes. A bacterial generation time of 1000 seconds was used in these calculations.

Release per THE tube ($cfu.ml^{-1}.tube^{-1}$)	Surface population measurement ($cfu.cm^{-2}$)	Actual Release per THE tube ($cfu.ml^{-1}.tube^{-1}$)	Predicted Release per THE tube ($cfu.ml^{-1}.tube^{-1}$)
Run 2 (Values from end of run – un-fouled surface)	$0.6x10^6$ (Swab)	$1.4x10^6$	$0.13x10^6$
Run 4 (Average values between 8-24 hours – un-fouled surface)	$0.36x10^6$ (impedance)	$0.71x10^6$	$0.077x10^6$
Run 5 (Average values between 8-16 hours – un-fouled surface)	$0.23x10^6$ (impedance)	$1.7x10^6$	$0.049x10^6$
Run 5 (Average values between 12-16 hours – fouled surface)	$48x10^6$ (impedance)	$2.7x10^6$	$10x10^6$

4.5.3. Un-Steady state contamination model theory

Models of un-steady state bulk stream microbial contamination are generally based on mass balances of the microorganisms (de Jong *et al.*, 2002; den Aantrekker *et al.*, 2003). The particular difference between this model and others is that only microorganisms at the surface of the wall layer are assumed to interact with the bulk flow. Therefore the population within the wall layer is not relevant. Langeveld *et al.* (1995), de Jong *et al.* (2002) and den Aantrekker *et al.* (2003) all used the population in the entire biofilm or fouling layer for their models. This premise is important since the number of bacteria in fouling layers is much larger than in biofilms as shown above (Section 4.1.5) and in Langeveld *et al.* (1995).

Consider the situation below in Figure 4.5.1 where milk flows through a pipe with thermophilic bacterial contamination.

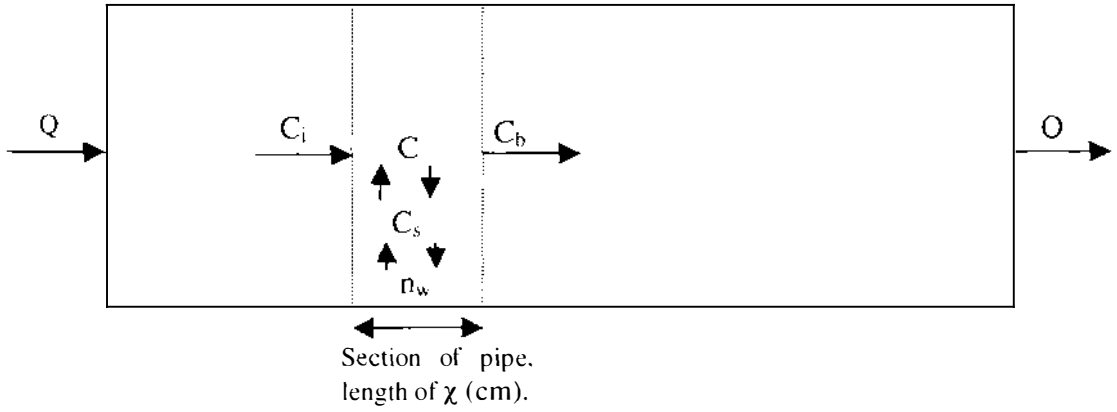


Figure 4.5.1. Diagram of milk flowing through a pipe with thermophilic bacterial contamination.

Where:

Q = Flow rate ($cm^3.s^{-1}$).

C_i = Inlet bulk thermophile concentration to pipe section ($cfu.ml^{-1}$).

C_b = Bulk thermophile concentration at end of section ($cfu.ml^{-1}$).

C = Average bulk thermophile concentration in section, $(C_i + C_b)/2$, ($cfu.ml^{-1}$).

C_s = Bulk thermophile concentration near surface ($cfu.ml^{-1}$).

n_w = Attached thermophile surface population ($cfu.cm^{-2}$).

Two basic equations are required to describe the system, the first to estimate the number of bacteria at the surface of the wall layer and the second to estimate the number of bacteria in the bulk fluid above the wall.

4.5.3.1. Estimate of wall surface population

The rate of increase in surface population $\frac{dn_w}{dt}$ equals the rate of accumulation $\mu.n_w.(1-\beta)$ from growth remaining at surface plus the rate of new adhesion to the surface $k_a.C_s$. Hence,

$$\frac{dn_w}{dt} = \mu.n_w.(1-\beta) + k_a.C_s \qquad 4.21$$

Where:

β is the proportion of thermophiles generated at the surface released into the bulk flow (see below).

k_a is the adhesion rate constant ($cm.s^{-1}$) (see below).

μ is the specific growth rate (s^{-1}) (see below).

4.5.3.2. Estimate of bulk stream population

The rate of change of the bulk bacteria population in the section of length χ, $\frac{dC_b}{dt}$, equals the rate of net flow of bacteria in and out of the section

$$\frac{Q}{V}(C_i - C_b)$$

plus the rate bacteria released from the surface area within the section

$$\frac{A}{V}(\beta.\mu.n_w)$$

minus the rate bacteria adhesion to the surface within the section

$$\frac{A}{V}(-k_a.C_s)$$

plus the rate of bulk growth within the section

$$C.\mu.$$

Hence

$$\frac{dC_b}{dt} = \frac{Q}{V}(C_i - C_b) + \frac{A}{V}(\beta.\mu.n_w - k_a.C_s) + C.\mu \qquad 4.22$$

Where:

V is the volume of the pipe section (cm^3) (see below).

A is the surface area of the pipe section (cm^2) (see below).

This mass balance results in the same equations as that generated by de Jong *et al.* (2002). Nomenclature has been kept consistent with that used by de Jong for easier comparison between the two applications of the model. However, in de Jong *et al.* (2002) the equation for bulk contamination with respect to time is only applied to tank reactor situations. A separate mass balance equation was generated for bulk contamination with respect to tube length in a plug flow reactor system, therefore providing three equations in de Jong's model (Section 2.2.4.2). In the solution of the

model presented here, Equation 4.22 above has been applied in differential elements along the tube length through the use of finite differences, therefore a differential equation describing bulk contamination with respect to tube length is not required.

4.5.3.3. Parameter estimation

A number of parameters required by Equations 4.21 and 4.22 must be estimated through independent experiments.

The growth rate of the bacteria can be calculated by:

$$\mu = \frac{\ln 2}{g} \qquad 4.23$$

Where g is the thermophile generation time (or doubling time) (s).
The rate of adhesion to the surface is given by:

$$k_a = \frac{n_w}{t_a . C_s} \qquad 4.24$$

This has been determined experimentally from adhesion experiments (Section 4.4) where surface samples were exposed to bacterial suspensions of different bulk concentrations for different lengths of time (denoted in the formula as the adhesion time, t_a (s)).

The parameter β, the proportion of thermophiles generated at the surface of the wall layer that are released into the bulk flow, cannot be readily measured. A general form for this parameter can however be formulated based on fundamental assumptions. Intuitively one would expect the rate of release of surface bacteria over time to depend on the number of surface bacteria, the rate of increase of surface bacteria and the convection strength of the flow stream.

A simple formulation, originally proposed by de Jong *et al.* (2002), has been applied in this model:

$$\beta = 1 - a.e^{t - k_r . n_s} \qquad 4.25$$

where:

k_r= release constant. This constant reflects the change in β with surface population n_w.

a = constant controlling initial release proportion (i.e. when $n_w = 0$).

Both a and k_r would be effected by convective forces which are a function of the Reynolds number (Re). In the present work, since only one flow rate is used, this Reynolds number effect does not need to be modelled explicitly.

The variations of the parameter β, described by Equation 4.25, with surface population are shown in Figure 4.5.2 and indicate that in the initial period of film build up most of the bacteria generated at the wall are retained there and few are released. As the bacteria at the surface of the wall become crowded, the proportion released increases until at steady state all the increase in surface population is released into the bulk stream.

The most suitable values for a and k_r must be obtained by fitting Equations 4.21 and 4.22 to data from experimental runs performed in the equipment and with the bacteria under investigation. It can then be used for predictions of subsequent runs.

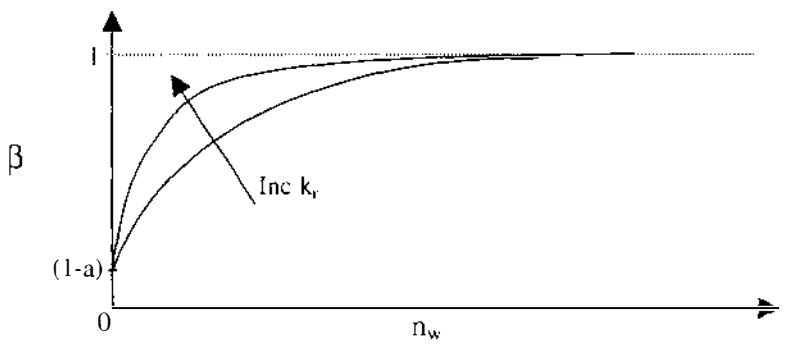


Figure 4.5.2. Change in the proportion of bacteria generated that are released from the surface (β) as the surface population increases.

It is also assumed in the model that as the adhesion rate is relatively small, adhesion does not noticeably deplete the concentration of bacteria near the surface. Hence C_s= C.

The surface area for a section of the double pipe heat exchanger system of the THE with the milk in the outer pipe was calculated as:

$$A = 2\pi.x.(R_1 + R_2) \qquad 4.26$$

Where:

R_1 = inner pipe O.D. radius (m)

R_2 = outer pipe I.D. radius (m).

Likewise the volume for a section of the double pipe heat exchanger system was calculated using:

$$V = \pi.x.(R_2^2 - R_1^2) \qquad 4.27$$

Temperature dependant growth kinetics were not incorporated into the model as the THE rig in the pilot plant was operated at a constant temperature. Therefore adding this into the model would add complexity that is not required for this situation.

4.5.3.4. Numerical solution

To enable predictions of surface and bulk populations Equations 4.21 and 4.22 were integrated numerically with the ode45 solver function available in Matlab version 5.2 (The Mathworks Inc. Natick, MA).

The numerical integration was carried out using finite differences (Benefield & Molz, 1985) and solved in Matlab. The Equations 4.21 and 4.22 were entered in the Matlab function file code in finite difference format (Matlab files can be found in Appendix E, pages E-1 to E-6). The finite difference equation formats and finite difference grid is given below in Figure 4.5.3. The numerical solution in Matlab was run with 6 nodes per THE tube (18 nodes in total, J=18), therefore each node spanned a pipe length of approximately 1/6 m.

Surface population equation (from Equation 4.21):

$$\frac{dn_{w_j}}{dt} = \mu.n_{w_j}.(1-\beta) + k_a.\frac{(C_{b_{j-1}} + C_{b_j})}{2} \qquad 4.28$$

Bulk population equation (from Equation 4.22):

$$\frac{dC_{b_j}}{dt} = \frac{Q}{V}(C_{b_{j-1}} - C_{b_j}) + \frac{A}{V}(\beta.\mu.n_{w_j} - k_a.\frac{(C_{b_{j-1}} + C_{b_j})}{2} + \frac{(C_{b_{j-1}} + C_{b_j})}{2}.\mu \qquad 4.29$$

Finite difference grid:

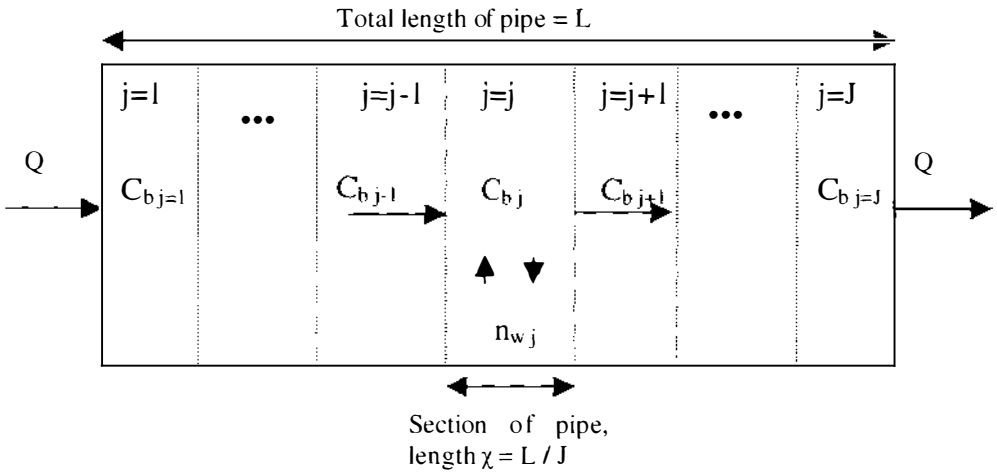


Figure 4.5.3. Diagram of finite difference grid applied in the numerical solution of the un-steady state model. The grid consists of J number of nodes spaced over the length (L) of the pipe.

4.5.4. Un-Steady state contamination model predictions

In the work on thermophile adherence above (Section 4.4), a value of $5x10^{-7} \pm 0.75x10^{-7}$ $cm.s^{-1}$ was estimated for the adhesion rate constant (k_a) of *B. stearothermophilus* to stainless steel. For the predictions below this has been increased by a decade to $5x10^{-6}$ $cm.s^{-1}$ to provide a better fit to the experimental data. The reason why the larger value gives a better fit is probably mainly due to the difficulty in assessing surface numbers that was mentioned earlier in Section 4.5.2 above. In the adherence work above the surfaces were rinsed before enumeration, thus the adhered surface numbers and therefore the adhesion rate constant would have been underestimated due to the removal of loosely adhered cells. As was used above in the steady state modelling (Section 4.5.2), a generation time of 1000 seconds was used in the unsteady state predictions. The other constants a and k_r were adjusted to provide the best fit for the experimental data and the values used below are 0.9 and $8x10^{-7}$ respectively. These estimates were obtained using an optimisation function within MATLAB® that enables optimisation of the model predictions against the experimental results by adjusting the value of the parameters over a series of iterations (Appendix E, page E-1 contains further details).

The model was used to predict the contamination to the bulk flow with different levels of inoculation of *B. stearothermophilus* to the milk vat and the increase in surface

population with time. The runs targeted for modelling are those where fouling was prevented by keeping the milk and hot water temperature at around 55°C. The effect of recontamination from residual bacterially active fouling layer is also predicted. The effect of modifying the attachment rate and bacterial doubling time are also simulated. These what-if scenarios allow insights into potential gains of surface treatments of equipment material and process modifications to change bacterial growth patterns. A comparison of contamination from clean stainless steel and fouled surfaces is also discussed.

4.5.4.1. Prediction of Bulk Numbers

Figure 4.5.4 shows the model predictions of thermophile numbers in the bulk stream exiting the THE over time for situations where the inlet bacterial bulk concentration varies and compares these predictions with experimental data points. The model predictions fit the pattern of the contamination profile of the experimental data quite well as shown on Figure 4.5.4. Steady state contamination concentrations are modelled closely however there are slight differences in the rate of build up. The model slightly under predicts the bacterial concentration of the two runs with higher inlet thermophile numbers (5000 and 30000 $cfu.ml^{-1}$) after 4 hours. The run with 200 $cfu.ml^{-1}$ in the inlet milk is fitted closely. The model prediction for the run with low numbers in the inlet milk (10 $cfu.ml^{-1}$) under predicts the bacterial concentration in the early stages of the run 2-6 hours).

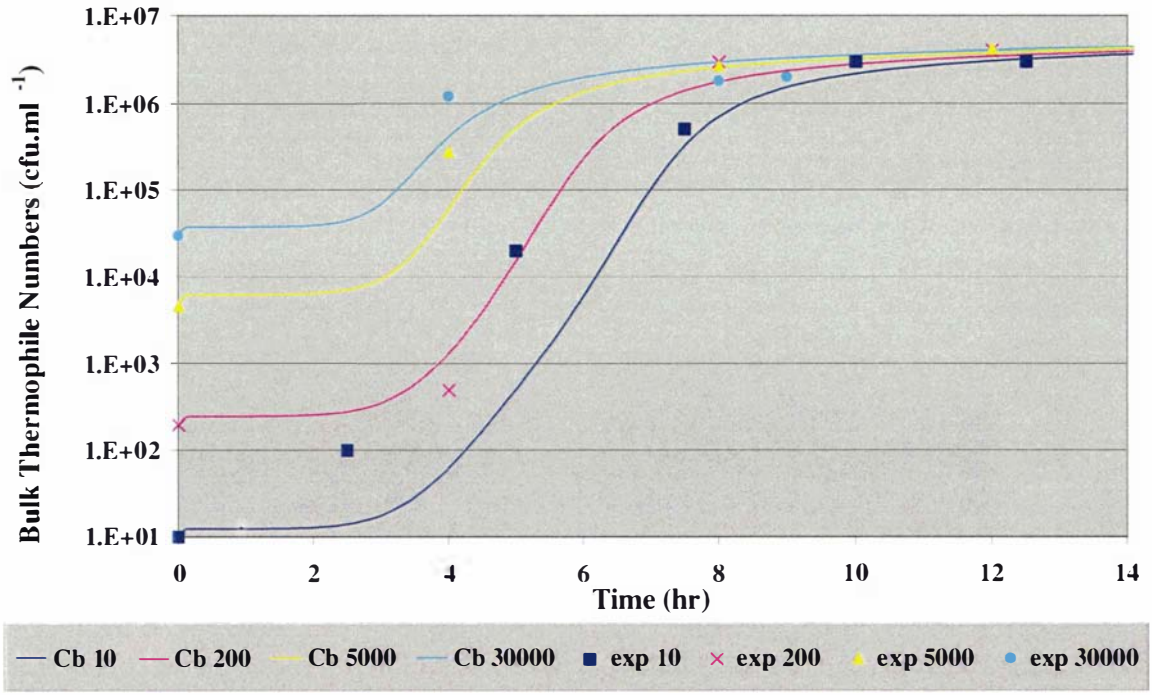


Figure 4.5.4. Predicted profile of thermophile contamination of bulk milk with varying inlet bulk concentrations of bacteria (Cb 10, Cb 200, Cb 5000 and Cb 30000) compared with experimental data (exp 10, exp 200, exp 5000 and exp 30000). The solid lines show model predictions while experimental data are shown as the single data points. Inlet bacterial concentrations of 10, 200, 5000 and 30000 cfu.ml^{-1} are shown (k_a = 5E-6, a = 0.9, k_r = 8E-7 and g = 1000 s).

One possible reason for this discrepancy at the low inlet concentrations could be that the plant was not completely clean at the beginning of the run. As the numbers of bacteria in the milk were low, the effect of this would be more noticeable than in the other runs, where higher inlet numbers would have masked the effect of a small number of bacteria remaining on the surface after cleaning. If the model is rerun with the same inlet concentration of 10 cfu.ml^{-1} but also with an added average of only 1 cfu.cm^{-2} of bacteria on the surfaces because of poor cleaning, the model quite closely fits the experimental data, as shown in Figure 4.5.5 below (dotted line).

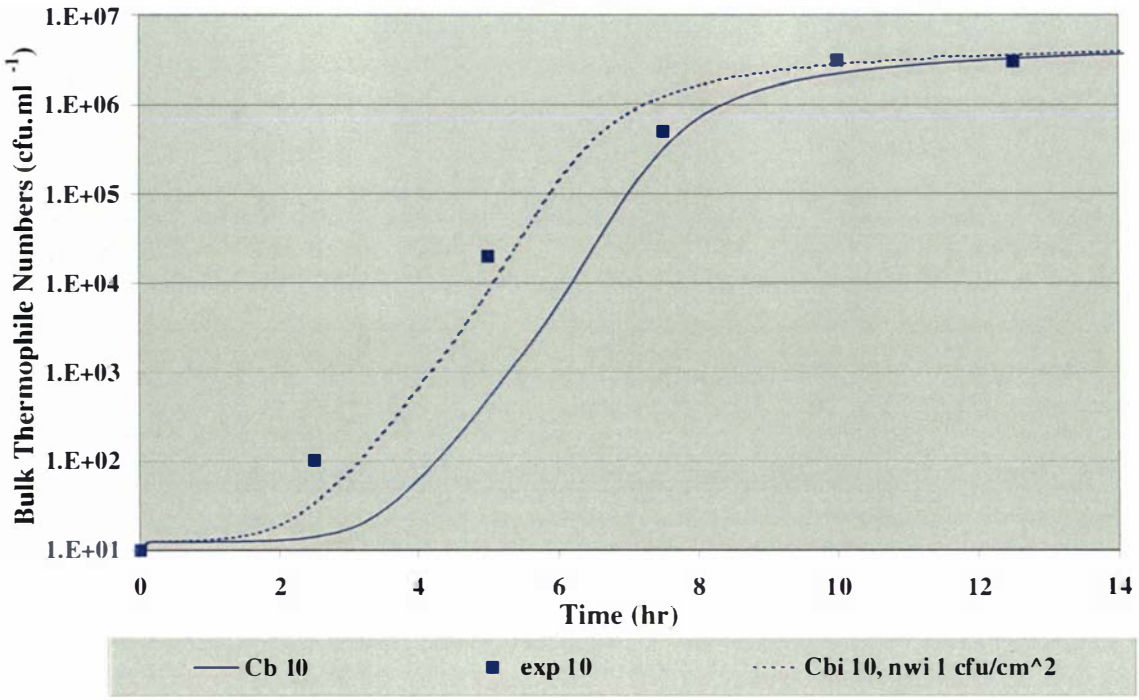


Figure 4.5.5. Predicted profile of thermophile contamination of bulk milk with an inlet bulk concentration of 10 cfu.ml^{-1} bacteria (Cb 10) compared to experimental data (exp 10). The solid line shows model predictions with no bacteria initially present on the surface and the dotted line with 1 cfu.cm^{-2} initially present on the surface (depicted as Cbi 10, nwi 1 cfu.cm^{-2}. The experimental data are shown as the single data points (k_a = 5E-6, a = 0.9, k_r = 8E-7 and g = 1000 s).

Experimental Run 5 compared the release of thermophilic bacteria into the bulk milk between clean surfaces and surfaces that started the run with a contaminated surface upstream of their location. This was conducted on the THE rig with one tube set completely clean and the other tube set with the first tube already contaminated with thermophiles. The contamination in the bulk milk exiting each side of the THE was compared over time. The model can also predict the contamination profile in this situation. The experimental and predicted values are compared in Figure 4.5.6.

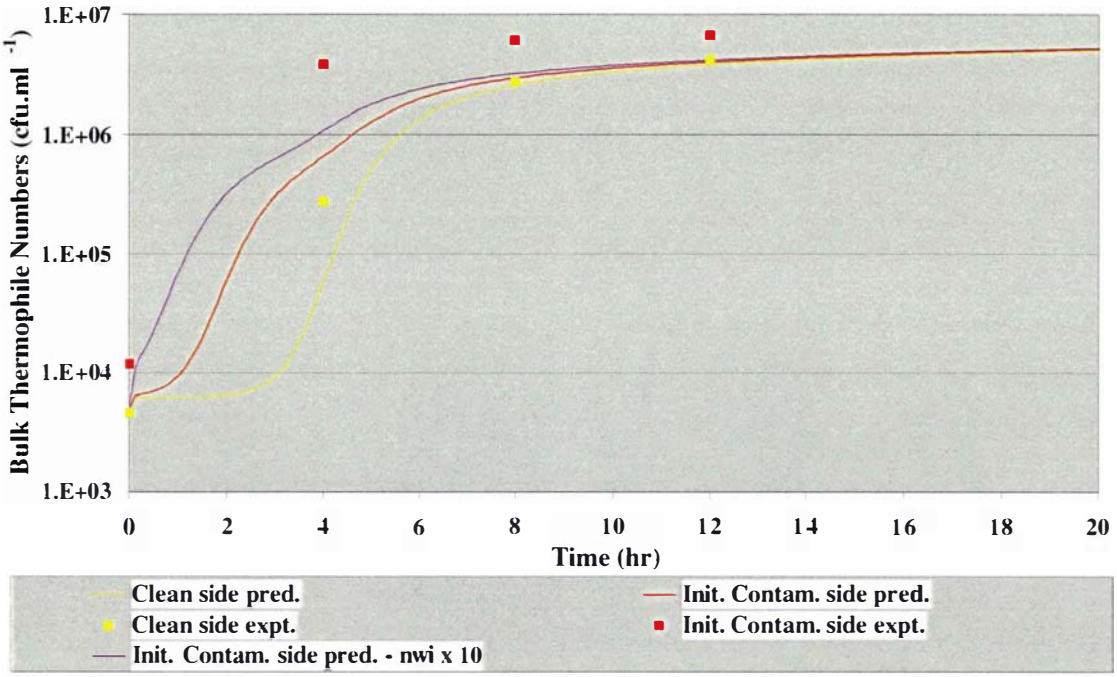


Figure 4.5.6. Experimental and predicted bulk milk contamination at the THE exit for a situation such as in Run 5 where the first tube on one side of the THE was initially contaminated with $4x10^4$ cfu.cm^{-2}. Also shown is the predicted contamination profile if the surface numbers were initially $4x10^5$ cfu.cm^{-2} (depicted in legend as nwi x 10). The inlet concentration of bacteria was 5000 cfu.ml^{-1} (k_a = 5E-6, a = 0.9, k_r = 8E-7 and g = 1000 s).

The model prediction follows the same pattern as the experimental data, however the contamination from the initially contaminated side of the THE is under predicted, particularly after 2 - 4 hours. If the surface numbers on the initially contaminated tube were under estimated this could result in under prediction by the model. If the initially contaminated surface numbers are increased 10 times ($4x10^5$ cfu.cm^{-2}) then the model prediction is closer to the experimental data.

4.5.4.2. Prediction of surface numbers

The surface thermophilic bacterial population over time was also predicted (Equation 4.21). Figure 4.5.7 shows the model predictions of surface numbers compared with experimental measurements from Runs 4 and 5, where the initial bulk concentration of bacteria was 200 and 5000 cfu.ml^{-1} respectively. As can be seen, the rate of build up between model predictions and experimental results is similar. However, the numbers predicted are a decade greater than those found experimentally. As mentioned

previously in regard to the steady state model, this could be due to the difficulty in measuring surface numbers accurately.

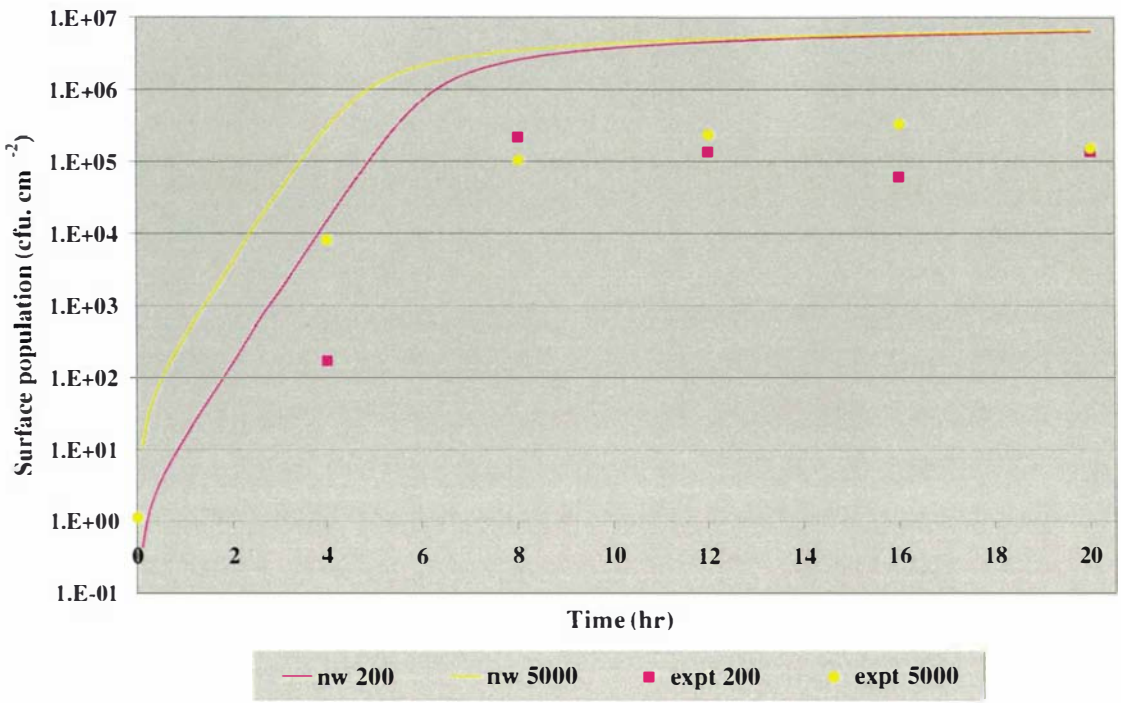


Figure 4.5.7. Model predictions of surface numbers (nw 100 and nw 5000) compared to experimental measurements from Runs 4 (expt 200) and 5 (expt 5000) where the initial bulk concentration of bacteria was 200 and 5000 cfu.ml^{-1} respectively. The solid lines show model predictions while experimental data is shown as the single data points (k_a = 5E-6, a = 0.9, k_r = 8E-7 and g = 1000 s).

Accurate enumeration of adhered surface numbers that contribute to the bulk milk contamination is a problem. The comparison between the model predictions of de Jong *et al.* (2002) and their swabbing results of wall population were even further underestimated than the work described above as underestimations of two to three decades were observed. Removing the test surfaces from the equipment and rinsing them before enumeration underestimates the numbers present. However, if they are not rinsed then thermophiles not adhered to the surface but present in the residual milk left on the surface may over estimate the count. This problem was resolved by den Aantrekker *et al.* (2003) where a combination of methods were used to get an estimate of loosely, reversibly and irreversibly attached cells in a test rig consisting of a series of tubes. After a liquid sample was taken from the tube under flowing conditions, the liquid from inside the tube was collected and the bacteria enumerated. This liquid

contained both loosely attached cells and bulk liquid cells. The number of loosely attached cells was determined by subtracting the bulk liquid cell numbers. To determine reversibly attached cell numbers the tube surfaces were rinsed and the bacteria in the rinsing liquid enumerated. Irreversibly attached cells were determined by exposing the tube surfaces to shaking with glass beads and enumerating the removed cells. All three values were then added together to obtain the total number of attached cells on the surface. These experimental values were predicted well by the model developed by den Aantrekker *et al.* (2003), thus showing that this is a more accurate way of determining total surface numbers and confirms that the numbers measured experimentally in this thesis are an underestimate.

4.5.4.3. Variation of attachment constant

The effect of altering parameters in the model was also investigated. One of these parameters was the adhesion constant, k_a. As can be seen on Figure 4.5.8, each ten fold reduction in the adhesion constant increases the initial four hour lag time of contamination by around one hour. For the first decrease in the adhesion constant this is a 25 % increase (from 4 hours to 5 hours), potentially providing a 25 % longer production run before high thermophile numbers would require the plant to be cleaned.

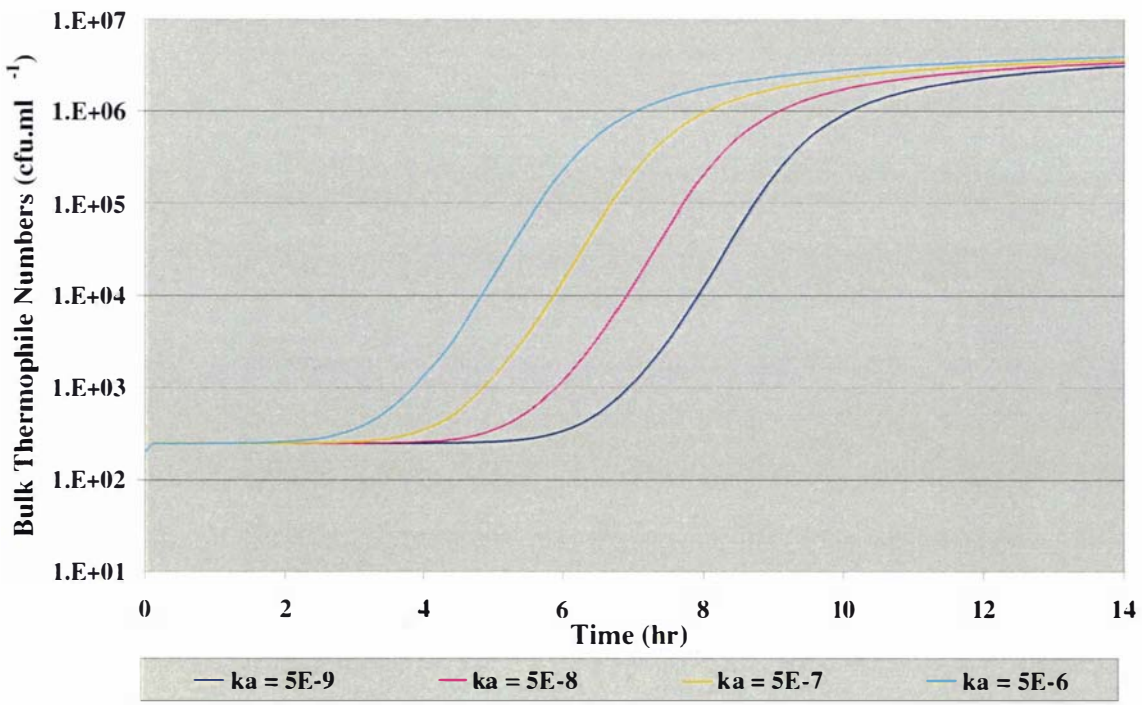


Figure 4.5.8. Model predictions showing the variation in the bulk contamination profile as the adhesion constant is reduced from 5E-6 to 5E-9 $cm.s^{-1}$ (Cbi (initial bulk numbers) = 200 $cfu.ml^{-1}$, a = 0.9, k_r = 8E-7 and g = 1000 s).

It is possible that the adhesion constant could be reduced through the use of different surface treatments (Parkar *et al.*, 2003) or in other ways such as increasing the shear stress at the wall (de Jong *et al.*, 2002) from using higher flow rates. However, to make significant gains in production time, quite large reductions in the adhesion rate of the bacteria to the surfaces (e.g. reduced by 10 times) would be required. This may be possible through the use of specially treated stainless steel or surface coatings (Parkar *et al.*, 2003) and is an area of research that has potential to provide an extension of operation times in milk powder plants.

The surface numbers on the wall were also predicted for different rates of adhesion as shown below in Figure 4.5.9. As can be seen, the rate of increase of build up of bacteria once an initial surface population is established, in each case is the same. This is because the growth rate at the surface, not the adhesion rate, is the greater and hence dominant process increasing the surface population. The difference between each prediction is in the time it takes to establish the initial surface population, which is dependent on the adhesion rate.

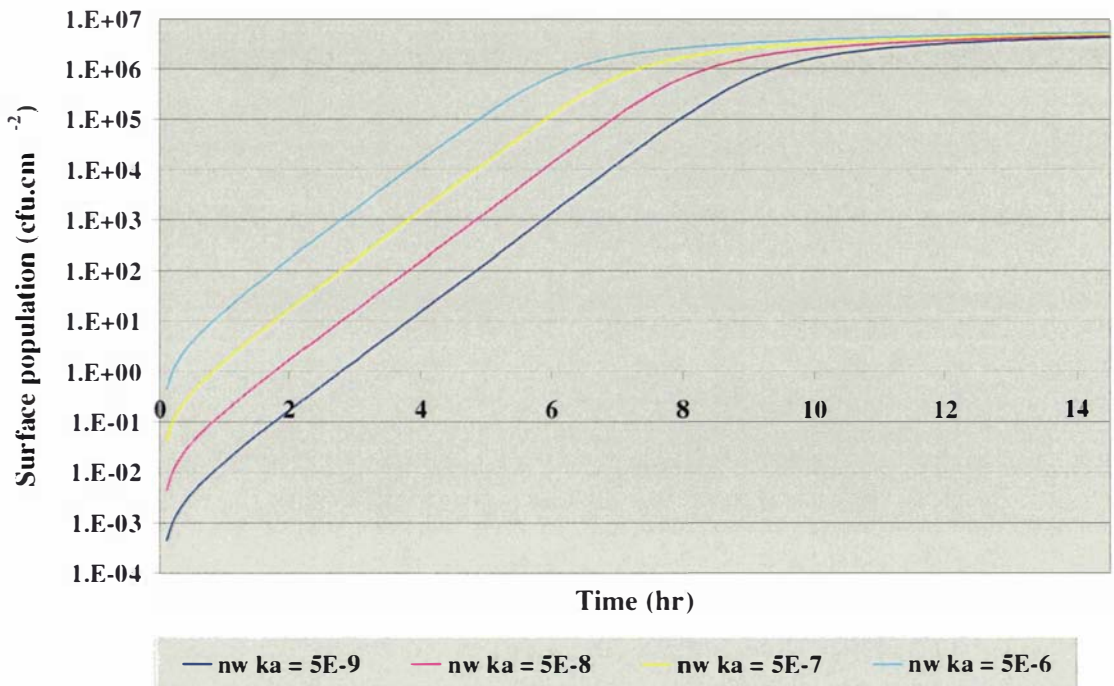


Figure 4.5.9. Model predictions showing the variation in the surface numbers over time as the adhesion constant is reduced from 5E-6 to 5E-9 $cm.s^{-1}$ (Cbi (initial bulk numbers) = 200 $cfu.ml^{-1}$, a = 0.9, k_r = 8E-7 and g = 1000 s).

4.5.4.4. Variation of generation time

As with the adhesion constant the effect of varying the generation time can also be predicted. Figure 4.5.10 shows that increasing the generation time also increases the lag time of contamination by around an hour for every 200-300 second increase in the generation time. As well as taking longer for large amounts of contamination to occur, the steady state level of contamination is also lower. This effect would be likely to occur in areas within the process where the thermophilic bacteria were in sub-optimal conditions, such as low or high temperatures, or in regions of high total solids concentrate. The bacteria are still potentially capable of causing a contamination problem in these areas, but the process will take longer and the amount of contamination produced at steady state will be less.

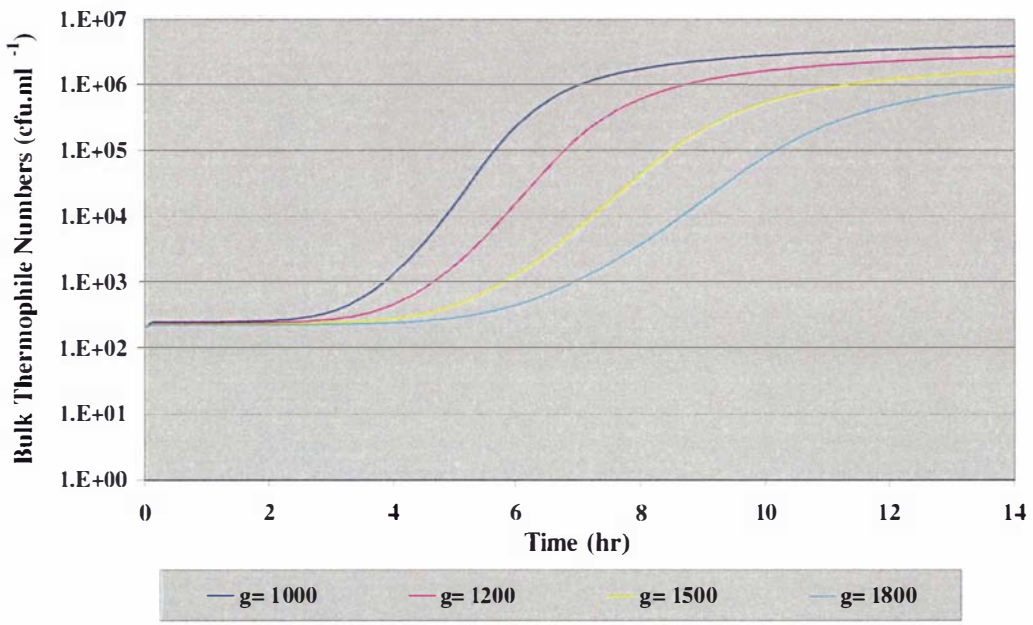


Figure 4.5.10. Model predictions showing the variation in the bulk contamination profile as generation time is increased (Cbi = 200 cfu.ml^{-1}, a = 0.9, k_r = 8E-7 and k_a = 5E-6).

Surface numbers were also predicted as shown in Figure 4.5.11. Unlike the effect of reducing the adhesion constant, increasing the generation time changes the rate of increase of numbers at the surface rather than the initial development of a surface population.

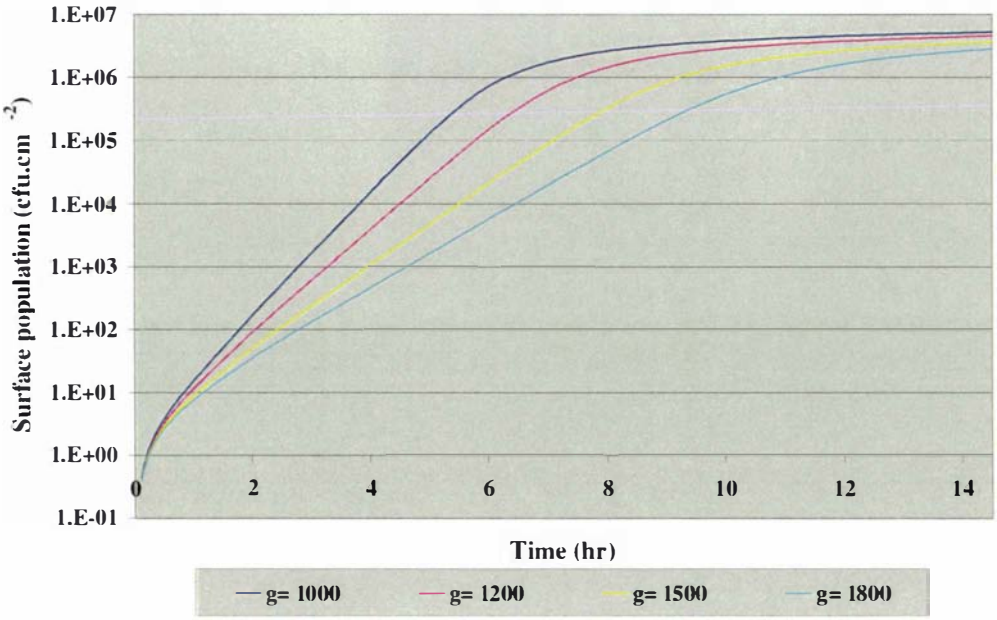


Figure 4.5.11. Model predictions showing the variation in surface numbers over time as generation time is increased (Cbi = 200 cfu.ml^{-1}, a = 0.9, k_r = 8E-7 and k_a = 5E-6).

4.5.4.5. Further Discussion

The steady state model provides a quick estimate of the level of bulk milk contamination that can be expected, however it is dependent on obtaining accurate measurements of the surface numbers.

The unsteady state model predicts the trends observed in the experimental data and provides reasonable estimates of the bulk contamination that can be expected over time from the tubular heat exchanger. Situations where parameters in the model such as adhesion rate and generation time are varied can also be modelled. This provides an insight to the magnitude of any gains that can be made by modifying different aspects of the system.

Before the unsteady state model could be applied in an industrial situation, such as for a plate heat exchanger, the model would have to incorporate temperature dependant growth kinetics. This is because industrial heat exchangers operate at a range of temperatures, unlike the pilot plant THE, in which the surfaces were isothermal. This would also require an expression for the temperature profile across the heat exchanger to be incorporated into the model. In addition to this, the effect of the greater flow velocity and turbulence present in an industrial plant compared to the pilot plant on

surface colonisation and contamination would have to be assessed. The Reynolds number in the pilot plant THE was typically 300, while in an industrial plant values of 150×10^3 are typical in pipes. This increase would be likely to reduce the adhesion constant (k_a) (de Jong *et al.*, 2001). Adjustment of the constants a and k_r in the function controlling the proportion of cells released (β) may also be required to fit predictions of industrial contamination to measured industrial data, due to the different flow conditions. However, the relative affect of varying the model parameters, as carried out above, will remain the same, as the basic properties of the system would be unchanged.

5. Conclusions and Recommendations

5.1. Conclusions

Experimental work conducted in the pilot plant has shown that there are 10 to 100 times greater numbers of thermophilic bacteria (*Bacillus stearothermophilus*) associated with the fouling deposits than found on the stainless steel surfaces, yet the number of thermopiles released into the bulk milk flow is similar in each case. This is possibly a result of thermophiles deeper within the deposit (i.e. further from the solid-liquid interface) having more difficulty being released into the bulk flow than bacteria closer to the interface. A smaller proportion of these thermophiles would then be released into the bulk flow. This would cause numbers to build up within the fouling layer faster than would occur on stainless steel where the thermophiles can be released into the bulk flow more easily. As colonisation progresses, eventually a steady state point seems to be reached as the surface numbers plateau. At this point the production of cells at the surface would be equal to the release of cells into the bulk flow. Therefore, to make it possible for the steady state release from the fouled and un-fouled surfaces to be similar, the number of bacteria generated from the two surfaces must be the same at this point. Since there are more bacteria on the fouled surface than the stainless steel surface the average generation time of the bacterial population on the fouled surface must be longer at steady state than on the stainless steel. This could be due to toxic products or nutrient limitation effects in the harder to access locations of the fouling layer. Thermophiles in the easier to access (that is higher and less sheltered) regions are likely to have similar generation times to the bacteria on stainless steel. But if nutrient limitation and toxic products are greater in the harder to access regions, then growth in these areas will be low. The higher proportion of spores per bacterial population on the fouling layer than on stainless steel may be one indicator that there is greater stress on the bacteria that are on the fouling layer.

This similarity between the bulk contamination from fouled and un-fouled surfaces shows that in regard to thermophilic bacterial contamination, fouling deposits do not increase the steady state amount of bulk contamination. The more important factor in determining the amount of contamination at steady state is the amount of surface area available for colonisation within the temperature growth range of the thermophiles.

The number of thermophilic bacteria released into the bulk milk stream was found to be linearly proportional to the amount of wetted surface contact area that the milk passes that is within the growth range of thermophilic bacteria of approximately 45 °C to 65 °C. The wetted surface contact area is defined as the amount of surface area of stainless steel pipe that is in contact with the milk as it flows past. The numbers of thermophilic bacteria released into the milk increased in approximately even increments after passing through each tube placed in series, with both the pre-fouled and un-fouled tube sets. Therefore, it is important to design milk powder plants with a minimum amount of surface contact area available in the parts of the process that are within the thermophilic bacteria growth temperature range. This should provide the maximum possible production length before thermophilic bacteria or spores in the milk stream reach specification limits.

It was also observed that the number of thermophiles released from a single heat exchange tube (with a wetted contact surface area of 1200 cm^2) seems to reach a maximum at around $2 - 3 \times 10^6$ $cfu.ml^{-1}$ in the pilot plant. This indicates that there is a maximum contamination potential that each heat exchange tube can provide. This would also hold true for industrial dairy processing equipment, however by the time this is reached the amount of contamination in the product stream is likely to be unacceptable due to the amount of surface area available for colonisation at temperatures suited to thermophile growth in industrial plants. However, if the amount of surface area available for thermophile growth was designed to be minimal, this maximum amount of contamination may be small enough that it is still acceptable in terms of product quality. This would then allow longer manufacturing runs as the product contamination would reach and maintain an acceptable maximum amount despite long operating times.

It has been shown that the protection against cleaning provided by milk fouling layers is greater than that provided by a biofilm alone. During milk powder production runs thermophiles are continuously attaching and detaching from the plant surfaces. Simultaneously, milk solids are continuously deposited. Thus necessarily, some bacteria will be embedded in the fouling matrix found at the end of the run. In the cleaning investigation carried out, the hard to clean areas in the plant were simulated, as it is

likely that cleaning procedures in the dairy industry are effective in the high flow areas and that it is the hard to clean areas that are the problem as far as residual contamination is concerned. These are areas of low or slow flow (including partially blocked evaporator tubes and plate heat exchangers) where fouling may remain after cleaning. The observed results indicate that caustic solution is unable to deactivate or remove the bacteria entrapped in the foulant as long as some of it remains. As foulant is removed by the caustic solution, the bacteria present at the newly exposed surface can then become active and could hence be available to contaminate the next production run.

This shows that fouling can allow thermophiles to survive during cleaning of dairy equipment, especially in hard to clean locations where fouling remains after cleaning. It is therefore important when cleaning dairy equipment to remove all traces of fouling to minimise the numbers of thermophiles in the plant after cleaning so that the amount of recontamination in the plant in the following production run is kept to a minimum.

These findings show that the importance of fouling deposits in thermophilic bacterial contamination is not to increase the steady state release of bacteria into the bulk milk, but to provide areas where thermophiles can be protected from cleaning and act as seeding points for future runs.

In addition to this, there is also another link between fouling and thermophile contamination. Since fouling acts as a thermal insulator it decreases the heat transfer coefficient. Therefore, to achieve the same level of heating throughout a production run the heat exchanger must be oversized to account for the development of fouling. As mentioned, is has been observed experimentally that the thermophilic bacterial contamination in the bulk stream will occur faster as a consequence of the increased surface area of the heat exchanger. Therefore if fouling can be minimised a smaller heat exchanger surface area can be used (for instance plates in a plate heat exchanger could be removed) which would thus reduce the potential for thermophile contamination.

It was found that the time taken for bulk contamination to become excessive (greater than $1x10^6$ $cfu.ml^{-1}$) was halved when residual contamination was present upstream of the tubular heat exchange rig, thus showing that run lengths can be shortened dramatically if active thermophiles are left on the surfaces within the plant. It was also

observed that as the initial bulk concentration of thermophilic bacteria fed into the pilot plant increased, the lag time of growth was reduced. In addition, the time for the bulk contamination to approach steady state was reduced as the initial bulk concentration increased. Therefore, thermophiles that survive cleaning or greater initial thermophile concentrations in the raw milk can reduce the plant production time available before concentrations of thermophiles in the bulk milk become excessive.

During the contamination process thermophilic bacteria are transported from their original locations via convection in the bulk flow to downstream areas of the plant. Experiments have shown that even in laminar conditions convection is able to transport detached bacteria much faster than bacterial motility or spreading of the biofilm across the surface (1300 $cm.hr^{-1}$ with convective transport under laminar flow compared to 8 $cm.hr^{-1}$ under static conditions). It was also shown that contamination from downstream to upstream does not occur quickly even under laminar flow (less than 1.3 $cm.hr^{-1}$), also showing that spreading of the biofilm along the surface is not a major factor in the contamination of milk processing equipment by thermophilic bacteria.

It is thus crucial to ensure that cleaning procedures in milk powder plants remove or destroy all traces of thermophiles so that none are present at the start of the run to allow the maximum possible run length. It is similarly important that milk fed into any thermal milk processing equipment with milk contact surfaces within the growth temperature range of thermophilic bacteria has the lowest possible thermophile concentration. This includes the processing operations in the early stages of the manufacturing process such as separation and pasteurisation, as any increases in thermophile concentration in the milk from these areas will potentially be amplified when they reach the downstream processes.

During adhesion studies the number of thermophilic bacteria adhering to stainless steel surfaces increased with bulk cell concentration and increasing contact time for adhesion. The whole milk foulant adhesion rate constant was found to be $4x10^{-6} \pm 1x10^{-6}$ $cm.s^{-1}$ and for stainless steel to be $5x10^{-7} \pm 0.75x10^{-7}$ $cm.s^{-1}$. Therefore the adhesion rate of thermophiles to whole milk fouling layers is around ten times higher than the adhesion rate to the stainless steel. This knowledge was applied in the thermophile modelling investigations.

Steady state and unsteady state thermophile contamination models were developed. The steady state model provides a quick estimate of the level of bulk milk contamination that can be expected, however it is dependent on obtaining accurate measurements of the surface numbers. The model was found to under-predict the bulk bacterial release into the bulk milk stream by approximately a decade. This under-prediction is most likely due to error in the measurements of surface numbers. The methods used for obtaining surface population measurements by removing the surface from the liquid stream and rinsing are likely to under estimate surface numbers by up to ten times as the loosely adhered cells are dislodged and not counted. This discrepancy would account for the difference between predicted and actual contamination. If the fouled surface numbers are used to predict the amount of bacteria released using the steady state model the release is over predicted by around 4 times. Therefore this indicates that not all of the bacteria associated with the fouling layer have equal ability to contribute to the numbers in the bulk milk stream. This would also provide further support for the explanation provided above regarding the similarity of the numbers of thermophiles released from clean and fouled surfaces despite 10-100 times greater thermophiles being present on the fouled surface.

The unsteady state model predicts adequately the trends in contamination of the bulk milk flowing through the tubular heat exchanger. The model proved to be quite flexible and could predict both the effect of increases to the initial population of thermophiles in the milk feed and the effect of recontamination from improperly cleaned surfaces. However the surface population was over estimated by a factor of 10. It is suspected that this discrepancy is due to the inadequacy of the enumeration techniques used for surface populations of wall layers as mentioned above.

Situations where parameters in the model such as adhesion rate and generation time are varied could also be modelled. This provides an insight to the magnitude of any gains that can be made. Decreasing the adhesion constant increased the lag time of bulk contamination. The lag time in the development of surface numbers was also increased. However, the rate of increase of build up of bacteria once an initial surface population is established is the same. This is because the growth rate at the surface, not the adhesion rate, is the greater and hence dominant process increasing the surface population.

Therefore, the difference between each prediction is in the time it takes to establish the initial surface population, which is dependent on the adhesion rate.

Practically it is possible that the adhesion constant could be reduced through the use of different surface treatments or in other ways such as increasing the shear stress at the wall from using higher flow rates. However, the model shows that to make significant gains in production time, quite large reductions in the adhesion rate of the bacteria to the surfaces (e.g. reduced by 10 times) would be required. This may be possible through the use of specially treated stainless steel or surface coatings, as suggested by the work of Busscher *et al.* (1996). This is an area of research that has potential to provide an extension of operation times in milk powder plants if these technologies can be applied to the areas in the process where milk contact surface area exists in the temperature range for thermophile growth. However the stability of any applied surface coating and its regulatory status if found in the final product will be important considerations to factor into such work.

Increasing the generation time increased the lag time of contamination and also reduced the steady state level of contamination. This effect would be likely to occur in areas within the process where the thermophilic bacteria were in sub-optimal conditions, such as low or high temperatures, or in regions of high total solids concentrate. The bacteria are still potentially capable of causing a contamination problem in these areas, but the process will take longer and the amount of contamination produced at steady state will be less. Unlike the effect of reducing the adhesion constant, increasing the generation time changes the rate of increase of numbers at the surface rather than the initial development of a surface population.

5.2. Recommendations

From the work described we can outline a number of practical steps that can be taken to reduce product and plant contamination with thermophiles:

1. Do not operate equipment in the temperature range of 40-70 °C unless necessary. This is particularly true for equipment found in the early parts of the

manufacturing process such as separators and pasteurisers, as these can speed up re-contamination of downstream processing equipment by increasing the initial thermophile numbers fed into the milk powder plants.

2. Minimise the contact surface area of thermal equipment. This can be done by utilising alternative heating technologies wherever possible, such as direct steam injection and steam infusion, following Refstrup (1998). Both of these minimise the amount of milk contact surface area involved relative to heat exchangers, as the milk is heated by direct contact with steam without the need for the heating surface required with indirect heaters. Steam infusion can potentially operated with very minimal surface contact as the milk can be heated as a falling curtain in a steam infused vessel. An alternative option to this is the use of low adherence construction materials such as specially treated stainless steel or surface coatings in those areas of the process where thermophilic biofilm formation is a problem.

3. Minimise fouling as much as possible through management of the milk quality, optimising processing conditions, hygienic design of the plant equipment and ensuring the product mix is suited to the plant. Successful reduction of fouling build-up will potentially allow for the use of smaller heaters, as the design fouling factor can be reduced and it will also help to prevent bacteria surviving the cleaning process.

4. Clean as thoroughly as possible. This may require different cleaning protocols and different clean-in-place (CIP) formulations for different product specifications. Also, the CIP procedure and equipment may need to be upgraded to better target fouling in hard to reach areas (e.g. installation of more CIP nozzles and greater capacity pumps to increase mechanical action during cleaning).

5. If adequate cleaning cannot be achieved, sanitising the remnants of fouling layers with an appropriate sanitising agent to kill off residual thermophiles may help to reduce thermophile contamination.

5.3. Future work

To further the understanding of the interactions occurring between fouling deposits thermophiles, more detailed studies on a microscopic level are required. These could involve the use of microprobes to measure concentration gradients of oxygen, carbon dioxide and waste metabolite across the depth of the fouling deposits where thermophiles are actively growing in a flowing situation. This would provide understanding of any nutrient limitation effects occurring and the rate of growth at different points within the fouling deposit.

The current surface enumeration techniques available are not accurate for situations where surfaces need to be removed from their environment. This seems largely due to the variety of adhesion strengths of surface associated bacteria on surfaces (e.g. loosely adhered, firmly adhered etc.). This needs to be improved.

Before the unsteady state model could be applied in an industrial situation, such as for a plate heat exchanger, the model would have to incorporate temperature dependant growth kinetics. This is because industrial heat exchangers operate at a range of temperatures, unlike the pilot plant tubular heat exchanger in which the surfaces were isothermal. This would also require an expression for the temperature profile across the heat exchanger to be incorporated into the model. An approach similar to that used in (de Jong *et al.*, 2001) could be used. However, as there is a wide range of different optimal temperature ranges for the thermophile strains that contaminate milk powder plants, further work would be needed to obtain accurate data on the growth kinetics of all the common strains. In addition to this, the effect of the greater flow velocity and turbulence present in an industrial plant compared to the pilot plant on surface colonisation and contamination would have to be assessed. The Reynolds number in the pilot plant was typically 300, while in an industrial plant values of 150×10^3 are typical in pipes. This increase would be likely to reduce the adhesion constant (k_a) (de Jong *et al.*, 2001). Adjustment of the constants a and k_r in the function controlling the proportion of cells released (β) may also be required to fit predictions of industrial contamination to measured industrial data, due to the different flow conditions.

The modelling work could also be improved through better measurements of the generation time used, as current measurements are based on either planktonic growth or on inaccurate estimates of wall growth since wall enumeration techniques are still deficient. The coefficient β that governs the release of bacteria from the wall also needs to be measured. At the moment it is estimated by fitting the model to data and therefore is restricted to the conditions of the system investigated.

The unsteady state model could also be applied to modelling of the contamination from fouled surfaces rather than just that from stainless steel surfaces. For this case as the coefficient β would remain much less than 1 even at steady state, as the release of thermophiles as a percentage of the total population is much less than that for stainless steel. Alternatively if more is known about how thermophiles behave at different depths of the fouling layer, this knowledge may be able to be incorporated into the model to simulate the inhibition effects that are thought to be occurring within the fouling layer.

Further work on surface coatings and stainless steel treatments could also be conducted. If large reductions in bacterial adherence can be achieved then this could be applied in process areas prone to contamination with thermophilic bacterial biofilms to help increase production run times.

There is also further cleaning investigations that could be carried out to determine the effect of different cleaning chemicals on thermophiles in fouling layers. This may highlight those chemicals that are best to use to attack fouling and thermophiles in fouling. A variety of strains of thermophilic bacteria could also be used in these studies to determine if any strains in particular are harder to remove or inactivate when associated with fouling due to increased spore formation or other properties.

The research reported in this thesis has confirmed that thermophile contamination and release is an important and complex issue in dairy powder processing. Research across many or all of various fields noted above will be required to provide the knowledge necessary to achieve significant increases in running time in industrial plants whilst meeting quality and safety constraints.

Appendix A- Equipment Drawings.

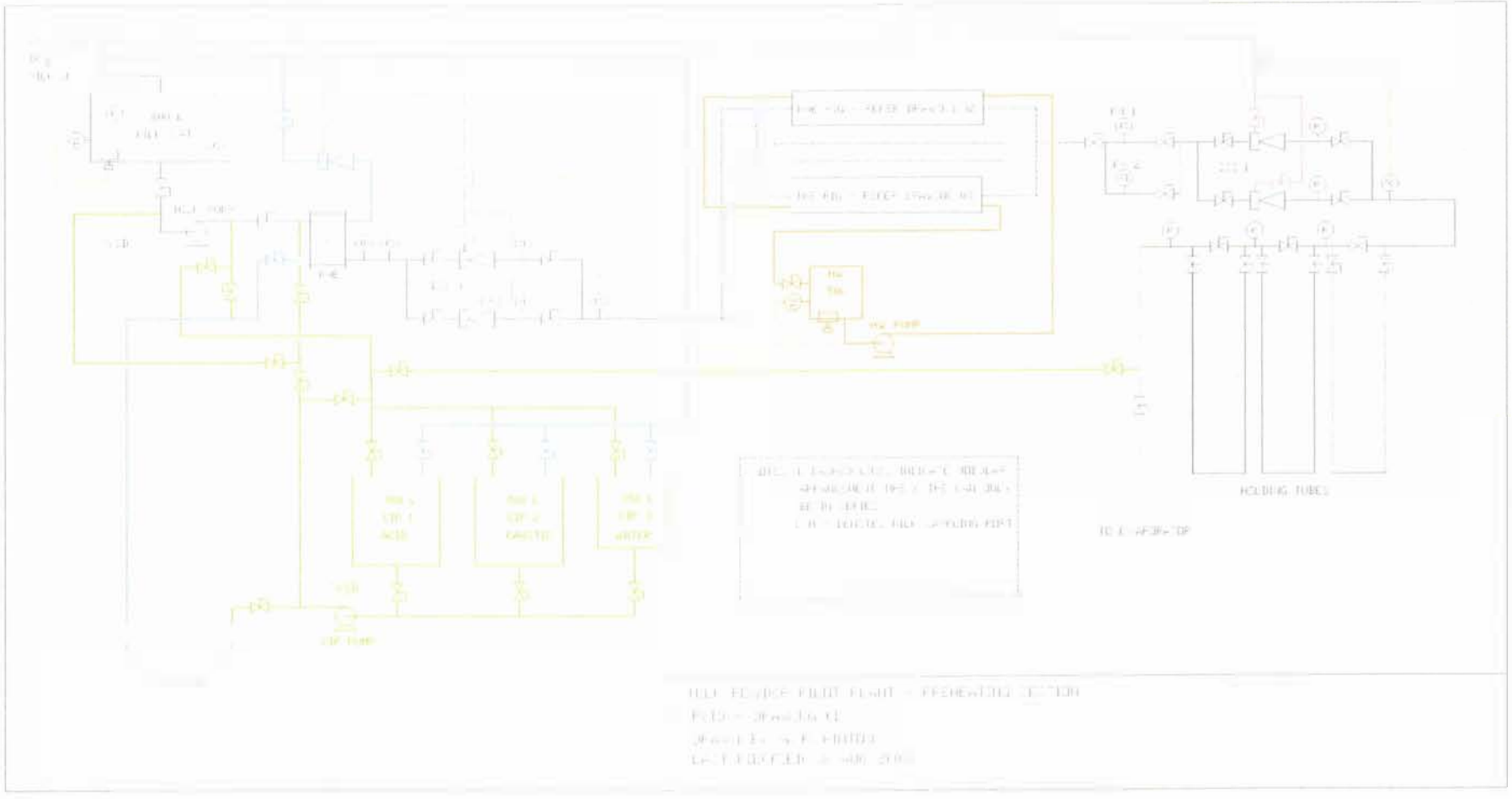


Figure A.1. Pilot plant piping and instrumentation drawing (P&ID).

Table A.1. List of commercial equipment details.

Commercial equipment details:			
Item	**Drawing Ref #**	**Description**	**Source**
Pumps:			
Milk Pump	P&ID Drawing 01 (Figure A.1.)	Centrifugal pump, Ebara, Model No. CDX70/05, 0.37kW.	Keith R.Norling Ltd., Palmerston North, N.Z.
CIP Pump	P&ID Drawing 01 (Figure A.1.)	Centrifugal pump, Model No. FP712KF, 1.1kW.	Fristam, Auckland, N.Z.
HW Pump	P&ID Drawing 01 (Figure A.1.)	Centrifugal pump, Ebara, Model No. CDX70/05, 0.37kW.	Keith R.Norling Ltd., Palmerston North, N.Z.
Heat exchangers:			
PHE	P&ID Drawing 01 (Figure A.1.)	Plate heat exchanger, Model No. U265R	APV, Denmark
Flow Meters:			
FM1 (low range flow)	P&ID Drawing 01 (Figure A.1.)	Magnectic flow meter, Endress-Hauser Picomag, Model No. 11 PM 165333.	EMC Industrial Instrumentation, Auckland, N.Z.
FM2 (high range flow)	P&ID Drawing 01 (Figure A.1.)	Magnectic flow meter, Endress-Hauser Promag, Model No. 30FT25-AA1AA11A21B	EMC Industrial Instrumentation, Auckland, N.Z.
FM3	P&ID Drawing 03 (Figure A.3.)	Paddle flow meter, Flow Sensor Dual Range, Model No. 256-225.	RS Components Ltd., Auckland, N.Z.
FM4	P&ID Drawing 03 (Figure A.3.)	Paddle flow meter, Flow Sensor Dual Range, Model No. 256-225.	RS Components Ltd., Auckland, N.Z.

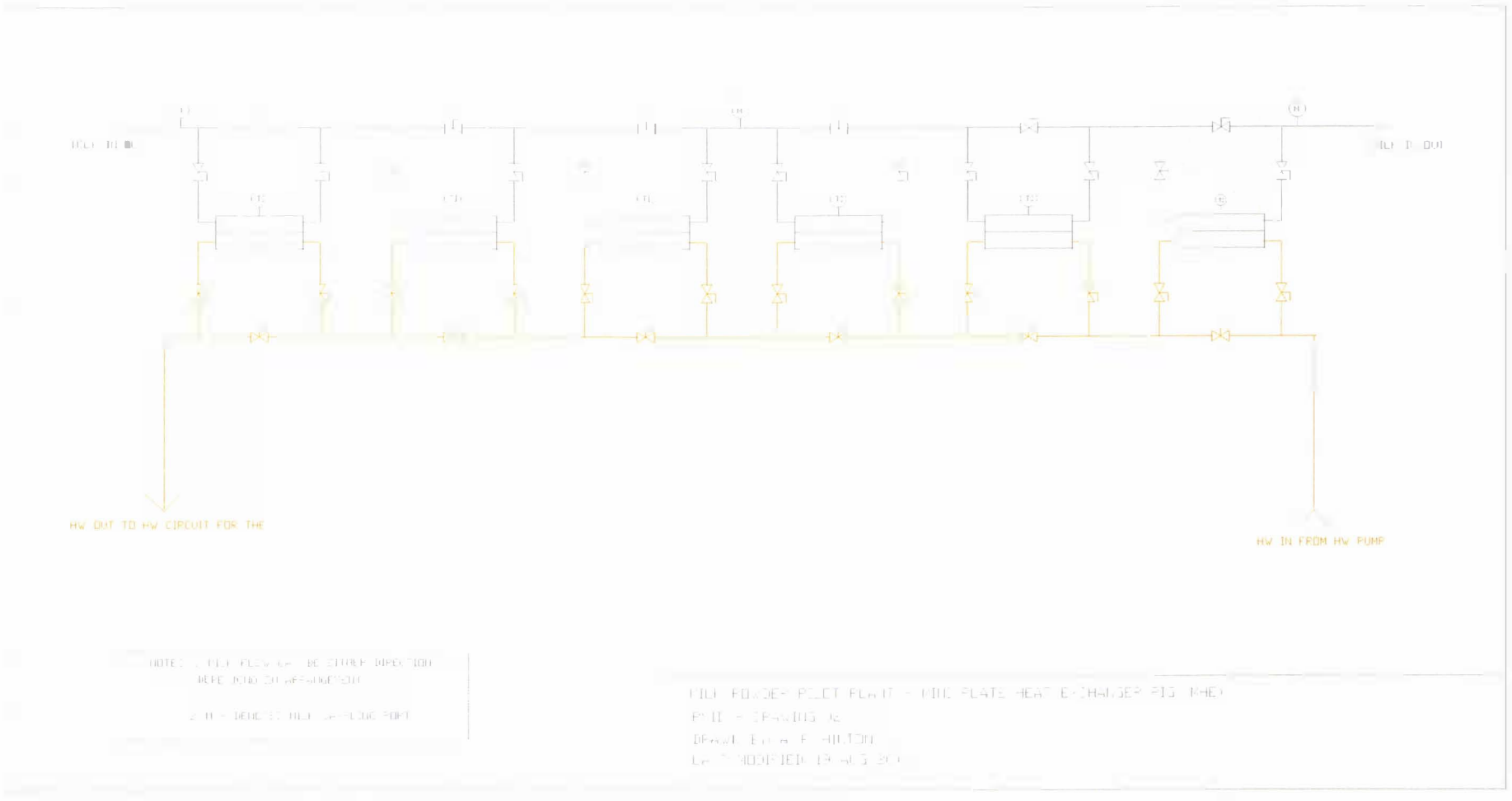


Figure A.2. Mini plate heat exchanger (MHE) rig piping and instrumentation drawing (P&ID).

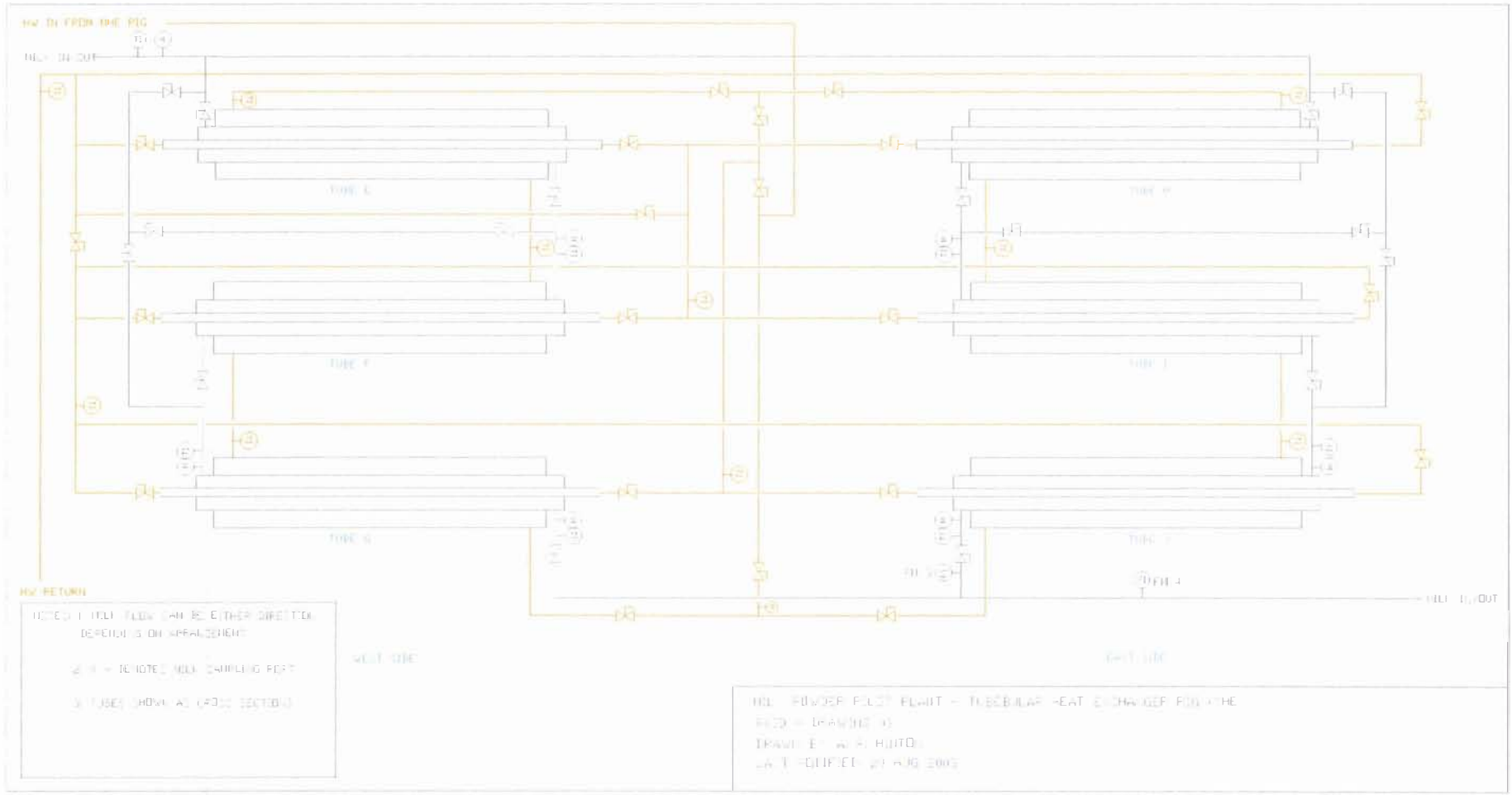


Figure A.3. Tubular heat exchange (THE) rig piping and instrumentation drawing (P&ID).

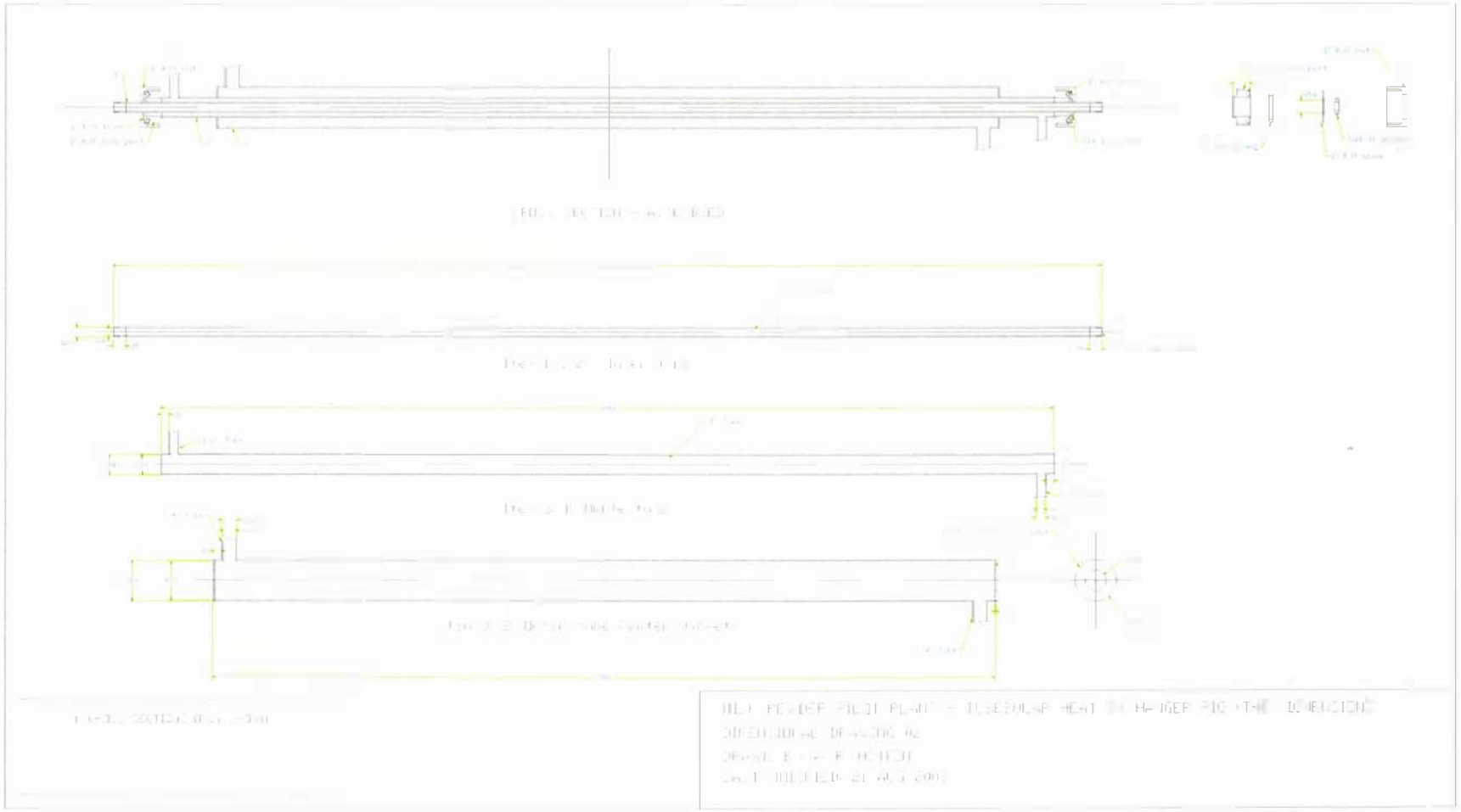


Figure A.4. Tubular heat exchanger (THE) dimensional drawing

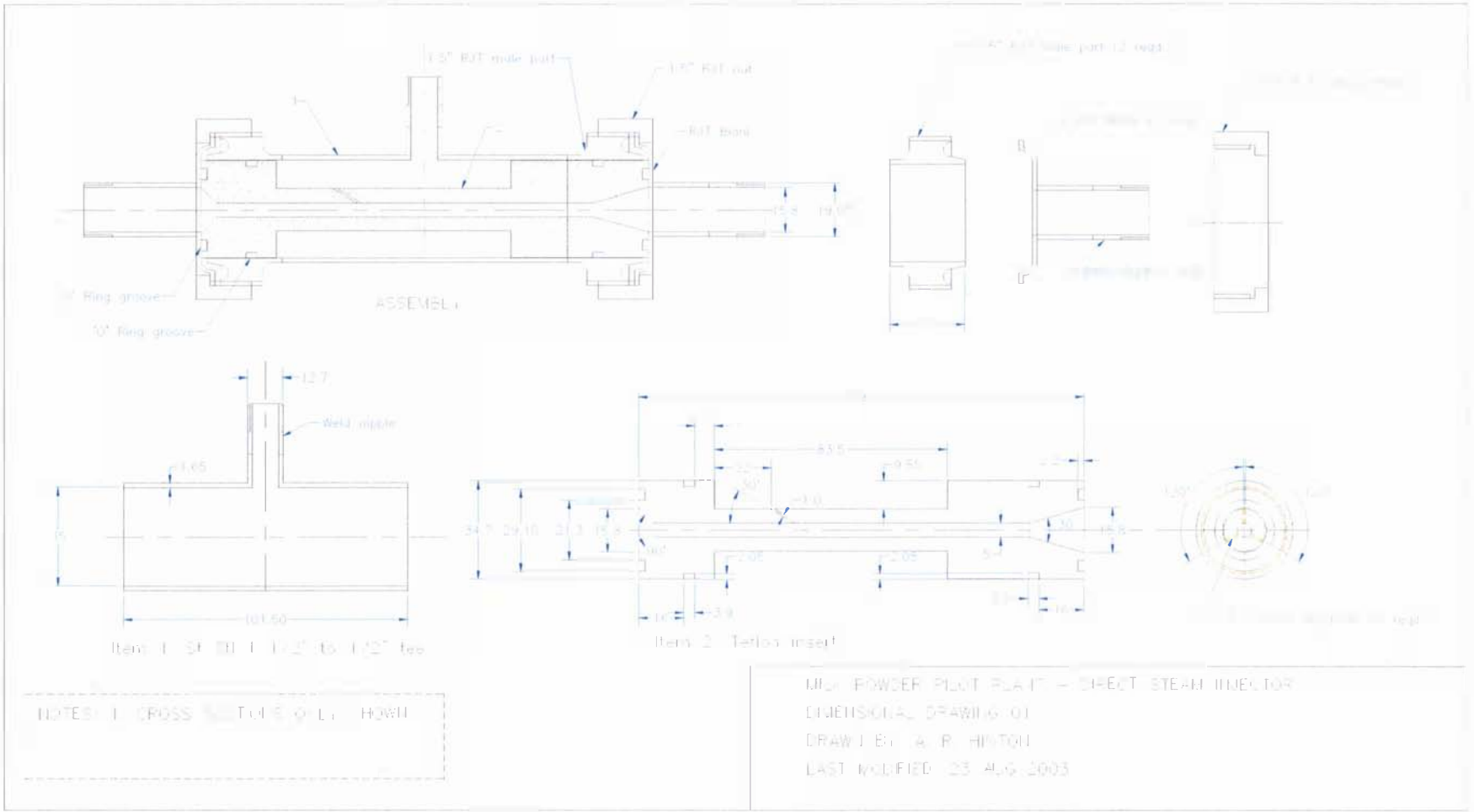


Figure A.5. Direct steam injector (DSI) dimensional drawing.

Appendix B- General Information

Direct steam injection design calculation

Table B.1. Example of direct steam injection design calculation.

Direct Steam Injection Design Calculation					
Constants:	**Symbol**	**Value**	**Condition**	**Formula #**	**Literature source**
Heat Capacity of Milk (kJ/kg.K)	C_{pm}	3.9	40°C		Wood, 1996
Density of Milk (kg/m^3)	ρ_m	1026	40°C		Wood, 1996
Latent Heat of steam (kJ/kg)	H_{fg}	2200	270kPa.a		Cooper and Le Fevre, 1969
Viscosity of Milk (Pa.s)	μ_m	0.002	40°C		Wood, 1996
Constant R	R	286.8			Vennard and Street, 1976
Density of steam (kg/m^3)	ρ_s	1.5	270kPa.a		Cooper and Le Fevre, 1969
Variables:					
Temperature Inc. across DSI (K)	ΔT_m	40			
Milk Flow (L/hr)	V_m	30			
Milk Flow (m^3/s)		8.33E-06			
Milk mass flow (kg/s)	m_m	8.55E-03		1	
Steam supply pressure (bar.g)		2			
Steam supply pressure (Pa.a)		303900		2	
Steam supply pressure (kPa.a)	P_s	303.9			
Max. pressure in milk pipe (kPa.a)	P_m	250			
Steam supply temperature (K)	T_s	416.5			Cooper and Le Fevre, 1969
Diameter of Milk Constriction (m)	d_c	0.005			
Diameter of steam nozzles (m)	d_N	0.001			
Heat Input Calculations:					
Heat Input to Milk Required (kJ/s):	Hm	1.3338		3	
Flow Rate of Steam Required (kg/s):	m_s	0.000606		4	
Flow Rate of Steam Required (kg/hr):		2.183			
Milk Aperture Calculations:					
Diameter of Milk Constriction (m)	d_c	0.005			
Area of constriction aperture (m^2)	A_c	1.963E-05			
Velocity of milk through Aperture (m/s)	u_m	0.424		5	
Reynolds Number through milk aperture	R_{em}	1088.62		6	

Steam Aperture Calculations:				
Steam Pressure (kPa.a)	P_s	303.9		
Max. pressure in milk pipe (kPa.a)	P_m	250		
Constant k	k	1.3		Vennard and Street, 1976
Critical pressure ratio	$(P_m'/P_s)_c$	0.546	7	Vennard and Street, 1976
Actual_pressure_ratio		0.823	8	Vennard and Street, 1976
Flow condition		subsonic flow		
For sonic flow:				
Area of steam aperture req. (m^2)	A_s	1.033E-06	9	Vennard and Street, 1976
For subsonic flow:				
Area of steam aperture req. (m^2)	A_s	1.689E-06	10	Vennard and Street, 1976
Diameter of steam nozzles (m)	d_N	0.001		
Area of one nozzle (m^2)	A_N	7.854E-07	11	
Area of steam ap. Req. (m^2)	A_s	1.689E-06		
Number of steam nozzles required	N_N	2.15	12	

Direct Steam Injection Design Formulae

Refer to calculation for description of terms.

$$m_m = \frac{V_m \cdot \rho_m}{1000 \times 3600} \quad 1$$

$$P_s(Pa.a) = ((P_s(bar.g) \times 101.3) + 101.3) \times 1000 \quad 2$$

$$H_m = m_m . C_{pm} . \Delta T_m \quad 3$$

$$m_s = \frac{H_m}{H_{fg}} \quad 4$$

$$u_m = \frac{V_m}{1000 \times 3600 \times A_m} \quad 5$$

$$Re_m = \frac{u_m . d_i . \rho_m}{\mu_m} \quad 6$$

Critical Pressure Ratio:

$$\left[\frac{P_m'}{P_s}\right]_{crit} = \left(\frac{2}{k+1}\right)^{k/(k-1)} \quad 7$$

P_m' = Pressure at outlet of steam hole (kPa.a)

$$Actual\_pressure\_ratio = \left(\frac{P_m}{P_s}\right) \quad 8$$

If Critical pressure ratio > Actual pressure ratio, then have sonic steam flow condition, otherwise have subsonic steam flow.

For sonic flow:

$$A_s = \frac{m_s \cdot \sqrt{T_s}}{P_s \sqrt{\frac{k}{R}\left(\frac{2}{k+1}\right)^{\frac{(k+1)}{(k-1)}}}} \qquad 9$$

For sub-sonic flow:

$$A_s = \frac{m_s}{\sqrt{P_s \cdot \rho_s \left(\frac{2k}{(k-1)}\right)\left[\left(\frac{P_m}{P_s}\right)^{\frac{(2)}{(k)}} - \left(\frac{P_m}{P_s}\right)^{\frac{(k+1)}{(k)}}\right]}} \qquad 10$$

$$A_N = \frac{\pi . d_N^{\,2}}{4} \qquad 11$$

$$N_N = \frac{A_s}{A_N} \qquad 12$$

Example of dilution series used for enumeration of thermophiles in bulk milk

Example dilution series used for each bulk milk sample in Experimental Run 5. Dilution prefix represents dilution series not overall dilution factor on plate, as 0.1 ml is used for each plate.

Table B.2. Example dilution series for each bulk milk sample taken during Experimental Run 5.

Pre start up samples	Vegetative cells	Spores
Milk reservoir	10^{-2}-10^{-4}	10^{0}-10^{-3}
Number agar plates	6	8
Number 9ml dilution bottles	8	6

Time (hr)	**Sample #**	Vegetative cells				
		Sample Point				
		A.	B.C	D	E.F.G	H.I.J.K.L
0	1	10^{0}	10^{0}	10^{0}	10^{0}	10^{0}-10^{-4}
4	2	10^{0}	10^{0}	10^{0}	10^{0}-10^{-3}	10^{-1}-10^{-4}
8	3	10^{0}	10^{0}-10^{-2}	10^{0}-10^{-2}	10^{-1}-10^{-4}	10^{-1}-10^{-4}
12	4	10^{0}	10^{0}-10^{-3}	10^{0}-10^{-3}	10^{-1}-10^{-4}	10^{-1}-10^{-4}
16	5	10^{0}	10^{0}-10^{-3}	10^{0}-10^{-3}	10^{-2}-10^{-5}	10^{-2}-10^{-5}
20	6	10^{0}	10^{0}-10^{-3}	10^{-1}-10^{-4}	10^{-2}-10^{-5}	10^{-2}-10^{-5}
24	7	10^{0}	10^{0}-10^{-3}	10^{-1}-10^{-4}	10^{-2}-10^{-5}	10^{-2}-10^{-5}
Number agar plates		14	84	42	150	290
9ml dil. bottles		0	56	28	138	280
9.9ml dil. Bottles		0	0	0	18	30

Time (hr)	**Sample #**	Spores			
		Sample Point			
		A.B	C.D	E.F.G.	H.I.J.K.L
0	1	10^{0}	10^{0}	10^{0}	10^{0}-10^{-3}
4	2	10^{0}	10^{0}	10^{0}-10^{-1}	10^{0}-10^{-3}
8	3	10^{0}	10^{0}	10^{0}-10^{-2}	10^{0}-10^{-3}
12	4	10^{0}	10^{0}-10^{-1}	10^{0}-10^{-3}	10^{0}-10^{-3}
16	5	10^{0}	10^{0}-10^{-1}	10^{0}-10^{-3}	10^{0}-10^{-3}
20	6	10^{0}	10^{0}-10^{-1}	10^{0}-10^{-3}	10^{0}-10^{-3}
24	7	10^{0}	10^{0}-10^{-1}	10^{0}-10^{-4}	10^{0}-10^{-4}
Number agar plates		28	44	138	290
9ml dil. bottles		0	16	96	220

Agar plate totals from Table B.2 above:

Vegetative cells: 6+14+84+42+138+290= 586
Spores: 8+28+44+138+290= 508
Spare plates (contingency for mistakes and contamination): 200 plates (~20 %)
Therefore total plates: 586+508+200 = 1294 plates.

Thermophile release data

Table B.3. Thermophilic bacterial and spore release data for Run 2 as plotted on Figure 4.1.8 and 4.1.10. Taken from linear regression of the amount of thermophilic bacteria released across side of THE rig, assuming each THE tube has a surface contact area of 1200 cm^2. Confidence intervals were calculated by Excel linear regression at 95 % level of significance.

Run 2	**Thermophile release per ml per cm^2**			
Bacteria				
Time	Pre-fouled Gradient	95 % CI	Un-fouled Gradient	95 % CI
(hr)	($cfu.ml^{-1}.cm^{-2}$)	($cfu.ml^{-1}.cm^{-2}$)	($cfu.ml^{-1}.cm^{-2}$)	($cfu.ml^{-1}.cm^{-2}$)
5	5.71	1.42	5.73	1.11
7.5	112.90	52.74	151.70	46.32
12.5	845.90	325.38	585.50	187.97
15	1252.00	317.26	1143.00	494.34
Spore				
Time	Pre-fouled Gradient	95 % CI	Un-fouled Gradient	95 % CI
(hr)	($cfu.ml^{-1}.cm^{-2}$)	($cfu.ml^{-1}.cm^{-2}$)	($cfu.ml^{-1}.cm^{-2}$)	($cfu.ml^{-1}.cm^{-2}$)
7.5	0.002	0.002	0.015	0.000
12.5	3.466	0.902	2.491	0.686
15	3.110	1.275	3.365	1.549

Table B.4. Thermophilic bacterial and spore release data for Run 4 as plotted on Figure 4.1.9 and 4.1.11. Taken from linear regression of the amount of thermophilic bacteria released across side of THE rig, assuming each THE tube has a surface contact area of 1200 cm^2. Confidence intervals were calculated by Excel linear regression at 95 % level of significance.

Run 4	**Thermophile release per ml per cm^2**			
Bacteria				
Time	Pre-fouled Gradient	95 % CI	Un-fouled Gradient	95 % CI
(hr)	($cfu.ml^{-1}.cm^{-2}$)	($cfu.ml^{-1}.cm^{-2}$)	($cfu.ml^{-1}.cm^{-2}$)	($cfu.ml^{-1}.cm^{-2}$)
0	0.01	0.00	0.01	0.00
4	0.05	0.00	0.11	0.00
8	156.38	19.13	730.75	78.58
12	770.83	282.42	985.42	108.90
16	995.42	486.38	596.25	131.12
20	301.25	89.14	345.42	110.90
24	157.08	66.65	286.46	83.96
Spore				
Time	Pre-fouled Gradient	95 % CI	Un-fouled Gradient	95 % CI
(hr)	($cfu.ml^{-1}.cm^{-2}$)	($cfu.ml^{-1}.cm^{-2}$)	($cfu.ml^{-1}.cm^{-2}$)	($cfu.ml^{-1}.cm^{-2}$)
12	0.004	0.009	0.013	0.008
16	3.418	1.251	1.705	1.255
20	2.975	2.178	5.246	2.326
24	3.114	1.876	3.198	1.209

Table B.5. Thermophilic bacterial release data for Run 5 as plotted on Figure 4.3.4. Taken from linear regression of the amount of thermophilic bacteria released across side of THE rig, assuming each THE tube has a surface contact area of 1200 cm^2. Confidence intervals were calculated by Excel linear regression at 95 % level of significance.

Run 5	**Thermophile release per ml per cm^2**			
Bacteria				
Time	Initially contaminated gradient	95 % CI	Initially clean gradient	95 % CI
(hr)	($cfu.ml^{-1}.cm^{-2}$)	($cfu.ml^{-1}.cm^{-2}$)	($cfu.ml^{-1}.cm^{-2}$)	($cfu.ml^{-1}.cm^{-2}$)
0	0.0208	0	0.0446	0
4	583.3333	178.2833	79.26083	20.69774
8	1731.004	297.3456	748.4583	188.1934
12	1922.721	315.8322	1137.221	229.9859
16	2512.5	780.6875	2302.5	691.5438

Appendix C - Fouling Photographs

Experimental Run 1

Figure C.1. Un-fouled inner tube before Run 1.

Figure C.2. Pre-fouled inner tube before Run 1.

Figure C.3. Un-fouled inner tube after Run 1.

Figure C.4. Pre-fouled inner tube after Run 1.

Experimental Run 2

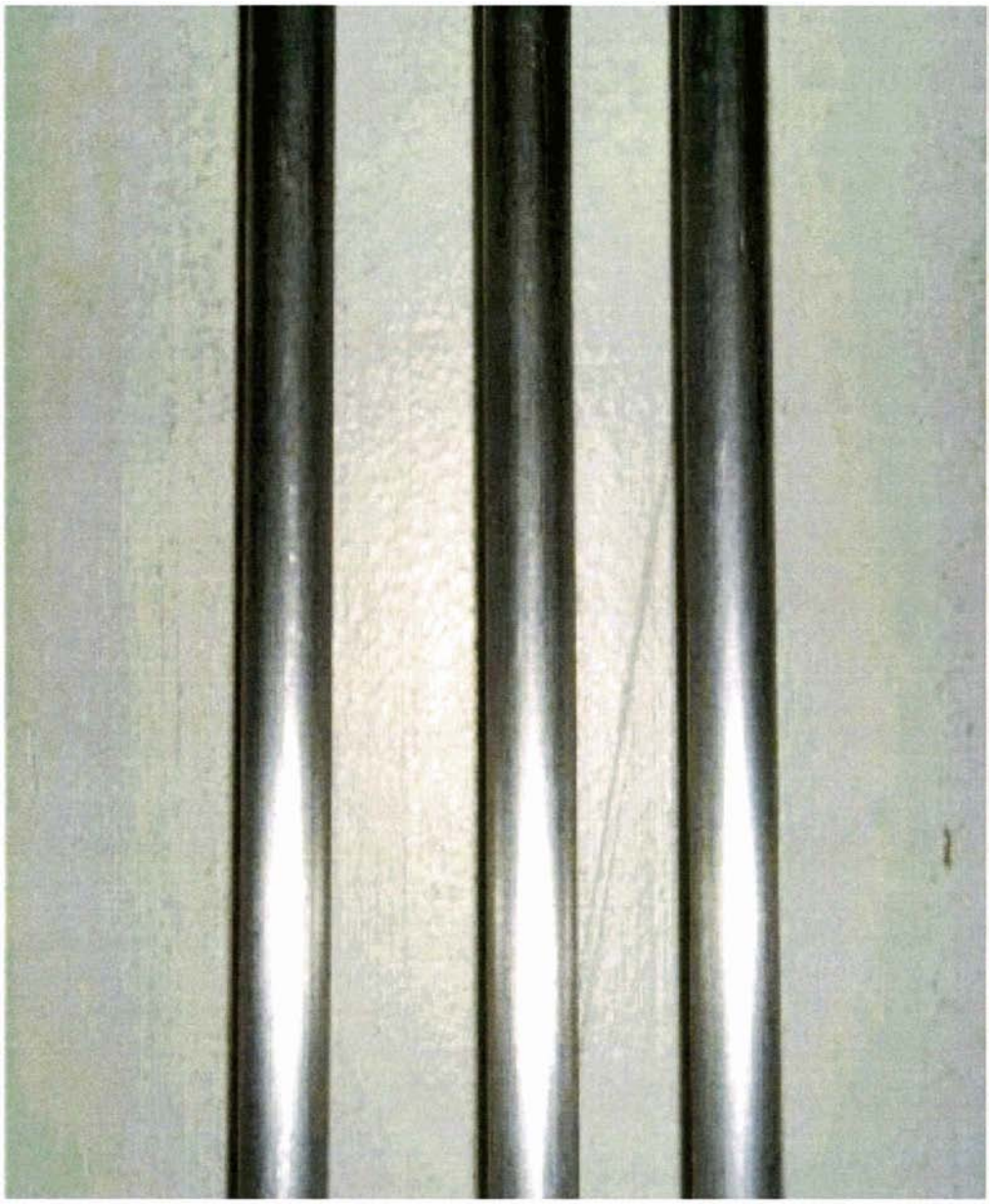


Figure C.5. Un-fouled inner tubes before Run 2 (in order from first tube to last tube in THE).

Figure C.6. Pre-fouled inner tubes before Run 2 (in order from first tube to last tube in THE).

Figure C.7. Un-fouled inner tubes after Run 2 (in order from first tube to last tube in THE).

Figure C.8. Pre-fouled inner tubes after Run 2 (in order from first tube to last tube in THE).

Experimental Run 3

THE inner tube photographs.

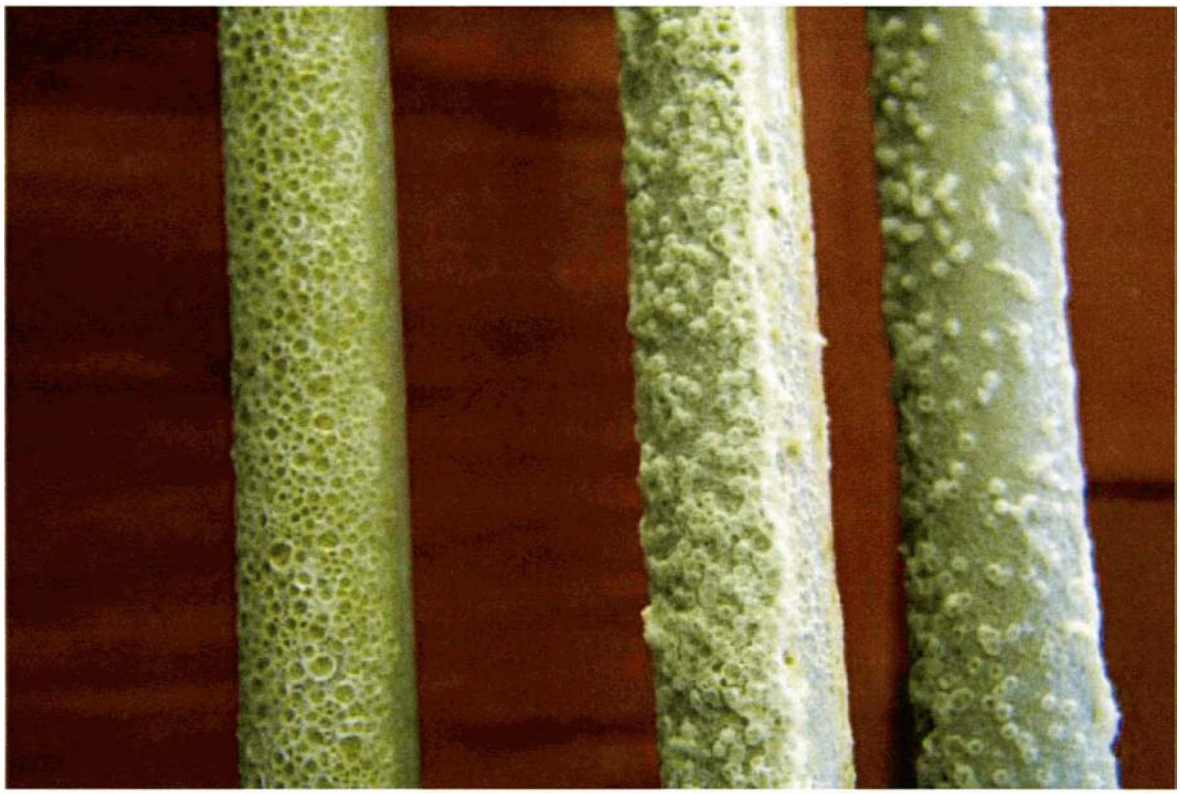


Figure C.9. Pre-fouled inner tubes before Run 3 (in order from first tube to last tube in THE).

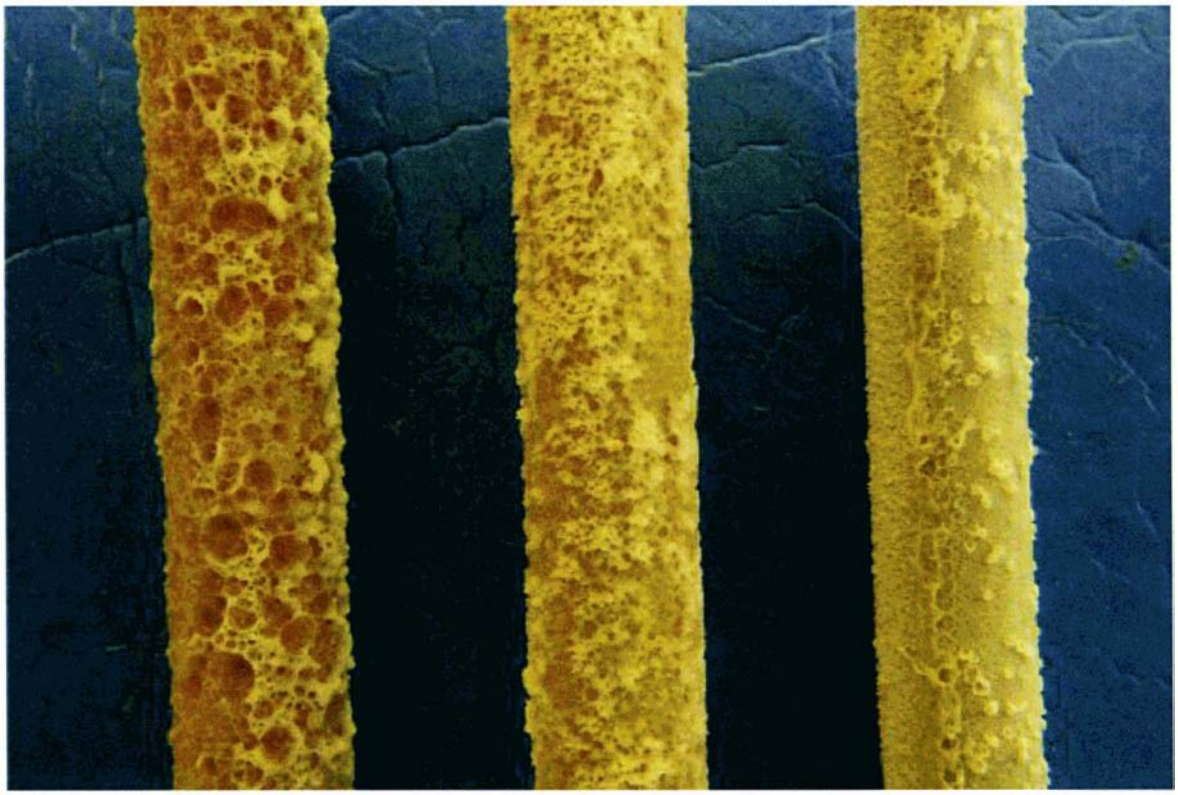


Figure C.10. Originally clean inner tubes after Run 3 (in order from first tube to last tube in THE).

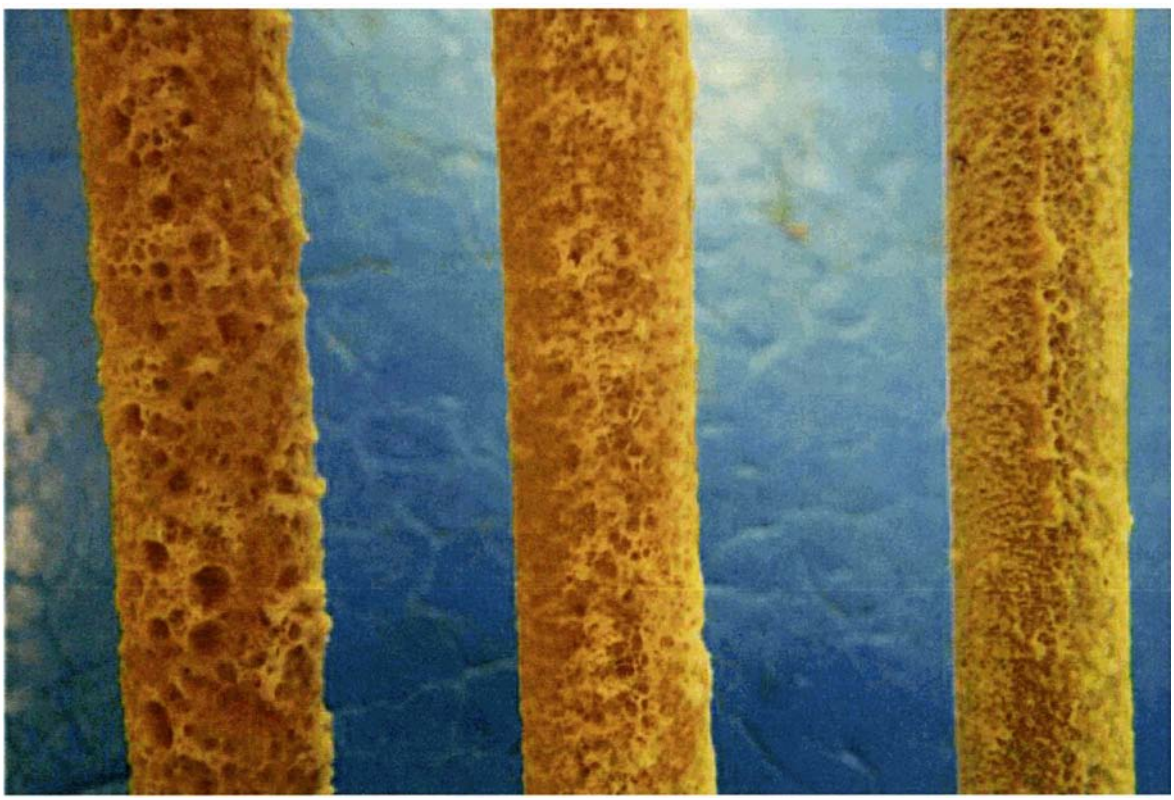


Figure C.11. Pre-fouled inner tubes after Run 3 (in order from first tube to last tube in THE).

MHE plate surface photographs.

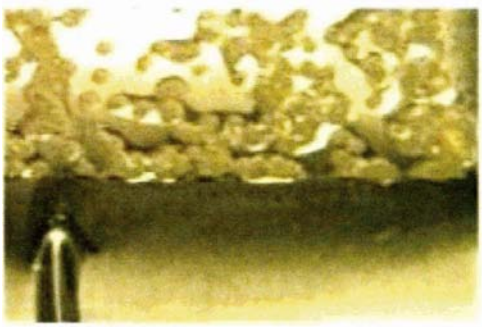


Plate 1 - before

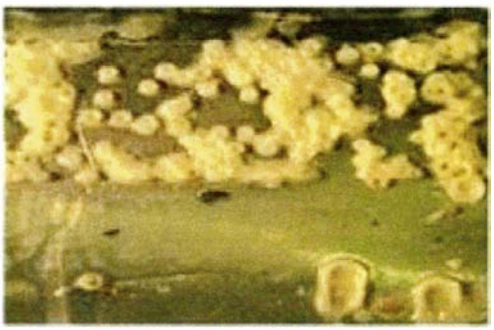


Plate 1 - after 5 hr

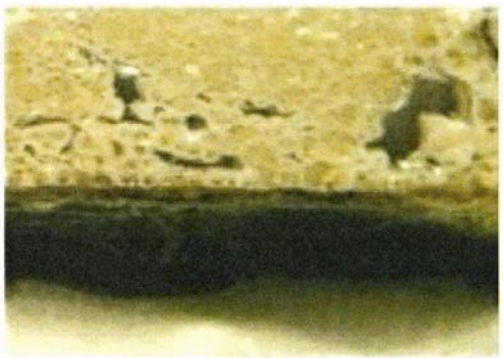


Plate 2 - before

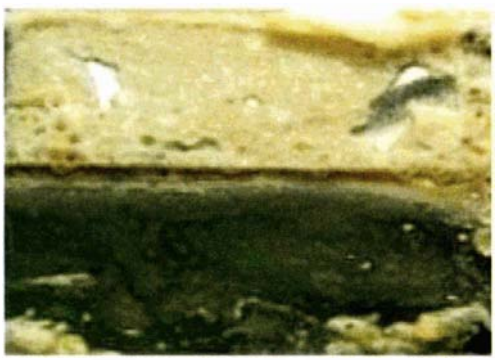


Plate 2 - after 7.5 hr

Plate 3 - before

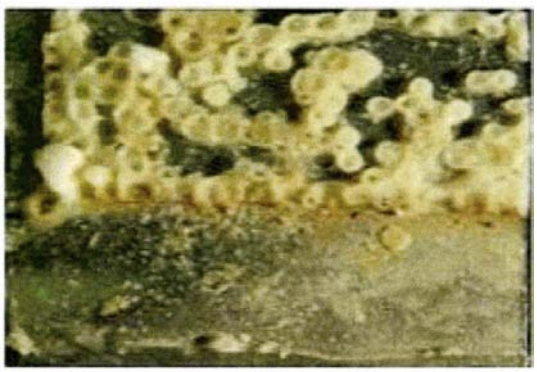


Plate 3 - after 10 hr

Figure C.12. MHE plate surfaces 1-3 before and after Run 3.

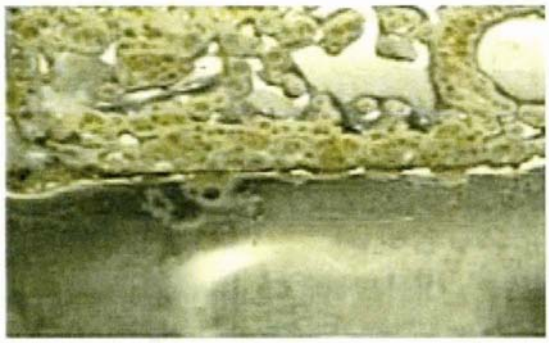


Plate 4 - before

Plate 4 - after 12.5 hr

Plate 5 - before

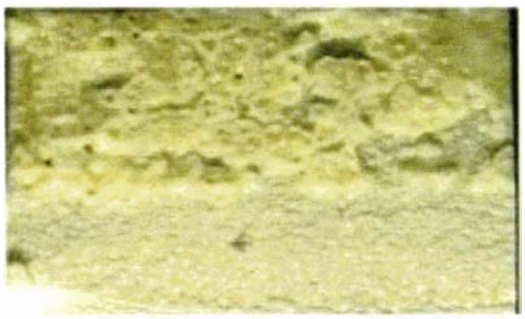


Plate 5 - after 15 hr

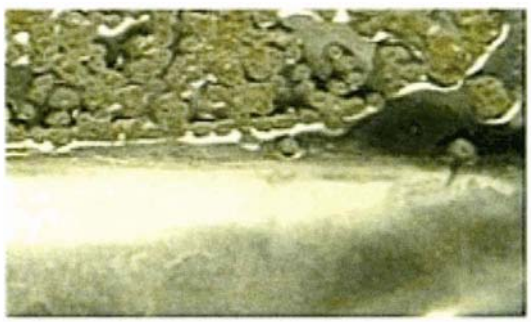


Plate 6 - before

Plate 6 - after 20 hr

Figure C.13. MHE plate surfaces 4-6 before and after Run 3.

Experimental Run 4

THE inner tube photographs.

Figure C.14. Pre-fouled inner THE tubes before Run 4 (in order from first tube to last tube in THE).

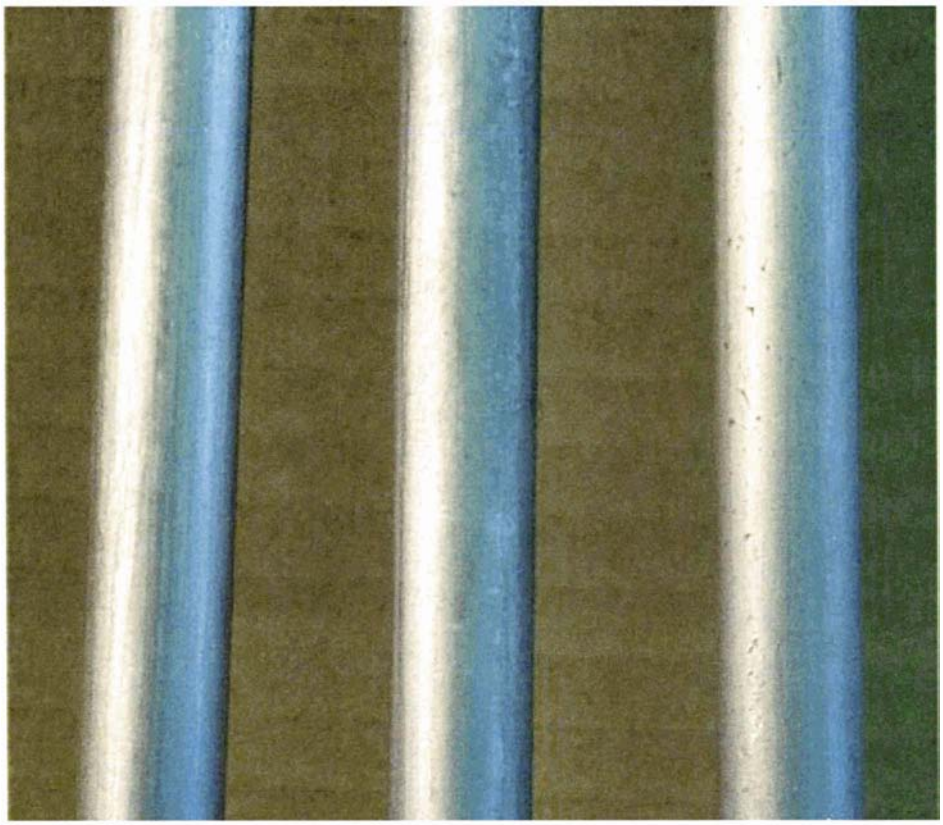


Figure C.15. Un-fouled inner THE tubes after Run 4 (in order from first tube to last tube in THE).

Figure C.16. Pre-fouled inner THE tubes after Run 4 (in order from first tube to last tube in THE).

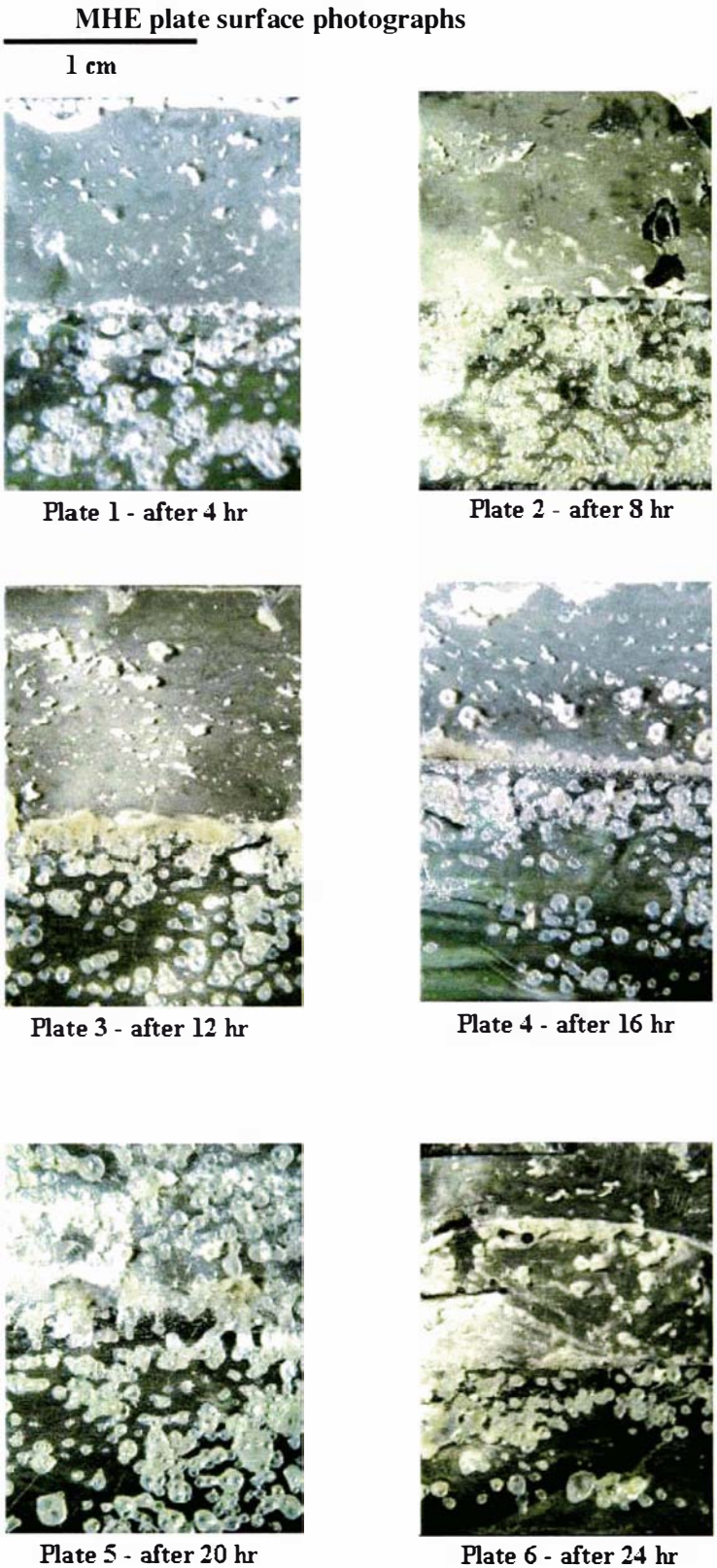


Figure C.17. MHE plate surface photographs after Run 4.

Experimental Run 5

THE inner tube photographs

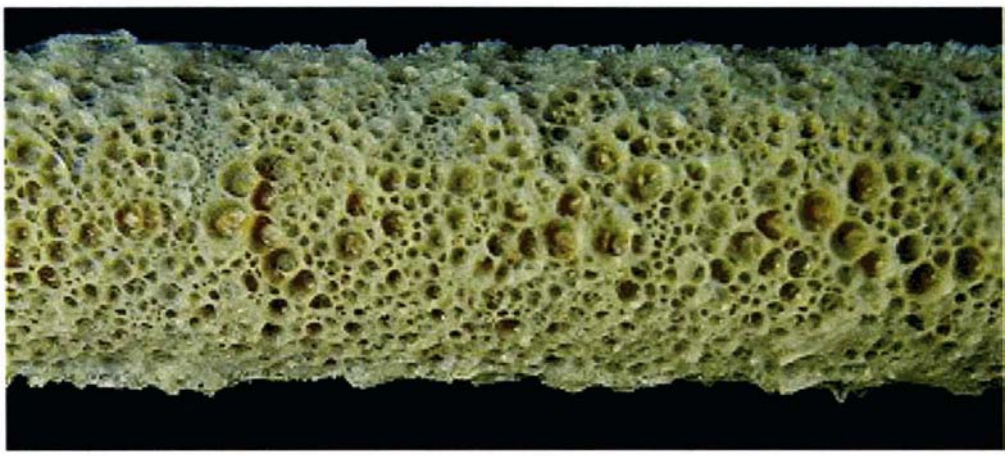


Figure C.18. Pre-fouled inner THE tube before innoculation and Run 5.

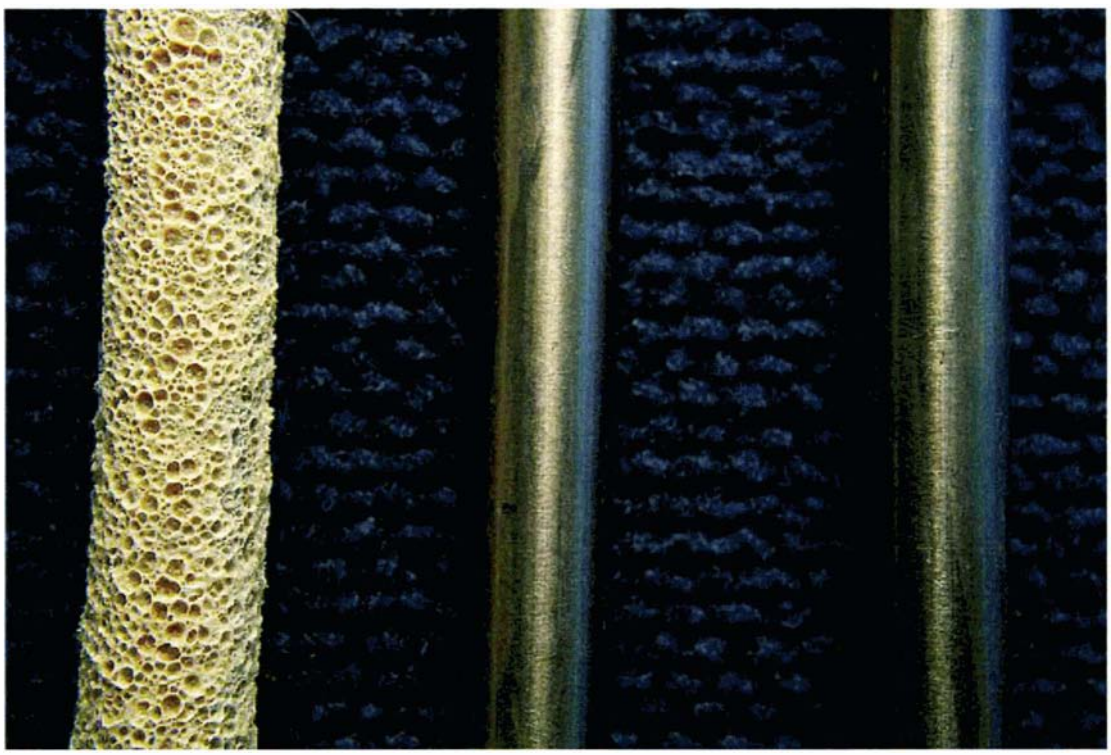


Figure C.19. Pre-fouled inner THE tube and the downstream un-fouled inner THE tubes (i.e. the initially contaminated side of the THE) after Run 5.

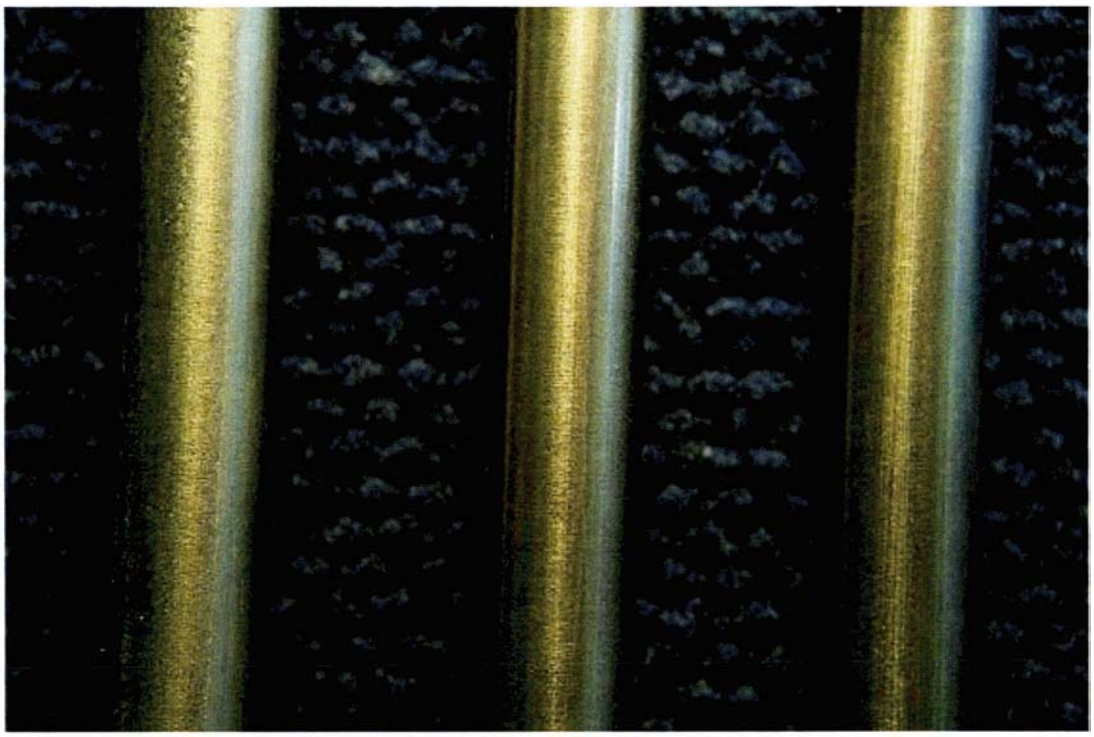


Figure C.20. Un-fouled inner THE tubes (i.e. the initially clean side of the THE) after Run 5.

MHE plate surface photographs

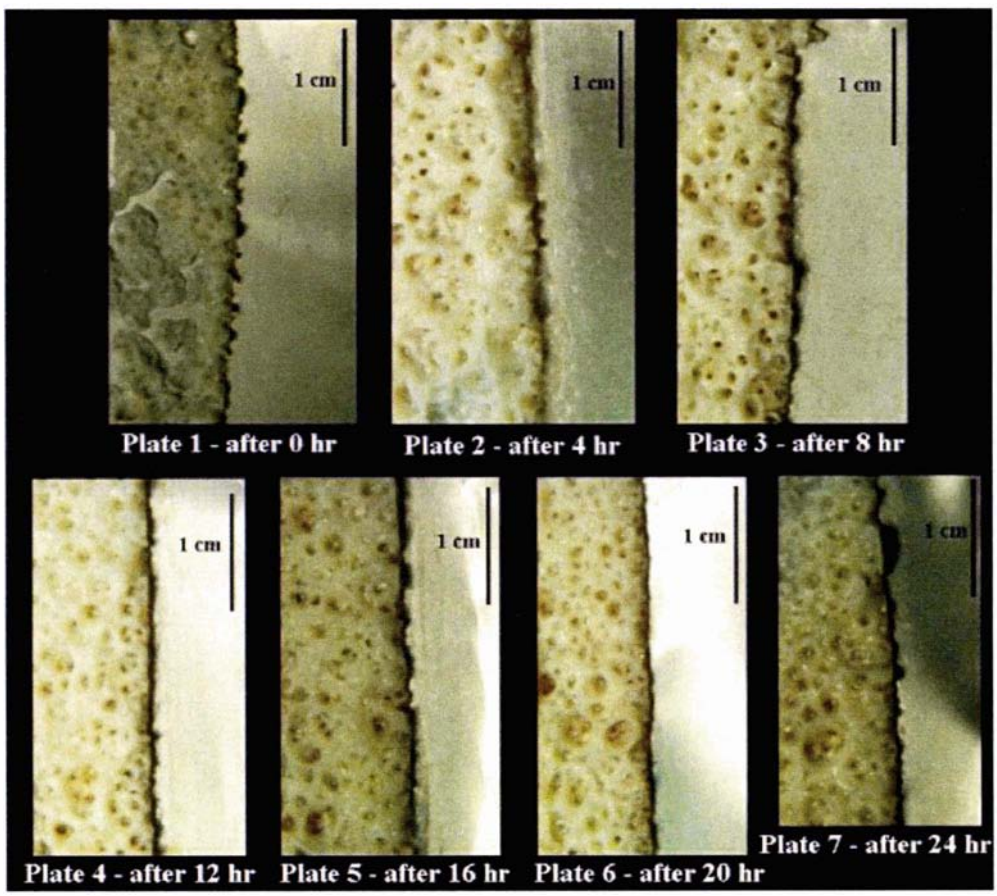


Figure C.21. MHE plate surface photographs after Run 5.

Appendix D- Pilot plant data.

This section contains graphs of the key data logged from the pilot plant during each experimental run.

Experimental Run 1

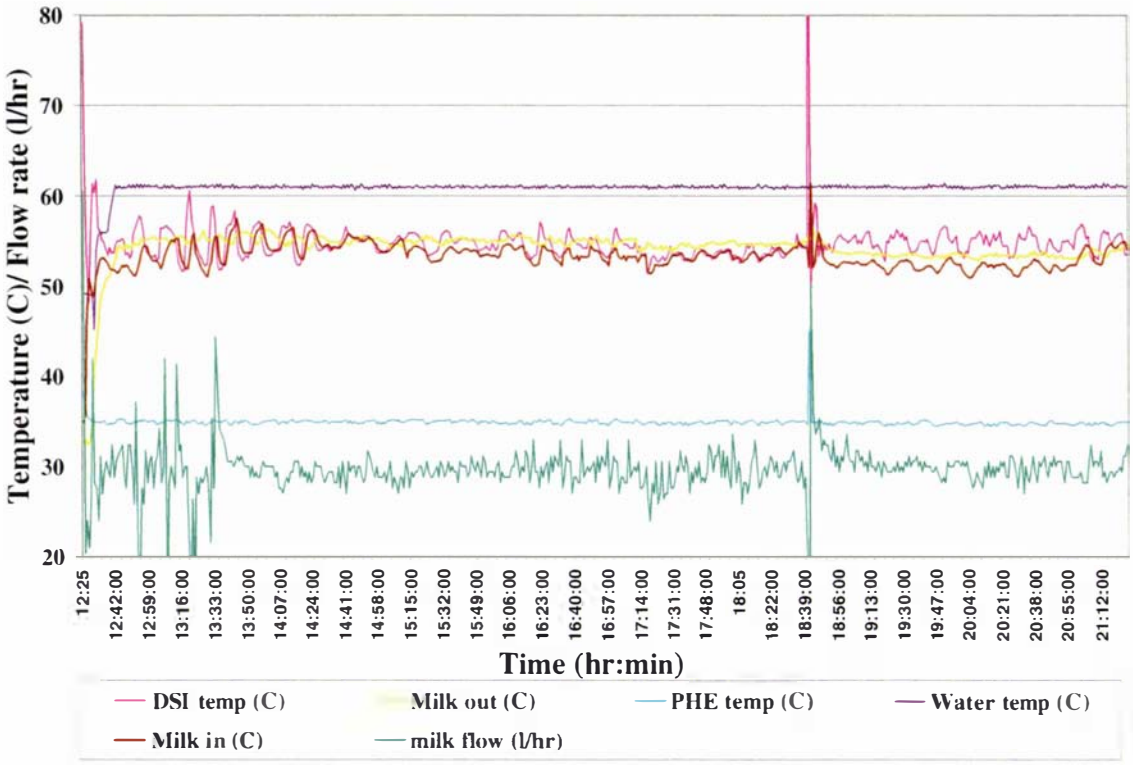


Figure D.1. Pilot plant data logged during experimental Run 1.

Experimental Run 2

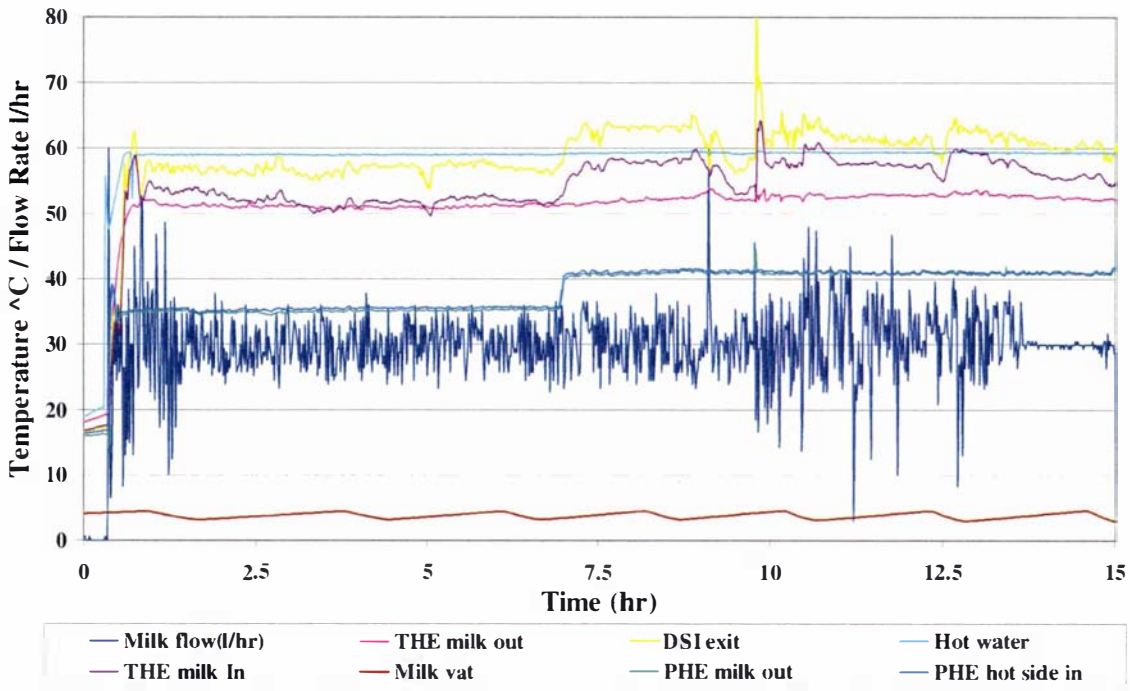


Figure D.2. Pilot plant data logged during experimental Run 2.

Experimental Run 3

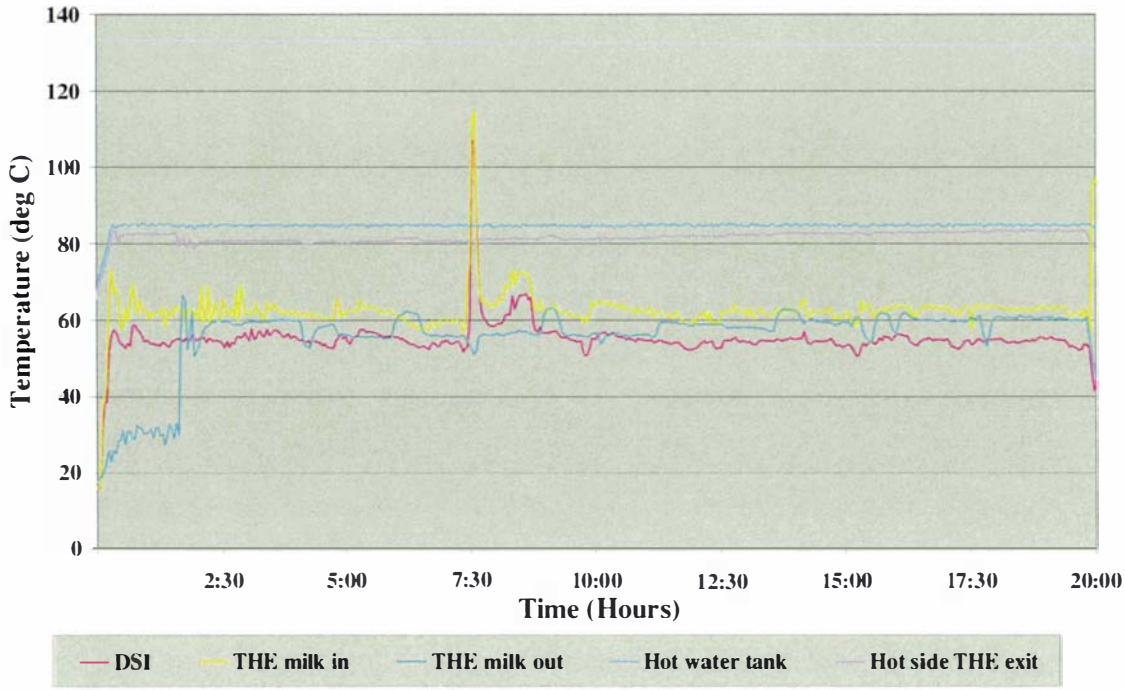


Figure D.3. Pilot plant data logged during experimental Run 3. Temperature data from THE inlet and outlet, DSI, and the hot water circuit are plotted.

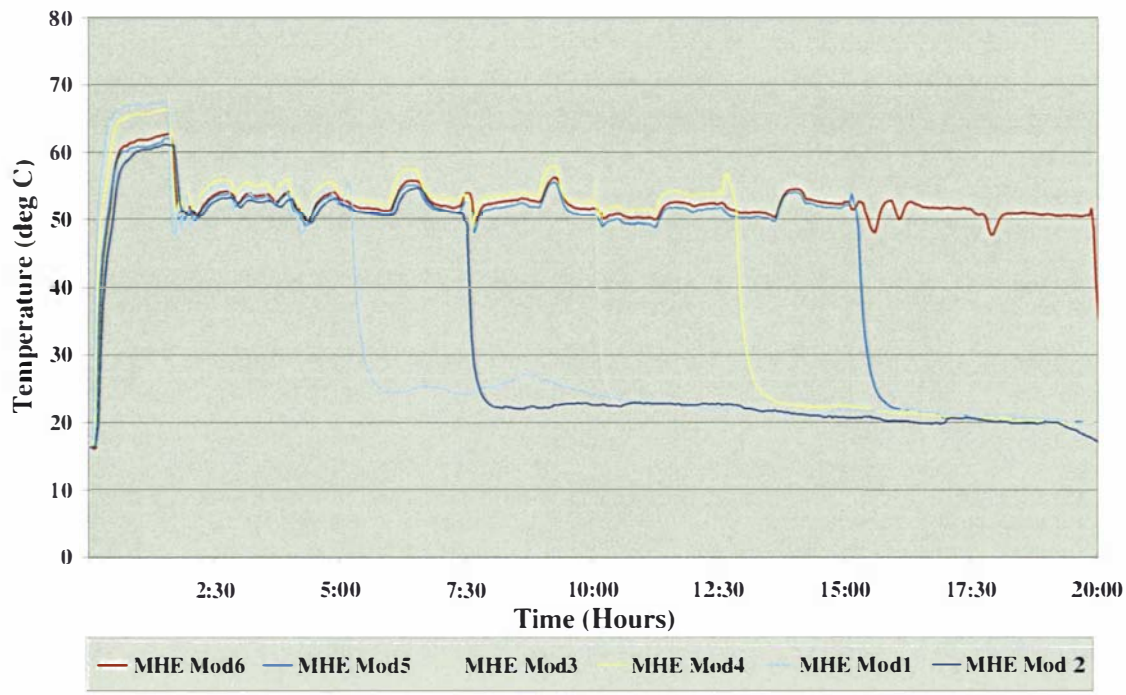


Figure D.4. Pilot plant data logged during experimental Run 3. Temperature data from the MHE modules are plotted. Note the drop in temperature as each plate surface is removed.

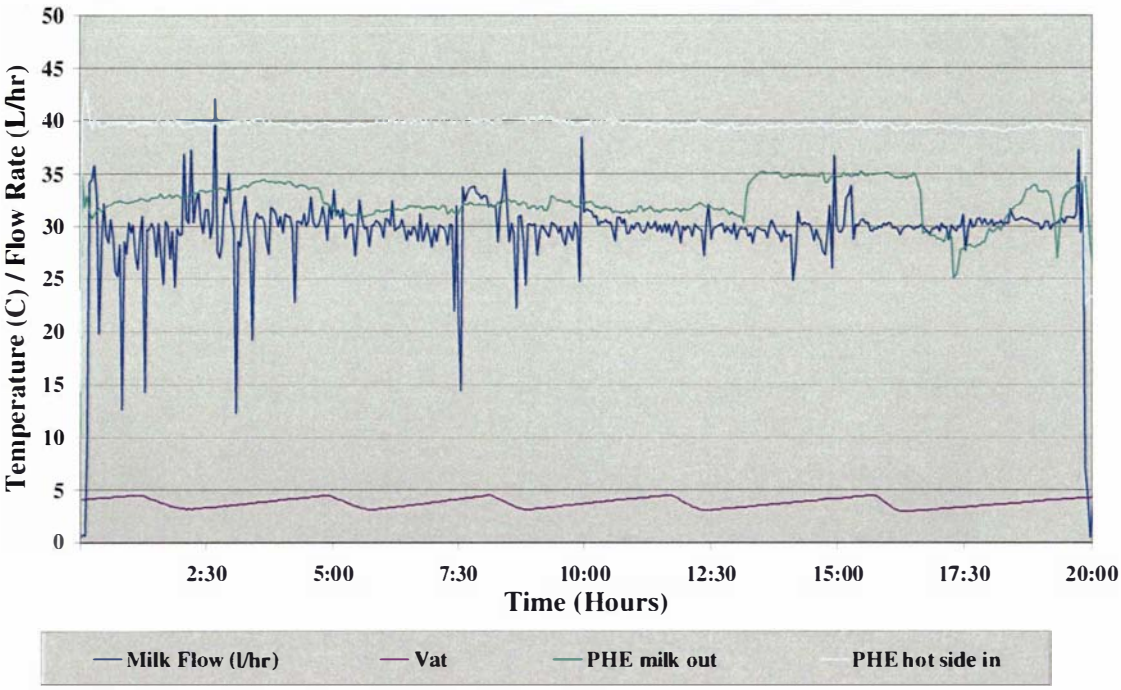


Figure D.5. Pilot plant data logged during experimental Run 3. The flow rate and temperature data from the PHE and milk vat are plotted.

Experimental Run 4

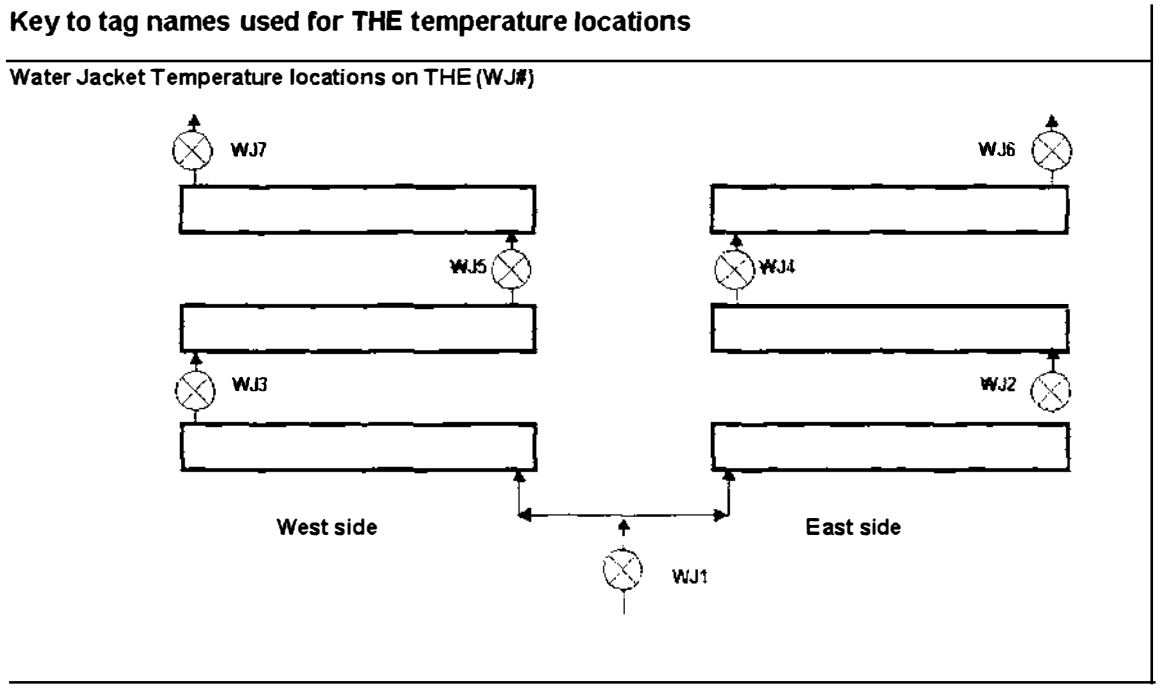


Figure D.6a. Key to tag names for temperature indicators (thermocouples) on the water jacket (outer tube).

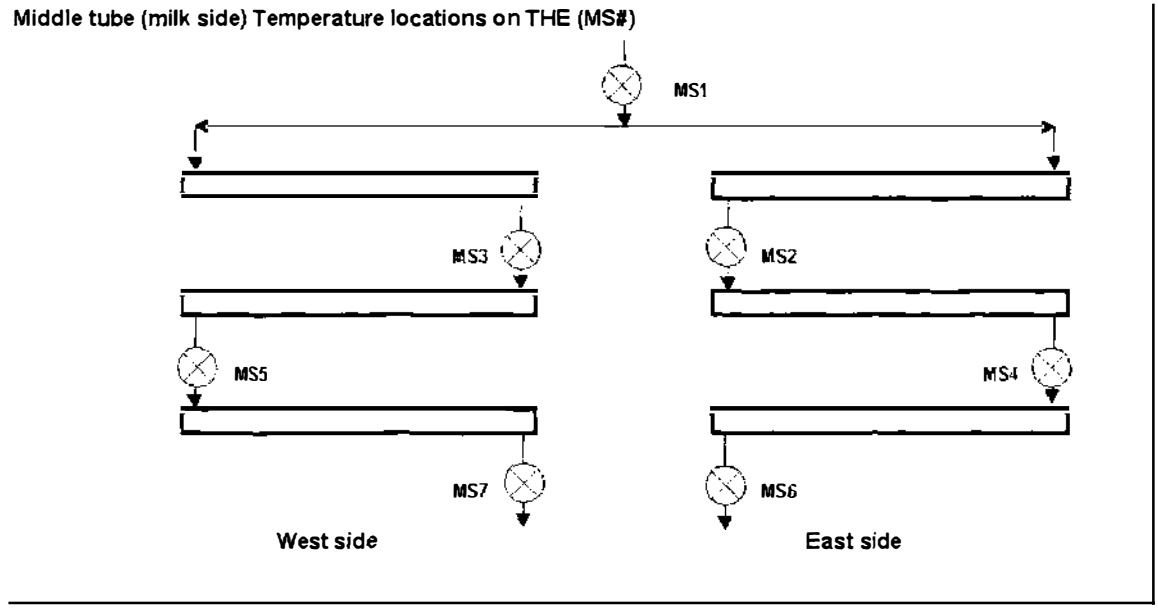


Figure D.6b. Key to tag names for temperature indicators (thermocouples) on the milk side (middle tube).

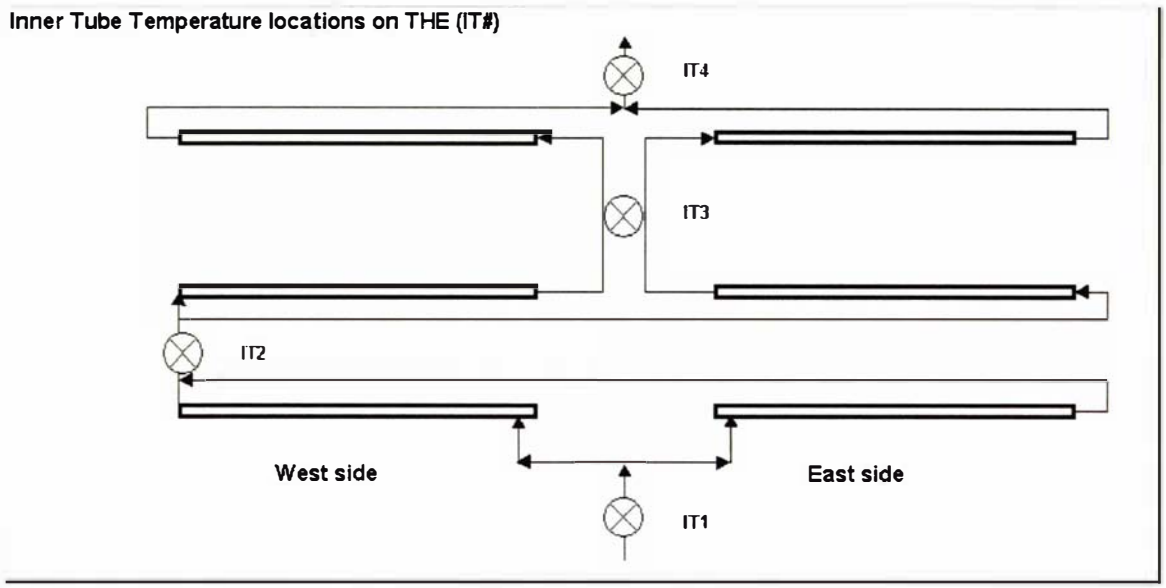


Figure D.6c. Key to tag names for temperature indicators (thermocouples) on the inner tube.

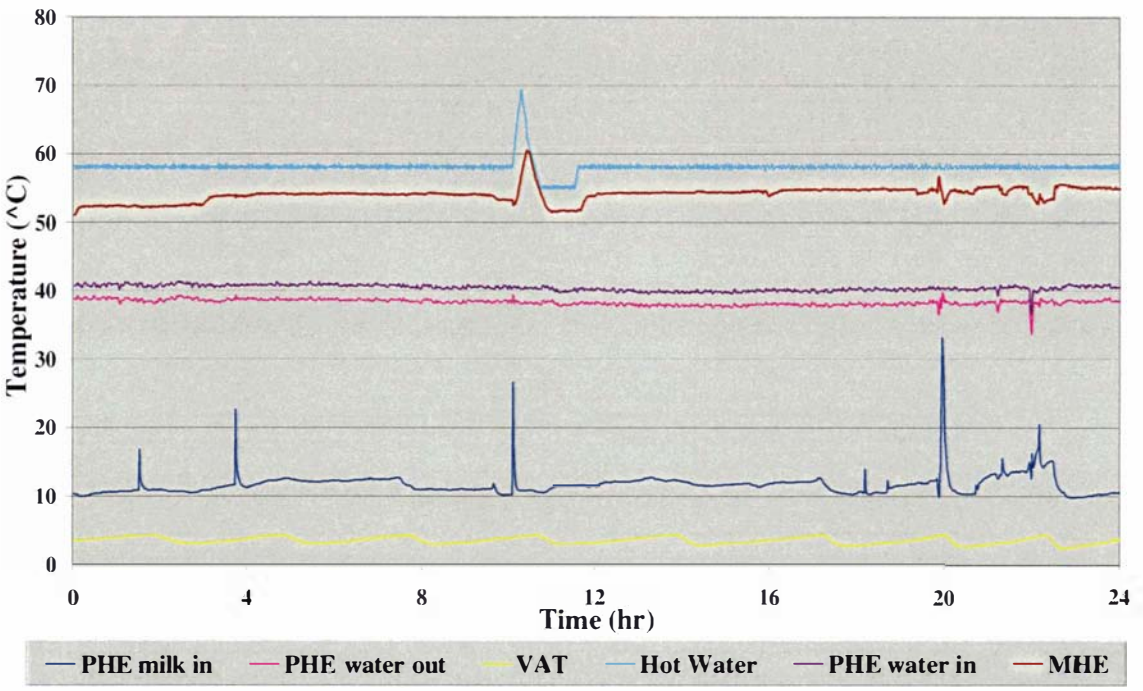


Figure D.7. Temperatures logged from the milk vat, PHE, hot water tank and MHE rig during Run 4.

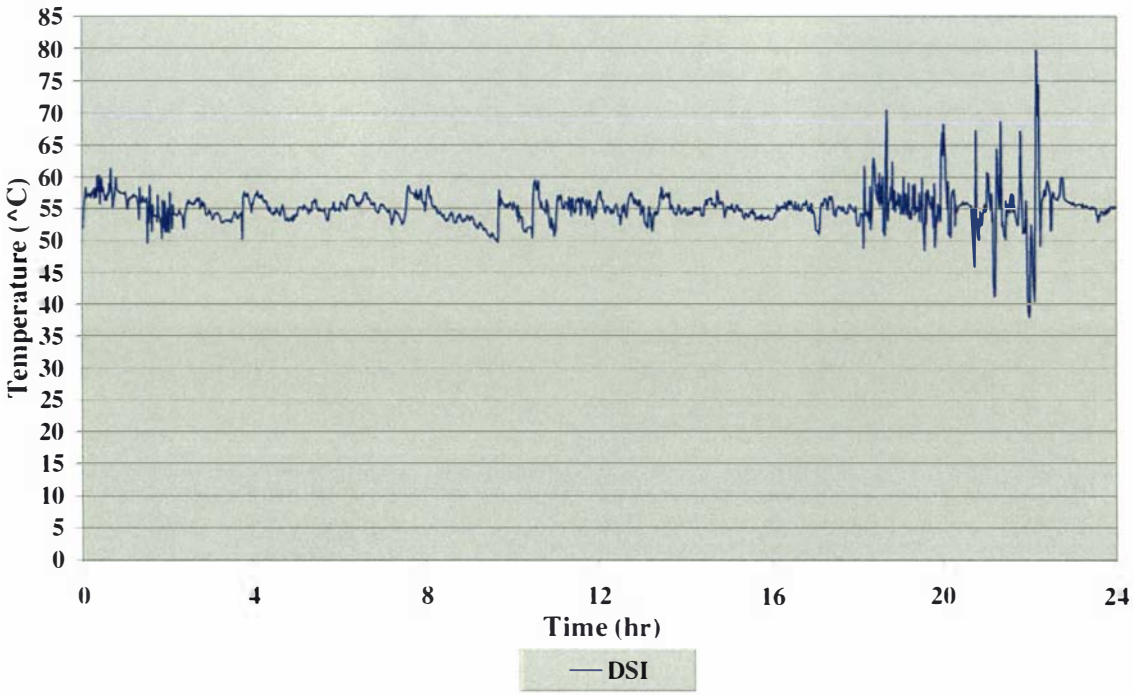


Figure D.8. Temperature logged from the DSI during Run 4.

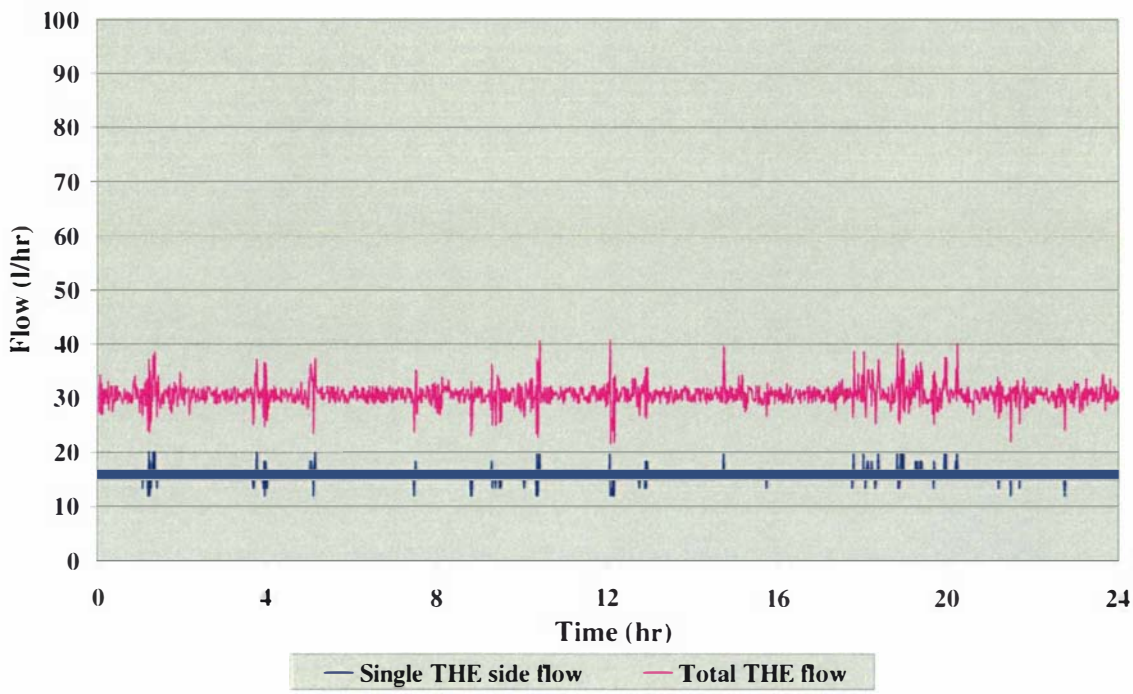


Figure D.9. Milk flow rate logged from pilot plant during Run 4.

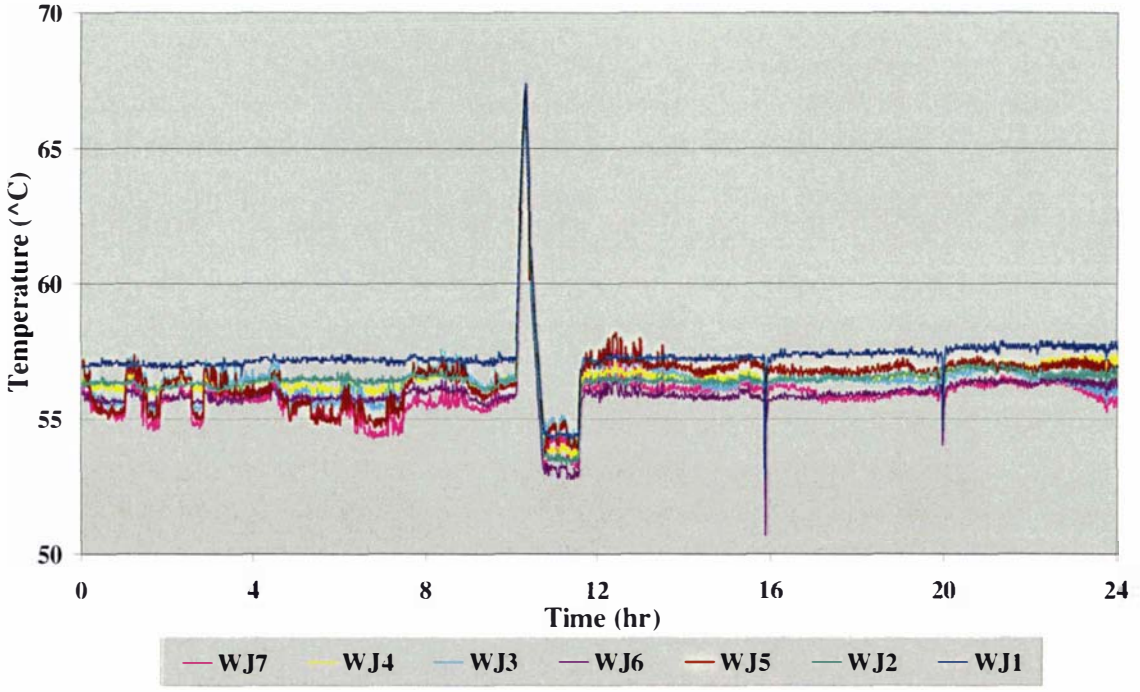


Figure D.10. Temperatures logged from the THE water jackets (outer tubes) during Run 4. See key in Figure D.6a for locations.

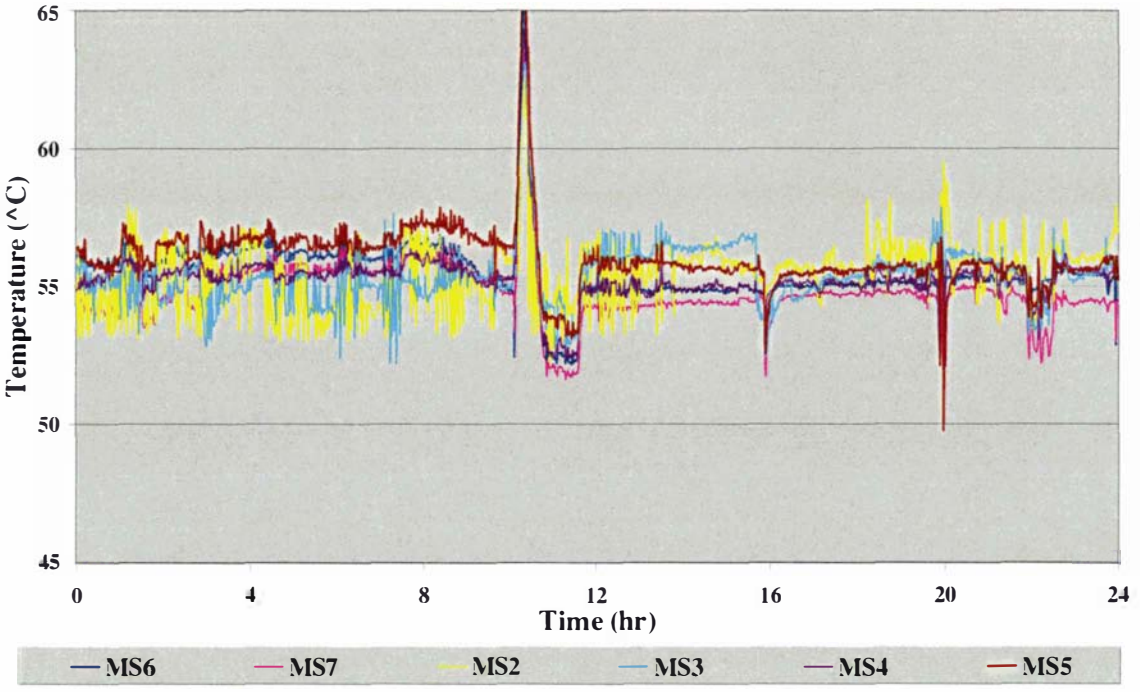


Figure D.11. Temperatures logged from the milk side of the THE (middle tubes) during Run 4. See key in Figure D.6b for locations.

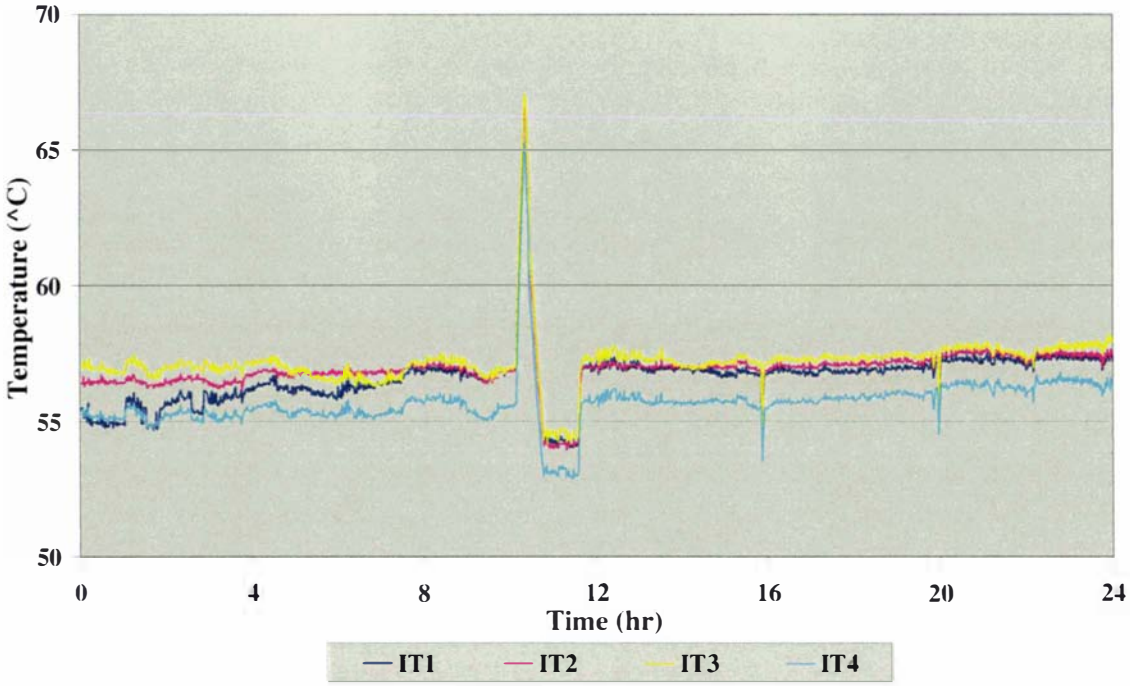


Figure D.12. Temperatures logged from the THE inner tubes during Run 4. See key in Figure D.6c for locations.

Experimental Run 5

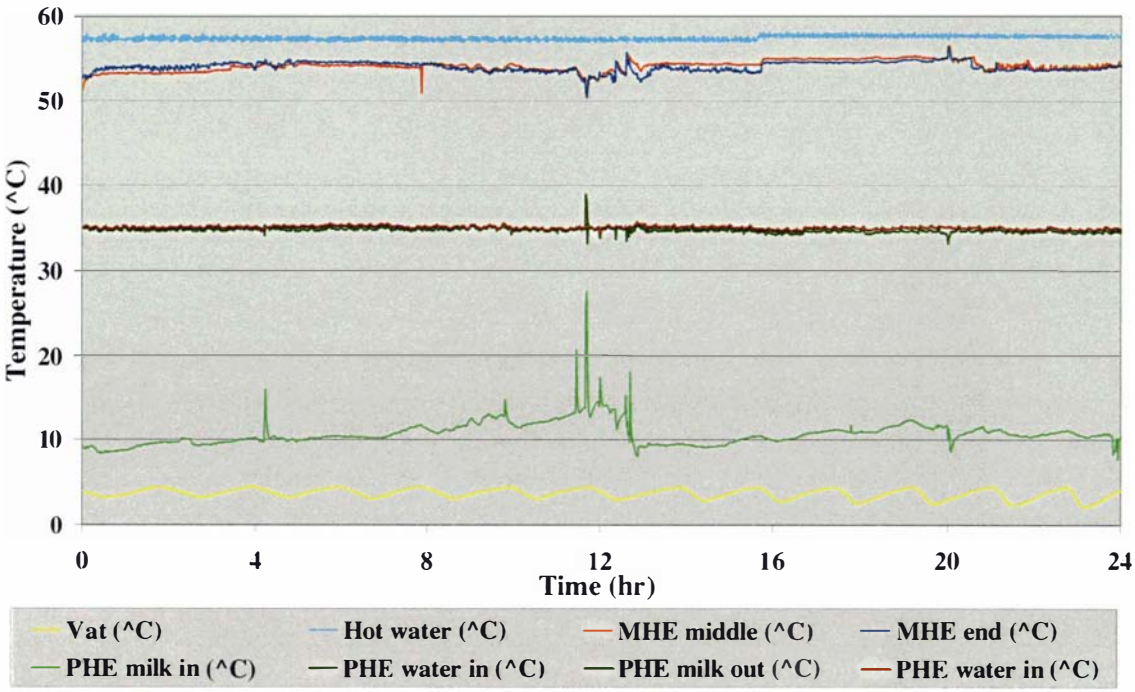


Figure D.13. Temperatures logged from the milk vat, PHE, hot water tank and MHE rig during Run 5.

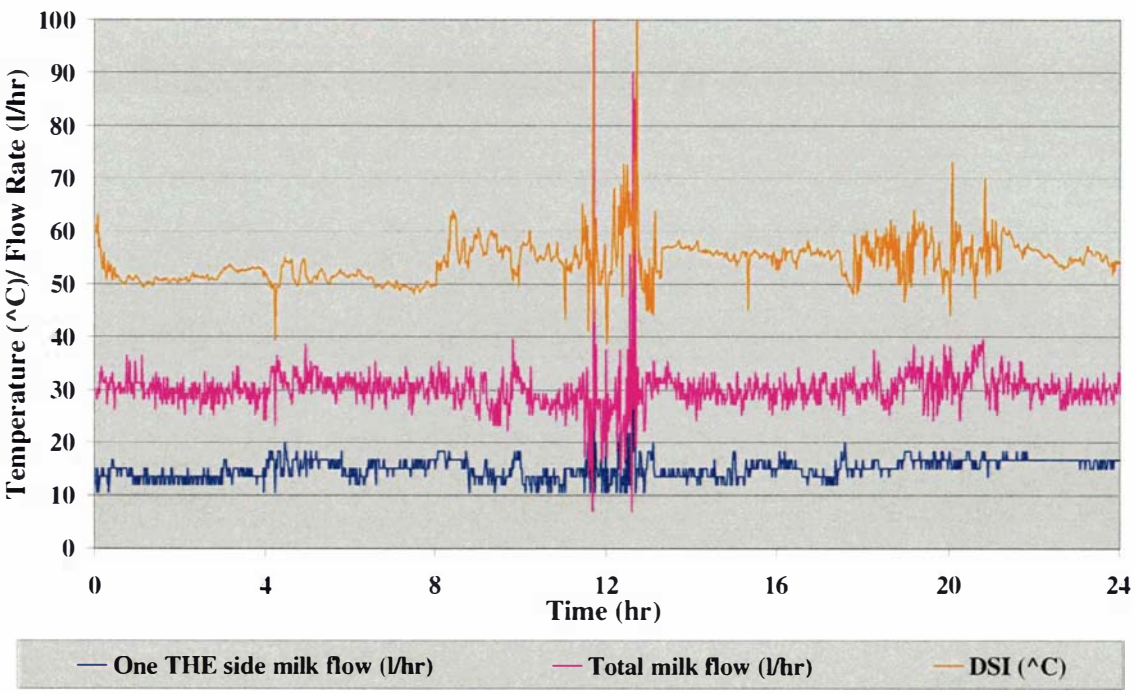


Figure D.14. Milk flow rate and DSI temperature logged from pilot plant during Run 5.

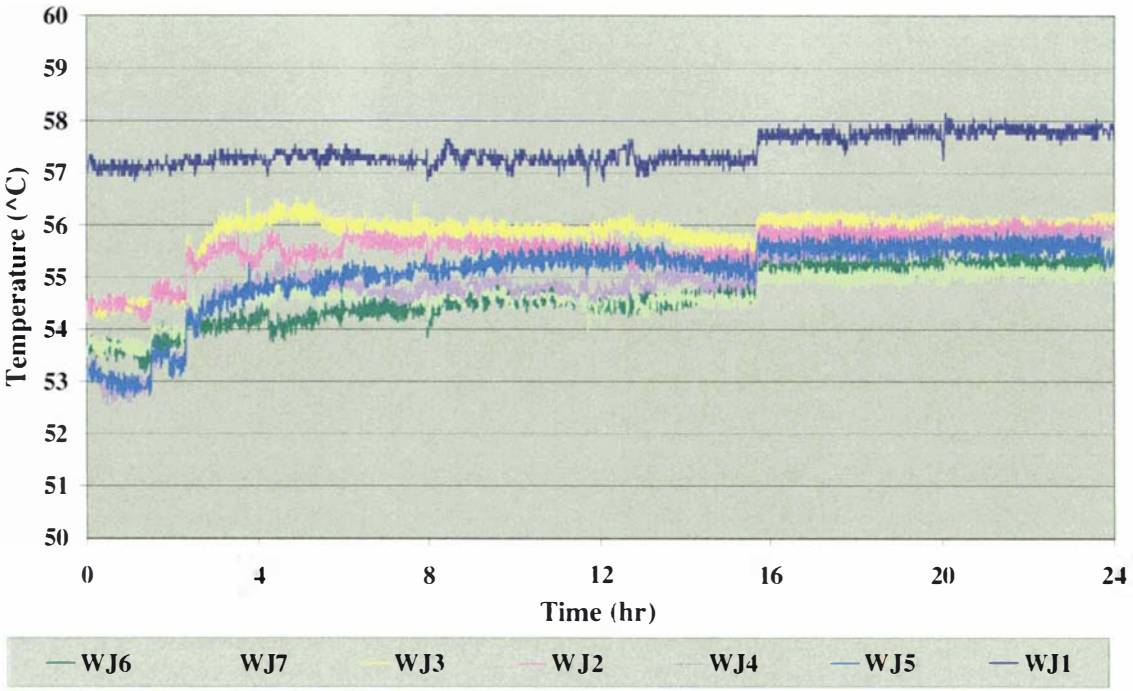


Figure D.15. Temperatures logged from the THE water jackets (outer tubes) during Run 5. See key in Figure D.6a for locations.

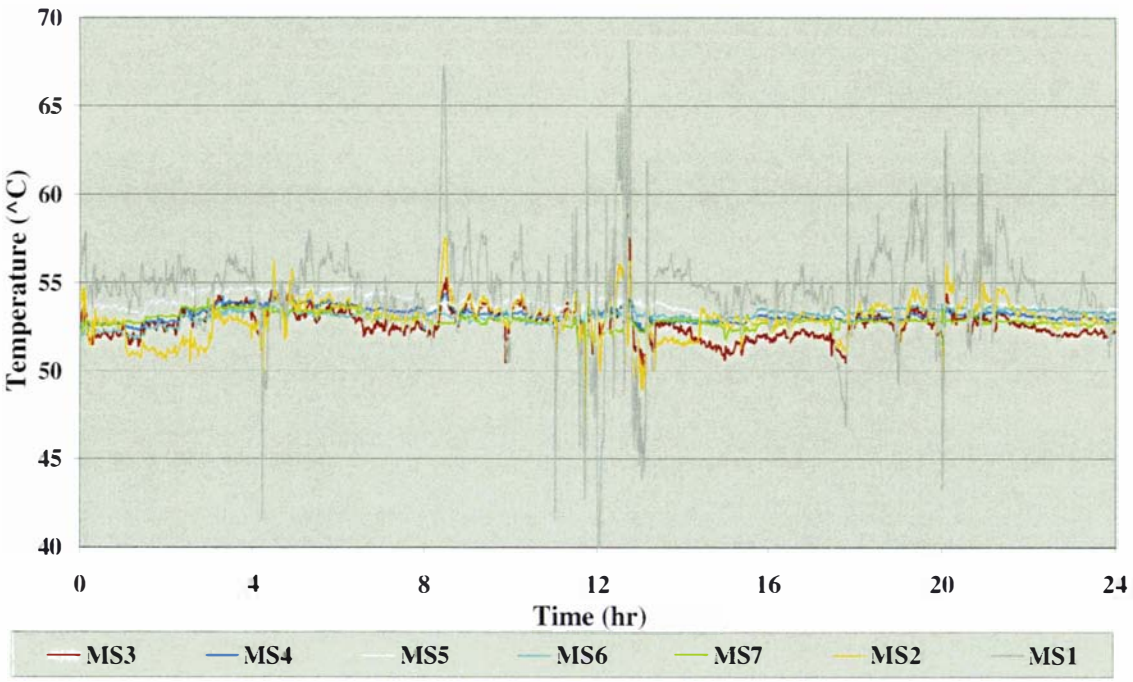


Figure D.16. Temperatures logged from the milk side of the THE (middle tubes) during Run 5. See key in Figure D.6b for locations.

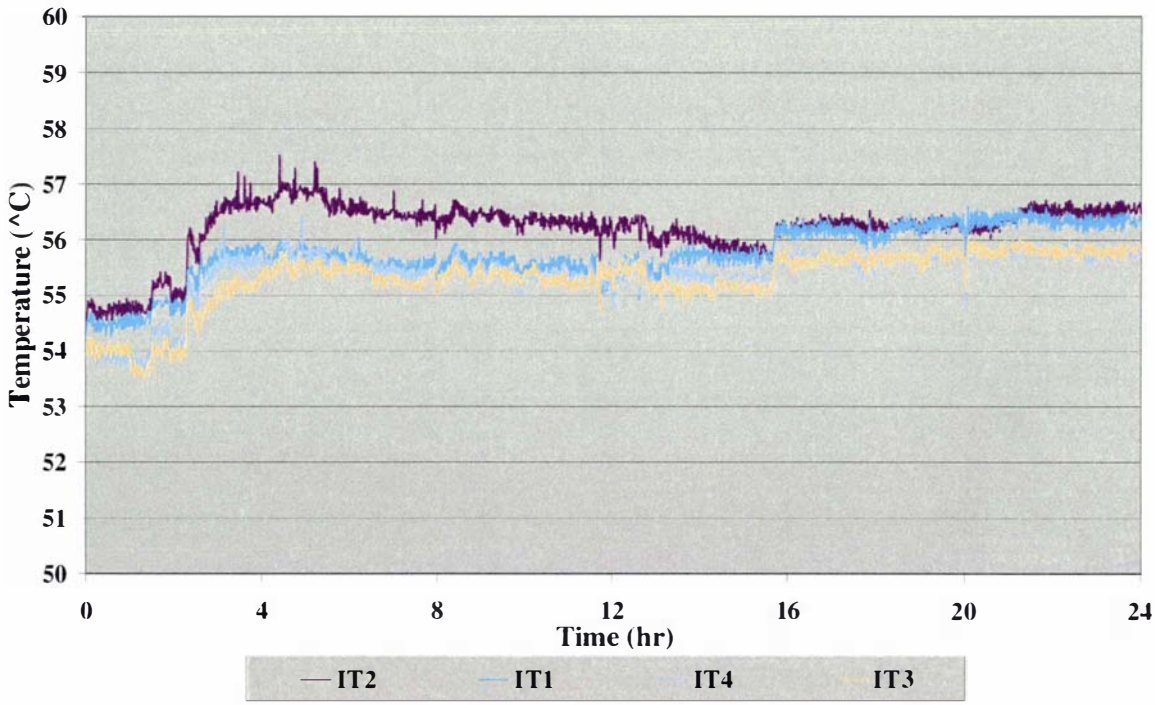


Figure D.17. Temperatures logged from the THE inner tubes during Run 5. See key in Figure D.6c for locations.

Appendix E - Other Modelling Information

Estimation of unknown model parameters

Unknown parameters in the model (a, k_r, k_a and g) were estimated using the optimisation toolbox in MATLAB® using the function 'lsqnonlin'. The MATLAB® files were rewritten so that comparisons of predicted and experimental results were output with the four unknown parameters as input arguments. The optimisation function modified the initial guesses of the four unknown parameters until the differences between the predicted and experimental results were minimised. The optimising function needed to be bound within sensible limits so that the solution converged. These upper and lower bounds are provided in Table E1.

This optimisation process was run for each set of experimental data that had different initial conditions. The best-fit estimates of the four parameters differed slightly depending on which set of experimental data was fitted. To obtain overall best estimates of the parameters, averages were taken of the estimates from the optimisation of each set of experimental data.

Table E.1. Best fit estimates of unknown model parameters and overall averages used in model predictions.

Parameter:	**k_r**	**a**	**G**	**k_a**
Lower bound	8E-9	0.5	1000	5E-9
Upper bound	8E-5	1.0	1800	5E-5
Run 1	1.07E-6	1.00	1000	6.70E-6
Run 2	8.04E-7	0.81	1000	*
Run 4	8.01E-7	1.00	1000	5.60E-6
Run 5	6.06E-7	0.87	1000	3.00E-6
Average	**8.2E-7**	**0.92**	**1000**	**5.10E-6**

*Model not is sensitive to changes in k_a when the initial population on surface > 1 cfu.cm^{-2}, as once initial surface population is established contribution from surface growth is much greater than the attachment rate.

Sensitivity Analysis

The parameters in the model were altered to determine the sensitivity of the model to changes in the parameters. The parameters were altered within the range of which they could be expected to potentially fall.

The results of varying adhesion constant and thermophile generation time are provided in Section 4.5.4.3 and 4.5.4.4.

Variation of constant 'a'

The constant 'a' in the term controlling the release proportion (β) was altered to determine the magnitude of its effect in changing the model predictions. Values for 'a' of between 0.5 and 1 were input into the model. Figures E1 and E2 below show the predictions for the bulk thermophile numbers and surface population over this range. The effect of decreasing 'a' is to reduce the rate of build up of thermophiles on the surface, which in turn also reduces the rate of increase in bulk thermophile numbers. Significant changes in the predictions can be seen with each 0.1 incremental change, showing that this parameter needs to be estimated accurately.

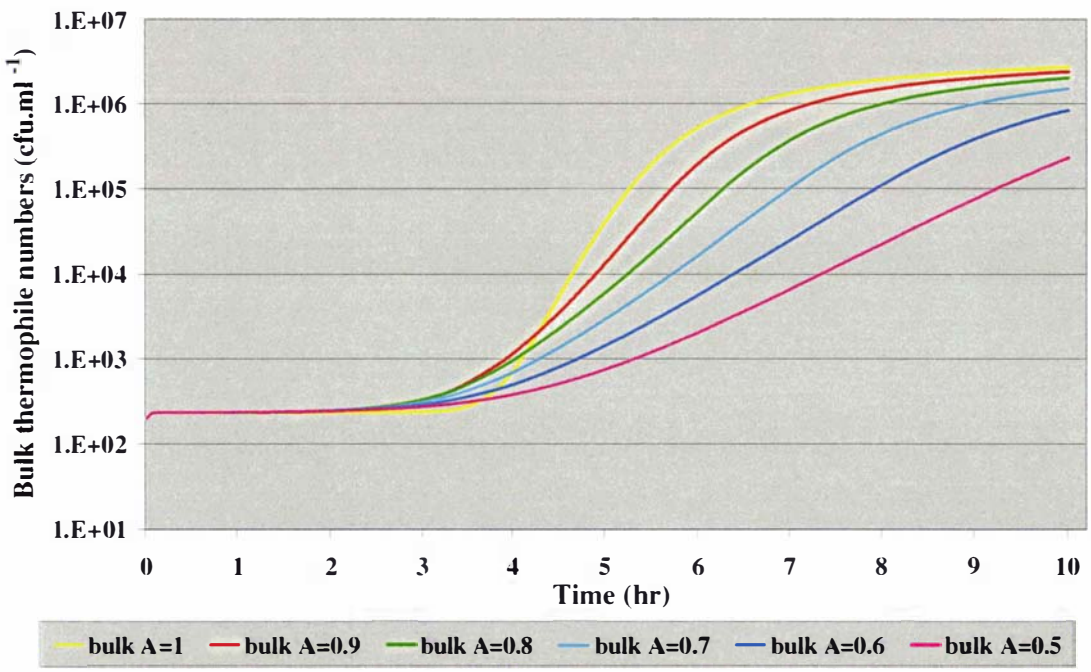


Figure E.1. Predictions of bulk thermophile numbers over time with varying values for the constant 'a' of 0.5 to 1.0 (Cbi = 200 cfu.ml^{-1}, k_a = 5E-6, k_r = 8E-7 and g = 1000 s).

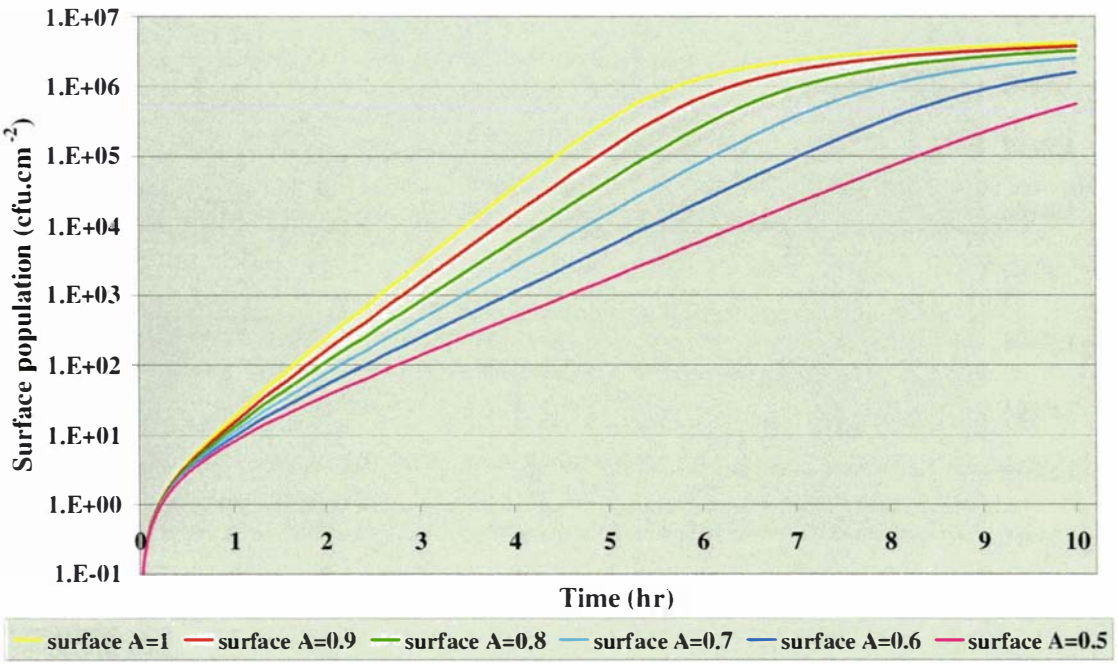


Figure E.2. Predictions of surface population over time with varying values for the constant 'a' of 0.5 to 1.0 (Cbi = 200 cfu.ml^{-1}, k_a = 5E-6, k_r = 8E-7 and g = 1000 s).

Variation of constant 'k_r'

The constant 'k_r' in the term controlling the release proportion (β) was also altered to determine the magnitude of its effect in changing the model predictions. Values for 'k_r' of 8E-9 to 8E-5 were input into the model. Figures E3 and E4 below show the predictions for the bulk thermophile numbers and surface population over this range. The effect of decreasing 'k_r' is to increase the steady state surface population and hence also the steady state bulk thermophile concentration. Significant changes in the predictions can be seen with each incremental change of a decade, showing that this parameter needs to be estimated accurately.

Table E.3. Prediction of surface population and bulk thermophile numbers using 1, 6 and 20 nodes per THE tube (predictions rounded to nearest whole number).

Nodes per THE tube:	1 node		6 nodes		20 nodes	
Time (s)	Surface # (cfu.cm^{-2})	Bulk # (cfu.ml^{-1})	Surface # (cfu.cm^{-2})	Bulk # (cfu.ml^{-1})	Surface # (cfu.cm^{-2})	Bulk # (cfu.ml^{-1})
0	0	200	0	200	0	200
3000	10	237	10	237	10	237
6000	75	241	77	241	77	241
9000	499	266	509	266	511	266
12000	3243	433	3315	434	3326	434
15000	20799	1636	21252	1643	21321	1648
18000	124208	13345	126716	13447	127094	13496
21000	547938	135322	555941	136007	557142	136453
24000	1355421	598629	1366501	596828	1368164	597989
27000	2164102	1202479	2175095	1194941	2176754	1194217
30000	2814660	1709623	2825894	1694394	2827601	1693329
33000	3329087	2107185	3341493	2086999	3343388	2086607
36000	3746878	2423403	3761208	2398209	3763406	2397876
Bulk diff. rel. to 1 node:		0		-25194		-25527
Surface diff. rel. to 1 node:	0		14330		16528	

Comparison with 1D model

The predictions of the output bulk thermophile concentration from the THE and the surface population from the 2D model (with 6 nodes per tube) were compared to those of a simpler 1D model. The predictions of the two models were very similar (Figure E5 and E6). This shows that the integration of finite differences into the numerical solution has not resulted in any numerical or mathematical errors.

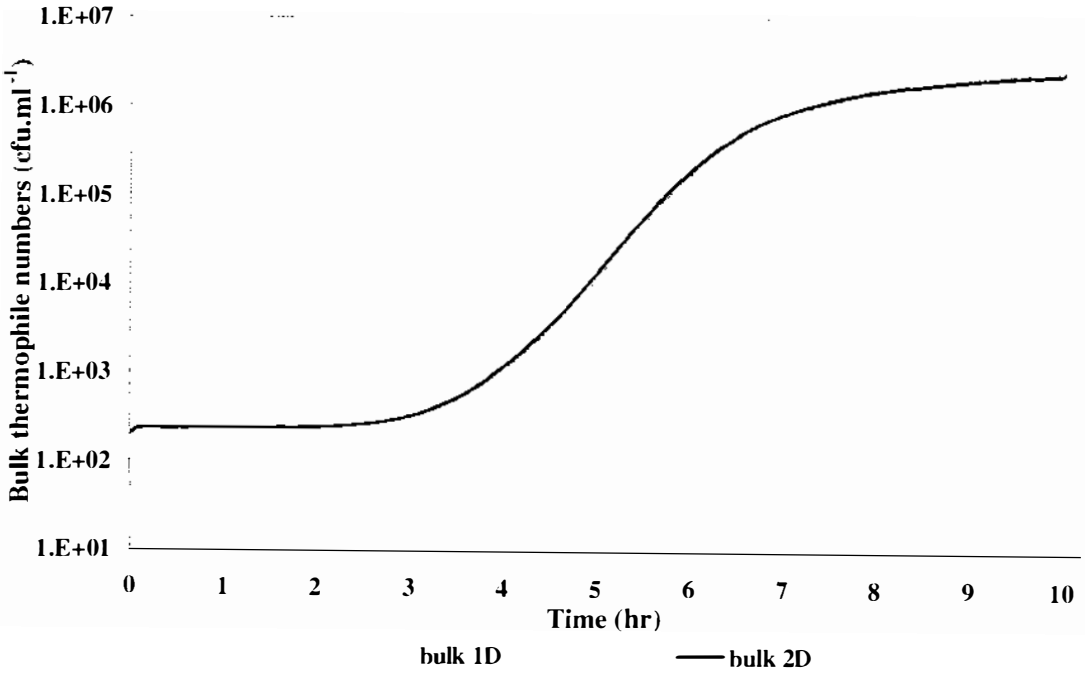


Figure E.5. Predictions of bulk thermophile numbers from 2D and 1D models using the same input parameters (g=1000 s, Cbi = 200 cfu.ml^{-1}, a = 0.9, k_r = 8E-7 and k_a = 5E-6).

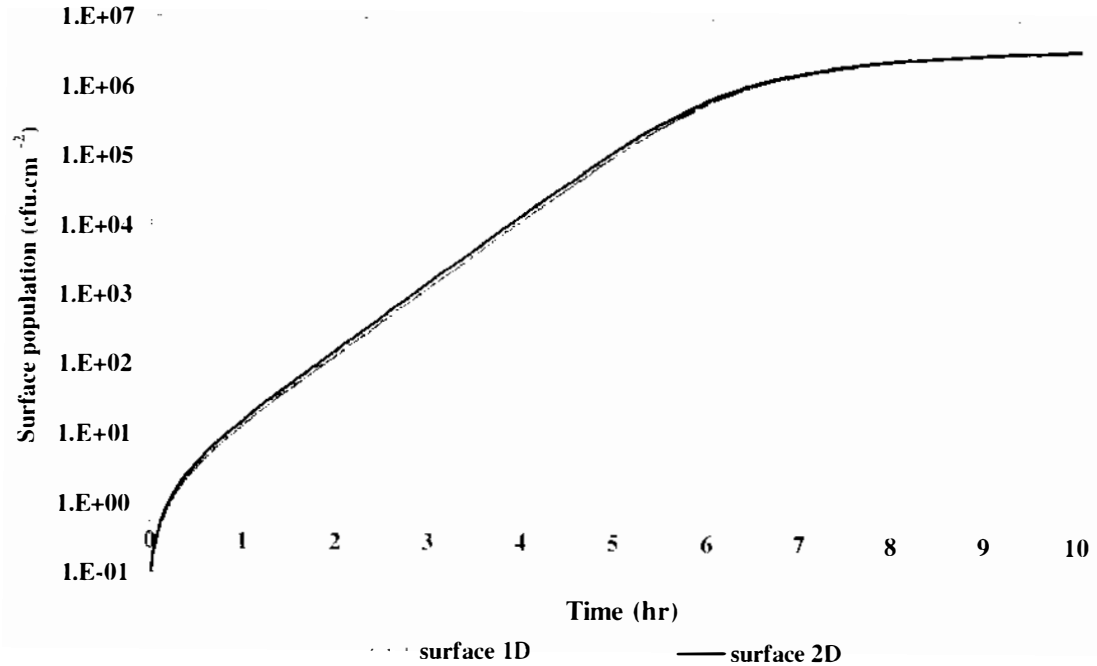


Figure E.6. Predictions of thermophile surface population from 2D and 1D models using the same input parameters (g=1000 s, Cbi = 200 cfu.ml^{-1}, a = 0.9, k_r = 8E-7 and k_a = 5E-6).

MATLAB® script and function files

Script file for 2D finite difference model

```
%Script file for thermophile release from pipe
%Andrew Hinton
%IFNHH

global Cbi;
global nwi;
global Simtime;
global j;
global J;
global tspace;
global l;
global g;
global Q;
global R1;
global R2;
global ka;
global kd;
global kr;
global A;
global pi;
global x;

%Variable inputs
disp(' ');
disp('Thermophile growth model for 3 tube tubular heat
exchanger');
disp(' ');
Simtimehr=   input('Enter simulation time (hr) (e.g.10) ');
Cbi=      input('Enter inlet bulk thermophile concentration
(cfu/ml) (e.g. 200) ');
nwi1=        input('Enter initial average wall population
on tube 1 (cfu/cm2) (e.g. 0) ');
nwi2=        input('Enter initial average wall population
on tube 2 (cfu/cm2) (e.g. 0) ');
nwi3=        input('Enter initial average wall population
on tube 3 (cfu/cm2) (e.g. 0) ');
Jt=       input('Enter number of nodes along each pipe
(e.g. 2) ');
Q=        input('Enter flow rate (l/hr) (e.g. 15) ');
disp(' ');
disp('----------Please wait----------');
disp(' ');

%Fixed inputs
l=330;
R1=0.635;
```

```
R2=1.18;
g=1000;
tspace=60;
pi=3.14159;
A=0.9;
kr=8e-7;
kd=0;
ka=5e-6;
J=Jt*3;

%initial conditions

yi=ones(1,2*J);
yi(1:Jt)=nwi1;
yi(Jt+1:2*Jt)=nwi2;
yi((2*Jt)+1:J)=nwi3;
yi(J+1:2*J)=Cbi;

Simtime=Simtimehr*3600;
Tspan=[0:tspace:Simtime];
Options=odeset('RelTol',1e-6);

%ODE call
[t,y]=ode45('Thermophilemodel2',Tspan,yi);

%Output stuff
figure;
whitebg('white');
semilogy(t./3600,y(:,1:Jt),'r-',t./3600,y(:,Jt+1:2*Jt),'b-
',t./3600,y(:,(2*Jt)+1:J),'g-');
title('Surface Numbers');
xlabel('Time [hr]');
ylabel('Surface Numbers [cfu/cm2]');

figure;
semilogy(t./3600,y(:,J+1:J+Jt),'r-
',t./3600,y(:,J+Jt+1:J+(2*Jt)),'b-
',t./3600,y(:,J+(2*Jt)+1:2*J),'g-');
title('Bulk Numbers');
xlabel('Time [hr]');
ylabel('Bulk Numbers [cfu/ml]');
```

Function file for 2D finite difference model

```
function odes=Thermophilemodel2(t,y)

%function file for thermophile release from pipe
%Andrew Hinton
%IFNHH

global R1;
global R2;
global ka;
global kd;
global kr;
global A;
global g;
global Q;
global u;
global V;
global sa;
global l;
global pi;
global J;
global j;
global Cbi;
global nwi;
global Simtime;
global tspace;
global ra;
global x;

%Node width, flowrate(ml/s) and growth rate
x=l/J;
q=(Q*5)/18;
u=0.693/g;


%area and volume calcs
sa=(2*pi*x*(R2+R1));
V=(pi*x*((R2*R2)-(R1*R1)));


%Update variable
   nw1=y(1);
    Cb1=y(1+J);

   %release proportion
   B=1-A*exp(-kr*nw1);

   %Attachment rate
   ra=ka*Cb1;
```

```
odes=zeros(2*J,1);
odes(1)=u*nw1*(1-B)+ra;
odes(1+J)=(q/V)*(Cbi-Cb1)+(sa/V)*((B*u*nw1)-ra)+(Cb1*u)-
(Cb1*kd);

j=2;

for j=j:J

   %Update variable
   nwj=y(j);
    Cbj=y(j+J);
    Cbp=y(j+J-1);

   %release proportion
    B=1-A*exp(-kr*nwj);

    %Attachment rate
    Cj=(Cbp+Cbj)/2;
   ra=ka*Cj;

   %ODE's
   odes(j)=u*nwj*(1-B)+ra;
   odes(j+J)=(q/V)*(Cbp-Cbj)+(sa/V)*((B*u*nwj)-ra)+(Cj*u)-
(Cbj*kd);

end
```

Example input and output

Input

```
»
Thermophile growth model for 3 tube tubular heat exchanger

Enter simulation time (hr) (e.g.10) 10
Enter inlet bulk thermophile concentration (cfu/ml) (e.g. 200) 200
Enter initial average wall population on tube 1 (cfu/cm2) (e.g. 0) 0
Enter initial average wall population on tube 2 (cfu/cm2) (e.g. 0) 0
Enter initial average wall population on tube 3 (cfu/cm2) (e.g. 0) 0
Enter number of nodes along each pipe (e.g. 2) 6
Enter flow rate (l/hr) (e.g. 15) 15

----------Please wait----------
```

Output

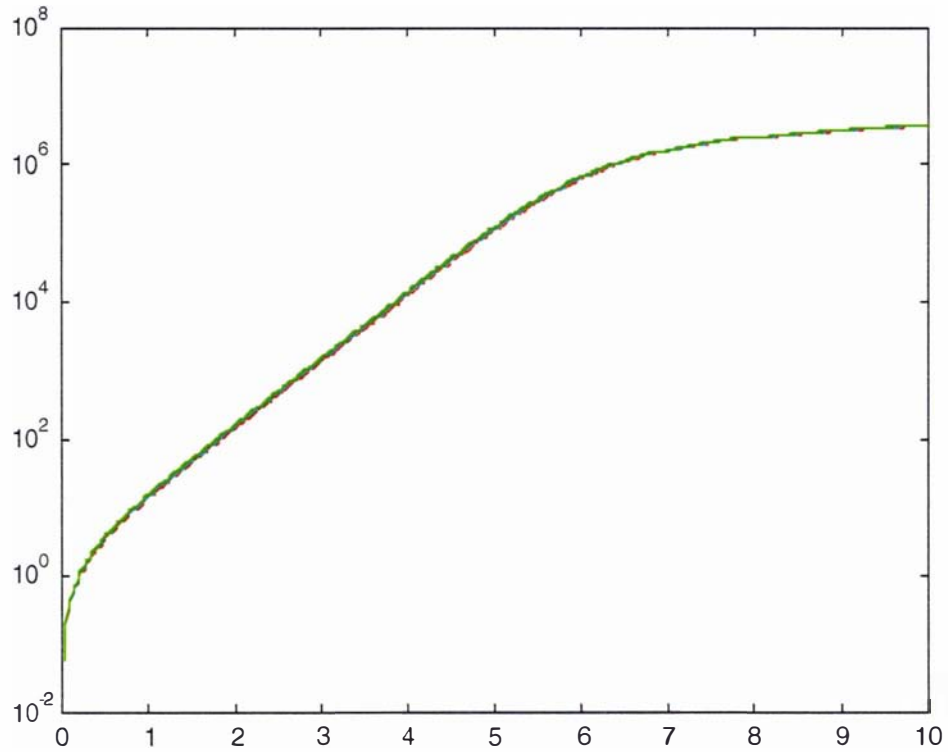


Figure E.7. First MATLAB output graph (surface population (cfu.cm^{-2}) vs time (hrs)) from un-steady model using example input above.

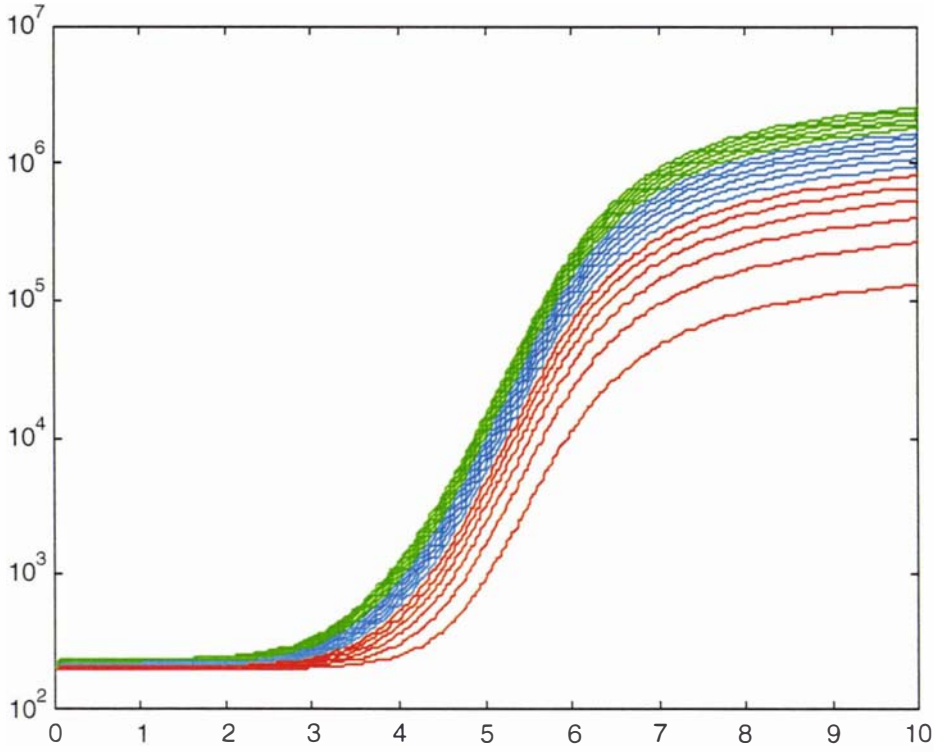


Figure E.8. Second MATLAB output graph (bulk numbers ($cfu.ml^{-1}$) vs time (hrs)) from un-steady model using example input above.

Script file for 1D model

```
%Script file for thermophile realease from pipe
%Andrew Hinton
%IFNHH

global g;
global q;
global Cbi;
global nwi;
global Simtime;
global l;

%inputs
Cbi=200;
nwi=0;
Simtime=36000;
l=330;
g=1000;
q=4.1666667;
tspace=60;

yi=[nwi,Cbi];
Tspan=[0:tspace:Simtime];

[t,y]=ode45('Thermophile1',Tspan,yi);

N=[y(:,1)];
C=[y(:,2)];

figure;
whitebg('white');
semilogy(t./3600,N(:,1),'b-');
title('Surface Numbers');
xlabel('Time [hr]');
ylabel('Surface Numbers [cfu/cm2]');

figure;
semilogy(t./3600,C(:,1),'g-');
title('Bulk Numbers');
xlabel('Time [hr]');
ylabel('Bulk Numbers [cfu/ml]');
```

Function file for 1D model

```
function dy=Thermophile1(t,y)

%function file for thermophile realease from pipe
%Andrew Hinton
%IFNHH

global R1;
global R2;
global ka;
global kd;
global kr;
global A;
global g;
global q;
global u;
global Cbi;
global nwi;
global V;
global sa;
global l;
global pi;
global nwamax;
global ra;

nw=y(1);
Cb=y(2);


nwamax=100000;
R1=0.635;
R2=1.18;
pi=3.14159;
A=0.9;
kr=8e-7;
kd=0;
ka=5e-6;
u=0.693/g;

B=1-A*exp(-kr*nw);

sa=(2*pi*l*(R2+R1));
V=(pi*l*((R2*R2)-(R1*R1)));

ra=ka*Cbi;

dnw=u*nw*(1-B)+ra;
dCb=(q/V)*(Cbi-Cb)+(sa/V)*((B*u*nw)-ra)+(Cb*u)-(Cb*kd);

dy=[dnw;dCb];
```

Appendix F - Peer reviewed papers

The peer-reviewed papers from this work are listed below. These can be found in *.pdf format on the enclosed compact disc, path: /Documents/Peer reviewed papers/.

Paper 1: 6th World Congress of Chemical Engineering, Melbourne 2001

Hinton, A. R., Trinh, K. T., Brooks, J. D., & Manderson, G. J. (2001). Thermophile growth in the preheating section of a milk powder pilot plant. Proceedings of 6th World Congress of Chemical Engineering (Paper 773).

Paper 2: 9th APCChE Congress and CHEMECA 2002

Hinton, A. R., Trinh, K. T., Brooks, J. D., & Manderson, G. J. (2002). Thermophile recontamination of the pre-heating section of a milk powder pilot plant. Proceedings of 9th APCCHE Congress and Chemeca 2002 (Paper 380).

Paper 3: 9th APCChE Congress and CHEMECA 2002

Hinton, A. R., Trinh, K. T., Brooks, J. D., Manderson, G. J., Osbaldiston, K. A. O. M., Ng, J. W. L., & Millward, S. (2002). Thermophile adhesion to stainless steel and milk fouling from static cultures. Proceedings of 9th APCCHE Congress and Chemeca 2002 (Paper 381).

Paper 4: Trans IChemE, Vol. 80, Part C, December 2002.

Hinton, A. R., Trinh, K. T., Brooks, J. D., & Manderson, G. J. (2002). Thermophile survival in milk fouling and on stainless steel during cleaning. Trans IChemE, 80, Part C(12), 299-304.

Appendix G - Index to attached compact disc

Table G.1. Index of information on enclosed compact disc.

Index to Compact Disc				
Level 1	**Level 2**	**Level 3**	**Level 4**	**Contents**
/Documents				
	/Other reports			Copies of other reports generated as from this PhD work
	/Peer reviewed papers			Copies of the peer reviewed papers in PDF
	/Thesis			Full copy of the thesis in MS Word format.
/Images				
	/Adhesion			
		/Skim milk		
			/CLSM images	CLSM image files from skim milk fouling adhesion studies
			/Fouling Photos	Photographs of skim milk fouling layers
		/Whole milk 1		
			/CLSM images	CLSM image files from adhesion studies using variable amount of whole milk fouling
			/Fouling Photos	Photographs of varying amount whole milk fouling layers
		/Whole milk 2		
			/CLSM images	CLSM image files from adhesion studies using constant amount of whole milk fouling
			/Fouling Photos	Photographs of constant amount whole milk fouling layers
	/Agar plate photos			Photographs of agar plates from bulk milk thermophile enumeration in experimental runs showing different colony morphologies present.
	/Equipment			
		/Drawings		Drawings of pilot plant equipment.
		/Photographs		Photographs of pilot plant equipment
	/Expt Run 1			
		/THE photos		Photos of THE inner tubes - Run 1
	/Expt Run 2			
		/THE photos		Photos of THE inner tubes - Run 2
	/Expt Run 3			
		/CLSM images		CLSM image files - Run 3 (includes index to file names "Run 3 CLSM file index")

	/CLSM slides		Photos of CLSM microscope slides – Run 3
	/MHE photos		Photos of MHE plate surfaces - Run 3
	/THE photos		Photos of THE inner tubes - Run 3
	/UV images		UV images from Run 3
/Expt Run 4			
	/MHE photos		Photos of MHE plate surfaces - Run 4
	/THE photos		Photos of THE inner tubes - Run 4
/Expt Run 5			
	/CLSM images		CLSM image files - Run 5 (includes index to file names "Run 5 CLSM file index.xls")
	/CLSM slides		Photos of CLSM microscope slides - Run 5
	/MHE photos		Photos of MHE plate surfaces - Run 5
	/THE photos		Photos of THE inner tubes - Run 5
/Survival			
	/CLSM images		CLSM image files from cleaning survival experiment
	/Fouling photos		
		/After clean	Photographs of fouled MHE plates after cleaning survival experiment
		/First foul	Photographs of fouled MHE plates after first fouling development for cleaning survival experiment
		/Final foul	Photographs of fouled MHE plates after final fouling development for cleaning survival experiment

Appendix H - Reference List

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