Rapid Methods for Assessing Surface Cleanliness within the Food Industry: Their Evaluation, Design and Comparison to Traditional Techniques.

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To Mum, Dad, Anna and Adam

for the love, the support and the chocolate cake!

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For what seems like a decade I have struggled to string enough words together to form a thesis. Three hundred pages and a gallon of tears later, it has come to this – the one page friends and colleagues *will* read!! So with a deep breath, the slight aroma of cheese and a rush of "have-just-come-back-from-holiday" endorphins, here goes…

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Summary

The cleanliness of the processing environment is an important factor in both assuring food quality and protecting the consumer from pathogens, consequently, food businesses should continually assess surface cleanliness in order that any problems associated with the 'cleaning' process can be identified and rectified rapidly. There is, however, widespread confusion regarding why, when and how cleanliness assessment should be carried out and, thus, a need to provide the food industry with guidance.

Laboratory and field studies were conducted in order to evaluate the performance characteristics of both microbiological and chemical-based cleanliness assessment methods. Limits of detection were determined together with factors affecting test efficacy and associated advantages, drawbacks and limitations.

The mechanical energy generated during sampling, the absorbency of the bud material and the swab-wetting solution used, all contributed to the efficacy of the traditional swabbing procedure. Overall however, it was the ease with which bacteria could be released from the bud that had the greatest effect and omitting this step via the use of dipslides increased the sensitivity of microbiological assessment. The benefits of using alternative microbiological techniques have also been highlighted, and a novel, swab-based, enzymatic method capable, in just 5 h, of detecting the presence of < 1 coliform colony cm^{-2} has been developed.

Unlike microbiological techniques, surface dryness had little effect upon the performance of non-microbiological test methods, yet, other factors, including the universality of the component residue being tested for and its intrinsic level within the food debris, did influence the results obtained, demonstrating that choice of method must depend upon the type of food produced. Results also identified key sectors of the food industry for which appropriate test methodology is currently lacking. To fulfil such requirements, an assay for use within high-fat processing environments has also been developed.

Taken collectively, the results demonstrate that given the variability in food debris and surface contamination, no one method is ideal for assessing cleanliness and rather than being interchangeable, test methods should be used in combination. An integrated cleaning assessment strategy has been devised and its implementation should enable food businesses to ensure their cleaning and disinfection procedures are effective and that the food produced is safe and of the highest quality.

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Chapter 1

Introduction

Every year there are approximately 4000 million cases of diarrhoeal disease worldwide. Nearly 50% of cases affect children under the age of 5 with 3 million of these dying as a direct result and an even greater number succumbing to diarrhoea-associated malnutrition. Safe and wholesome food is a basic human right, yet 70% of all diarrhoeal disease cases can be attributed to an illness resulting from a foodborne disease (Motarjemi and Käferstein, 1999).

Foodborne disease is defined as "a disease of an infectious or toxic nature caused by or thought to be caused by the consumption of food or water" (Tirado and Schmidt, 2000) and can be associated with microbial pathogens such as bacteria, viruses and parasites, biotoxins and chemical contaminants. Preservation techniques, such as drying, salting, heating and fermentation have been employed for centuries as a means of inactivating both pathogenic and food spoilage organisms. However, despite advances in refrigeration, chemical preservatives and packaging techniques and a greater understanding regarding the deterioration of food, 25% of all foods produced globally is still lost post-harvest or post-slaughter because of microbial spoilage (Gram *et al.* 2002).

While most foodborne diseases are sporadic and often not reported, foodborne disease outbreaks may also take on massive proportions (Asao *et al.* 2003) and although mainly occurring in developing countries, any area, regardless of its stage in development can be affected and it is estimated that in the industrialised world, 10% of the population suffer from foodborne disease each year (Motarjemi and Käferstein, 1999). Not only is general

health and well-being affected but illness may also have severe economic consequences for the individual, their families, communities and businesses and in addition can impose a substantial burden on a country's health-care system (Olsen *et al.* 2001; Abe *et al.* 2002; Roberts *et al.* 2003). Consequently, in October 2000, the UK Food Standards Agency announced that by April 2006, its aim was to have reduced the incidence of UK foodborne disease by 20% (Food Standards Agency, 2000). However, in a great number of developing countries, public health authorities, either because of a lack of resources or a lack of awareness, undertake very few measures to investigate or prevent foodborne disease unless it becomes an obstacle to effective trade or tourism. Consequently, and tragically, in many parts of the developing world, foodborne disease occurs so frequently, it is almost considered a part of everyday life (Motarjemi and Käferstein, 1999).

In contrast, in the industrialised world, programmes have been implemented that involve the continuous and systematic collection, collation and analysis of data. This information is used to identify prominent or potential pathogens and/or faults within a production process, which could contribute to microbial spoilage and/or foodborne disease and also to facilitate the development of appropriate intervention strategies (Wall *et al.* 1996). *Listeria monocytogenes* for example, causes severe illness in the elderly, the immuno-compromised and the unborn foetus; 25% of all recognised infections result in death (Tauxe, 2002). However, in the U.S., the active surveillance of foodborne listeriosis followed by effective control measures has led to a 48% reduction in the mortality attributable to this infection (Käferstein and Abdussalam, 1999). Similarly, *Salmonella typhi* has been all but eliminated from industrialised nations and is now largely associated with international travel to developing countries where typhoid fever remains a significant cause of morbidity and mortality (Olsen *et al.* 2001).

However, despite making substantial advances, the industrialised world cannot afford to become complacent. By 2020 the human population is predicted to reach 8.5 billion, 80% of which is expected to be in developing countries and preventing the spoilage of a diminishing supply of fresh food will become ever-more important. In the industrialised world, the proportion of people aged 60 years and over is predicted to rise from 17% now, to 25% by 2025 (Käferstein and Abdussalam, 1999). A population that is aging and subject to more immuno-compromising conditions will be at higher risk and "vulnerable" to any illness including foodborne disease. Globally, therefore, food spoilage and foodborne disease will continue to be of considerable economic importance and a major public health concern and, with expanding international trade, international co-operation will be required in order to minimise, prevent and control the contamination of food (Buchanan, 1997; Motarjemi and Käferstein, 1999). Consequently, whilst industry, government and the consumer must share the responsibility for safe and wholesome food, the food industry as a whole is becoming increasingly involved in developing both food safety and quality plans and standards.

The ultimate aim of the food industry is to produce and supply a quality product, which in microbiological terms encompasses safety, acceptability/shelf-life and consistency. The food produced must not contain levels of pathogens or associated toxins at levels likely to cause illness or levels of microorganisms sufficient to reduce shelf-life or render the product organoleptically spoiled (Adams and Moss, 1995). However, spoilage organisms such as *Pseudomonas* spp. can become indigenous to processing plants and pathogenic organisms such as *Staphylococcus aureus* and *L. monocytogenes* can easily colonise processing equipment (Notermans *et al*, 1994). Thus, the inadequate cleaning and disinfection of these and all product contact and environmental surfaces can increase the risk of such organisms becoming dislodged, contaminating the final product and contributing to its microbial load.

High standards of cleanliness can, therefore, help to ensure that shelf-life is maintained and public health protected and many food companies see food hygiene not only as a food safety and quality issue but also as a potential selling point – standards set by a retailer, for example are likely to be imposed upon their suppliers (Aston, 2000; Tauxe, 2002). Nevertheless, "shiny surfaces" are no longer an acceptable indication that the surfaces are clean and free from contamination. Scientific evidence is now required that demonstrates categorically, that the cleaning and disinfection procedures have been effective (Oosterom, 1998). However, despite a number of methods being available to the food industry, no standard protocol has been adopted and this has led to confusion within the industry with regard to how best to assess surface cleanliness.

The aim of applied food safety research is to provide the food industry with answers and/or guidance with regard to specific problems or issues that may prevent them from producing safe and wholesome food and fulfilling their responsibility to the consumer. Research is necessary in order that strategies can be developed that will enable food businesses to simply, reliably and effectively assess the cleanliness of surfaces and thus the efficacy of the cleaning and disinfection procedures applied and in doing so help ensure that the safety and quality of the product is maintained.

1.1. Survival and Growth of Microorganisms on Food Contact Surfaces

When supplied with sufficient nutrients and its optimal growth temperature, pH, oxygen level and solute concentration, any microorganism will grow at its characteristic maximum growth rate. However, such ideal conditions are rarely found outside the laboratory and consequently, the ability of a bacterium to respond to impromptu alterations in its environment is crucial to its survival (Moat *et al.* 2002). Different bacteria exhibit a variety of physiological and genetic responses to a range of environmental stresses (Abee and Wouters, 1999) and as a consequence, microorganisms are capable of surviving within any food processing environment, despite conditions often not being ideal.

The ability of both food spoilage and pathogenic microorganisms to attach to a wide variety of materials used within the food industry has been well documented (Pontefract, 1991; Andrade *et al.* 1998a; Beresford *et al.* 2001; Flint *et al.* 2001). Bacteria derive particular advantages from adhesion, not least because, in comparison to any bulk fluid, nutrient molecules are likely to be at a higher concentration on, or in close proximity to, a surface. Furthermore, water, which is essential for microbial growth, may, even on a visibly dry surface, remain in surface cracks and crevices (Gabis and Faust, 1988). Thus, the ability to achieve a close association with a surface enables the bacteria to readily scavenge available water and nutrients and it has been proposed that the attached state is the predominant form of microorganism survival in natural and man-made ecosystems (Zottola and Sasahara, 1994; Lindsay and von Holy, 1997).

Bacterial adhesion involves the solid surface, the suspending medium and the microorganisms themselves and a change in the characteristics of any one of these will induce changes in the adhesion process (Boulangé-Petermann, 1996).

1.1.1. Surface Conditioning and the Significance of Residual Food Debris

In any food processing environment, both food contact and environmental surfaces come into contact with fluids containing various levels of food component. The organic and inorganic molecules comprising the surrounding medium physically adsorb to a surface – a process known as "conditioning" and one thought to take place very rapidly, before bacterial attachment occurs. Surface conditioning is likely to change the physiochemical properties of the substratum and, as a result, the interaction between bacterium and surface (Hood and Zottola, 1995; Kumar and Anand, 1998; Barnes *et al.* 1999). *Listeria monocytogenes* for example, is reported to readily adsorb to hydrophilic surfaces, such as clean stainless steel and consequently, the use of hydrophobic surfaces has been recommended as a means to minimise *L. monocytogenes* adherence levels (Chavant *et al.* 2002). However, proteins adsorb very rapidly to hydrophobic surfaces and at very low concentrations (Cunliffe *et al.* 1999) and whilst their hydrophobic moieties interact with the surface their hydrophilic sites are left in the aqueous phase and, thus, the hydrophobic surface becomes hydrophilized (Michalski *et al.* 1999). Conversely, during adsorption to hydrophilic surfaces, it is the hydrophilic regions of the protein that irreversibly adsorb, whilst the hydrophobic moieties are those that remain at the interface, thus, increasing surface hydrophobicity - microorganisms have also been demonstrated to attach very firmly to hydrophobic surfaces (Cunliffe *et al.* 1999). Nevertheless, the adsorption of certain molecules, specifically particular types of protein, seemingly impairs bacterial attachment.

Milk residues reportedly reduce the adherence of a variety of microorganisms (Hood and Zottola, 1997a; Hood and Zottola, 1997b; Barnes *et al.* 1999; Parkar *et al.* 2001). McGuire (1989) postulated that this was due to adsorbed proteins establishing an equilibrium with the proteins in the bulk fluid, resulting in a "passive" surface unable to further adsorb

particles, including microorganisms. This model is not specific to milk proteins and similar reductions in bacterial attachment have been observed in the presence of tryptone soya broth and bovine serum albumen (Almakhlafi *et al.* 1994; Dewanti and Wong, 1995; Cunliffe *et al.* 1999). However, the presence of such food residues should not be relied upon to prevent microbial attachment and growth. Not only can adsorbed proteins provide ample nutrients for those bacteria that do attach but, over time, their continual denaturation may result in a greater number of sites being made available for further adsorption of food particles and microorganisms (McGuire, 1989).

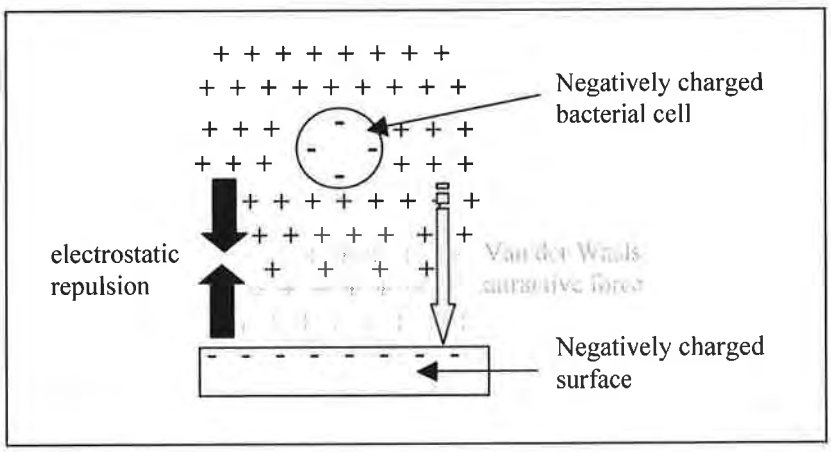
There are many regions along a polymer chain where interactions between surface and macromolecule and macromolecule and bacterium can occur (Stainsby, 1986). However, it is not yet known whether microorganisms interact only with the conditioning film, or if there are interactions through the film directly with the original surface (Carpentier and Cerf, 1993). Nevertheless, in many cases, the presence of food residues and/or modifications to substratum hydrophobicity and electrostatic charge has been shown to facilitate the attachment of microorganisms (Hood and Zottola, 1997b; Bagge *et al.* 2001).

1.1.2. Reversible Microbial Attachment

The two most common processes by which microorganisms approach a surface are via sedimentation (i.e. solely due to the forces of gravity) or, as is often the case in extensive pipeline systems, via the turbulence associated with the suspending medium (Boulangé-Petermann, 1996). Although bacterial cells and almost all non-biological surfaces are negatively charged, in both cases, they attract oppositely charged ions from the surrounding fluid, so forming an "electric double layer". When a bacterial cell approaches a surface the double layers start to overlap and, being like charged, repel each other. The

closer the two charged surfaces get, the greater the repulsive force experienced. However, a significant attractive force – van der Waals force – is also known to be present and according to DLVO (Derjaguin, Landau, Verwey, and Overbeek) theory, at any one distance, by adding these two opposite forces together, it is possible to describe the overall force acting upon the bacterial cell (Figure 1.1) (Boulangé-Petermann, 1996; McClaine and Ford, 2002).

Figure 1.1. DLVO theory and the attachment of a bacterial cell to a surface



Although van der Waals forces are very powerful, they only operate over a small distance and at distances of between 10 and 20 nm, the attractive force is significantly less than the repulsive force experienced by the bacterium. As a result, the cell is only weakly attached to the surface and can easily be dislodged. Nevertheless, if the cell can get close enough to the surface, then the van der Waals forces will hold it very tightly indeed and, thus, to increase their chances of becoming firmly attached, bacteria are thought to employ a variety of mechanisms in order to exploit the brief time they are weakly held away from the surface (Zottola and Sasahara, 1994; Hood and Zottola, 1995).

1.1.3. Irreversible Microbial Attachment

Although gram-positive and gram-negative organisms are both regarded as negatively charged particles, variation in the nature, quantity, conformation and distribution of component material within the cell surface, can affect the interaction between the substratum and different bacterial species (Speers and Gilmour, 1985). Spores, for example, generally attach to surfaces at a greater rate than vegetative cells, a process facilitated by their relatively high hydrophobicity, which may be due to the comparative abundance of proteins within the sporal outer coat (Flint *et al.* 2001; Parkar *et al.* 2001). Similarly, the synthesis and/or presence of specific cell surface proteins reportedly increases bacterial cell hydrophobicity and enhances the ability of a range of bacterial species to attach to a variety of different materials (Leyer and Johnson, 1993; Toledo-Arana *et al.* 2001). In addition, many bacteria, when nutrients are limited, undergo a variety of cellular changes, including cell shrinkage and increased hydrophobicity and thus, during starvation, display enhanced adhesive characteristics (Brown *et al.* 1977; Kjelleberg *et al.* 1983; James *et al.* 1995).

The surface properties of microorganisms can, therefore, govern their adhesion to an inert surface and although cell surface hydrophobicity and charge are considered the principal physiochemical forces involved, the presence of particular cell appendages, such as flagella and fimbriae, as well as extracellular polysaccharide, are also thought to influence bacterial attachment.

Cells which achieve the highest levels of adherence have been documented as being those that are capable of persisting, within any given processing environment, for a period of months or even years (Lundén *et al.* 2000). The main reasons for this will be discussed in Section 1.3. However, during their study, Lundén *et al.* (2000) also observed that the

poorest adherence levels were exhibited by non-motile bacterial strains. The presence of flagella is reported to be important in the attachment of both spoilage and pathogenic bacteria (Dickson and Daniels, 1991; Lindsay and von Holy, 1997; McClaine and Ford, 2002). The kinetic energy produced by flagellated cells may overcome the electrostatic repulsion forces enabling the cells to migrate closer to the surface (Norwood and Gilmour, 2001). However, Speers and Gilmour (1985) stated that motility does not have a consistent effect on bacterial adhesion. Their conclusion is supported by Vatanyoopaisarn *et al.* (2000) who, by preventing the motility of *Listeria monocytogenes,* via nutrient limitation, demonstrated that flagellated but non-motile cells, attached at levels 10-times higher than non-flagellated cells. Thus, it would appear, it is the presence of flagella, independent of cell motility, which aids the attachment of *L. monocytogenes* and, therefore, the flagella associated with this particular bacterial species appear to act as adhesive structures. This is particularly relevant to the food industry, where relatively low temperatures (< 22°C) are frequently encountered. Under these conditions, *L. monocytogenes* possesses multiple peritrichous flagella (Smoot and Pierson, 1998), which will aid attachment to food contact and environmental surfaces, even if the surroundings do not provide enough nutrients to allow the organism to be fully motile (Vatanyoopaisarn *et al.* 2000). Nevertheless, further studies have concluded that the presence or absence of flagella does not affect the final levels of attachment, which are achieved after longer periods of time (Lundén *et al.* 2000; Heydorn *et al.* 2002). This suggests that flagella facilitate initial attachment only and that other factors also contribute to bacterial adhesion.

As early as 1971, Marshall *et al* (cited in Donlan, 2002), using scanning electron microscopy, showed that attached bacteria could be associated with a surface via fine, extracellular polymeric fibrils. These polymer bridges are thought to increase the tendency of some organisms to attach by either drawing the cells closer to the surface and/or reducing the electrostatic repulsion between substratum and cell (Speers and Gilmour,

1985; Donlan, 2002). Although the exact nature of these structures is unknown, specific staining methods have indicated that the material is often an acidic polysaccharide (Hood and Zottola, 1995). Further evidence for this was provided by Hood and Zottola (1997b), who demonstrated that *Pseudomonas fragi* always adhered in higher numbers when grown in the presence of sucrose. It was hypothesised that the inclusion of the sugar provides the bacteria with additional carbohydrate, which can then be utilised for the production of polysaccharide. In addition, the presence of compounds capable of binding or disrupting carbohydrates were shown to cause a reduction in the adherence levels of *P. fragi*.

In contrast, Parkar *et al.* (2001) concluded that the initial attachment of vegetative cells does not involve the biosynthesis of new molecules or structures and that extracellular polysaccharide contributes little to the attachment of thermophilic bacilli to stainless steel. Furthermore, these authors noted that by removing cell surface polysaccharides the attachment process can, in fact, be increased. These findings are supported by Smoot and Pierson (1998), who observed that in the presence of the proteolytic enzyme trypsin, the attachment of *L. monocytogenes* to rubber or stainless steel, was reduced by 99.9%, suggesting that proteins rather than polysaccharides play a major role in the attachment of *L. monocytogenes*.

Nevertheless, bacteria are unlikely to be present on a surface in the form of a pure culture and interactions may occur between different bacterial species. It has been demonstrated that when *L. monocytogenes* is grown in a mixed culture, with bacteria such as *Flavobacterium* or *Pseudomonas* spp, the number of *L. monocytogenes* cells attaching to stainless steel increases significantly, compared to when this organism is present as a pure culture (Sasahara and Zottola, 1993; Kumar and Anand, 1998; Bremer *et al.* 2001). It has been postulated that the extracellular polysaccharide produced by so-called "primary colonisers" can incorporate *L. monocytogenes* cells, so enhancing the adherence and

growth of the pathogen (Bremer *et al.* 2001). However, the competition for nutrients is also thought to influence bacterial adhesion. Members of the staphylococci are reported to produce extracellular polysaccharides that contain antagonistic compounds, which may prevent the attachment of pathogens such as *L. monocytogenes* (Norwood and Gilmour, 2001).

There are, therefore, a variety of means by which different bacteria attempt to initiate firm, stable and irreversible surface adhesion. However, common to many is that once attached and under favourable conditions, cells can multiply, form microcolonies and produce extracellular polysaccharides and eventually develop into highly complex and dynamic biofilms.

1.1.4. Biofilm Formation

Biofilms are defined as "cells, immobilised at a substratum, frequently embedded in an organic polymer matrix of microbial origin which is not necessarily uniform and may be composed of a significant fraction of inorganic or abiotic substances" (Characklis and Marshall, 1990). They are fundamentally different from populations of suspended cells, in terms of metabolism, chemical structure, cell surface characteristics, antimicrobial resistance (see Section 1.3.3.3) and architecture (Pavey *et al.* 2001). However, although two-, three- and five-stage models have been proposed, biofilm formation is still poorly understood (Zottola and Sasahara, 1994; Hood and Zottola, 1995). Notermans *et al.* (1991) describes a three-stage process involving: i) the initial adsorption of the microorganisms to the surface (Section 1.1.2); ii) consolidation, during which, the organisms produce thin polysaccharide fibres that thicken over time leading to irreversible attachment (Section 1.1.3) and iii) colonisation, when cells start to metabolise nutrients,

release waste products and produce additional structures and components, such as extracellular polymeric substances (EPS), which determine the adhesiveness, structure and cohesive strength of the biofilm (Tuompo *et al.* 1999; Ghigo, 2003).

Biofilms formed by *Pseudomonas aeruginosa* have been extensively studied and results have demonstrated the importance of both twitching-motility and cell-to-cell signalling (quorum sensing) both of which illustrate the complexity of biofilm development. Twitching motility is a flagellum-independent movement involving the extension and retraction of type IV pili, which enables the organism to propel itself across and colonise an entire surface area (Heydorn *et al.* 2002). Quorum sensing enables an entire cellular population to initiate and synchronise a collective action (Whitehead *et al.* 2001) and in the case of *P. aeruginosa* biofilms, this may result in the differentiation of surface-associated microcolonies into a mature biofilm consisting of tower- and mushroom-shaped microcolonies interspersed with water channels (Heydorn *et al.* 2002). Such water channels not only enable the extracellular polymeric matrix to remain highly hydrated but they also allow for the diffusion of nutrients, oxygen and cell-signalling molecules (Donlan, 2002).

1.1.4.1. The food industry biofilm

Food processing environments provide a variety of conditions, which might favour the formation of biofilms. These include the presence of water, nutrients, suitable attachment sites and microorganisms, either originating from raw materials or the environment (Elvers *et al.* 1999). Although some structural attributes can generally be considered universal, every microbial biofilm community is unique and its characteristics are likely to depend upon its immediate surrounding environment (Jones, 1994; Donlan, 2002). For example,

within any production area, nutrient supply may vary from continuous and dilute to intermittent and concentrated. Biofilm development has been shown to occur faster when nutrient availability in the medium is low and, in addition, be accompanied by the formation of thicker EPS (Dewanti and Wong, 1995). Similarly, the transfer from a complex to a low nutrient medium results in adherent bacteria developing from single cells to biofilms that become associated with an extensive polymeric matrix.

Shear forces also vary and can range from highly turbulent environments through low-shear, uni-directional flows to static systems (Jones, 1994). Under flow conditions, biofilm formation has been demonstrated as being significantly slower than that which occurs in a static system (Bagge *et al.* 2001). However, it has been suggested that the lack of shear stress associated with the latter results in the bacteria only having a loose association with the EPS and, thus, weaker overall levels of attachment (Chae and Schraft, 2000). In comparison, the biofilms formed under high shear forces have been shown to be thinner and denser and generally more compact in nature (Liu and Tay, 2001).

The humidity within a food processing environment may also vary from continually wet sites to those that alternate between wet and dry (Jones, 1994). Peters *et al.* (1999), by sampling conveyor belts wetted either by the continual seepage of whey from cheeses or via the formation of condensation due to local chilling of a processing line, demonstrated that both scenarios can support biofilm growth.

However, although the initial stage of surface adsorption is virtually instantaneous, biofilm formation is a time-dependent process (Notermans *et al.* 1991) and although sugar refineries are known to harbour thick films of *Leuconostoc* spp. and thick biofilms are also known to exist within the permanently wet washing tanks of flour mills and malthouses (Carpentier and Cerf, 1993), the cleaning and disinfection procedures employed by many

food industries means, that in many cases, the time dimension for biofilm development is usually relatively short. Consequently, Holah and Gibson (1999) have defined a food industry biofilm as being either "a core consortium of microorganisms developing within a defined time period, dependent on the cycle of cleaning and disinfection programmes" or "the core consortium, surviving at low population densities, remaining after such cleaning cycles have been completed". Within the food industry, therefore, and for the purposes of this thesis, the term biofilm is more associated with the attachment and growth of microorganisms on surfaces, rather than the development of thick biological films over long periods of time (Holah *et al.* 1994)

1.1.5. Microbial Detachment

It has been established that biofilms do exist within the food industry and are particularly prevalent on environmental surfaces, which are likely to be cleaned less often than food contact sites (Rahkio and Korkeala, 1997; Gibson *et al.* 1999). In order to colonise new surfaces, individual cells must be able to disperse from a mature biofilm and reattach elsewhere (Eginton *et al.* 1995). *P. aeruginosa* for example, is known to produce the enzyme alginate lyase – alginate being the major component of its EPS. It is thought that enzymatic cleavage of the matrix polymers causes a release of cells from the solid surface aiding their dispersal (Donlan, 2002). Nutrient levels can also influence detachment. Dewanti and Wong (1995) demonstrated that *Escherichia coli* O157 cells, which adhere under low-nutrient conditions, readily dissociate from the surface when supplied with a nutrient rich medium. This is of particular significance to the food industry, where such a situation could potentially occur at the start of each post-clean production run. Detachment is also caused by physical forces such as shearing, the continual removal of small portions

of the biofilm via fluid dynamic forces and abrasion caused by the collision of particles from the bulk fluid (Donlan, 2002).

The ability of cells to transfer from an adherent biofilm to a previously un-colonised surface is not only imperative for their long-term survival but is also central to the problem of product contamination (Eginton *et al.* 1995). It has been reported for example, that the adherence of *Streptococcus thermophilus* to post-pasteurisation regions of a pasteuriser can result in the inoculation of milk at a rate of 10^6 cells ml^{-1} (Carpentier and Cerf, 1993). Consequently, biofilms present in areas associated with ready-to-eat products or those likely to undergo minimal further processing, pose a significantly higher risk to the safety and quality of the final product than those present in areas associated with raw ingredients and/or foods which will be further processed (Holah and Gibson, 1999).

However, despite laboratory studies demonstrating the ability of a variety of pathogenic and spoilage organisms to form biofilms (Dewanti and Wong, 1995; Lindsay and von Holy, 1997; Bagge *et al.* 2001; Chae and Schraft, 2000; Chavant *et al.* 2002; Heydorn *et al.* 2002), there is little evidence to suggest that significant pathogen populations are associated with biofilms occurring in high-risk processing areas and it has been suggested that the presence of a biofilm merely threatens the quality rather than the safety of a product (Peters *et al.* 1999). Nevertheless, any organism associated with a surface has the potential to proliferate and/or contaminate the final product and consequently, single adherent microorganisms may be considered as significant as those that exist within a well-developed biofilm (Hood and Zottola, 1995). Many food companies, therefore, have a zero tolerance level for pathogens such as *Salmonella* or *Listeria* spp. and identify such organisms as food safety hazards that must continually be controlled.

1.2. Food Safety and Quality Management

To provide both an indication of product quality and shelf-life and a safeguard for consumers, food microbiologists have traditionally determined the number of spoilage organisms and confirmed the absence of pathogens in a food, via cultivation-based analysis of the final product (van der Zee and Huis in't Veld, 1997). However, such procedures require large amounts of time, money and media.

Traditional methods for the microbiological analysis of foods are laborious and time-consuming, especially if it is necessary to detect a specific organism type, such as *Salmonella* spp., where 4-7 d may elapse before a result is obtained. However, many food products today are produced at high speed and in huge quantities and are shipped almost immediately after production to distribution centres or chain warehouses and, thus, may be purchased by consumers within a very short period of time (Bauman, 1994). It is quite conceivable, therefore, that a food could be in a shop or even consumed before results of some microbiological tests are known or, alternatively, should a positive-release system be operated then it may become necessary to utilise a large volume of expensive warehouse space (Stannard and Gibbs, 1986). Furthermore, to ensure representivity a significant proportion of the final product has to be taken for analysis and even then food safety can only be assured at the point of test (Ropkins and Beck, 2000). Thus, batch testing of the final product does not prevent the manufacture of a sub-standard product and in addition places the responsibility for food safety upon a relatively small component of the workforce, namely the quality assurance and quality control personnel (Ropkins and Beck, 2000). The alternative, therefore, is to control the entire production process.

1.2.1. Hazard Analysis Critical Control Point (HACCP)

Hazard Analysis Critical Control Point (HACCP) is a systematic approach to food safety management. By identifying hazards that are likely to occur and by establishing measures that will prevent them from happening it can control any area or point in the food supply chain considered critical to ensuring the safety of the food (Bauman, 1994; Notermans *et al.* 1994; Mortimore and Wallace, 2001). Monitoring procedures, designed to ensure that such control measures are working effectively, enable remedial action to be implemented before control of a product or process has been lost. Thus, unlike the microbiological testing of the final product, HACCP is considered a pro-active, preventative system of food control.

The application of HACCP to food production was pioneered by the Pillsbury Company with the cooperation and participation of the National Aeronautic and Space Administration (NASA), Natick Laboratories of the U.S. Army and the U.S. Air Force Space Laboratory Group. Their aim was to produce food for the United State's space programme, which they could consistently and absolutely guarantee would not be contaminated with bacterial or viral pathogens, toxins or chemicals, any of which if ingested by astronauts could cause illness and result in an aborted or catastrophic mission (Bauman, 1994). The need to eliminate end product testing resulted in the development of the original three-stage HACCP procedure (Table 1.1).

In the 40 years since its conception many businesses have implemented HACCP and as a result, in order to meet specific industry needs, the concept has had to evolve (Ropkins and Beck, 2000). Published guidelines, such as those produced by the Codex Alimentarius Commission (1997) and the National Advisory Committee for the Microbiological Criteria for Foods (1998) have, therefore, recommended seven basic steps or "principles" (Table

1.1) that can be used by the food processing industry to reduce, prevent or eliminate biological, chemical and physical hazards that might occur in the final product (Brashears *et al.* 2002). Nevertheless, the three original components remain inherent in all contemporary HACCP plans and such an approach has been recognised by the World Health Organisation (WHO) as being the most effective means of controlling foodborne disease. Consequently, international legislation is moving more and more towards making HACCP a mandatory requirement in the food industry (Mortimore and Wallace, 2001).

Table 1.1. The Hazard Analysis Critical Control Point system

The original Pillsbury HACCP procedure (conceived 1959)	The "seven universal HACCP principles"
1. identification and assessment of all hazards associated with the final foodstuff	**1.** conduct hazard analysis, considering all ingredients, processing steps, handling procedures and other activities in food stuff production
2. identification of the steps or stages within food production at which these hazards may be controlled, reduced or eliminated: the Critical Control Points (CCPs)	**2.** determine the CCPs
3. the implementation of monitoring procedures at these CCPs	**3.** define critical limits for ensuring the control of each CCP
	4. establish a system to monitor control of each CCP
	5. establish the corrective action to be taken when monitoring indicates that a particular CCP is not under control
	6. establish procedures for verification to confirm that the HACCP plan is working effectively
	7. establish documentation concerning all procedures and records appropriate to these principles and their application

(WHO, 1997; Ropkins and Beck, 2001)

The Food Safety (General Food Hygiene) Regulations were introduced in the UK in 1995, in response to EU Directive 93/43 EEC, which ordered a common food hygiene approach to be implemented across all EU member states (Ropkins and Beck, 2001). The increase in legislation has meant that UK food businesses are now legally required to identify any step in their activities critical to ensuring food safety and ensure that adequate safety procedures are identified, implemented and maintained (Mortimore and Wallace, 2001). Thus, the onus of ensuring that the food produced is safe and wholesome has been placed firmly on the individual food business (Wheelock, 1994). Consequently, many books, regarding how to implement HACCP procedures have since been written and are aimed specifically at those working within the food industry (Dillon and Griffith, 2001; Mortimore and Wallace, 2001).

The process begins with a hazard analysis (Table 1.1) comprising hazard identification to determine potential hazards and hazard evaluation to determine which identified hazards are of such significance that a critical control point (CCP) is required in order to control it (Sperber, 2001). A hazard can be defined as "a physical, chemical or biological agent with the potential to cause an adverse health effect" (WHO, 1997), its significance will depend upon the likelihood of it occurring and should it occur, the severity of the outcome. Thus, as the elderly, very young, sick or immuno-compromised can be much more susceptible to specific hazards, consideration must also be given to the target consumer group (Dillon and Griffith, 2001). Once identified, those actions or activities that can be used to simply yet effectively control the hazard must also be determined and must be applied at those steps in a production process where it is essential to prevent, eliminate or reduce the hazard to an acceptable level (WHO, 1997). So, in the production of ready-to-eat cooked meats for example, the presence and subsequent transfer of pathogens to the final product from post-process surfaces, equipment or utensils (Section 1.1.5) is likely to be identified as being a potential hazard and since any contaminating organisms won't be eliminated during a later

point in the production process, the cleanliness of these food contact surfaces will be designated a CCP with their effective cleaning and disinfection the specified control measure.

It has been reported that the implementation of the HACCP system within large and medium-sized businesses has been relatively successful, hence the apparent control of pathogens in high-risk post-process areas of production plants (Section 1.1.5). In addition, those food industries operating with full commitment and understanding of the HACCP system are unlikely to be implicated in a foodborne disease outbreak (Motarjemi and Käferstein, 1999). However, although HACCP provides the food industry with a powerful tool to combat foodborne disease, if it is expanded to include quality parameters, it can lead to large numbers of extra controls being defined as CCPs. This in turn can result in an unwieldy, time-consuming system that detracts from the essential safety aspects of food production (Wallace and Williams, 2001). Furthermore, although regulatory agencies have an obligation to control food safety, they maintain they cannot regulate any form of food quality (Adams, 1998). Thus, HACCP is the process control for food safety and food safety alone whilst product suitability, which includes the prevention of food spoilage and the extension of product shelf-life, can only be managed by other means, including the implementation of pre-requisite programmes (Adams, 1998; Heggum, 2001).

1.2.2. Pre-requisite Programmes and Good Manufacturing Practice

Many pre-requisite programmes (PRPs) are based upon current good manufacturing practices (GMP) such as cleaning, operator and environmental hygiene, plant and building design and preventative maintenance (Wallace and Williams, 2001). They frequently function across product lines and unlike HACCP systems, which are product or process

specific, are often managed as facility-wide or company-wide programmes (Sperber *et al.* 1998). Occasional deviation from a PRP requirement would not by itself be expected to create a food safety hazard. However, when foodborne disease outbreaks do occur, they are most likely to be caused by poorly developed or poorly performed PRPs, even when HACCP systems are in place (Heggum, 2001). Thus, reliance in the HACCP approach should not result in neglecting important pre-requisite practices. In contrast, reliance on well-developed and consistently performed PRPs can simplify the HACCP process and as a result, within an overall food safety management programme, GMP and PRPs are generally considered the foundations upon which the HACCP plan is built (Sperber *et al.* 1998; Wallace and Williams, 2001). In principle, therefore, HACCP is an extension of GMP and, thus, it will fail if GMP within a processing plant is insufficient (Notermans *et al.* 1994).

Such issues have since been highlighted by Samelis and Metaxopoulos (1999) during a study conducted in Greece. Here, due to its relatively recent introduction, the HACCP philosophy is not yet fully understood and it was noted that many manufacturers tend to underestimate the importance of keeping high standards of hygiene within the pre-cook areas of meat production plants. Instead they rely upon pasteurisation to destroy potential hazards and priority is given to cleaning those areas where the final product is handled. Nevertheless, during the study the authors isolated *Listeria* spp. from many of the final meat products. It was subsequently concluded that rather than being the result of post-process contamination, the pathogen was being transferred from contaminated surfaces associated with the pre-cook stages of production and surviving the heating process.

In an attempt to prevent such occurrences, Wallace and Williams (2001) have recommended the formalisation of PRPs alongside HACCP to control general hygiene and quality issues, allowing the HACCP plan to concentrate on controlling significant hazards.

Such a system already operates in some countries. In North America for example, the United States Department of Agriculture (USDA) requires Sanitation Standard Operating Procedures (SSOPs) to be written and implemented by meat and poultry processors as part of HACCP regulations. Such SSOPs must at minimum address the cleaning of direct food contact surfaces, equipment and utensils and should describe those procedures conducted before and during operations to prevent direct product contamination (Adams, 1998). Similarly, the Food and Drug Administration (FDA) requires that seafood companies address eight hygienic control points prior to the development of HACCP programmes (Ropkins and Beck, 2000).

Microbial attachment and biofilm formation can, therefore, be prevented. However, it requires constant attention to cleaning and disinfection procedures, proper training of personnel and the commitment of management to produce safe and wholesome food (Krysinski *et al.* 1992; Zottola and Sasahara, 1994).

1.3. Cleaning and Disinfection

Microorganisms, present on any food contact or environmental surface, exist in a complex environment where the surface itself, food and detergent residues, moisture, temperature, the population density of the organisms and various other factors each interact, one with another. Such a system maintains an equilibrium controlling microbial survival and growth (Chaturvedi and Maxcy, 1969). Thus, to prevent any physical, chemical or biological contamination of the final product, it is imperative that cleaning and disinfection procedures, which together are known as sanitation, are undertaken in order to remove all

undesirable material (food debris, microorganisms, foreign bodies and cleaning chemicals) from the surface (Holah, 1992).

Sanitation, therefore, is the major control measure associated with surface hygiene and if implemented correctly, and providing the processing environment and production equipment are hygienically designed, can control biofilm growth (Wirtanen *et al.* 1996; Elvers *et al*, 1999; Gibson *et al.* 1999). If sanitation procedures are not effective, microorganisms and food residues will remain at concentrations that could affect the safety and quality of the product. Thus, although required at a number of stages in food preparation and sometimes considered to be an integral part of a HACCP system, cleaning is an essential element of Good Manufacturing Practice and, therefore, demands the same degree of attention as any other key process in the production of safe and wholesome food (Holah, 1992; Bagshaw, 2001).

1.3.1. Hygienic Design of Machinery and Equipment

The contribution of effective cleaning and disinfection to product safety is such, that there is a legal requirement for the proprietor of any food business to ensure that the premises are kept clean and maintained in good repair and that its layout, design, construction and size permits adequate cleaning and/or disinfection. Furthermore "where food will come into contact with articles, fittings or equipment, these items must be kept clean and be so constructed and be of such materials, and be kept in such good order, repair and condition, as to minimise any risk of cross contamination" (Food Safety (General Food Hygiene Regulations) 1995). Sanitation, therefore, has become of primary concern to the food industry and in response the manufacturers of food equipment are placing greater emphasis on the hygienic design of production premises, machinery, equipment and surfaces.

Nowadays, it is common for designers, when constructing production equipment, to deliberately incorporate features, the sole purpose of which, rather than to increase productivity, is to assist in the cleaning of the machine or parts of it. Such active measures include the addition of clean-in-place components or improving the accessibility of operators to component parts (DeFrancisci, 2002). It is important that machinery and equipment are easy to dismantle, as this ensures that sanitation procedures can achieve their aims and reach all surfaces in contact with the product (Holah, 1992). In addition, the purpose of the more traditional hygienic design features, common to many pieces of equipment, is to minimise or prevent microbial colonisation. The use of continuous welds as opposed to bolted joints and junctions and the elimination of dead ends and crevices all aid in reducing areas where food debris and microorganisms can accumulate. Similarly, the structure of the machinery can be configured so as to avoid the 'pooling' of dirt and liquid and those surfaces in contact with the product can and should be corrosion-resistant and be easy to clean (Orth, 1998; Russell *et al.* 1999; DeFrancisci, 2002).

1.3.2. Surface Cleanability

Environmental surfaces, such as floors and walls must also, when in areas where food is prepared, "be maintained in a sound condition and be easy to clean and, where necessary, disinfect" (Food Safety (General Food Hygiene Regulations, 1995). This raises specific issues with regard to appropriate flooring materials. It has been reported that the food industry is one of the worst manufacturing industries in terms of injuries per 100,000 employees (Taylor and Holah, 1996). Wet and soiled floors can increase the risk of slipping and, thus, not only must floors be easy to clean, they must also possess anti-slip properties (Mettler and Carpentier, 1998). It is important, therefore, to consider the slip-resistance of any flooring material and this can be assessed using its average surface

roughness (R_a) measurement. However, it is generally believed that an increase in surface roughness enhances the retention of microorganisms (Speers and Gilmour, 1985; Wirtanen *et al.* 1996). Materials that retain fewer microorganisms after cleaning present the least risk of cross contamination. Thus, food manufacturers, believing they must strike a balance between personnel and product safety, can find it difficult to decide upon the most appropriate flooring material to utilize.

Despite these suppositions, laboratory studies have determined that R_a value has no effect upon the cleanability of a variety of materials used within the food industry, including those used for floors (Taylor and Holah, 1996; Mettler and Carpentier, 1998), walls (Taylor and Holah, 1996) and food contact surfaces (Kaufmann *et al.* 1960; Steiner *et al.* 2000; Frank and Chmielewski, 2001). Nevertheless, with continuing use a surface can become physically abraded or corroded and such damage *has* been shown to affect cleanability. Rubber and rubber-like materials for example, whilst initially having a smooth surface can become mechanically or chemically abraded resulting in the appearance of surface cracks. Such surface deterioration has been shown, over a period of continuous soiling-washing cycles, to result in the accumulation of soil at levels 10-times that on steel or glass (Dunsmore *et al.* 1981). Likewise, Holah and Thorpe (1990) when comparing the cleanability of worn surfaces to that of the new, un-used material, concluded, that regardless of R_a value, the greater the degree of surface irregularities caused by abrasion or impact damage, the greater the chance of bacterial retention. Similarly, Steiner *et al.* (2000) demonstrated that after cleaning, the number of organisms retained on stainless shot-peened steel was significantly lower than that on sandblasted stainless steel. Scanning electron microscopy revealed that whilst the surface of the former was relatively smooth, the surface of the sandblasted stainless steel, despite its comparatively lower R_a value, comprised numerous jagged peaks and crevices.

Thus, the R_a value does not necessarily reflect the true topographical profile of a material and it is the microscopic irregularities, such as peaks, crevices and pits, rather than the gross topographical features, that have the greatest effect upon the cleanability of a surface (Holah and Thorpe, 1990; Frank and Chmielewski, 2001; Faille *et al.* 2002). Despite the availability of a variety of different surface finishes, stainless steel is, in general, much more resistant to abrasion and impact damage than many other materials (Holah and Thorpe, 1990) and consequently, is more cleanable than rubber, aluminium and mineral-resin polymers (Dunsmore *et al.* 1981; Boulangé-Petermann, 1996; Frank and Chmielewski, 1997). In addition, it is strong, stable, and inert and possesses a surface-associated oxide film making it resistant to corrosion (Covert and Tuthill, 2000). Stainless steel has, therefore, become the most widely used material within the food industry.

However, when assessing surface cleanability, the majority of previous studies have employed traditional cultivation techniques as a means to monitor the change in bacterial numbers. Recent studies have employed more modern techniques, such as atomic force microscopy and the results of these investigations have shown, that although the topography of a worn stainless steel surface does not affect microbial attachment *per se*, soil elements do tend to be retained in defects the dimensions of which, do not provide microbial cells with adequate protection from shear stress or cleaning processes (Boyd *et al.* 2001; Verran *et al.* 2001a). Retained soil may facilitate the attachment of microorganisms (Section 1.1.1) and Mettler and Carpentier (1998), have demonstrated that a microbial population can develop over time and can stabilise at high levels, even on a smooth surface. Regardless of the cleanability of stainless steel, therefore, the level of food debris and microorganisms removed from food contact surfaces will depend upon the efficacy of the sanitation procedures employed.

1.3.3. Design of Appropriate Sanitation Programmes

Unlike many industries, the food industry has routine, defined, sanitation programmes that utilise a specific sequence of detergents and disinfectants, applied by defined physical techniques (Zottola and Sasahara, 1994). Implemented correctly, such programmes can maintain food contact and environmental surfaces in a condition that ensures they do not impair the safety and quality of the product (Dunsmore *et al.* 1981). Consequently, during a recent investigation into the development and control of biofilms in the food industry, all the companies involved in the study, identified "cleaning" as an important control measure in the prevention of product contamination. However, although all expended a considerable amount of time and money on cleaning and disinfection, in the majority of cases, sanitation was not effective (Elvers *et al.* 1999). These findings and those of others (Griffith *et al.* 2001) support the hypothesis of Dunsmore (1981), who stated that despite the importance of cleaning and disinfection procedures to the food industry, there is, in general, a lack of understanding with regard to the factors which affect their efficacy.

The reasons for ineffective sanitation are numerous and will be discussed subsequently. However, good, professional cleaning and disinfection practices start with well-planned, communicated and documented procedures (Loghney and Brougham, 2001). It is imperative they take into consideration the nature of the soil – its chemical composition and adherence, the type of material to be cleaned, the water quality and in some cases the compatibility of the cleaning and disinfecting chemicals with other materials or substances (Boulangé-Petermann, 1996). For sanitation procedures to be successful, therefore, they must be influenced by the product, the process and the environment.

The principle stages involved in the routine sanitation of a food production plant are outlined in Table 1.2. Although the number of operations can be extended, reduced or

combined, it has been suggested that the extent to which microbial contamination is reduced, correlates with the number of stages within the sanitation procedure applied (Kaufmann *et al.* 1960; Michaels *et al.* 2001a). In addition, the detergents and disinfectants can be selected from a wide range of available cleaning chemicals and the timing of each application can vary. The number of different sanitation procedures employed within the food industry is, therefore, potentially huge. Nevertheless, to optimise the cleaning and to minimise cost, it is essential that sanitation procedures should be developed as a whole and the practical requirements of efficiency, reliability, ease of use and economy limit the selection greatly (Dunsmore *et al.* 1981; Wirtanen *et al.* 1996). However, whatever the application method, it is the sequence in which the cleaning and disinfecting procedures are conducted that has the greatest effect upon overall sanitation efficacy (Krysinski *et al.* 1992).

Table 1.2. Principle stages of a sanitation programme applied within a food plant

1. Preparation	**5.** Inter-rinse
2. Gross soil removal	**6.** Disinfection
3. Pre-rinse	**7.** Post-rinse
4. Cleaning	**8.** Dry / Inter-production conditions

1.3.3.1. Cleaning

The primary aim of the cleaning phase is to remove the tenacious layer of soil that remains after completion of the gross clean and pre-rinse. A number of stages are involved: the wetting of the soil and surface by the cleaning chemical, the reaction of the chemical to facilitate the removal of the soil from the surface and the prevention of the re-disposition

of the dispersed soil back onto the cleaned surface (Gibson *et al.* 1999). To achieve this, it is necessary to apply chemical, mechanical and thermal energy to the surface.

A cleaning solution or detergent is blended from a range of typical components (Table 1.3) and is likely to be formulated to perform a specific type of task. It is necessary, therefore, to ensure that the chosen agent suits the surface to be cleaned, does not cause corrosion, removes the type of soil present without leaving any sort of residue and is compatible with the water supply (Russell *et al.* 1999). An example of how a general purpose cleaning solution may attack and remove residual food debris is illustrated in Figure 1.2.

Mechanical energy is required in order to physically remove soils from the surface (Figure 1.2c) and is also recognized as being highly effective in eliminating biofilm (Wirtanen *et al.* 1996; Michaels *et al.* 2001a). In fact, previous studies have implied that in the absence of food debris, the chemical contribution made to cleaning by a detergent has, in terms of reducing the level of microbial contaminants, no increased effect over the physical action of spraying alone (Gibson *et al.* 1999; Verran *et al.* 2001b). However, the presence of residual food debris affects the attachment and retention characteristics of the surface and its subsequent interaction with contaminating microorganisms (Verran *et al.* 2001a). Consequently, in addition to their direct attachment to the surface, microorganisms may also be attached to food particles (Section 1.1.1) and studies involving atomic force microscopy and the XPS (X-ray photoelectron spectroscopy) analysis of contaminated stainless steel have revealed that not only is proteinaceous material more easily removed using a detergent but that the application of a detergent reduces the size of the force required to remove any attached bacterial cells (Verran *et al.* 2001a; Verran *et al.* 2001b). Many authors, therefore, have concluded that, although responsible for removing contaminating material, the cleaning phase of a sanitation programme is also the most important stage for minimising microbial colonisation (Table 1.4).

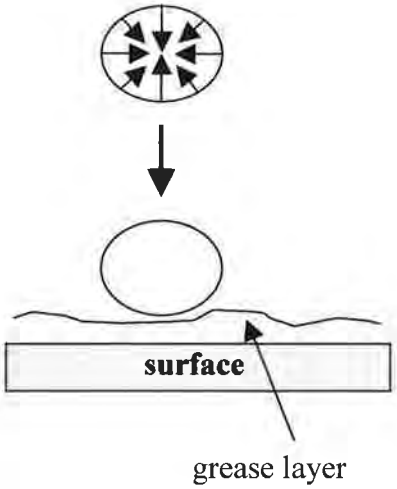
Table 1.3. Components of a typical cleaning solution

Component		Function	Example	Additional Information
Surface Active Agents (Surfactants)				
	Anionic	possesses strong detergent (i.e. cleaning) properties (Figure 1.2)	sodium stearate (soaps) alkylbenzene sulphate (soapless)	*anionic and cationic compounds must not be used together. However, their detergent properties (anionic) and their bactericidal properties (cationic) are combined in* ***amphoteric*** *compounds(e.g. dodecyl-di(aminoethyl)-glycine)*
	Cationic	possesses strong bactericidal properties but weak detergent properties	quaternary ammonium compounds	
	Non-ionic	possesses good wetting ability (Figure 1.2)	alkylphenol ethoxylate polysorbates (tweens)	
Inorganic Alkalis (Degreasing Agents)		Converts fats to soaps (saponification) and if chlorinated also breaks down/solubilises proteins	trisodium phosphate	
Inorganic Acids		removal of limescale	phosphoric acid	*must not be used in conjunction with hypochlorite-based disinfectants as they react to form chlorine gas*
Organic Acids		general cleaner	acetic acid	*generally used cold*
Chelating Agents		binds and removes deposited minerals	ethylenediamine tetraacetic acid (EDTA)	*particularly important in hard water areas*
Rinsing and deflocculating agents		Prevents the re-disposition of soil		

(Dillon and Griffith, 1999; Russell *et al.* 1999)

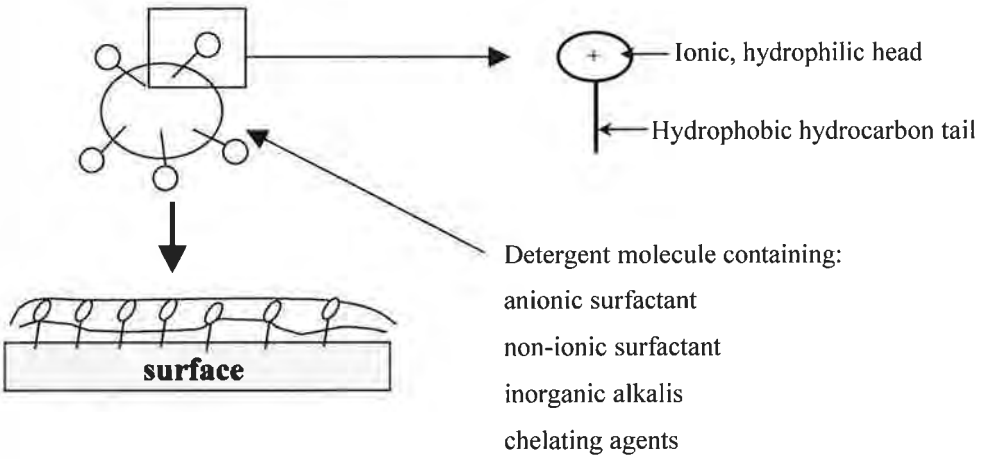
Figure 1.2. Action of a general purpose cleaning solution

a. the addition of water to a grease layer



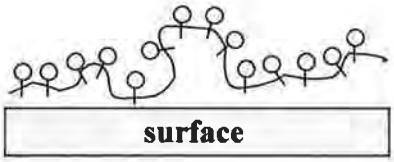
The intermolecular forces at the water surface pull inward but are not counterbalanced by forces exerting outward. This net inward pull creates the unusually high surface tension associated with water. The surface area of a water drop is, therefore, reduced to a minimum resulting in it taking on a spherical shape. Conversely, grease molecules are relatively non-polar, thus, nothing in a greasy surface has enough polarity to attract the water molecules to make the water spread out and wet the surface.

b. the addition of detergent to a grease layer



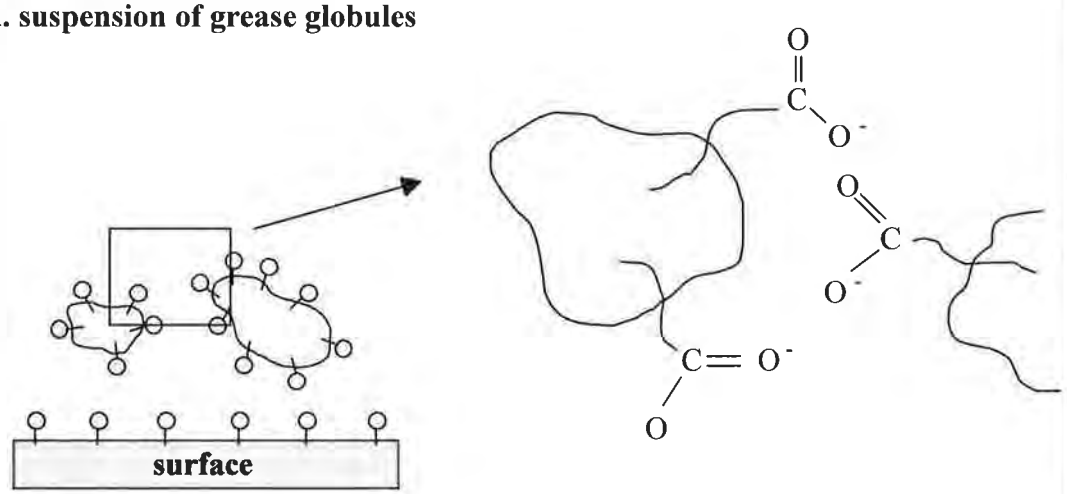
The presence of a non-ionic surfactant reduces the surface tension of the water molecule enabling the cleaning solution to wet the entire surface area. The ionic, hydrophilic heads of the anionic surfactant remain in the liquid phase, whilst the hydrophobic tails burrow into the grease layer, which becomes "pin-cushioned" with electrically charged sites.

c. the break down of the grease layer



The presence of alkaline phosphates in the cleaning solution facilitates the break down of the grease layer. Its action is aided by means of manual agitation or scrubbing.

d. suspension of grease globules



Grease globules, studded with ionic groups, become suspended and, being like-charged, repel each other. Surfactant molecules also become associated with the surface and the same repulsion forces prevent the re-attachment of the grease droplets back onto the cleaned surface. In hard water areas, re-disposition can also result via the precipitation of "hardness ions". The inclusion of chelating agents within the cleaning solution, which bind and remove calcium and magnesium ions, can prevent such scum formation.

The detergent-soil complexes can then be rinsed from the surface.

(Holum, 1994; Dillon and Griffith, 1999)

Table 1.4. Reduction in surface bacteria count after application of a detergent.

Log Reduction	Study
(86%)	Kaufmann *et al.* (1960)
3 (99.9%)	Dunsmore, 1981
2 – 6	Schmidt and Cremling, 1981 (cited in Holah, 1992)
4	Holah, 1992
3 – 5	Schmidt 1989 (cited in Reuter, 1998)
1.18	Gibson *et al.* 1999
2 – 3	Verran *et al.* 2001a
4.5 – 5.8	Michaels *et al.* 2001a

An increase in temperature has been shown to increase the efficacy of cleaning procedures (Michaels *et al.* 2001a). In general this increase is linear. However, to facilitate the removal of fats and oils, a temperature above their respective melting points is required and for more complex soils, particularly those containing proteins, there is an optimal detergent temperature – for the removal of milk soil for example, the temperature of the detergent should be 65°C (Dunsmore *et al.* 1981; Holah, 1992).

For cleaning processes involving mechanical, chemical and thermal energies, the longer the application time, the more efficient, in general, the process (Holah, 1992). Foam cleaners were introduced in the 1970's – until then detergents were dispensed, via high pressure, as dilute sprays. Although this provided good mechanical action, the spray was not in contact with the surface long enough to provide optimal cleaning (Banner *et al.* 1999). The increased surface adhesion of foam cleaners provides a longer retention time and thus, a longer cleaning time. However, as with all aspects of food safety, the development of new and improved cleaning chemicals is the subject of continual research and gel cleaners have recently been produced which, via their increased viscosity, remove significantly more soil from surfaces than foams (Banner *et al.* 1999). The authors of this report also concluded that in addition to superior efficacy, gels are more readily rinsed

from a surface and, thus, their use, via 25% labour savings and 27% water savings, can reduce the cost of cleaning.

1.3.3.2. Inter-rinse

The inter-rinse is an important stage of a sanitation programme and assists in the removal of detergent-soil complexes, thus, helping to prevent the accumulation of microorganisms. It is imperative that care be taken to minimise the amount of splash and/or aerosolisation, which may re-contaminate the previously cleaned surfaces. Hygiene operations are often responsible for spreading contamination, particularly from surrounding floors, to previously un-contaminated surfaces (Mettler and Carpentier, 1998). It is essential, therefore, that the cleaning and rinsing of environmental surfaces occurs prior to the cleaning and rinsing of food contact surfaces (Holah and Gibson, 1999). The use of high-pressure hoses should also be avoided as these have been shown to significantly increase aerosol generation (Banner *et al.* 1999), the droplets of which can contain viable microorganisms, which can be transported to heights in excess of 2 m and distances in excess of 7 m (Taylor and Holah, 1996). For optimum cleaning, therefore, and to minimise the generation of aerosols, it has been suggested that high mechanical energy should be combined with a detergent that aids soil removal *and* reduces microbial viability (Gibson *et al.* 1999).

Combination detergent-disinfectants (sanitisers) are available to the food industry and, due to their ability to remove and inactivate microorganisms their application has been shown to be more effective in reducing bacterial numbers than the use of a detergent alone (Dunsmore *et al.* 1981). However, their use is only successful where light soiling occurs and a relatively low level of microbial contamination has to be removed - the presence of

organic material can severely compromise the efficacy of the antimicrobial component (Russell *et al.* 1999). Cleaning chemicals used within food manufacturing plants are, therefore, generally developed to remove particular types of food soil (fats, proteins, mineral deposits; Table 1.3) rather than for the destruction of microorganisms and although many microbial contaminants can be removed during the cleaning phase of a sanitation programme, no detergency step is ever totally effective (Dunsmore *et al.* 1981). Thus, after the removal of food residues, additional measures may be needed to further reduce the number of microorganisms present. Such measures, known as terminal disinfection or microbiological cleaning, are especially important in food handling environments where food contact surfaces must only have minimum levels of microbial contamination, for example in the production of ready-to-eat foods. Thus, a major function of the inter-rinse stage is to ensure that all organic debris has been removed from the surface so assuring optimal disinfection can occur.

1.3.3.3. Disinfection

Within the food industry, disinfection – the reduction in microorganisms to an acceptable level - is traditionally achieved by means of heat in the form of hot water or steam. However, its use is often too expensive and impractical for use with large-scale industrial machinery, equipment and surfaces and liquid chemicals are instead employed (Russell *et al.* 1999). A wide range of chemical disinfectants, including the quaternary ammonium compounds (QACs), biguanides and chlorine-releasing compounds, are available and they and their respective modes of action have been extensively reviewed (Reuter, 1998; Russell *et al.* 1999). However, regardless of type and action, those microorganisms that are exposed to chemical disinfectants will be those that remain after the cleaning stage has been completed and, thus, are those likely to be surface attached (Holah, 1992).

Surface attached (sessile) bacteria, particularly those associated with a biofilm, are more resistant to disinfectants than their freely suspended (planktonic) counterparts (Andrade *et al* 1998b; Eginton *et al.* 1998; Lindsay and von Holy, 1999; Joseph *et al.* 2001; Stewart *et al.* 2001; Stopforth *et al.* 2002). Bower and Daeschel (1999) suggest this is due to the disinfectant molecules being able to approach and target a planktonic cell from all sides and angles, in comparison, an organism attached to a surface is susceptible from just one side only. Indeed, removing adherent cells from a surface has been shown to increase their susceptibility to disinfectants to levels equivalent to that of planktonic cells (Frank and Koffi, 1990), re-emphasising the need for adequate mechanical energy to be applied throughout the sanitation process. However, once attached, microbial and soil contaminants become progressively more difficult to remove. Relatively strong mechanical forces only partially reduce the level of contamination and are even less effective if the microtopography of the surface is such that organisms and/or food debris have become entrapped (Notermans *et al.* 1991; Section 1.3.2). Under these circumstances, bacterial resistance is usually attributed to the failure of the biocide to penetrate food debris and/or the biofilm matrix.

The presence of organic matter within the surface layers of a biofilm can protect the innermost cells by reacting with the antimicrobial agent, thus, reducing its bioavailability (Gilbert and McBain, 2001). It is postulated, that if this neutralisation occurs faster than the biocide can diffuse into the biofilm interior, then its ability to penetrate the biofilm will be severely compromised (Stewart *et al.* 2001). This "reaction-diffusion theory" is thought to form the basis for biofilm resistance to strong oxidising agents such as chlorine (de Beer *et al.* 1994; Xu *et al.* 1996) and peracetic acid (Gilbert *et al.* 2001) and suggests that a less reactive biocide that penetrates a biofilm effectively would outperform, in terms of microbial killing, a stronger disinfectant that fails to penetrate fully. Indeed, Stewart *et al.* (2001) demonstrated that despite chlorosulfamate being the weaker disinfectant, it

penetrated biofilms approximately eight times faster than alkaline hypochlorite. The presence of food debris has been shown to provide a similar "organic challenge" to disinfectant molecules (Fatemi and Frank, 1999) and emphasises the importance of ensuring all food residues are removed from the surface prior to disinfection. Nevertheless, by increasing the in-use concentration, and given time, antimicrobial agents can successfully diffuse throughout a biofilm (Stewart *et al.* 2001) yet, even after this occurs, they remain unable to effectively destroy sessile microorganisms, implying that bacteria in a biofilm are protected by some mechanism other than the simple shielding by the biofilm matrix (Stewart *et al.* 2001).

Nutrients and oxygen are more readily available to those cells at the surface of a biofilm and, thus, the cells within the core grow more slowly and express starvation phenotypes (Kumar and Anand, 1998). These phenotypes are generally more resistant to antimicrobials (Gilbert and McBain, 2001). Rand *et al.* (2002) demonstrated that slow-growing *E. coli*, even in the absence of antimicrobial compounds, may up-regulate expression of *acrAB*. The *acrAB-tolC* operon encodes for the inner membrane transporter AcrB, and the outer membrane channel TolC, which together comprise a multi-drug efflux system capable of expelling a variety of antimicrobial agents from the cell (Rand *et al.* 2002). In the presence of biocides, therefore, those bacteria with active efflux pumps will be those more likely to survive and multiply. Nevertheless, selection is not the only means of promoting resistance within a microbial population – microorganisms are also capable of adaptation.

Aase *et al.* (2000) reported that after the continual exposure of *L. monocytogenes* to sub-lethal concentrations of the quaternary ammonium compound, benzalkonium chloride, all isolates originally susceptible to this disinfectant became resistant. These adaptation conditions, which could arise *in situ* via the disinfection of very wet surfaces, inadequate

rinsing after disinfection or dosage failure, were shown to induce an efflux pump conferring resistance to both benzalkonium chloride and ethidium bromide. Another important way in which bacteria can adapt to become resistant to the action of a biocide is by reducing their permeability. Cationic biocides, for example polyhexamethylene biguanide (PHMB), enter the gram-negative bacterial cell via "self-promoted uptake", whereby the biocide displaces cations from the lipopolysaccharide present within the cell membrane (Gilbert and McBain, 2001). However, following the attachment of cells to a surface, PHMB is reportedly subject to a rapid and significant attenuation of action which, strongly suggests that major phenotypic differences may exist with regard to the number and/or nature of the cation binding sites associated with biofilm and planktonic bacteria (Gilbert *et al.* 2001).

Despite such resistance mechanisms, biocides, at in-use concentrations tend to act at numerous biochemical target sites. Thus, unlike antibiotic resistance, whereby small modifications to a single target can alter the susceptibility of an organism to such an extent that the therapeutic dose can no longer be achieved, complete resistance to a biocide would require the initiation of multiple resistance mechanisms (Gilbert and McBain, 2001). Multiple target sites mean, therefore, that complete resistance is considered highly unlikely. However, Mokgatia *et al.* (2002) have recently reported the isolation of a hypochlorous acid-tolerant *Salmonella* sp., its resistance, they state, being due to a combination of physiological adaptations, which lead collectively to an enhanced degree of tolerance to this widely used antimicrobial agent.

Bacterial resistance, by whatever mechanism, can, therefore, contribute to ineffective disinfection. Dillon and Griffith (1999) suggest that rotating the disinfectants used within a sanitation programme will help to eliminate the build-up of resistant populations. However, stock rotation is unlikely to ameliorate a further problem associated with

increased biocide tolerance, and one that has been highlighted by a number of authors (Assanta *et al.* 1996; Andrade *et al.* 1998b; Lindsay and von Holy, 1999; Gilbert *et al.* 2001). Surface attached bacteria, which are those likely to have survived the cleaning process, are also likely to survive exposure to disinfectants applied at their normal, recommended in-use concentrations. This reduction in activity will be exacerbated should the chemicals be made up at an incorrect pH or applied to the surface for an insufficient period of time. Furthermore, there are growing environmental concerns over the presence of chemical by-products that are formed when chlorine is used as a disinfectant (Richardson *et al.* 1998) and such issues have prompted an increasing interest in the use of additional or alternative disinfectants, for example chlorine dioxide gas, electrolysed oxidizing water and ozone (Han *et al.* 1999; Venkitanarayanan *et al.* 1999; Moore *et al.* 2000) as well as natural biocides such as chitosan and carvacrol (Knowles and Roller, 2001).

1.3.3.4. Inter-production condition

Effective cleaning and disinfection procedures, although not necessarily removing all food debris and destroying all microorganisms, should reduce them to levels that are harmful neither to health nor to the quality of the final product (Orth, 1998). Procedures then need to be undertaken to prevent the growth of microorganisms on food contact surfaces, and/or surface re-contamination, in the period up until the next production process (Holah, 1992). Dry surfaces are generally considered the most hygienic (Boulangé-Petermann, 1996; Mettler and Carpentier, 1998; Loghney and Brougham, 2001) and as a result, surfaces are normally left to air-dry. However, the drying process and the absence of visible water, cannot always be relied upon to prevent microbial survival and growth (McEldowney and Fletcher, 1988; Tebbutt, 1999). Furthermore, laboratory studies have demonstrated that

microbial survival in aerosols can be as long as 210 min and within a meat processing facility, the aerosolisation of Enterobacteriaceae was shown to result in the deposition of viable bacteria for up to 3 h after cleaning and disinfection procedures had been completed (Jones, 1994; Zottola and Sasahara, 1994). Fielding *et al.* (2002) have described a successful use of gaseous ozone, whereby exposing the high-risk area of a cheese production unit to relatively low levels of ozone overnight, progressively reduced the number of organisms isolated from the surfaces prior to the start of the next working shift.

Despite sanitation being the major control measure associated with surface hygiene, there are numerous factors that can contribute to it failing to control soil accumulation and microbial contamination effectively. "Cleaning" costs the food industry many millions of pounds per year. However, if ineffective, businesses can incur further losses either through the purchase of unsuitable cleaning chemicals or via the build-up of organic debris within equipment and machinery leading to a reduction in heat transfer and inefficient processing (Dillon and Griffith, 1999). Furthermore, contamination of the finished product may lead to product recalls, which, with associated adverse publicity may result in loss of customers, sales and profits (Holah *et al.* 1994). It is essential, therefore, that the efficacy of cleaning and disinfection procedures is continually assessed and, should a surface be inadequately cleaned, the problem identified quickly and the correct remedial action implemented.

1.4. The Assessment of Surface Cleanliness

The effective cleaning and disinfection of food processing surfaces, equipment and plants in general is a basic requirement in the production of safe and wholesome food. It plays a vital role within both GMP and HACCP (Section 1.2) and its importance, coupled with the costs of cleaning, means that sanitation procedures should be validated, monitored and verified to ensure maximum effectiveness at minimum cost (Dillon and Griffith, 1999).

1.4.1. Validation, Monitoring and Verification Procedures

The validation of a HACCP plan provides assurances that, prior to its implementation, the specified control measures effectively control the identified hazards (i.e. the HACCP plan works) (Swanson and Anderson, 2000). The validity of supporting PRPs also needs to be determined so as to ensure they are effective in providing the level of control and prevention necessary for the HACCP plan to be effective (Kvenberg and Schwalm, 2000). If the validity of either is compromised, the provision of safe and wholesome food cannot be achieved. It is imperative, therefore, that prior to their implementation, those sanitation procedures intended for use either as part of a PRP or as a control measure within the HACCP system are shown to reduce surface contamination to levels deemed acceptable in terms of production area, product and process. Over time, microorganisms occurring within the environment may become more resistant (Section 1.3.3.3), more able to produce spoilage or more virulent and/or may gain entry to the product via a previously unrecognised control point (Waites, 1997). Consequently, the re-validation of both HACCP and PRP systems is recommended on an annual basis but is also considered essential should a business experience a HACCP failure or should significant changes

occur with regard to the product or process (Kvenberg and Schwalm, 2000). However, post-implementation, it is the regular monitoring of both systems that demonstrate the control of food safety hazards and hygiene on a day-to-day basis (Wallace and Williams, 2001).

Monitoring is defined as "the act of conducting a planned sequence of observations or measurements of control parameters to assess whether a CCP is under control" (WHO, 1997). Monitoring procedures not only enable food businesses to determine when and if there has been an abrupt system failure, they also provide information and an indication as to general trends relating to a gradual loss of control (NACMCF, 1998). In addition, the results of monitoring activities should be recorded and thus, provide documented evidence that the process was under control and that the food was produced in accordance with the critical controls identified as those which ensure safe food (Mortimore and Wallace, 2001). In the UK, the monitoring records are legal documents and thus, if necessary, can be used to support a claim of due diligence. This defence, contained within the Food Safety Act 1990, can be provided for a person, should he be charged with an offence, provided he can prove he "took all reasonable precautions and exercised all due diligence to avoid the commission of the offence by himself or by a person under his control" (Jukes, 1997). Thus, monitoring serves three main purposes, which although have been considered in relation to a HACCP system are just as relevant with regard to the application of supporting PRPs.

Documented monitoring records are also required for verifying that the HACCP and pre-requisite programmes are being performed, monitored and recorded in the manner originally intended (Sperber *et al*, 1998). Verification is the application of methods, procedures, tests and other evaluations, in addition to monitoring, to determine compliance with the HACCP or PRP plan (Dillon and Griffith, 2001). Verification is an on-going

process and the continual assessment of the adequacy of the plans and the efficacy of their elements in achieving set objectives is a major reason for both HACCP and PRP systems improving the production process and enhancing food safety and quality (Motarjemi and Käferstein, 1999). Nevertheless, although current legislation requires UK food businesses to adopt a HACCP-type approach, neither documentation nor verification (Table 1.1) is legally required, due to perceived difficulties of their application by small and medium-sized businesses (Walker *et al.* 2003). However, as in the United States, where the Department of Agriculture (USDA) has placed all seafood, juice, meat and poultry processing facilities, regardless of size, under a HACCP mandate, the EU is now proposing the implementation of *full* HACCP programmes in *all* food businesses (Quinn *et al.* 2002; Walker *et al.* 2003).

1.4.2. Methods for Assessing Surface Cleanliness

To effectively validate, monitor and verify the sanitation programmes used within a production environment, the efficacy of the cleaning and disinfection procedures applied needs to be accurately and reliably determined.

1.4.2.1. Microbiological methods

There are a variety of microbiological techniques that can be conducted within the laboratory to assess the efficacy of cleaning and disinfection strategies. These include the use of bioluminescent bacteria, either recombinant derivatives of important foodborne microorganisms, such as *Listeria monocytogenes* (Walker *et al.* 1993), or natural bioluminescent bacteria, for example *Photobacterium leiognathi*, which unlike

recombinants do not require genetic manipulation and the addition of a *lux* expression plasmid (Wirtanen *et al.* 1995). In both cases bacterial bioluminescence is directly related to cell viability and, thus, damage caused by the applied cleaning and disinfection procedures can be observed via a change in light emission. Microscopic techniques, which involve the direct examination of contaminated surfaces, have also been described (Pontefract, 1991; Yu *et al.* 1993; Griffiths, 1997; Bredholt *et al.* 1999; Wirtanen *et al.* 2001). Test surfaces contaminated and 'cleaned' either artificially under controlled conditions or by being placed within a production environment are stained and examined with an epifluorescent image analyser enabling researchers to directly determine cell viability and/or metabolic activity. However, such techniques although providing valuable information, are completely impractical in terms of the facilities and expertise available to an individual food business. Nevertheless, a number of methods are available, which allow those working within the food industry to regularly and easily assess the cleanliness of food contact and environmental surfaces.

The most common technique employed by the food industry and, thus, the method most widely recognised, is the detection of viable microorganisms via the use of conventional hygiene swabs. A sterile cotton swab is moistened and rubbed over the surface to be sampled. The tip of the swab is then aseptically placed into a tube containing a sterile diluent, shaken and the rinse fluid plated with an appropriate culture medium (Favero *et al.* 1968). An estimate of the microbial load per unit area of plant is obtained which can then be compared to pre-determined specifications (Griffiths, 1997). This sampling procedure not only provides quantitative information but also can easily be adapted to incorporate a range of different culture media. Thus, the use of hygiene swabs is particularly useful during the validation and verification of sanitation procedures where the detection of indicator organisms or specific microbial hazards such as *L. monocytogenes* is likely to be required (Eisel *et al.* 1997; Kohn *et al.* 1997). Although fairly specialised media may be

used, the swabbing procedure itself is relatively inexpensive, can be used to sample any size or shape of surface and in addition is quick and easy to perform (Griffith *et al.* 1997). Consequently, Copan Diagnostics Inc. recently estimated that in the past year, 25 million environmental swab samples were taken in the U.S. alone (N. Sharples; personal communication) contributing significantly to the estimated 144 million microbiological tests performed annually for the U.S. Food Industry (Fung, 2002).

However, the acquisition and interpretation of microbiological data is method-dependent. A variety of different methods can be used to swab a surface and this can dramatically influence the results obtained. Although standardised methodology can be employed, a number of 'standards' are likely to be available and are likely to have been produced by a number of different organisations. Consequently, these methods may also vary and techniques adopted as official methods in one country may differ from those used in other parts of the world (Buchanan, 2000).

The same is true with regard to the level of microorganisms considered indicative of a clean surface (Table 1.5). However, it is acknowledged that this value will most likely be influenced by the risk associated with the surface(s) sampled. Studies have demonstrated that after the implementation of a recommended cleaning protocol, general microbial values of < 2.5 cfu cm^{-2} can be achieved for a range of surfaces (Griffith *et al.* 2000). However, whilst achieving such levels of 'cleanliness' may be deemed necessary within those areas of a production plant where the cleanliness of food contact surfaces has been designated critical to food safety, higher levels of microbial contamination may be considered 'acceptable' within 'low-risk' production areas. Nevertheless, regardless of surface sampled, it should be remembered that the degree of pressure applied to a swab during sampling and the speed of the swabbing action itself can vary from person to

person. This can lead to variable microbial counts and incorrect estimates of the numbers of organisms present on the swabbed surface (Pontefract, 1991).

Table 1.5. Previously published and/or recommended microbiological criteria for the acceptable sanitation of product contact surfaces

Acceptable microbial counts	Reference
< 2 cfu cm^{-2}	Compendium of methods for the microbiological examination of foods (Sveum *et al.*1992)
< 2.5 cfu cm^{-2}	Mossel *et al.* (1999)
< 3 cfu cm^{-2}	Swedish Food Agency (SLV SFS 1998:10, the Swedish Statute Book)
< 5 cfu cm^{-2}	United States Department of Agriculture guidelines for reviewing microbiological control and monitoring programs (1994)
< 10 cfu cm^{-2}	Meat (Hazard Analysis and Critical Control Point) (England) Regulations 2002
< 12.5 cfu cm^{-2}	Rahkio and Korkeala (1997)
< 25 cfu cm^{-2}	Joint committee of the milk marketing board and the dairy trade federation of England and Wales (1985). Code of practice for the assessment of milk quality (cited in Bell *et al.* 1994)
< 40 cfu cm^{-2}	Environmental procedure manual, Ontario Ministry of Health (cited in Seeger and Griffiths, 1994)
< 600 cfu/bottle	Guidelines for the bacteriological cleanliness of milk bottles (cited in Roberts and Greenwood, 2003)
< 1000 cfu cm^{-2}	Herbert *et al.* (1990)

Dipslides, comprise a double-sided hinged paddle with nutrient or selective agar attached to both sides. The dipslide is placed on the surface to be tested, removed, replaced in the accompanying sterile tube and incubated. Although the applied pressure can still vary, their use does go someway towards standardising both methodology and the size of area sampled. In addition, although dipslides do not reduce the time taken to obtain results they do facilitate sampling and eliminate the need for media preparation and, thus, it has been reported that their use results in savings of approximately 40% over the traditional

swabbing technique (Griffiths, 1997). This together with simplicity of use makes the dipslide a highly desirable field test, particularly in situations where routine and replicate samples are required (Angelotti *et al.* 1958). However, as dilution of the sample is not possible, only small numbers of contaminants can be enumerated. In the case of confluent growth, it becomes necessary to interpret the results using a key provided by the manufacturer (Salo *et al.* 1999) and, thus, the results obtained are only semi-quantitative. Nevertheless, it has been suggested, that since resulting microbial counts are simply compared to a pre-determined value, that which represents the acceptable safety and quality standard (Table 1.5), relevance should only be placed on whether the levels lie above or below this 'critical limit' and, thus, the exact measure of the microbial population is of little importance (Bautista *et al.* 1995).

The advantages associated with the simplicity and sensitivity of traditional microbiological surface sampling methods have until recently outweighed the need to obtain results rapidly (Hawronskyj and Holah, 1997). However, the HACCP approach focuses on prevention and control (Section 1.2) and although verification that a process is safe must involve microbiological testing, the length of time required to generate microbial data means that microbiological methodology is now considered unsuitable for the routine monitoring of CCPs (Griffiths, 1996). Furthermore, as with all cultivation-based sampling methods, hygiene swabs and dipslides only provide information regarding the microbiological aspects of poor cleaning. It has already been established that product residues remaining on food contact surfaces can not only facilitate the attachment of microorganisms and provide them with a source of nutrients (Section 1.1.1) but may also protect them from the action of disinfectants (Section 1.3.3). Thus, it has been argued that in many situations, it is the absence of biological contamination in general that is the more relevant measure of cleanliness; particularly, with regard to monitoring procedures, if results can be obtained rapidly (Lundin, 1999).

1.4.2.2. Non-microbiological methods

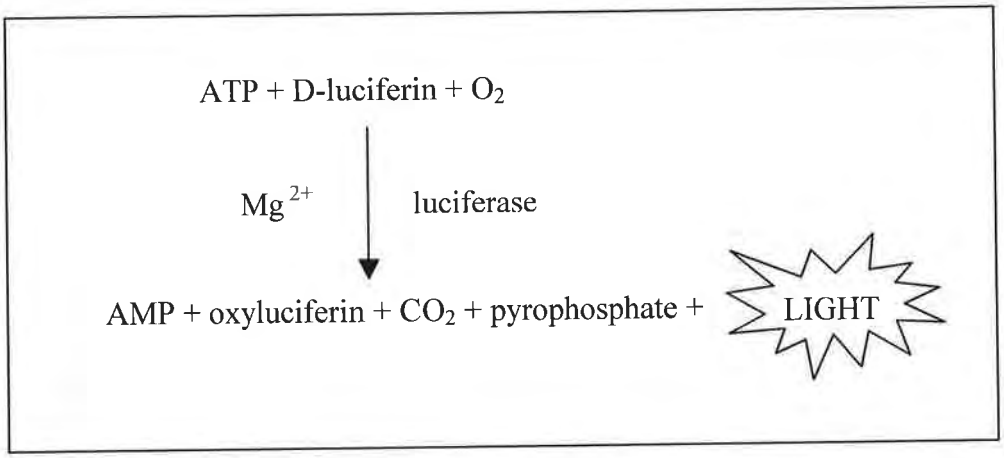
The results of a recently conducted, self-administered quantitative attitude survey revealed that 70% of respondents considered food manufacturers to be ultimately responsible for the safety of their food (Redmond, 2002). Nonetheless, the majority of food handlers from 52 small to medium sized businesses admitted to not always carrying out all the food safety practices, including cleaning, they knew they should be implementing (Clayton *et al.* 2002). Safety and quality is the responsibility of all, including production and cleaning staff and Griffith *et al.* (1994) have stated that in terms of the effective execution of sanitation procedures, this philosophy can be encouraged by empowering people at all levels within a company with the ability to assess surface cleanliness.

Point of production, instant-result, test kits have been developed which enable non-technically trained staff to conduct the routine assessment of surface cleanliness. However, it has been argued that the introduction of these non-microbiological test methods has bridged the important divide between production and quality assurance personnel and as a result, production staff have become involved in issues in which they do not fully appreciate all the ramifications (Anon, 2000). Indeed Clayton *et al.* (2002) reported that only 66% of food handlers identified the effective cleaning and disinfection of equipment, utensils and surfaces as being something that they could do to prevent food poisoning. Nevertheless, studies have demonstrated that when production staff are taught to use simple cleanliness assessment methods, improvements in environmental cleanliness can result. It has been reported that the use of such non-microbiological techniques provides staff with a strong incentive firstly to clean properly and then to improve their cleaning technique (Ogden, 1993; Mossel *et al.* 1999; Worsfold and Griffith, 2001).

1.4.2.2.1. ATP bioluminescence

Adenosine triphosphate (ATP) is present in all actively metabolising cells and its detection via ATP bioluminescence was first described in the 1960s by NASA scientists who developed the assay as a means of detecting extraterrestrial life (Chappelle and Levin, 1968). ATP bioluminescence is based on the reaction that occurs naturally in the tail of *Photinus pyralis* – the North American firefly, whereby the enzyme luciferase uses the chemical energy associated with ATP to drive the oxidative decarboxylation of luciferin (Hawronskyj and Holah, 1997) – a reaction that results in the production of light (Figure 1.3).

Figure 1.3. The ATP bioluminescence reaction



Firefly luciferase is almost entirely specific for ATP and since the latter is present within both viable microorganisms and a variety of foodstuffs, ATP bioluminescence results in the dual detection of both these sources of contamination (Hawronskyj and Holah, 1997). Furthermore, the underlying premise of the assay is that for every molecule of ATP present in the sample, one photon of light is emitted and, thus, the amount of light produced is directly proportional to the levels of microorganisms and/or food residues present on the surface (Griffiths, 1996). Light output, usually quantified by means of a portable, hand-

held luminometer, is measured in relative light units (RLU) and rather than staff having to calculate the actual amount of ATP or colony forming units present, these arbitrary units can be used as a direct measure of surface cleanliness. In addition, results are obtained rapidly, allowing real-time control of the process environment, and as a result, ATP bioluminescence has been used for monitoring surface cleanliness in a variety of processing environments including cheese plants (Kyriakides *et al.* 1991), fruit juice operations (Bautista *et al.* 1993), breweries (Ogden, 1993), bakeries (Illsley *et al.* 2000) and meat processing plants (Chen, 2000).

As with microbiological methods, when implementing ATP bioluminescence, it is essential to establish the levels of ATP that are indicative of effective or ineffective cleaning. However, even with the same ATP concentration in the final assay mix, the measured light signal can vary in response to a number of different parameters (Lundin, 1999). The luciferase activity of a variety of ATP reagents has, for example been shown to differ and luminometers, particularly different models, can vary by several orders of magnitude with regard to the units used for presenting the results (Lundin, 1999). Each of these issues highlight the importance of a documented monitoring procedure, preventing in-coming managerial staff from implementing their preferred ATP system. Previously developed baseline data become worthless if new testing protocols do not provide equivalent results (Swanson and Anderson, 2000). Additionally, as with any enzyme-based assay, external factors such as pH and chemicals can affect the activity of the firefly luciferase. Factors affecting the stability of the bioluminescence reagents include salts, metal ions, preservatives and cleansing solutions (Calvert *et al.* 2000).

ATP extractants are often similar to those used as cleaning agents and, therefore, detergents and/or disinfectants can either degrade the enzyme or have an additive effect upon the ATP measurement. Several studies have demonstrated, that a number of

commonly used cleaning chemicals, when applied at in-use concentrations, can cause the quenching or, in some cases, the enhancement of the ATP light signal (Simpson and Hammond, 1991; Velazquez and Feirtag, 1997; Green *et al.* 1999; Lappalainen *et al.* 2000) and ultimately result in a false-negative or false-positive reading. It has also been suggested that with regard to many commercially available ATP kits, it is the potential effect that the extractants themselves can have upon the luciferase reaction, rather than their ability to extract intracellular ATP, that has the greater bearing upon which is actually incorporated within the test (Lundin, 1999). Consequently, studies, conducted under controlled conditions, have suggested that when used to detect the presence of microbial contaminants on a wet surface, the sensitivity of ATP bioluminescence is considerably lower than that of traditional microbiology (Davidson *et al.* 1999).

In order to improve the sensitivity of the bioluminescent technique, an alternative approach, which involves the amplification of ATP has been proposed. Intracellular adenylate kinase (AK) is extracted and this then converts available ADP (adenosine diphosphate) to ATP and AMP (adenosine monophosphate). The ATP bioluminescence assay is then used to detect the ATP generated from AK activity and a detection limit of approximately 4 *Escherichia coli* cells has been reported (Corbitt *et al.* 2000). Tanaka *et al.* (2001) have since expanded upon this assay and have increased the amplification of ATP by converting the AMP produced by both AK activity and the bioluminescent reaction itself (Figure 1.3) to initially ADP and then, via AK, to ATP. As already mentioned, ATP bioluminescence detects both microorganisms and food debris and, depending upon the foodstuff present, this latter assay can reportedly increase the sensitivity of ATP bioluminescence by up to 177,000-times (Tanaka *et al.* 2001).

Despite the possibility of detecting very low levels of surface contamination, many potential customers desire the ability to differentiate between microbial and non-microbial

surface contamination (Griffiths, 1996). However, in order for ATP bioluminescence to solely detect microorganisms, the assay procedure must include steps to either segregate microbial from somatic ATP or to destroy the somatic ATP so that all that remains in the sample is that which is associated with microbial contaminants (Cutter *et al.* 1996). The level of microbial contamination on beef, pork and poultry carcasses has successfully been determined using an assay procedure that incorporates a filtration step (Bautista *et al.* 1995; Siragusa *et al.* 1995; Cutter *et al.* 1996). Although the described methodology could be adapted in order to detect the presence of microbial contaminants on food contact surfaces, the extra equipment, facilities and technical expertise required would prevent production staff from conducting the analyses. It has also been proposed that the ATP bioluminescence assay could be made specific by using a host-specific lytic phage to selectively lyse target organisms (Griffiths, 1996). The lysis would then be detected via ATP bioluminescence and Wu *et al.* (2001) have since described an assay that allows the detection of *E. coli* and *Salmonella enteritidis* at levels of 10^3 cfu ml^{-1} within 2 h of sampling. However, for the reasons previously discussed, at present the detection of specific organism types is restricted to laboratory personnel. Furthermore, such enhancements to the ATP bioluminescence technique would naturally increase the cost of a test method, already considered by some as being too expensive to implement.

1.4.2.2.2. Instrument-free, food residue detection methods

The perceived high running costs associated with ATP bioluminescence are often an important obstacle limiting its use (Kyriakides *et al.* 1991). The results of an industry survey conducted by Griffith *et al.* (1994) revealed that whilst 25% of respondents preferred traditional microbiology, the majority cited the then high cost of testing as the main reason for not using ATP bioluminescence. Luminometers can cost thousands of

pounds and the total labour and material costs associated with ATP bioluminescence is approximately 50% greater than traditional swabbing (Kyriakides *et al.* 1991). However, it has been argued that although material costs may be high, the labour involved in performing the ATP bioluminescence assay is considerably lower than that associated with microbiological methods. In addition, if the cost benefits of obtaining results rapidly are also considered, including economic labour use, savings from reduced product wastage and improved cleaning, then ATP bioluminescence is in fact likely to be more cost effective than the traditional swabbing technique (Ogden, 1993; Griffith *et al.* 1994).

Nevertheless, this reasoning could be true for any rapid test method and with the high initial expenditure normally associated with the purchase of one, or many, luminometers often proving too great for many smaller businesses, there has been an increased interest in the design and development of instrument-free cleanliness assessment methods. Methods are now available that allow those working within the food industry to rapidly detect the presence of food debris, including reducing sugars, carbohydrate and/or protein residues that are left behind on an inadequately cleaned surface. As with ATP bioluminescence, non-technically trained staff can use these methods, yet, because of their relatively recent introduction, very little information is currently available with regard to other associated advantages, disadvantages, drawbacks or limitations.

1.5. Aims of Research

Controlling the production of safe and wholesome food requires the systematic collation of reliable data relating to the occurrence, elimination, prevention and reduction of identified hazards (Kvenberg and Schwalm, 2000). Environmental sampling is a key tool in achieving this aim, the power of which is frequently overlooked, particularly, as currently there is no standard method, technique or protocol for assessing surface cleanliness (Griffith *et al.* 1997). Sampling, is at present often only conducted simply because it must be done and frequently there is little reasoning or logic behind the choice of sampling method and, as the data is rarely used for trend analysis, no clear understanding as to why the results are being collected (Buchanan, 2000). Nevertheless, the desire to standardise methodology must be carefully weighed against the benefits of providing industry with the flexibility to choose methods that meet their specific needs (Swanson and Anderson, 2000). However, it must be appreciated that whilst 'clean' is defined as being "free from soil" (Dillon and Griffith, 1999), 'clean' can only truly be interpreted in terms of the component residues being tested for. If sampling is to be used effectively, therefore, it is critical that the individuals performing the analyses and those interpreting the results have a clear understanding of the goals of the different types of testing, the principles underlying the sampling techniques and the limitations of the methods employed (Buchanan, 2000). Sampling should be directed towards process improvement and should not be done solely for the sake of generating data. Industry, academia and government need to work together to develop reasonable, scientifically based strategies for determining appropriate methodology (Swanson and Anderson, 2000).

The aims of the work reported in this thesis are, therefore, to:

- Evaluate those methods currently available to the food industry for assessing surface cleanliness and determine their limits of detection together with factors influencing their efficacy.
- Provide the food industry with the information necessary to identify which test method(s) is best suited for any given processing environment.
- Devise a generic cleaning assessment strategy and establish key areas where appropriate methodology is currently lacking.
- Design, develop and evaluate novel cleanliness assessment techniques to fulfil these requirements.

Objectives

- Determine those factors influencing the recovery of microorganisms from food contact surfaces using the traditional swabbing technique.
- Assess, under controlled laboratory conditions, the ability of the new generation of rapid, instrument-free test kits to detect the presence of a variety of different food residues.
- Compare and contrast the performance of these test methods to that of ATP bioluminescence and traditional microbiology.
- Assess, *in situ*, the ability of microbiological and non-microbiological methodology to evaluate surface cleanliness.
- Identify, design, develop and evaluate an appropriate non-microbiological surface sampling method.
- Identify, design, develop and evaluate an appropriate microbiological surface sampling method.

Chapter 2

Factors Influencing the Recovery of Microorganisms from a Stainless Steel Surface by use of Traditional Hygiene Swabbing.

2.1. Introduction

For nearly a century, microbiologists from public health agencies, research laboratories and a wide range of other industries and disciplines, including the arts have been concerned with the detection and enumeration of microorganisms on surfaces (Walter, 1955; Laiz *et al.* 2003). Within the food industry, microbiological samples are frequently taken from both food contact and environmental surfaces to assess the efficacy of the cleaning and disinfection procedures applied (Section 1.4.2.1).

The conditions necessary for microbial growth are nearly always present in the majority of food processing environments (Gabis and Faust, 1988). Food spoilage and pathogenic organisms can easily colonise processing surfaces, equipment and machinery and inadequate sanitation can increase the risk of such organisms becoming dislodged, contaminating the final product and contributing to its microbial load (Chapter 1). Thus, the numbers and specific types of organisms present on food contact surfaces will directly relate to the safety and quality of the product (Buchanan, 2000; Salo *et al.* 2000). As a result, the detection and enumeration of specific pathogens and/or indicator microorganisms (see Chapters 6 and 7) remains an important means of assessing the hygienic status of a variety of processing environments (Fernandes *et* al. 1996; Rahkio and Korkeala, 1997; Russell *et al.* 1997; Brown *et al.* 2000; Miettinen *et al.* 2001).

Numerous investigations have been conducted to fill the need for a simple, reliable, bacteriological test to determine, quantitatively, the sanitary quality of food contact surfaces (Angelotti *et al.* 1958; Clark, 1965; Scheusner, 1982; Fung *et al.* 2000). However, the recommended procedure and the technique most commonly employed, remains one based upon the swab-rinse technique originally developed by Manheimer and Ybanez in 1917 (Favero *et al.* 1968; Mossel *et al.* 1995).

A sterile cotton swab is moistened and rubbed over the surface to be tested. Contaminants are picked up and transferred directly to a nutrient medium (swab plate) or to an intermediate diluent, which can be quantitatively assayed (pour plate). Although the swab plate can be used to make a gross estimate of surface contamination, vortexing the swab in a diluent is a more effective means of breaking up clumps of bacteria and, therefore, is more likely to measure the number of individual bacterial cells present on a surface (Gilbert, 1970).

Thus, the accurate detection and enumeration of microbial contaminants by use of the traditional swabbing technique relies initially upon the ability of the swab to remove microorganisms from a surface, followed by their effective release from the swab bud and subsequent recovery and cultivation. However, it has been reported that bacteria become increasingly difficult to remove once they have adhered to a surface, particularly if they have become associated with a biofilm (Bredholt *et al.* 1999; Salo *et al.* 1999). Furthermore, the buds of cotton-tipped swabs are thought to retain some of the microorganisms removed from the surface, again resulting in an apparent reduction of recovery (Favero *et al.* 1968). Additionally, surface hygiene swabbing is subject to a number of inherent errors (Angelotti *et al*, 1958; Greene and Herman, 1961, Silliker and Gabis, 1975). It is, for example, very difficult to standardise either the swabbing pattern or the angle and degree of pressure applied to the swab during sampling. Thus, no two people

use a swab exactly alike and the inability to control both the reproducibility and the repeatability of the swabbing technique can lead to extreme variability in the results obtained.

These acknowledged shortcomings have led to continual attempts to improve the swabbing procedure and as a result, there is no one universally accepted swabbing protocol. Variations exist with regard to the type, number and dryness of the swab(s) used, the composition of the diluent and, if applicable, the swab-wetting solution and, in addition, the state (i.e. wetness) and size of the surface area sampled. Different methods will likely provide different results and this, in turn, can make it very difficult to relate microbial data obtained from one plant to that from another and is particularly relevant considering microbiological criteria set by one company are likely to be imposed upon their suppliers. An important step in developing a standard method for detecting microorganisms on food contact surfaces is, therefore, to optimise the swabbing procedure (Nedoluha *et al.* 2001). However, in order to improve a system, there must first be a clear understanding as to why that system should fail, yet, in general, information is currently lacking with regard to the variables that affect the accuracy of the swabbing technique.

The aim of this chapter is, therefore, to:

- Determine those factors, which influence the recovery of microorganisms from food contact surfaces using the traditional swabbing technique.

Objectives

- Design an experimental protocol to enable the systematic evaluation of each individual component of the swabbing procedure.

- Determine the efficiency of the traditional cotton-tipped hygiene swab when used to sample a wet and dry stainless steel surface.
- Determine whether a relationship exists between swabbing efficiency and microbial viability.
- Determine whether a relationship exists between swabbing efficiency and the ability of a cotton swab to remove bacteria from a surface.
- Determine whether a relationship exists between swabbing efficiency and the ability of a cotton swab to release bacteria into a diluent.
- Assess whether one or more of these component stages can be significantly improved in order to optimise the traditional swabbing protocol.
- Evaluate a range of swab types and swab-wetting solutions and assess their effect upon bacterial removal, release and overall recovery.

2.2. Materials and Methods

2.2.1. Microorganisms

The microorganisms used during this study were selected on the basis of their association with foodborne disease and/or food spoilage.

Listeria monocytogenes represents an important foodborne pathogen and the isolation of any *Listeria* spp. is indicative of its presence (Peters *et al.* 1999; Samelis and Metaxopoulos 1999). Consequently, the accurate detection of *Listeria* spp. in

environmental samples can form a critical component of HACCP validation and verification programmes (Kohn *et al.* 1997).

The *Listeria* sp. used during the current investigation was isolated from a food processing environment and was provided, on Listeria Selective Medium (Oxford Formulation), by South Wales Food Labs. Although black zones were seen to surround the colonies, suggesting that the isolate was *Listeria monocytogenes*, no independent identification was conducted. Consequently, the culture is subsequently referred to as being *Listeria* sp.

The potential pathogenicity of the Enterobacteriaceae and their wide use as indicator organisms meant that, in terms of this study, this group of bacteria was also of particular interest.

A Gram negative, oxidase negative rod was isolated from a food environment and identified, using biochemical test strips (API 20E; bioMérieux), as being *Salmonella* sp.

Pseudomonads have been documented as being good producers of extracellular polymeric substances, which help anchor the cells to a surface and to trap and retain nutrients (Section 1.1.3). Thus, *Pseudomonas* spp. can play an important role in initiating and maintaining biofilm growth. Additionally, many species of psychrotrophic pseudomonads are important low temperature spoilage organisms (Mossel *et al.* 1995) and studies have shown that they can be readily transferred from processing equipment and surfaces to the final product (Bagge *et al.* 2001).

A Gram negative, oxidase positive rod was isolated from the mains water supply. Growth on Pseudomonas Agar (Oxoid, Basingstoke, UK; 24.2 g 500 ml^{-1}) with added C-F-C selective supplement (Oxoid; 1 vial 500 ml^{-1}) was taken as presumptive evidence of

Pseudomonas sp. Identification was confirmed using biochemical test strips (API 20 NE; bioMérieux).

2.2.2. Preparation and Maintenance of Bacterial Cultures

To reduce the risk of mutations which may alter culture phenotype, pure cultures were suspended in a cryopreservative and stored at -20°C on porous ceramic beads (Protect bacterial preservation system; Fisher Scientific, UK). Every 4-6 weeks, the cultures were sub-cultured onto Tryptone Soya Agar (Oxoid; 40 g l^{-1}) (TSA) plates and maintained at 5°C.

For many microorganisms, their effective dispersal throughout the environment depends upon their ability to survive outside the host for long periods of time. This survival frequently takes place under conditions of adverse pH, osmolarity and temperature. When bacteria are starved of nutrients, they enter a stationary-phase of growth and undergo a radical physical adaptation to ensure that they can combat such physical stresses, despite remaining in a relatively dormant state (Rees *et al.* 1995). Cells grown to stationary phase under laboratory conditions are also likely to be more resilient to a range of stresses (Humphrey *et al.* 1995) and, consequently, are more likely to resemble environmentally adapted microorganisms. During this investigation, the Miles and Misra technique (Harrigan, 1998) was employed to generate growth curves for each of the three different bacteria (Appendix I). These curves were then used to determine the length of time required by each microorganism to reach their stationary-phase of growth.

Bacterial cultures were prepared by aseptically transferring a single colony of the *Salmonella, Listeria* or *Pseudomonas* strain into a 250 ml conical flask containing 100 ml

of sterile Nutrient Broth No. 2 (Oxoid; 25 g l^{-1}) (NB). Stationary phase cultures were obtained by incubating the bacteria at 30°C in an orbital shaking incubator (100 revolutions min^{-1}; Model 4518, Forma Scientific Inc., Ohio, USA) for 18 h. For the *Salmonella* and *Pseudomonas* strains used in this investigation, these culture conditions were found to yield approximately 1 x 10^9 colony forming units (cfu) ml^{-1}, whereas for the *Listeria* strain, these conditions resulted in approximately 1 x 10^8 cfu ml^{-1}. After incubation, a five-fold dilution series of each bacterial culture was prepared using ¼ strength Ringer solution (Oxoid; 1 tablet 500 ml^{-1}).

2.2.3. Preparation of Test Surfaces

It has been demonstrated that pristine surfaces are altered significantly after one 'soiling' event and, thus, argued that new, clean surfaces should not be used in laboratory trials (Verran *et al.* 2001a). During the current investigation, therefore, new squares (5 cm x 5 cm) of food-grade stainless steel (type 304; Food Quality Engineering, Cardiff) were conditioned before use. This initially involved them being placed in acetone and sonicated for 15 min using a Sonicleaner (Lucas Dawe Ultrasonics, London, UK) before being soaked in a sodium hypochlorite solution to remove any grease associated with the manufacturing process (Hood and Zottola, 1997a). To mimic the effects of a protein-based food soil, the coupons were then immersed, overnight, in Tryptone Soya Broth (Oxoid; 30 g l^{-1}) before finally being rinsed, dried and, prior to use, autoclaved at 121°C for 15 min.

Thereafter, between each set of experiments, the coupons were immersed overnight in Virkon (see Section 3.2.4.1) at the manufacturer's recommended usage level (1% solution; Antec International, Suffolk, UK), before being rinsed, dried and, prior to use, autoclaved.

2.2.4. Swabs and Swabbing Protocol

2.2.4.1. Swabs and swab-wetting solutions

Although cotton-tipped hygiene swabs are traditionally used during the microbiological examination of surfaces, other swab types are available. Of particular interest in terms of the current study were dacron swabs, which are commonly incorporated within ATP bioluminescence systems, polyurethane foam swabs, which being tipped with a much rougher material may improve the removal of bacteria from a surface and alginate swabs, the fibres of which dissolve in Calgon Ringers, reportedly improving bacterial recovery.

Thus, the surfaces were sampled using swabs tipped with cotton (TSA-6; Technical Service Consultants Limited, Lancashire, UK), dacron (TS19-M; Fisher Scientific, UK), polyurethane foam (Hardwood Products Company, Guilford, Maine) or alginate (TS7; Technical Service Consultants Limited). The swabs were used either dry or after they had been pre-moistened with a range of swab-wetting solutions (Table 2.1).

2.2.4.2. Swabbing protocol

In all experiments, the stainless steel coupons were sampled using a previously described swabbing protocol (Davidson *et al.* 1999). The swab, held by the handle rather than the applicator stick, was passed, in a zig-zag pattern (approximately 20 strokes), over the surface to be sampled. This process was then repeated at an angle of 90° to the first swabbing. In both cases, the swab was rotated constantly, thus, ensuring that the entire swab bud came into contact with the test surface.

Table 2.1. Swab-wetting solutions and their components

Solution	Formula
¼ strength Ringer solution	***isotonic diluent***
0.1% agar solution (Swedish Food Agency)	Maximum Recovery Diluent (Oxoid; 9.5 g l^{-1}) (MRD) Bacteriological Agar (0.1% w/v)
MES-buffer based solution	MES buffer (0.01M; pH 6.8) Tween 80 (0.03% w/v): ***neutralises quaternary ammonium compounds*** Sodium thiosulphate (0.025% w/v): ***neutralises hypochlorites***
TRIS-buffer based solution (Tuompo *et al.* 1999)	TRIS-acetate buffer (0.02M; pH 6.7 (using acetic acid)) Ethylenediamine tetraacetic acid (0.1% w/v) (EDTA): ***chelating agent*** Triton-X-100 (1% w/v): ***non-ionic detergent***
3% Tween solution (Bloomfield, 1991)	MRD Tween 80 (3% w/v): ***neutralises quaternary ammonium compounds*** Sodium thiosulphate (0.1% w/v): ***neutralises hypochlorites*** Lecithin (0.3% w/v): ***in combination with Tween 80 neutralises biguanides***
Spraycult® (Orion Diagnostica, Espoo, Finland)	***commercially produced "biofilm-disintegrating agent"***

2.2.5. Assessing the Removal of Bacteria from a Stainless Steel Surface

If an enumeration technique involves the removal of cells, then to enable the effectiveness of cell recovery to be calculated, it is desirable to include a step that allows the number of bacteria on the surface to be assessed both before and after the removal process (Bremer *et al.* 2001). The methodology used during the current study was based upon the direct surface agar plate (DSAP) technique described by Angelotti and Foter (1958) and, thus, it was necessary to conduct a preliminary set of experiments to determine those dilutions, which when inoculated onto the stainless steel coupons, would result in a countable number of survivors.

Sterile coupons were aseptically transferred to sterile petri dishes (90 mm diameter; Bibby Sterilin Ltd, UK) and 12.5 µl of the dilution appropriate to the organism and surface treatment was inoculated onto each square and spread evenly over the surface using a sterile, disposable "hockey stick" shape spreader (Technical Service Consultants Limited). The surfaces were sampled using the previously described swabbing protocol (Section 2.2.4.2) immediately after inoculation while still wet, or after they had been allowed to air-dry for 1 h under ambient conditions. The theoretical number of bacteria inoculated onto the test surface was calculated using the results obtained via the conventional cultivation of the bacterial suspension.

It has been speculated that low nutrient systems may enhance adherence (Hood and Zottola 1997a). Thus, when assessing the ability of swabs to remove bacteria from a 'biofilm' (Section 1.1.4.1), the sterile coupons were placed in sterile petri dishes and immersed in 20 ml of $^{1}/_{5}$ strength NB (Dewanti and Wong, 1995) containing approximately 50 *Pseudomonas* cfu ml^{-1}. The coupons were left in static conditions for 4 h at room temperature (Gibson *et al.* 1999). After incubation, sterile forceps were used to remove the

coupons from the bacterial suspension and whilst being held vertically, to drain any excess liquid, the coupons were rinsed with 5 ml of sterile phosphate buffered saline (Oxoid; 1 tablet 100 ml^{-1}) (PBS) to wash away any unattached cells. The coupons were then placed in sterile petri dishes and sampled as described in Section 2.2.4.2.

Once they had been sampled, all coupons were directly overlaid using molten, tempered (45°C) Plate Count Agar (Oxoid; 17.5 g l^{-1}) (PCA). Control coupons were prepared identically to the test coupons but were directly overlaid without having first been swabbed. All plates were incubated at 30°C for 48 h, after which time, the number of colonies present on the surface of those coupons that had been swabbed was compared to the number present on the surface of the control coupons. Each experiment was based on 10 replicates and the percentage of colony forming units removed from the surface during swabbing was calculated using equation 1.

$$N_{rem} = \left[\frac{N_{cc} - N_{tc}}{N_{cc}} \right] \times 100 \qquad (1)$$

Where:

N_{rem} = the percentage of colony forming units removed from the surface

N_{cc} = the mean number of colony forming units counted on the surface of the control (i.e. the un-swabbed) coupons

N_{tc} = the number of colony forming units counted on the surface of the test (i.e. the swabbed) coupons

2.2.6. Assessing the Release of Bacteria from the Swab Bud and Overall Recovery

The swabs used to sample the coupons were snapped off into either 10 ml ¼ strength Ringer solution or, in order to dissolve the alginate swabs, 10 ml Calgon Ringers (Oxoid; 1 tablet 10 ml^{-1}). The swabs were vortexed for 20 s to release the bacteria from the bud before 1 ml of the bacterial suspension was pipetted into a petri dish. Approximately 15 ml of PCA was added and the contents mixed well. Once set the plates were incubated at 30°C for 48 h.

After incubation, the colonies present on the agar plates were counted and the percentage of colony forming units released from the swab bud was calculated using equation 2. The efficiency of the sampling method (i.e. the overall percentage recovery) was calculated using equation 3; a method previously described by Whyte *et al.* (1989).

$$N_{rel} = \left[\frac{N \times d}{\left(\frac{N_{rem}}{100} \right) \times I} \right] \times 100 \qquad (2)$$

$$E = \left[\frac{N \times d}{I} \right] \times 100 \qquad (3)$$

Where:

N_{rel} = the percentage of colony forming units released from the swab bud

N = the mean number of colony forming units counted on replicate plates

d = dilution factor

N_{rem} = the percentage of colony forming units removed from the surface (as calculated in Section 2.2.5)

I = the number of colony forming units theoretically inoculated onto the surface

E = the efficiency of the bacterial surface sampling technique

Modifications to the above procedures will be discussed in relation to the results to which they apply.

2.2.7. Assessing the Change in Microbial Viability Over Time

The *Salmonella* and *Listeria* strain were suspended in either ¼ strength Ringer solution or bovine serum albumen (Sigma-Aldrich, Poole, Dorset, UK) (BSA). A 12.5 µl aliquot containing approximately 10^3 cells was inoculated onto the stainless steel coupons, spread evenly over the surface and allowed to air-dry for 1 h. At 10 min intervals throughout this 60 min period, control (un-swabbed) coupons (n = 10) were overlaid with PCA. The plates were incubated at 30°C for 48 h, after which time, any colonies present on the surface of the coupons were counted.

2.2.8. Statistical Analysis

To determine whether parametric or non-parametric techniques should be used to analyse the data, a normal probability plot of each set of results was constructed using MINITAB for Windows version 12. If the results followed a normal distribution then statistical analysis was performed using Microsoft Excel 2000. Statistical significance set at a level of $p < 0.05$ was determined by means of either t-tests or the analysis of variance (ANOVA). When the ANOVA indicated that differences between means existed, Tukey's Multiple Comparison Test was used to determine which of these means differed

significantly from one another (Hassard, 1991). If the data did not appear normally distributed then MINITAB was used to perform the non-parametric Mann-Whitney test.

2.3. Results

The efficiency of a bacterial surface sampling technique can be defined as its ability to recover microorganisms from a surface. The results presented in Table 2.2 confirm that the efficiency of the traditional swabbing technique is poor. When a wet stainless steel surface was sampled using a pre-moistened cotton-tipped hygiene swab, just 6% of the original inoculum was recovered (Section 2.2.6). Nevertheless, this was significantly greater ($p < 0.05$) than when the surface sampled was dry. Under these circumstances the efficiency of the swabbing technique did not exceed 0.2%.

Table 2.2. The effect of surface dryness (n = 5) upon the efficiency of the traditional swabbing technique.

Efficiency of swabbing technique* **(mean % ± 2 SE)**	
Wet surface†	**Dry surface†**
6.32 ± 2.52	0.15 ± 0.30

* cotton-tipped hygiene swabs pre-moistened with ¼ strength Ringer solution

† test organism: *Salmonella* sp. suspended in ¼ strength Ringer solution

The main objectives of the current investigation were to investigate the factors that may influence the recovery of microorganisms using the traditional cotton-tipped hygiene swab and to assess whether swabbing efficiency could be improved by altering swab type and/or

swab-wetting solution (Section 2.1). Thus, the following results are both presented and discussed in two sections and relate to each of these objectives in turn.

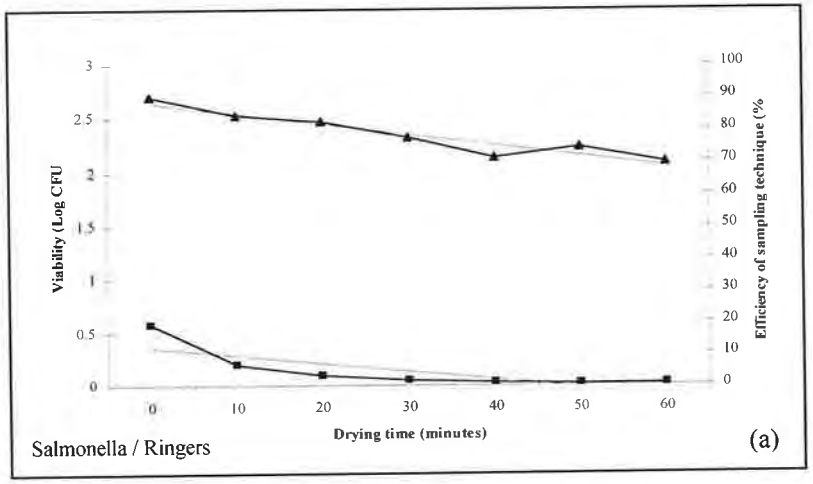
2.3.1. Factors Influencing the Recovery of Microorganisms using the Traditional Cotton-tipped Hygiene Swab

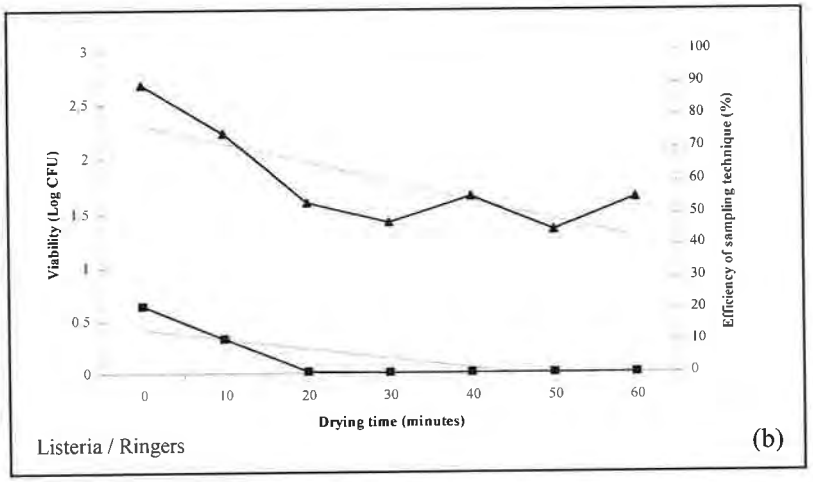
2.3.1.1. The relationship between microbial viability and swabbing efficiency

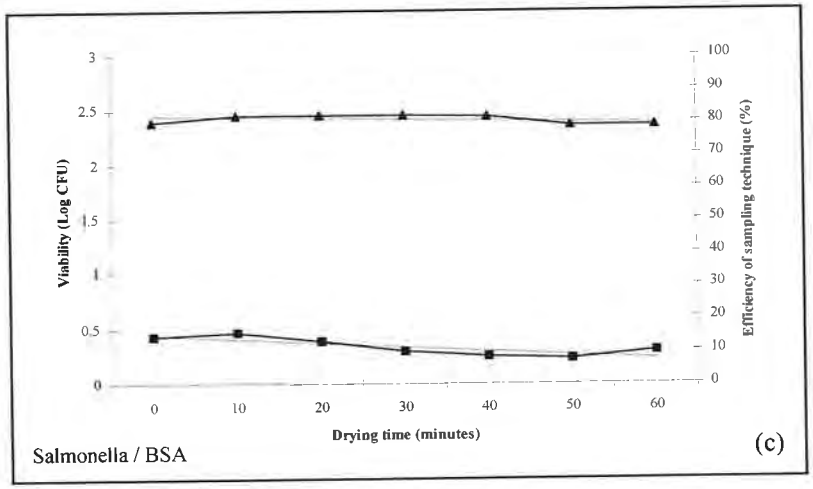
The results presented in Figure 2.1 illustrate the change in microbial viability over time (Section 2.2.7). When suspended in ¼ strength Ringer solution, the number of viable *Salmonella* colonies present on a wet surface (Time 0) was approximately 2.7 log values (Figure 2.1a). After being allowed to air-dry for 60 min, this number fell by approximately 0.6 log values to 2.09 log values. In comparison, the *Listeria* strain (Figure 2.1b) appeared more sensitive to the effects of drying and the number of viable colonies present on the surface was observed to range from 2.68 to 1.64 log values after a 0 min and 60 min drying time respectively. The addition of nutrients to the suspending medium appeared to increase the ability of both organisms to survive over the 60 min drying period. When suspended in BSA, there was only a minimal loss in *Salmonella* viability (Figure 2.1c) and a reduction of 'only' 0.6 log values in the viability of the *Listeria* strain (Figure 2.1d).

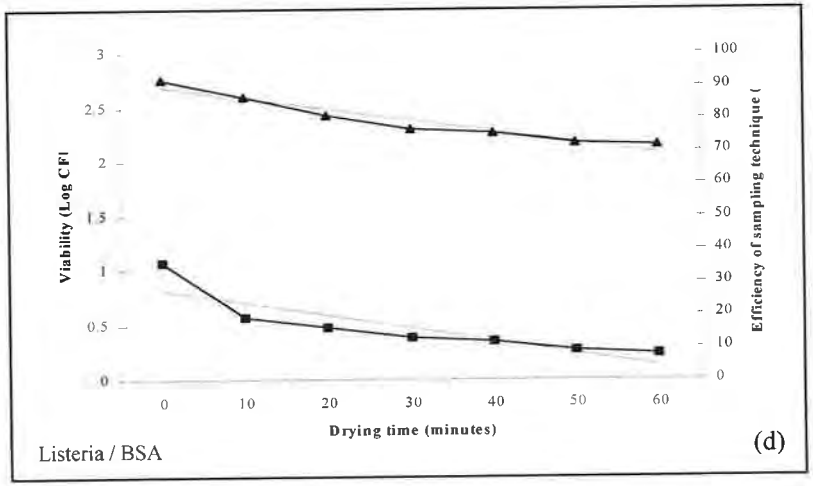
The corresponding change in sampling efficiency (Section 2.2.6), when pre-moistened cotton swabs were used to sample the stainless steel surfaces, is also illustrated (Figure 2.1). In all cases, optimal sampling efficiency was achieved by swabbing a wet surface (Time 0) which, when the *Salmonella* (Figure 2.1a) and *Listeria* (Figure 2.1b) strains were suspended in ¼ strength Ringer solution, equated to just 19% and 21% respectively. When the *Salmonella* strain was suspended in BSA (Figure 2.1c), this optimal sampling

Figure 2.1. The change in mean viability (-▲-) of a *Salmonella* ((a), (c)) and *Listeria* ((b), (d)) strain after each had been suspended in either ¼ strength Ringer solution ((a), (b)) or bovine serum albumen ((c), (d)), inoculated onto a stainless steel surface (n = 10) and allowed to air-dry for 60 min. The corresponding change in mean sampling efficiency is also illustrated (-■-).









efficiency was significantly reduced ($p < 0.05$). Conversely, altering the suspending medium significantly improved ($p < 0.05$) the efficiency of the swabbing technique when it was used to sample for *Listeria* (Figure 2.1d).

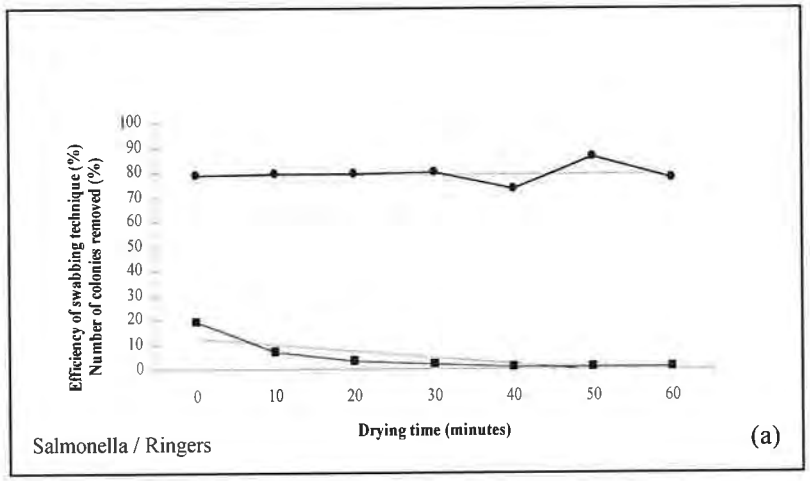
In all cases, sampling efficiency was seen to fall steadily over time (Figure 2.1) and this reduction appeared to correspond with the observed reduction in microbial viability - minimal reductions in swabbing efficiency occurred when there were minimal losses in microbial viability. When the *Salmonella* and *Listeria* strains were suspended in BSA and allowed to dry for 60 min, the sampling efficiency was 9.5% and 7.8% respectively (Figure 2.1c and d). In comparison, sampling a dry surface for *Salmonella* and *Listeria* colonies originally suspended in ¼ strength Ringer solution, resulted in a swabbing efficiency of only 0.52% and 0.12% respectively (Figure 2.1a and b).

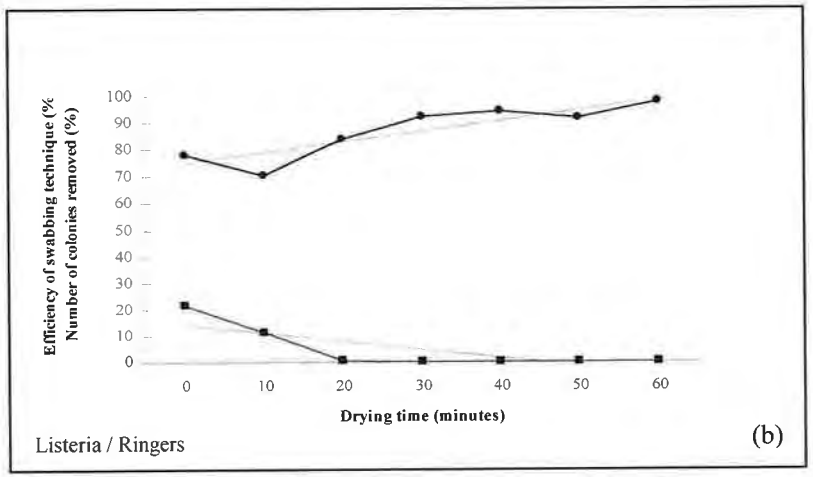
2.3.1.2. The relationship between swabbing efficiency and the ability of a cotton swab to remove bacteria from a surface

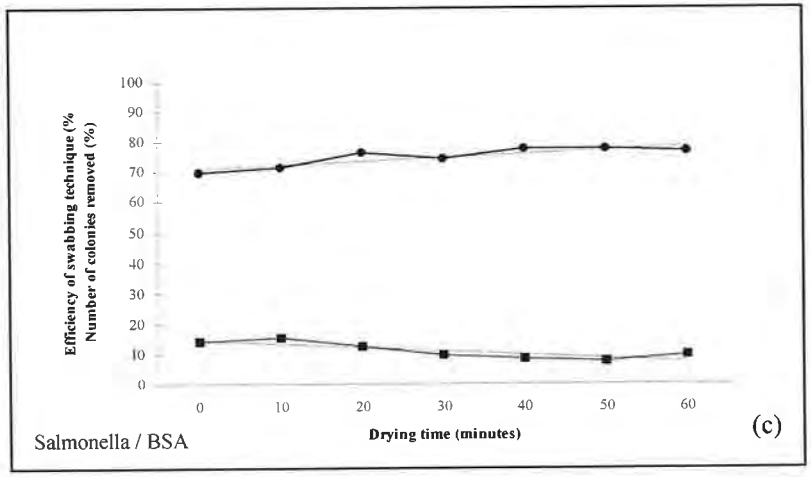
Poor sampling efficiency could be due to insufficient numbers of microorganisms being picked up from a surface. Figure 2.2 illustrates the mean percentage of bacteria that was removed from a stainless steel surface using a sterile pre-moistened cotton swab (Section 2.2.5). How bacterial pick-up changed over time (i.e. as the surface was allowed to dry) is also shown, as is the corresponding change in sampling efficiency.

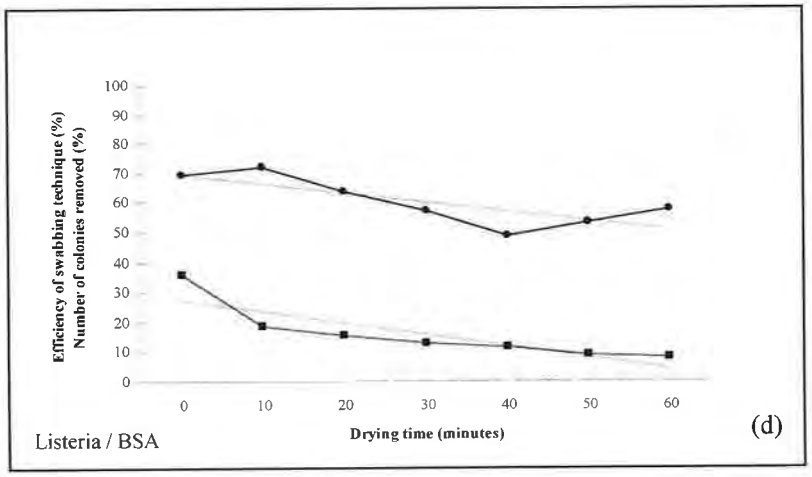
Pre-moistened cotton swabs were capable of removing, from a wet surface, approximately 79% of *Salmonella* colonies, which prior to inoculation had been suspended in ¼ strength Ringer solution (Figure 2.2a). Although, this level of bacterial pick-up remained relatively consistent throughout the 60 min period ($p > 0.05$), overall swabbing efficiency was seen

Figure 2.2. The mean percentage of a *Salmonella* ((a), (c)) and *Listeria* ((b), (d)) population, suspended in either ¼ strength Ringer solution ((a), (b)) or bovine serum albumen ((c), (d)) that was removed from a stainless steel surface (n = 10) using a pre-moistened cotton swab (-●-). The corresponding change in mean sampling efficiency is also illustrated (-■-).









to fall. Likewise, despite approximately 98% of similarly suspended *Listeria* colonies being removed from a dry surface, which in turn was significantly higher ($p < 0.05$) than that removed when the surface sampled was wet (Figure 2.2b), swabbing efficiency was also observed to decrease over time.

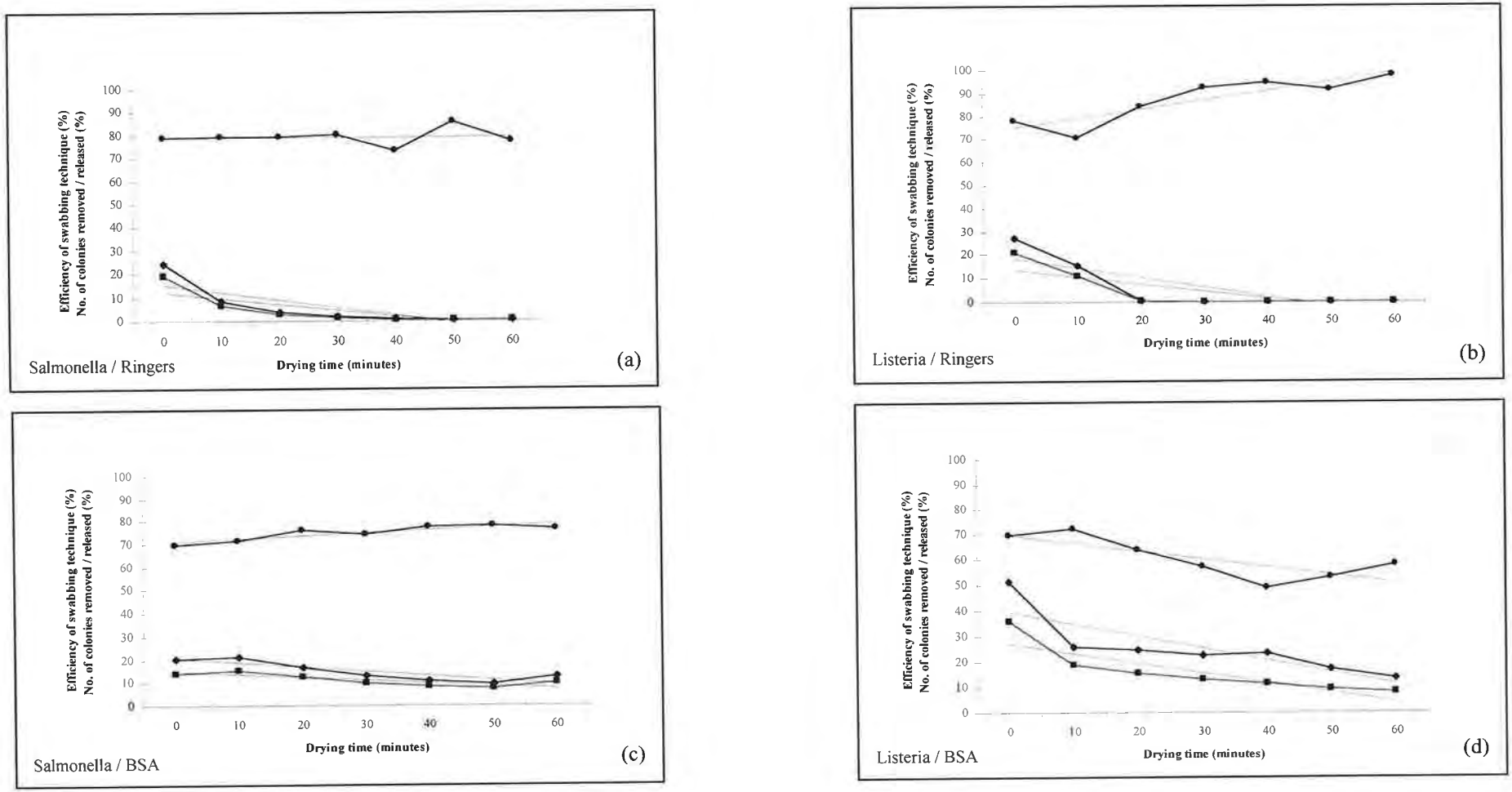
Inoculating the coupons with the *Salmonella*/BSA suspension (Figure 2.2c) also appeared to significantly increase ($p < 0.05$) the number of colonies that could be removed from the surface over time. In this case, the number of colonies removed from a wet and dry surface was approximately 69% and 77% respectively. Conversely, when the *Listeria* strain was suspended in BSA, the percentage of colonies removed from a wet and dry surface was approximately 69% and 58% respectively (Figure 2.2d). This difference was also significant ($p < 0.05$) and suggests that under these conditions, the *Listeria* colonies had become more difficult to remove over time and, in this case, may have contributed to the reduction in sampling efficiency.

2.3.1.3. The relationship between swabbing efficiency and the ability of the cotton swab to release bacteria into a diluent

Poor sampling efficiency may also result from the retention of microorganisms within the swab bud itself. The results presented in Figure 2.3 take into consideration the percentage of the original inoculum that was removed from the surface during sampling and, therefore, represent the mean percentage of bacteria theoretically present on the swab, which was released from the cotton bud during vortexing.

Highest bacterial release was achieved after a wet surface (Time 0) had been sampled. Nevertheless, when the *Salmonella* (Figure 2.3a) and *Listeria* (Figure 2.3b) strains were

Figure 2.3. The mean percentage of a *Salmonella* ((a), (c)) and *Listeria* ((b), (d)) population, suspended in either ¼ strength Ringer solution ((a), (b)) or bovine serum albumen ((c), (d)), that was removed from a stainless steel surface (n = 10) during swabbing (-●-). The mean percentage of these bacteria that were subsequently released from the cotton bud (-◆-) and the corresponding change in mean sampling efficiency (-■-) are also illustrated.



suspended in ¼ strength Ringer solution, this was still only 24% and 27% respectively. In this case, there was no significant difference ($p > 0.05$) in the ability of the cotton swab to release these two different organism types. However, when suspended in BSA, 51% of those *Listeria* colonies removed from a wet surface were released (Figure 2.3d), compared to just 20% of similarly suspended *Salmonella* colonies (Figure 2.3c).

Irrespective of organism type or suspending medium, as surface drying time increased, the percentage of bacteria released from the swab bud decreased. In all cases, this reduction was significant ($p < 0.05$) but was especially marked when the bacteria were suspended in ¼ strength Ringer solution. In this case, the percentage of *Salmonella* and *Listeria* colonies released from those swabs used to sample a dry surface (Time 60) was just 0.67% and 0.12% respectively (Figure 2.3a and b). Although this reduction was less pronounced when the bacteria were suspended in BSA, the proportion of *Salmonella* and *Listeria* colonies released from the bud was still only 12% and 13.5% respectively (Figure 2.3c and d.)

2.3.2. Means of Improving the Efficiency of the Traditional Swabbing Technique

2.3.2.1. Effect of swab type and swab-wetting solution upon the number of bacteria removed from a stainless steel surface

Table 2.3 shows the percentage of bacteria that were removed from a stainless steel surface using a variety of different swab types. Depending upon which swab was used, the number of *Salmonella* colonies removed from a wet and dry surface ranged from approximately 47% to 82% and from 41% to 79% respectively. Thus, regardless of swab type, the number of *Salmonella* colonies removed from a surface did not appear to be

greatly influenced by moisture level. In both cases, there were no significant differences ($p > 0.05$) between the number of colonies removed by the cotton, foam and alginate swabs. However, dacron swabs removed significantly fewer ($p < 0.05$) *Salmonella* colonies, from both a wet and dry surface, than any of the other three swab types.

Table 2.3. The percentage of bacterial colonies removed from a stainless steel surface using a range of different swab types.

	mean ± 2 SE (n = 70) / median number of colonies removed (%)			
	Swab type			
	COTTON	**DACRON**	**FOAM**	**ALGINATE**
Wet surface*				
Salmonella	82.41 ± 2.26	47.00 ± 11.69	78.74 ± 2.47	73.03 ± 2.17
Listeria	58.64 ± 3.81	31.03 ± 4.95	33.14 ± 6.85	40.32 ± 4.80
Dry surface*				
Salmonella	73.73 ± 4.42	40.97 ± 5.80	72.68 ± 3.84	79.49 ± 2.57
Listeria	79.66 ± 4.20	56.23 ± 11.51	63.24 ± 8.60	73.77 ± 5.13
Biofilm†				
Pseudomonas	18.84	28.95	76.65	60.58

* bacterial colonies suspended in ¼ Ringer solution

† see Section 2.2.5

The number of *Listeria* colonies removed from a wet surface was again (Figure 2.2b) significantly lower ($p < 0.05$) than that from a dry surface and, depending upon swab type used, was seen to range from approximately 31% to 58% and from 56% to 79% respectively (Table 2.3). When a wet surface was sampled, there were no significant differences ($p > 0.05$) between the number of *Listeria* colonies removed by the dacron, foam and alginate swabs but all removed significantly fewer colonies than the cotton swabs ($p < 0.05$). The cotton swabs also removed a significantly greater percentage

($p < 0.05$) of those *Listeria* colonies present on a dry surface than both the dacron and foam swabs but not, in this case, the alginate swabs ($p > 0.05$).

When the swabs were used to sample a surface that had been kept wet for 4 h, the foam swabs were capable of removing approximately 77% of those *Pseudomonas* colonies that had been encouraged to form a 'biofilm'. In this case, this was significantly greater than that removed by the alginate swabs ($p < 0.05$) and both these swab types removed a significantly greater number of colonies than either the cotton or dacron swabs ($p < 0.05$).

Table 2.4 illustrates the percentage of bacteria removed from the stainless steel surfaces using swabs pre-moistened with a variety of different swab-wetting agents. In general, swabbing solution had little effect upon the numbers of bacteria removed from either a wet or dry surface. However, swabs pre-moistened with the Spraycult® removed the lowest ($p < 0.05$) number of *Listeria* colonies from a wet surface and significantly fewer *Salmonella* colonies than those swabs used dry or pre-moistened with either ¼ strength Ringer solution, the 3% Tween solution or the TRIS buffer-based solution ($p < 0.05$). In addition, this latter swab-wetting agent removed a significantly greater number of *Salmonella* colonies than both the 0.1% agar solution and the MES buffer-based solution but significantly fewer *Listeria* colonies than those swabs used dry or pre-moistened with ¼ strength Ringer solution ($p < 0.05$).

When a dry surface was sampled, a significantly greater number of *Listeria* colonies were removed when the swabs were used wet rather than dry, although the 0.1% agar solution removed significantly fewer colonies than swabs pre-moistened with ¼ strength Ringer solution, the TRIS buffer-based solution or the Spraycult® ($p < 0.05$). The latter two swab-wetting agents were also the most effective at removing *Pseudomonas* colonies after this organism had been allowed to attach to the surface for 4 h ($p < 0.05$)

Table 2.4. The percentage of bacterial colonies removed from a stainless steel surface using swabs pre-moistened with a variety of swab-wetting agents.

	mean ± 2 SE (n = 40) / median number of colonies removed (%)						
	Swab-wetting solution						
	Dry swab	**¼ strength Ringer solution**	**0.1% agar solution**	**MES-buffer based solution**	**TRIS-buffer based solution**	**3% Tween solution**	**Spraycult®**
Wet surface							
Salmonella	73.64 ± 4.43	71.73 ± 3.58	65.66 ± 5.60	68.77 ± 2.91	87.27 ± 2.95	74.09 ± 2.10	50.36 ± 21.23
Listeria	51.87 ± 6.99	56.16 ± 5.62	43.49 ± 3.98	44.85 ± 4.40	32.34 ± 5.27	42.55 ± 6.75	14.60 ± 10.57
Dry surface							
Salmonella	62.38 ± 6.55	62.77 ± 8.90	73.26 ± 6.81	61.97 ± 7.09	72.66 ± 6.61	65.26 ± 8.98	68.69 ± 6.05
Listeria	34.13 ± 17.34	85.53 ± 3.54	59.61 ± 13.85	64.90 ± 7.43	85.07 ± 4.04	68.82 ± 6.78	80.43 ± 4.89
Biofilm							
Pseudomonas	65.31	59.79	-38.20	20.91	89.42	20.81	90.62

2.3.2.2. Effect of bud material and swab-wetting solution upon the number of bacteria released from a swab into a diluent

The percentage of bacteria released from a swab naturally relates to the number theoretically present on the bud (i.e. the number initially removed from the surface). However, during this investigation, particularly when a wet surface was sampled, certain swab/solution combinations resulted in a higher number of colonies being present after swabbing than before. In some cases, this appeared to result in a 'negative pick-up' (Table 2.4), which in turn, created a slight problem in terms of interpreting some of the results obtained during this study (see Section 2.4.2.1). For this reason, the results relating to the number of bacteria released from swabs used to sample a 'biofilm' have been omitted from the following tables.

Similarly, when used to sample a wet surface, swabs pre-moistened with the Spraycult® appeared to release a negative number of colonies (Table 2.5). These nonsensical results were mainly due to the dacron swab/Spraycult® combination increasing the number of bacteria on the surface during swabbing. When the figures associated with the Spraycult® were omitted from the overall results, the percentage of *Salmonella* and *Listeria* colonies released from the dacron swabs increased from approximately 7% and 9% to approximately 14% and 39% respectively (Table 2.6). Thus, dacron swabs were as effective in releasing *Listeria* colonies as the other three swab types and released a significantly similar percentage of *Salmonella* colonies as the alginate swabs ($p > 0.05$); significantly greater ($p < 0.05$) than those swabs tipped with either cotton or foam. With regard to the effect of the other swab-wetting agents, when used to sample a wet surface there were very few differences that proved significant (Table 2.5).

Table 2.5. The mean percentage of bacterial colonies released from the bud of swabs that had been pre-moistened with a variety of swab-wetting agents and used to sample a wet and dry stainless steel surface.

	mean ± 2 SE (n = 40) or median number of colonies released (%)						
	Swab-wetting solution						
	Dry swab	**¼ strength Ringer solution**	**0.1% agar solution**	**MES-buffer based solution**	**TRIS-buffer based solution**	**3% Tween solution**	**Spraycult ®**
Wet surface							
Salmonella	8.72 ± 2.31	14.81 ± 3.18	18.98 ± 3.51	12.42 ± 1.49	2.41 ± 0.84	12.87 ± 3.01	-4.74 ± 6.69
Listeria	17.79 ± 5.35	37.45 ± 5.56	58.16 ± 10.10	63.60 ± 9.51	74.38 ± 12.26	60.02 ± 13.53	-55.77 ± 41.02
Dry surface							
Salmonella	0	0.32	0.13	0.36	0.18	0.37	0
Listeria	0	0	0.02	0.08	0	0.06	0.02

Simply pre-moistening the swabs resulted in a significantly greater percentage of *Listeria* colonies being released than if the swabs were used dry and using swabs that had been pre-moistened with the TRIS buffer-based solution led to the release of significantly fewer *Salmonella* colonies ($p < 0.05$).

Table 2.6. The percentage of bacterial colonies released from the bud of a range of different swab types after they had been used to sample a wet and dry stainless steel surface.

	mean ± 2 SE (n = 70) / median number of colonies removed (%)			
	Swab type			
	COTTON	**DACRON**	**FOAM**	**ALGINATE**
Wet surface*				
Salmonella	8.49 ± 1.48	14.36 ± 3.13 (7.23 ± 5.34)†	6.64 ± 1.10	14.90 ± 2.44
Listeria	57.17 ± 7.35	39.39 ± 7.88 (9.03 ± 22.29)†	39.67 ± 22.66	38.82 ± 6.26
Dry surface*				
Salmonella	0.18	0	0.34	0
Listeria	0.02	0	0.07	0

* bacterial colonies suspended in ¼ Ringer solution

† calculation includes the results that were obtained when dacron swabs pre-moistened with the Spraycult® were used to sample the stainless steel surfaces

The number of colonies released from swabs used to sample a dry surface was significantly ($p < 0.05$) lower than after a wet surface was sampled (Table 2.6). Nonetheless, foam swabs released a significantly greater percentage of both *Salmonella* and *Listeria* colonies than cotton swabs, which in turn, released significantly more bacterial colonies than either the dacron or alginate swabs ($p < 0.05$). Swab-wetting solution again had little effect upon the extent of bacterial release (Table 2.5). However, in general, those swabs pre-moistened with solutions containing Tween 80, released significantly more bacteria than when any of the other swab-wetting agents were used ($p < 0.05$).

2.3.2.3. *Effect of different swab types and swab-wetting solutions upon the overall efficiency of the swabbing technique*

When sampling a wet surface for *Salmonella*, optimum sampling efficiency was just 10% and was achieved using either a dacron or alginate swab (Table 2.7). Conversely, when a dry surface was sampled, the use of either of these swab types resulted in a significantly poorer sampling efficiency ($p < 0.05$) than when either the cotton or foam swabs were used. The use of a foam swab also resulted in the highest swabbing efficiency when sampling a dry surface for *Listeria*. However, in this case, when a wet surface was sampled, optimum sampling efficiency could be achieved by using either a foam or cotton swab and equated to approximately 25% and 31% respectively. When sampling a surface that had become associated with a 'biofilm', the use of cotton swabs enabled approximately 8% of the original inoculum to be recovered; a significantly greater number than when foam swabs were used ($p < 0.05$). The use of either of these swab types resulted in a significantly greater sampling efficiency than when either the dacron or alginate swabs were used ($p < 0.05$).

In general, swab-wetting solution did not affect swabbing efficiency (Table 2.8). However, when sampling a wet surface for either *Listeria* or *Salmonella*, the respective use of dry swabs or those pre-moistened with the TRIS buffer based solution, resulted in the lowest sampling efficiency ($p < 0.05$). When a dry surface was sampled, optimum sampling efficiency was achieved using swabs that had been pre-moistened with solutions containing Tween 80. Nevertheless, regardless of organism type, this did not exceed 0.3%. The addition of Tween to a solution also appeared to improve the efficiency of the swabbing technique when it was used to sample a 'biofilm', as did the use of swabs, which had been pre-moistened with the 0.1% agar solution. Nevertheless, this optimum sampling efficiency did not exceed 8%.

Table 2.7. The effect of swab type upon the efficiency of traditional hygiene swabbing.

	mean ± 2 SE (n = 70) / median number of colonies removed (%)			
	Swab type			
	COTTON	**DACRON**	**FOAM**	**ALGINATE**
Wet surface*				
Salmonella	6.72 ± 1.17	9.63 ± 1.85	4.97 ± 0.78	10.63 ± 1.75
Listeria	31.48 ± 3.63	12.16 ± 2.08	25.14 ± 2.69	13.68 ± 1.75
Dry surface*				
Salmonella	0.15	0	0.22	0
Listeria	0.02	0	0.04	0
Biofilm†				
Pseudomonas	7.72	2.63	5.42	3.28

* bacterial colonies suspended in ¼ Ringer solution

† see Section 2.2.5

2.4. Discussion

2.4.1. Factors Influencing the Recovery of Microorganisms using the Traditional Cotton-tipped Hygiene Swab

2.4.1.1. The relationship between microbial viability and swabbing efficiency

Although the pour plate technique is widely used and accepted, previous studies have highlighted problems associated with the recovery of bacteria using traditional hygiene swab methodology (Section 2.1). Davidson *et al.* (1999) have reported that swabbing performance is particularly affected when a dry surface is sampled and it has been

Table 2.8. The effect of swab-wetting solution upon the efficiency of the traditional hygiene swabbing technique.

	mean ± 2 SE (n = 40) / median efficiency (%)						
	Swab-wetting solution						
	Dry swab	**¼ strength Ringer solution**	**0.1% agar solution**	**MES-buffer based solution**	**TRIS-buffer based solution**	**3% Tween solution**	**Spraycult ®**
Wet surface							
Salmonella	6.40 ± 1.67	10.39 ± 1.97	11.68 ± 1.92	8.48 ± 0.97	1.73 ± 0.67	9.42 ± 2.17	7.82 ± 2.63
Listeria	8.81 ± 2.46	20.85 ± 3.30	26.60 ± 5.53	27.87 ± 4.27	23.02 ± 3.46	21.57 ± 4.43	16.20 ± 3.31
Dry surface							
Salmonella	0	0.15	0.11	0.22	0.15	0.23	0
Listeria	0	0.06	0.02	0.06	0	0.04	0.01
Biofilm							
Pseudomonas	3.28	3.28	7.72	6.56	1.63	6.84	3.67

suggested that the observed reduction in swabbing efficiency is due to a loss in microbial viability (Davidson *et al.* 1999; Gehrig *et al.* 2000). Bacteria are unable to perform normal cell functions without water (McEldowney and Fletcher, 1988). Most microorganisms require a medium with a water potential greater than –10 MPa for growth. For lower water potentials, such as those that are obtained during drying, microorganisms can no longer grow and only survive in anabiosis (Marechal *et al.* 1999). Death can occur due to alterations in membrane properties resulting in damage to the cytoplasmic and/or outer membrane and subsequent loss of essential cell components (Hurst, 1977).

Bacteria often regulate their response to environmental stresses through the activation or induced expression of specific transcription factors (O'Byrne and Booth, 2002). In both gram-negative and gram-positive organisms, the products of the genes transcribed by the alternative sigma factors, RpoS and SigB (σ^B) respectively, act to protect the cells from a diverse range of environmental stresses, which include for example, oxidative stress, osmotic stress, low pH stress and starvation (Ferreira *et al.* 2001). Specific environmental triggers, which lead to elevated levels of RpoS or σ^B in the cell have, therefore, the potential to confer cross protection against multiple stresses (O'Byrne and Booth, 2002). For instance, the increased tolerance of *Salmonella enteritidis* phage type 4 to elevated temperatures is reportedly accompanied by an increased ability to survive in the presence of acid, hydrogen peroxide and on surfaces (Humphrey *et al.* 1995).

Cells in the stationary phase of growth are more resistant than those in the exponential phase to a number of stress factors, including those associated with drying (Barnes *et al.* 1996). In gram-negative organisms, a key factor in producing this increased resistance is the maximum induction of RpoS on entry into stationary phase (Dodd and Aldsworth, 2002). The growth curves associated with the *Salmonella* and *Listeria* strains used during this investigation, illustrate that 18 h incubation was sufficient for both cultures to reach a

stationary phase of growth. However, the rate of exponential growth associated with the *Salmonella* strain was faster and, therefore, after 18 h this organism had reached mid-stationary phase as opposed to the early stationary phase reached by the *Listeria* strain (Appendix I). Possible differences may have existed, therefore, between the cellular levels of translated stress response proteins and this may have been the reason why, unlike previous studies (Hirai, 1991; Lemmen *et al.* 2001), the gram-positive organism appeared to lose its viability faster under drying conditions, than did the gram-negative *Salmonella* sp. Nevertheless, the results presented in Figure 2.1, strongly suggest that both the *Salmonella* and *Listeria* strains tolerated the dry conditions and that substantial numbers of bacteria initially present on the surface did survive drying.

Despite appearing smooth to the unaided eye, stainless steel when viewed under a microscope is very rough, with distinct flaws that can harbour bacterial cells (Stone and Zottola, 1985). Should water and/or nutrients also be present then microbial survival may be enhanced and Scott and Bloomfield (1990) have reported that microbial survival on soiled surfaces can range from 4 to 24 h. The presence of macromolecular nutrients may alleviate desiccation damage by providing some protection against dehydration (McEldowney and Fletcher, 1988) and a further study has demonstrated that in the presence of proteins, the viability of bacteria in the dry state increases. Hirai (1991) stated that this "protein effect" can be marked, suggesting that it is possible to detect viable *Salmonella typhimurium* and *Escherichia coli* cells after 5 and 10 days respectively. Such extensive survival is likely to depend upon a high level of microbial contamination initially being present on the surface. However, the results presented in Figure 2.1c and d concur with these previous studies and illustrate that when the *Salmonella* and *Listeria* strains were suspended in bovine serum albumen (BSA), the number of organisms surviving the 60 min drying period was greater than when they were allowed to dry suspended in ¼ strength Ringer solution (Figure 2.1a and b). This comparative increase in viability was

accompanied by a slight increase in swabbing efficiency and suggests that losses in microbial viability can contribute to the reduced efficiency of surface hygiene swabbing. However, even when a loss in viability did not appear to occur (Figure 2.1c), the efficiency of the swabbing technique did not exceed 15%. This concurs with the findings of Abrishami *et al.* (1994), who reported that more than 90% of an *E. coli* inoculum, which had been allowed to dry for 2 h on the surface of a cutting board, could not be recovered, despite 75% of these cells remaining viable. Thus, a loss in microbial viability is not the only contributing factor in reducing the sensitivity of cotton hygiene swabs and other factors must be influencing the recovery of microorganisms from the surface.

2.4.1.2. The absorption of the cotton bud and its ability to remove microorganisms from a surface

For organisms to survive and to persist within food processing plants, it is important that they adhere to surfaces, preferably in high numbers, before cleaning and disinfection procedures take place (Chapter 1). As a result, this initial adsorption can be rapid and previous studies have reported that the attachment of bacteria to a variety of different materials can occur within minutes, with some listeriae even becoming instantaneously bound (Mafu *et al.* 1990; Notermans *et al.* 1991; Lundén *et al.* 2000; Beresford *et al.* 2001). The accurate detection of microbial contaminants, using the traditional swabbing technique, relies initially upon the ability of the swab to remove such microorganisms from the surface. Consequently, often cited as a reason for a low microbial recovery, is the inability of cotton swabs to pick up sufficient numbers of organisms from a surface (Bredholt *et al.* 1999; Salo *et al.* 1999; Taku *et al.* 2002). However, the results presented in Figure 2.2a and b, suggest that pre-moistened cotton swabs are capable of removing approximately 80% of a bacterial population from a surface. Furthermore, rather than the

bacteria appearing to adhere more firmly to a dry surface, as was suggested by Davidson *et al.* (1999), the results imply that they are as easy (Figure 2.2a), if not easier (Figure 2.2b) to remove, as those from a wet surface.

It is hypothesised that the ability of a cotton swab to remove a high proportion of bacteria from a surface is due to the natural absorbency of cotton fibres. The primary factor determining moisture absorption is the presence in the fibre molecule of any group that strongly attracts water, for example the hydroxyl groups of cellulose (Hearle, 1963). Cotton, a natural fibre is composed primarily of cellulose (Bailey *et al.* 1963), thus, it is likely to be capable of absorbing a relatively high volume of a liquid present on a surface, together with any bacteria contained within it, which become dislodged from the surface during swabbing. However, this 'absorption theory' implies that a lack of moisture on a surface would impede the removal of microbial contaminants. Nevertheless, the ability of a cotton swab to absorb and hold a relatively large volume of swab-wetting solution would naturally lead to a large quantity of this solution coming into contact with the surface during swabbing. This liquid would then help in detaching organisms and also be available for re-absorption hence the removal of bacteria from a dry surface can be significantly improved by using a wet swab (Salo and Wirtanen, 1999).

In the food processing environment, stainless steel surfaces come into contact with fluids containing various levels of food components. Within minutes, these molecules adsorb to the surface and form a conditioning film, which is likely to change the physiochemical properties of the substratum (Section 1.1.1). Meat juice for example, has been shown to reduce the negative charge of a clean stainless steel surface, thus, improving the interaction with negatively charged microorganisms (Zottola and Sasahara, 1994). Fouled surfaces can, therefore, facilitate attachment (Bagge *et al.* 2001) and have been shown to attract 10-100 times more vegetative cells than clean surfaces (Flint *et al.* 2001).

Conversely, the number of *E. coli* cells that attach to a stainless steel surface after being suspended for 1h in a complex medium, has been shown to be significantly lower than that when the bacteria are suspended in a minimal salts medium (Dewanti and Wong, 1995). Similarly, in comparison to ¼ strength Ringer solution, the inclusion of milk components to the suspending medium has been shown to reduce the adherence to stainless steel of both *Listeria monocytogenes* and *Salmonella typhimurium* (Hood and Zottola, 1997a). In both cases, it was hypothesised that higher protein levels may have impaired attachment and results from XPS (X-ray photoelectron spectroscopy) analysis of stainless steel have intimated that as the amount of nitrogen at the surface increases, bacterial attachment decreases (Barnes *et al.* 1999). Thus, whether reducing or increasing the level of microbial attachment, the presence of organic residues may improve or impair the ability of a swab to remove bacteria from a surface.

The results presented in Figure 2.2c and d, suggest that cotton swabs are capable of removing approximately 70% of a bacterial population from a wet, protein-soiled surface; approximately 10% fewer bacteria than that removed in the absence of protein (Figure 2.2a and b) implying that the presence of food components may affect the number of organisms removed from the surface during swabbing. Indeed, a steady reduction in the number of *Listeria* colonies removed from the surface was observed to occur over time (Figure 2.2d), suggesting that the cells may have been adhering more firmly and had become more difficult to remove. However, in the presence of proteins, gram-positive organisms have been shown to adhere in higher numbers than gram-negative organisms (Barnes *et al.* 1999) and the proportion of *Salmonella* colonies removed from a dry surface was, again, similar to that removed when the surface sampled was wet (Figure 2.2c).

These results, together with those presented in Figure 2.2a and b, strongly suggest that cotton swabs can and do remove a significant proportion of those bacteria present on a

surface. Furthermore, despite the possibility of bacteria adhering more strongly to a dry surface, it is believed that in the majority of cases, the reduced efficiency of the swabbing technique cannot be attributed to a reduction in bacterial pick-up alone.

2.4.1.3. The absorption of the cotton bud and its effect upon bacterial release

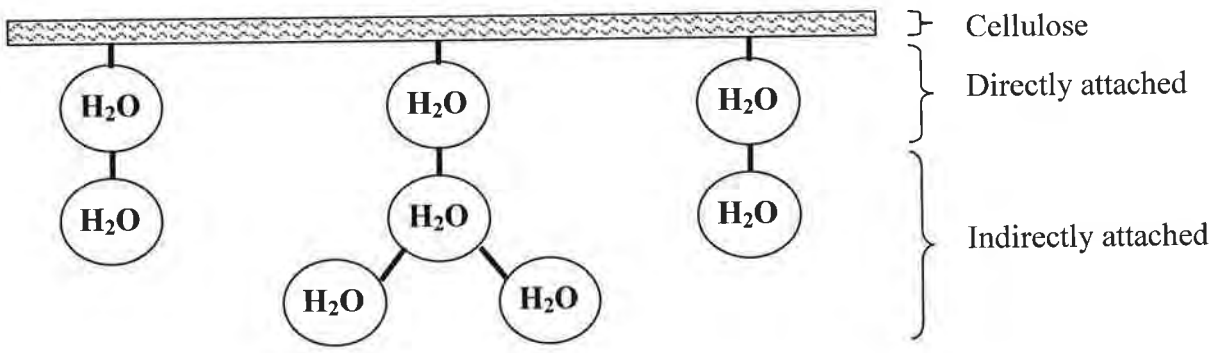
Reliable plate counts will only be obtained if the microorganisms that have been removed from the surface are effectively released from the swab bud. The results presented in Figures 2.3a to d, clearly indicate that the percentage of bacteria released from a cotton swab is low.

The secondary wall of the cotton fibre consists of concentric rings of cellulose. When cotton is wet with water, the fibres undergo limited swelling resulting in the cellulose rings separating into well-defined lamellae. It has been demonstrated that the spaces between these lamellae are the preferred sites for the entry of water and similar reagents (Bailey *et al.* 1963). Approximately 95% of the cellulose of cotton is located in the secondary wall of the fibre. It can be presumed, therefore, that water molecules will be primarily attracted to the cellulose rings (Section 2.4.1.2), leading to the possible entrapment, between the lamellae, of any bacteria removed from the surface. Furthermore, a single cotton fibre possesses a high length-width ratio together with numerous wrinkles, folds and convolutions (Bailey *et al.* 1963), thus, increasing the surface area available for absorption. Those characteristics that may enable a cotton swab to remove a high proportion of bacteria from a surface may, therefore, be the same characteristics that prevent the bacteria from being released from the swab bud.

After a wet surface was sampled, the *Listeria* cells appeared to be released more readily from a cotton swab than the *Salmonella* cells. Typically, *Listeria* cells are narrower than *Salmonella* cells and this size difference may have prevented a greater number of the gram-positive organisms from becoming trapped between the lamellae. These results highlight an issue of potential significance. Under most circumstances, bacteria are rarely present in the form of a pure culture. Differential release of organisms from a cotton swab could, therefore, lead to false impressions being made regarding the microbial population present within any food processing environment.

During absorption, the first water molecules are strongly adsorbed onto the active groups within a fibre. Thereafter, as illustrated in Figure 2.4, as absorption continues, successive layers of water molecules are added. This indirectly attached water is the most easily evaporated (Hearle, 1963) and suggests that the bonds are weaker. Thus, these molecules are also likely to be the most easily removed during vortexing. Consequently, maximum bacterial release, irrespective of organism type or suspending medium, was seen to occur after the swab had been used to sample a wet surface (Figure 2.3a to d).

Figure 2.4. How water molecules attach to a cotton fibre (Hearle *et al.* 1963)



Despite the level of bacteria removed from a surface remaining, in general, relatively consistent, the percentage of these bacteria released from the swab bud was observed to

decrease steadily over time. This reduction in bacterial release appeared to correspond to the reduction in swabbing efficiency. Unlike previous studies, therefore, which have suggested that the original detachment of surface bound organisms is the limiting factor in the swabbing procedure (Salo *et al.* 1999), this present study postulates that the effective removal of organisms from the swab bud is a more important contributing factor with regard to swab sensitivity.

These findings are supported by those of Buttner *et al.* (2001) who demonstrated that whilst the overall efficiency of the swabbing technique *could* be affected by the efficacy of removing bacteria from a surface, the majority of losses occurred during the 'processing steps' (i.e. the release steps). In an attempt, therefore, to optimise the swabbing protocol, the second part of this investigation involved identifying if and how, bacterial pick-up, release and thus, overall recovery could be significantly improved.

2.4.2. Means of Improving the Efficiency of the Traditional Swabbing Technique

2.4.2.1. Effect of swab type and swab-wetting solution upon the number of bacteria removed from a surface

As already discussed, an important factor influencing the number of bacteria that can be removed from a surface appears to be the natural absorbency of the bud material. Many differences between most natural and most synthetic fibres can be explained by the fact that the latter have no active groups and, thus, absorb little or no water (Hearle, 1963). Dacron is a polyester, and polyester fibre is one of the least absorbent of all fibres (Corbman, 1985). Consequently, the results presented in Table 2.3 are not entirely unexpected and indicate that the dacron swabs removed significantly fewer ($p < 0.05$)

Salmonella colonies from a wet surface than either the cotton, foam or alginate swabs. Nevertheless, although this low-level pick-up was likely due, in part, to the bud material, an equally important factor was probably the greater flexibility, in comparison to the other swab types, of the applicator stick associated with the particular dacron swab used during this study.

Any condition or practice that increases the amount of mechanical energy generated has been shown to improve the hygienic efficiency of handwashing (Michaels *et al.* 2001b). Likewise, during swabbing, a greater shear force will remove more cells from a surface (Hood and Zottola, 1995), yet, the flexibility of the dacron swab enabled less pressure to be applied and, consequently, less mechanical energy and a lower shear force to be generated. Thus, the two main factors influencing the number of bacteria that can be removed from the surface appear to be the inherent properties of the swab bud itself and the degree of pressure that can be applied to the swab during sampling. The latter has, in fact, long been recognised and in 1955, during a symposium for determining bacterial contamination on surfaces, it was suggested that to permit a firmer swabbing action, swabs with a stainless steel applicator stick could be used (Walter, 1955).

Nevertheless, even a high level of mechanical energy, generated via vigorous swabbing, reportedly detaches only a small proportion of cells within a biofilm (Wirtanen *et al.* 1999). However, this previous study was conducted using cotton swabs. During handwashing, the use of a coarse paper towel results in a greater proportion of the resident flora being removed from the hands than when a softer cloth towel is used (Michaels *et al.* 2001b). Similarly, during the present investigation, the use of a coarse foam swab resulted in the removal of a greater proportion of those bacteria associated with a 'biofilm' than when swabs tipped with a softer material were used (Table 2.3). In fact, it was possible, through the use of foam swabs, to remove nearly 85% of those *Pseudomonas* colonies that

had been allowed to attach to the surface for 4 h. 'Biofilms' of this age were used to mimic those typically generated during a food-manufacturing run (Willcock *et al.* 2000). However, as already acknowledged, true biofilms include not only the adherent cells but also a matrix of extracellular material, which helps anchor the cells to the surface (Section 1.1.4). The strength of this attachment has been shown to substantially increase with biofilm aging (Eginton *et al.* 1995) and, therefore, other means, in addition to mechanical forces may be needed to remove biofilm effectively (Tuompo *et al.* 1999).

Various substances can be used to improve the detachment of bacteria from surfaces. The addition of a surfactant for example, to a swabbing solution lowers the surface tension of that solution, increasing its ability to contact the entire surface area being sampled (its wetting effect) and helping it to detach cells to be flushed from the surface (its rinsing effect) (Chapter 1; Figure 1.2). Furthermore, the incorporation of a detergent prevents the re-deposition and re-attachment of lifted organisms back onto the surface (Tuompo *et al.* 1999). Similarly, cations, calcium in particular, are thought to play a part in bonding the polymer molecules within a biofilm. The absence of these ions or their chelation by, for example, ethylenediamine tetraacetic acid (EDTA) can, therefore, lead to the biofilm becoming detached (Carpentier and Cerf, 1993). Indeed swabs pre-moistened with either the commercially produced 'biofilm disintegrating reagent' (Spraycult®) or the TRIS buffer-based solution which contained both Triton-X-100 (a non-ionic detergent) and EDTA, removed a significantly greater ($p < 0.05$) proportion of biofilm-associated bacteria than swabs pre-moistened with any of the other swab wetting agents (Table 2.4).

The results presented in Tables 2.3 and 2.4 illustrate that the number of bacteria removed from a surface can be strongly influenced by either the degree of mechanical energy or the type of chemical energy applied to the surface. However, a closer inspection of some of the individual data highlights the importance of using a suitable combination of swab and

swab-wetting agent. The results presented in Table 2.9 illustrate that pre-moistening the least absorbent of the four swabs (i.e. the dacron and foam swabs) with solutions containing a relatively high level of surfactant, significantly reduced the number of *Listeria* colonies removed from a wet surface. Similarly, significantly fewer bacteria associated with a 'biofilm' were removed when these swab types were pre-moistened with solutions containing high levels of surfactant but no chelating agent. In fact, in some cases the results imply that more bacteria were present after swabbing than before strongly suggesting that a relatively high degree of cellular aggregation existed on the steel surfaces.

Table 2.9. Effect of certain swab and solution combinations upon the number of bacteria removed from a surface

Swab-wetting solution	Mean ± 2 SE (n = 10) number of colonies removed (%)			
	***Listeria* (wet surface)**		***Pseudomonas* (biofilm)**	
	Dacron	**Foam**	**Dacron**	**Foam**
¼ strength Ringer solution	47.57± 9.31	67.20± 5.49	34.61± 13.84	74.87 ± 9.64
0.1% agar solution	36.61 ± 5.66	48.71 ± 7.07	-162.73 ± 44.37	39.37 ± 25.28
MES buffer-based solution	51.10 ± 6.24	38.61 ± 7.81	-234.85 ± 64.19	71.29 ± 17.19
TRIS buffer-based solution	24.73 ± 6.64	23.76 ± 7.63	90.75 ± 4.92	99.57 ± 0.70
3% Tween solution	23.55 ± 5.86	27.47 ± 8.84	-255.23 ± 49.42	46.27 ± 11.05
Spraycult®	-3.63 ± 6.92	-14.22 ± 20.06	84.99 ± 4.39	94.09 ± 2.70

Previous studies have used a 1% Tween 80 solution in an attempt to prevent cell clumping (Franz and von Holy, 1994). During the present study, the amount of Tween 80 present on the bud of the swabs may have been sufficient to break up clumps of bacterial cells present on the surface. Additionally, the Tween may also have reduced the surface tension of the liquid on the surface. This enhanced wetting effect may have reduced the mechanical energy generated by the swabbing action and, thus, reduced the number of bacteria removed from the surface. Both instances would have been exacerbated by the inability of these swab types to absorb a large volume of moisture and, as a consequence, any bacterial

cells contained within it. Similar results were observed when the swabs were pre-moistened with the 0.1% agar solution, suggesting that the addition of agar to a swab-wetting solution could have a similar effect to that of a surfactant.

2.4.2.2. Effect of swab type and swab-wetting solution upon the number of bacteria released into a diluent

As already discussed, although the absorbency of a cotton swab appears to play an important role in facilitating the removal of bacteria from a surface, it also seems to hinder their release from the bud. Dacron swabs have been shown to remove significantly fewer bacteria than cotton swabs (Table 2.3). Despite this, after being used to sample a wet surface, they appear to release a statistically similar proportion ($p > 0.05$) of *Salmonella* colonies (Table 2.6) and, thus, the use of a dacron swab can significantly improve overall sampling efficiency (Table 2.7). The small absorption that occurs in a synthetic fibre is believed to be limited to the fibre surface (Hearle, 1963). Consequently, during sampling almost all moisture, rather than penetrating the dacron bud, will lie on its surface. As a result, fewer bacteria may become trapped within the bud, thus, allowing vortexing to remove a greater proportion of them. These results are supported by the findings of Salo *et al.* (1999) who demonstrated, via direct microscopy, that during swabbing, cotton swabs removed a greater proportion of bacteria from a surface than did dacron swabs, yet, the results that were obtained after traditional pour plate methodology was employed, suggested that the opposite was in fact true. Unlike previous studies (Fernandes *et al.* 1996), therefore, the results from this current investigation suggest that the type of swab used to sample a surface can have a significant effect upon the number of bacteria released into a diluent.

How the absorbency of the swab together with the entrapment of bacteria within the bud appears to influence the percentage of bacteria released, is further illustrated in Table 2.6. Researchers have previously reported on the advantages of placing a calcium alginate swab in a sodium hexametaphosphate solution (Calgon Ringers). After a period of vigorous shaking, the calcium alginate dissolves, thereby freeing trapped organisms and resulting in bacterial counts higher than those obtained with a cotton swab (Walter, 1955). In the present study, when used to sample a wet surface, the percentage of *Salmonella* colonies released from an alginate swab was significantly greater ($p < 0.05$) than that released from the other three swab types. Thus, as observed during previous studies (Favero *et al.* 1968), despite alginate swabs removing fewer colonies than cotton swabs (Table 2.3), their use also significantly improves the overall efficiency of the swabbing technique (Table 2.7).

Although the use of alginate swabs resulted in maximum *Salmonella* release, this was still only 15%. In comparison, the number of *Listeria* colonies released from this swab type was approximately 39%, which in this case, was lower than that released from the cotton swabs. However, because of the variability in the number of bacteria recovered from the replicate samples, this difference was not significant. Close agreement between the numbers of bacteria released from cotton and from alginate swabs has been observed in a previous study (Angelotti *et al.* 1958). It was hypothesized that calcium alginate or sodium hexametaphosphate may exhibit some inhibitory properties. The treatment of gram-positive bacteria with 1% sodium hexametaphosphate has since been demonstrated to cause a leakage of cell components (Fukao *et al.* 2000). Such damage reportedly does not occur with gram-negative organisms and it has been speculated that the outer membrane may offer these bacteria protection from the effects of solutions such as Calgon Ringers.

In order to ensure maximal bacterial release, the swabs should be used pre-moistened. However, in the main, when sampling a wet surface, the type of swabbing solution had

little effect upon the number of bacteria released (Table 2.5). Nevertheless, as with swab absorbency, those solutions that appeared to aid the removal of bacteria appeared to hinder their release from the bud. This was particularly apparent when the TRIS buffer-based solution was used to moisten the swabs. Although 87% of the *Salmonella* colonies were removed from a wet surface (Table 2.4), only 2.4% of them were released (Table 2.5).

2.4.3. Limitations of the Experimental Protocol and the Possible Problems Associated with Bacterial Injury

The experimental protocol employed during this study makes the assumption that the reduction in the number of colonies present on the coupons after sampling is due to their removal by the swab. Thus, a further assumption is made in that these bacteria are present on the swab bud. Characteristics of a swabbing solution must not alter the microbial population between swabbing and enumeration. The presence of antimicrobial substances for example, could reduce microbial numbers by causing injury or death (Fernandes *et al.* 1996). To investigate the hypothesis that the swabbing solutions used during the investigation had no beneficial or detrimental properties, microcosms containing each of the solutions together with aliquots of the *Salmonella, Listeria* or *Pseudomonas* strain were analysed (Abrishami *et al.* 1994). In the majority of cases, no adverse effects were imparted upon the viability of the cells. However, the TRIS buffer-based solution and the Spraycult® were both observed to significantly reduce the number of *Salmonella* colonies recovered.

It has been reported that EDTA has no effect upon the growth of either *Salmonella typhimurium* or other gram-negative organisms (Payne *et al.* 1994; Skandamis *et al.* 2001). Furthermore, Triton-X-100 has been documented as being non-toxic to bacteria across a

wide range of dilutions (Tuompo *et al.* 1999). However, other studies have implied that both these components of the TRIS buffer-based solution can cause cellular damage and affect the growth of gram-negative bacteria (Wells *et al.* 1998; Helander and Mattila-Sandholm, 2000). It is possible, therefore, that use of the TRIS buffer-based solution may have resulted in bacterial injury and may have contributed to both the high proportion of *Salmonella* colonies apparently removed from the surface and to the low percentage of them that were apparently released. Conversely, the use of the TRIS buffer-based solution resulted in the highest proportion of *Listeria* colonies being released from the swab bud (Table 2.5). These results support the conclusions of Nedoluha *et al.* (2001) and imply that sampling methods may need to be chosen on the basis of which method best recovers the organism(s) of interest, not which method recovers the most bacteria overall.

Similarly, it has been suggested that plate count methods may not detect all viable cells, particularly those injured by environmental stresses (Yu *et al.* 1993). Although Figure 2.1 suggests that a loss in microbial viability may not be the main contributory factor in reducing sampling efficiency, the drying of the inoculum over time may, nonetheless, have caused sub-lethal damage to the cellular membranes. Such injury can increase the sensitivity of the cells to substances and conditions, such as detergents or excessive agitation and strong shear forces, which may otherwise be tolerated by fully viable organisms (Hurst, 1977; Brashears *et al.* 2001).

Selective agar is known to inhibit the growth of damaged cells (Norwood and Gilmour, 2001), thus, throughout this study a non-selective agar (PCA) was used to overlay the coupons and to culture the bacteria present in the diluent. Nonetheless, the stresses to which the bacteria were subjected during swabbing were likely to be greater than those associated with the DSAP method (Barnes *et al.* 1996). Consequently, whilst the experimental protocol may have allowed those bacteria present on the control (un-

swabbed) coupons to remain culturable, injury to the drying cells, caused by the sampling process itself, may have resulted in them being unable to grow in the medium provided in the cultivation procedure. This would have given the impression that sufficient numbers of bacteria had been removed from the dry surface (Gilbert *et al.* 2001) and contributed to the marked reduction in both the number of bacteria released from the swab and the overall efficiency of the sampling technique.

2.5. Conclusion

In the past year, 25 million environmental swab samples were taken in the U.S. alone (Section 1.4.2.1), yet, no previous investigation has attempted to identify the reason(s) for the acknowledged, and it would seem acceptable, limitations and poor performance of the traditional swabbing technique.

This chapter has discussed a study, innovative in its design that involved the evaluation of each individual component of the swabbing procedure. Whilst the results confirm that the efficiency of the swabbing technique, regardless of swab type, is poor, they also highlight the ineffective release of bacteria from the swab bud as being the most important contributory factor with regard to the recovery of microorganisms from a surface. However, despite evaluating the effects of both sonication and increased vortex time, this investigation was unsuccessful in discovering a more effective means of releasing the bacteria. This finding is supported by previous studies (Lindsay and von Holy, 1997; Sanglay *et al.* 2002), which have speculated that ultrasonic treatment in particular, may lead to bacterial stress or injury, suggesting that such measures may, in fact, further reduce the number of bacteria recovered.

Nevertheless, it *can* be concluded that optimum swabbing efficiency can be achieved by sampling a wet surface with a pre-moistened swab. Under these conditions, the absorbency of the bud material is critical in terms of the number of bacteria removed from the surface. However, the results demonstrate, as in a previous study (Tuompo *et al.* 1999), that when bacteria are effectively removed from a surface, fewer bacteria are recovered. It can be concluded, that in general, the greater the absorbency of the bud material, the higher the number of bacteria that become trapped within the swab fibres. Thus, when testing for *Salmonella* sp., bacterial release and, thus, overall sampling efficiency could be significantly improved by substituting a cotton for a dacron or alginate swab, despite the lower absorbency of the latter swab types resulting in significantly fewer bacteria being removed from the surface. However, in contrast, when sampling for *Listeria* sp. cotton swabs proved the most effective, implying, therefore, that the type of swab used should, perhaps, be chosen on the basis of the organism being tested for.

Regardless of organism type and when sampling a 'biofilm', the most effective swab-wetting agent appeared to be the 0.1% agar solution which, when used to moisten the swab was observed to 'coat' the surface of the bud with a viscous film. This, it is hypothesised, had the effect of minimising the absorption of bacteria into the bud material, which although reducing the number of colonies removed from the steel coupon, kept those that were removed, close to the surface of the bud, thus, allowing them to be more readily eluted from the swab. However, within the food industry it is advisable that solutions used to pre-moisten swabs include agents capable of neutralising the effects of residual detergents and/or disinfectants that may be picked up by the swab during sampling. The results imply, therefore, that a solution similar in formulation to that of the MES buffer-based solution may be a more appropriate, universal, swab-wetting agent.

Nevertheless, the hypothetical 'agar coating' of the swab bud is similar in theory to that of an exciting new swab design. Copan Diagnostics have recently developed a swab that is produced by spraying a layer of absorbent material onto a solid, plastic swab 'bud'. It can be envisaged how such a design would prevent bacteria from becoming trapped within the swab, but, in addition, it is claimed that the bud 'covering' also allows strong capillary action, thus, unlike an 'agar coating', high numbers of bacteria are able to be removed from the surface. A swab allowing superior absorption *and* superior release could revolutionise the traditional swabbing procedure and this new swab device should be evaluated and validated at the earliest opportunity.

Additionally, a solid plastic 'bud', albeit coated with a softer material, is likely to be much harder than a bud composed entirely of material fibres. It is anticipated therefore, that the swabbing action associated with this new swab design would also generate a greater level of mechanical energy than would the use of traditional hygiene swabs and, thus, it may also prove more effective in recovering bacteria associated with a biofilm. However, no explanation has been found as to why the problems associated with bacterial release and recovery appear to be exacerbated when a dry surface is swabbed, but it is strongly suspected that cellular damage, caused by the swabbing action itself, could be an important influencing factor. In comparison, therefore, to those swabs tipped with a softer bud, it is speculated that the new Copan swab may prove less effective when used to sample a dry surface.

Further research is, therefore, warranted. This could involve for example, the use of staining techniques coupled with epifluorescent or confocal microscopy to determine the viability of the cells present on a dry surface before and after swabbing and within the swab bud before and after vortexing. Atomic force microscopy could also be employed as

a means to detect any cell surface defects that occur as the bacteria are allowed to dry on to a surface (Dufrêne, 2002).

Although perhaps unable to provide definitive answers, many of the conclusions drawn from this investigation have since been substantiated and are further discussed in Chapter 7. Furthermore, this study also demonstrates quite clearly that traditional microbiology should not necessarily be presumed either the 'gold standard' or the optimum means to assess the efficacy of a company's sanitation programme. Nonetheless, convenience, simplicity and indeed convention, means that microbial sample data will continue to be used, not least to assess the likelihood of the occurrence of microbial hazards, to establish critical limits and to assess the validity of a HACCP plan (Kvenberg and Schwalm, 2000). Work must continue, therefore, on the design and development of novel swab-based devices and bud materials in an attempt to improve the recovery of microorganisms from both wet and dry food contact and environmental surfaces.

However, regardless of potential and perhaps imminent, improvements in the accuracy and reliability of the traditional swabbing technique, the time involved in obtaining microbial data means that within HACCP, it is not feasible to use conventional microbiological methods for the routine assessment of surface cleanliness. Consequently, an extensive range of non-microbiological test methods, capable of detecting the presence of food residues within minutes, has recently become available to the food industry. However, there appears, at present, no reasoning or logic behind the choice of method used and, thus, no real appreciation as to when, why or how these methods should be employed. This shall be further discussed in Chapters 3 and 4.

Chapter 3

A Laboratory-based Comparison of Traditional and Recently Developed Methods for Assessing Surface Cleanliness within the Food Industry

3.1. Introduction

The food industry has a legal and moral obligation to supply a safe, fresh and organoleptically acceptable product. However, it is now well recognised that the microbiological safety and quality of food cannot be assured using microbiological testing alone (Blackburn, 1999).

The role of the food microbiologist has traditionally centred on end-product analysis. However, although results of such tests can indicate that problems have occurred during processing, they cannot establish the causes of microbial contamination (te Giffel *et al.* 2001). Microbiological methods are typically media and cultivation based and can take 48 h or more to complete. As a result and of particular relevance with regard to 'high-risk' products with a short shelf-life, by the time a defect is discovered a large amount of unsatisfactory or unsafe food may have been produced, distributed, sold and even consumed.

World wide, in an attempt to maintain shelf-life and to reduce the incidence of foodborne disease, food legislation commonly requires the implementation of general good manufacturing practices and, in addition, is increasingly incorporating the Hazard Analysis Critical Control Point (HACCP) philosophy, a proactive approach to food safety based upon the identification and control of specific hazards (Section 1.2). The use of such food

safety management systems has led to greater emphasis being placed upon the real-time monitoring of in-process preventative control measures (Kvenberg and Schwalm, 2000).

The Centre for Disease Control and Prevention (CDC) in Atlanta has identified contaminated equipment and surfaces as being one of five major categories of risk factors that contribute to foodborne disease (FDA Retail Food Program Steering Committee, 2000). On any food contact surface, the high levels of organic material which may be present, can result in the formation of a conditioning film onto which microorganisms may become attached and/or provide an environment for microbial survival and growth (Section 1.1). Failure to remove organic debris is, in turn, a common cause of ineffective disinfection (Section 1.3.3.3) and, thus, dirty surfaces may be a source of both pathogens and food spoilage organisms. Consequently, the inadequate cleaning and disinfection of these surfaces represents a significant risk factor for contamination.

Cross contamination has been identified as being an important contributory factor in a significant proportion of general foodborne disease outbreaks in the UK (Evans *et al.* 1998), Europe (Midelet and Carpentier, 2002) and the USA (Kassa *et al.* 2001). Thus, whilst GMP calls for the regular cleaning of *all* equipment and environmental surfaces within a food production area, for many foods, especially those eaten without further processing, the hygienic status of food contact surfaces may be identified as being critical to food safety. Appropriate cleaning may, therefore, also be designated a control measure that requires monitoring (Section 1.4.1).

Within the food industry, sanitation programmes are designed to reduce the levels of food debris and microorganisms to levels that pose the minimal risk to the safety and quality of the product (Section 1.3.3). However, as yet, no ideal method exists to determine the cleanliness of surfaces and as such, there is no standard method, technique or protocol for

assessing the efficacy of the cleaning and disinfection procedures used (Griffith *et al.* 1997).

Traditionally, the effectiveness of sanitation procedures has been evaluated using immediate visual assessment or microbiological methods, such as hygiene swabs or agar contact plates. However, although the visual inspection of surfaces can reveal gross deficiencies caused by the presence of visible food debris, most food operations require information on surface cleanliness that extends far beyond the sensitivity of this test (Mackintosh, 1990). Furthermore, although conventional hygiene swabbing is widely used, this method, as discussed throughout the previous chapter, recovers only a small proportion of the bacteria present on a surface (Chapter 2). In addition, the time required for microbial growth means that those results that are provided are retrospective and as such have limited value in preventative food safety management systems such as HACCP (Griffith *et al.* 1997).

However, as previously alluded to, although the presence of microorganisms is important, the hygienic status of a surface also depends on the presence or absence of product residues (Mackintosh, 1990). If a surface is unclean because of food debris, then this can soon become a source of both pathogens and food spoilage organisms. 'Modernists' argue, therefore, that when assessing surface cleanliness, it is important to consider *total* organic soil (i.e. microorganisms *and* residual food debris), especially if results can be obtained rapidly (Griffith *et al.* 1997). Such test methods are available to the food industry and their introduction has meant that results can be obtained within minutes allowing remedial action to be implemented before control of a product or process has been lost.

The use of adenosine triphosphate (ATP) bioluminescence as a means to provide, in real-time, an estimate of total surface contamination has been well documented (Cutter *et al.* 1996; Griffiths, 1996; Hawronskyj and Holah, 1997; Lundin, 1999; Chen, 2000) and the

technique has proved particularly beneficial in large manufacturing plants where its regular and frequent use can provide management with data on trends in levels of hygiene (Ogden, 1993; Powell and Atwell, 1997). However, the main reason given, particularly by smaller businesses, for not using ATP analysis is the perceived high-cost of testing – the price of a single luminometer can be as much as £2000. There has, therefore, been an increased interest in the development and use of rapid low-cost and/or instrument-free test methods, many of which detect the presence of chemical residues, such as proteins and/or reducing agents left behind on an inadequately cleaned surface. Low cost instrumentation, or tests requiring no equipment allow cleanliness assessment to be carried out without a burdensome initial expenditure and, as with ATP bioluminescence, by staff with little technical training.

The increasing number of rapid tests being made available to the food industry has increased the importance of the validation, endorsement and international acceptance of these new methods of assessing surface cleanliness (Blackburn, 1999). Not only should their design and application prove advantageous to users, but they must also be as accurate and reliable as traditionally used and accepted methods (Mackintosh, 1990). However, the possible variation in the type and level of organic debris that may be present in any production area can contribute to the problems associated with trying to determine the sensitivity and repeatability of such test methods *in situ* and as result, which test method is best suited for use within any given processing environment (see Chapter 4).

The aims of this chapter are, therefore, to:

- Determine, under controlled laboratory conditions, the limits of detection of a range of recently developed, rapid, low-cost and/or instrument-free test methods for a variety of different food types.
- Compare the performance characteristics of these new methods to those of both ATP bioluminescence and traditional microbiological methods.

Objectives

- Review trade literature and identify the rapid, cleanliness assessment methods currently available to the food industry.
- Select a range of appropriate traditional microbiological techniques.
- Select, on the basis of set criteria, a range of different food types to be used during the comparison study.
- Assess the ability of each test method to detect the presence of decreasing levels of each food residue on a wet surface.
- Assess the ability of each test method to detect the presence of decreasing levels of each food residue on a dry surface.
- Assess the ability of each test method to detect the presence of microbial contaminants on a wet and dry surface.
- Determine the minimum detection limit of each test method for each type of organic debris.

3.2. Materials and Methods

3.2.1. Preparation of Bacterial Cultures

Gram-positive and gram-negative organisms are thought to differ with regard to their susceptibility to the natural drying process (Hirai, 1991; Lemmen *et al.* 2001) and, thus, their ability to survive on food contact surfaces and their recovery and/or detection by various test methods (Davidson *et al.* 1999). For the purposes of this investigation, therefore, one bacterial strain was chosen to represent each of these two organism types.

The recovery of *Listeria* (gram-positive) and *Salmonella* (gram-negative), using the traditional swabbing technique, was studied in detail in Chapter 2. However, these pathogens, if detected on a surface, are likely to be present in very low levels and, consequently, their isolation usually requires a lengthy enrichment process. An alternative, therefore, is to look for an associated indicator organism – a concept, which will be discussed in greater detail in Chapters 6 and 7.

The detection of staphylococci and coliform bacteria can be used to indicate the possible presence of *Listeria* spp (Frank *et al.* 1990) and enteric pathogens (Adams and Moss, 1995) respectively. However, in addition, the ability of these organisms to colonise a range of materials within a variety of different processing environments (Mettler and Carpentier, 1998) means their detection can also provide a general indication as to the overall efficacy of the sanitation procedures applied.

A gram-positive coccus was isolated from a food sample and its growth on Baird-Parker Agar (Oxoid; 63 g l^{-1}) as grey-black, shiny, convex colonies surrounded by a zone of

clearing was taken as presumptive evidence of *Staphylococcus aureus*. Identification was confirmed by testing for the production of coagulase (Staphylase Test; Oxoid).

A gram-negative, lactose-fermenting rod was isolated from the environment and identified using biochemical test strips (API 20E; bioMérieux) as being *Escherichia coli*.

Bacterial cultures were prepared and maintained as described in Section 2.2.2.

It was necessary to ensure that when used to sample the inoculated surfaces, the various test methods would be detecting the bacteria as opposed to the growth medium. Thus, after incubation, a 5 ml volume of the overnight culture was centrifuged at 3000 *g* for 30 min (Mistral *3000i*). The supernatant was removed and discarded and the resulting pellet re-suspended in 5 ml ¼ strength (ATP and protein free) Ringer solution (Oxoid). The bacterial suspension was mixed well and a logarithmic dilution series was prepared, again using ¼ strength Ringer solution. Conventional cultivation of these dilutions was performed to obtain the theoretical number of bacteria inoculated onto the surface.

3.2.2. Preparation of Food Samples

Bovine serum albumen (BSA, Sigma-Aldrich) and the liquid-based food samples were serially diluted 2-fold and 5-fold respectively, using sterile deionised (ATP and protein free) water.

Ten grams of each solid food sample was placed in a stomacher bag (Fisher Scientific, Loughborough, UK) with 90 ml sterile deionised water and homogenized at medium speed

in a Stomacher 400 laboratory blender (Seward, London, UK) for 30 s. A 5-fold dilution series for each sample suspension was then prepared, again using sterile deionised water.

3.2.3. Microbial and Biochemical Analysis of Food Samples

3.2.3.1. Microbiological analysis

One millilitre of each sample dilution was pipetted into a petri dish and approximately 15 ml of molten, tempered (45°C) Plate Count Agar (PCA, Oxoid) was added. The contents of the plate were then mixed and the agar allowed to set before being incubated at 30°C for 48 h. Plates containing a minimum of 30 colonies but no more than 300 colonies at two consecutive dilutions were used to determine the number of colony forming units (cfu) per gram or ml of test sample. This was calculated using equation 1 (Harrigan, 1998).

$$N = \frac{\Sigma c}{(n_1 + 0.1n_2) \times d} \qquad (1)$$

Where:

N = the number of cfu per gram or ml of sample
Σc = the sum of all colonies counted on all the dishes
n_1 = the number of dishes retained in the first dilution
n_2 = the number of dishes retained in the second dilution
d = the dilution corresponding to the first dilution counted

3.2.3.2. Protein determination

The soluble protein content of each of the homogenized food samples was determined, depending upon the amount of protein likely to be present, by use of either the biuret (range: 1 – 20 mg protein) or Lowry (range: 25 – 500 μg protein) assay procedure (Frais, 1972).

3.2.4. Preparation and Inoculation of Test Surface

A food-grade stainless steel table marked with eighty-four 10 cm x 10 cm squares was used for the majority of this investigation. Additional studies were carried out using sterile, food-grade stainless steel coupons (5 cm x 5 cm), which were prepared as described in Section 2.2.3.

3.2.4.1. Preparation of stainless steel surface

Prior to inoculation, the table was pre-sanitised for 30 min using 1% Virkon (Antec International). Virkon is a fast acting oxidising system based on the peroxygen compound, potassium peroxomonosulphate and combined with an anionic detergent, which aids penetration of the oxidant and allows simultaneous cleaning and disinfection. However, a disadvantage of such a combined cleaning agent and of particular relevance to this study, specifically between sets of experiments involving the inoculation of homogenized food extracts, is that the antimicrobial component of a combined detergent-disinfectant may have to operate in relatively high amounts of soil, thus, reducing its efficacy (Dunsmore *et al.* 1981).

Combining mechanical or kinetic energy with the use of a detergent, not only increases the ease in which tenacious layers of soil, particularly those associated with proteinaceous material, can be removed from food contact surfaces (Section 1.3.3.1) but, in addition, is also responsible for the removal of the majority of microorganisms present (Chapter 1; Table 1.4). Thus, after being rinsed with boiling water, the surface was cleaned thoroughly using a clean, un-used rayon cloth, a detergent (< 5% amphoteric, 5-15% non-ionic, and 15-30% anionic surfactants) and boiling water. Kinetic energy was applied to the surface for approximately 2 min before it was rinsed three times, again with boiling water, to remove all traces of soil and detergent before finally being left to air dry at room temperature.

This in-house validated protocol, has been shown to consistently give ATP bioluminescence readings of 0 Relative Light Units (RLU) or < 100 RLU (depending on the system used), microbiological results of < 1 cfu 100 cm^{-2} and negative results using protein detection techniques.

3.2.4.2. Inoculation of stainless steel surface

Once the surface was completely dry, 0.1 ml of each sample dilution was inoculated onto five of the 100 cm^2 stainless steel areas and spread evenly over the surface using a sterile, disposable "hockey-stick" shaped spreader (Davidson *et al.* 1999). This surface conditioning represented the potential soiling of stainless steel under different food-processing conditions (Hood and Zottola, 1997b), with the different inoculum concentrations simulating varying degrees of poor cleaning. Clean surfaces were represented by means of control assays, which were performed by inoculating the surface with 0.1 ml of sterile, ATP and protein free, deionised water. The surfaces were sampled

immediately after inoculation, whilst still wet, or once they had been allowed to air-dry, under ambient conditions, for 1 h, after which time no visible liquid remained.

Each experiment was carried out using five replicates and repeated to validate the end points.

3.2.5. Microbiological Sampling of the Stainless Steel Surface

3.2.5.1. Hygiene swabs

Sterile dacron swabs were pre-moistened with sterile ¼ strength Ringer solution and, using the previously described swabbing protocol (Section 2.2.4.2), used to sample the test surfaces. The swabs were then either streaked directly onto the surface of pre-poured PCA plates (swab plates) or snapped off into 10 ml ¼ strength Ringer solution and vortexed, to release the bacteria from the bud, before 1 ml PCA pour plates were prepared (Section 2.2.6). All plates were incubated at 30°C for 24 h.

3.2.5.2. Dipslides

Dipslides are similar to contact plates and are pressed directly onto the surface to be sampled; any microorganisms present will contaminate the agar and subsequently grow. Although this method of sampling, by eliminating the need to release bacteria from a swab bud, would appear to have a significant advantage over the traditional swabbing technique (Chapter 2), there are disadvantages in neither vortexing nor diluting the sample. Colonies removed from a surface are unlikely to be broken up into smaller fragments of few or

single bacteria, thus, whether comprising 10 or 1000 cells, they will appear after incubation, as a single colony forming unit (Whyte *et al.* 1989). Furthermore, as these colonies can only be counted if present in relatively low numbers, in the case of confluent growth it becomes necessary to interpret the results using a key provided by the manufacturer (Salo *et al.* 2000).

PCA dipslides (PC2, Dimanco Ltd, Henlow, UK) were used to sample sterile, stainless steel coupons (5 cm x 5 cm), which had been inoculated with 25 µl of sample dilution. Each side of a dipslide measures approximately 2 ½ x 5 cm and both sides were pressed firmly onto the coupon so as to sample the entire 25 cm^2 surface area. The dipslides were then incubated at 30°C for 48 h.

3.2.6. Non-microbiological Sampling of the Stainless Steel Surface

3.2.6.1. ATP measurement

Two single-shot ATP bioluminescence systems were used during this study - the Clean-Trace ™ Rapid Cleanliness Test (UXL 100, Biotrace, Bridgend, UK) and the Charm PocketSwab Plus system (Charm Sciences Inc, Malden, MA, USA). In both cases the 100 cm^2 surface area was swabbed in accordance with the manufacturers' instructions and readings were taken using the Biotrace Uni-Lite® and the Charm Firefly® luminometer respectively. The latter is a small, specifically designed, low cost instrument for detecting ATP within food handling environments, and, at the time this study was conducted, was in its developmental stages.

3.2.6.2. Detection of specific component residues: Protein detection

Four protein detection kits were evaluated (Table 3.1). In all cases the surface was sampled in accordance with the manufacturers' instructions.

Table 3.1. Comparative description of the four different protein detection methods evaluated

Test and supplier	Test description	Test principle	Colour change ('clean' → 'dirty')	Time before results are obtained
Check-It (Biotrace Ltd)	self-contained* test strip	Protein error indication (Section 3.2.6.2.1)	yellow → green/blue	instant
Check Pro (DiverseyLever Ltd, Northampton, UK)	multi-shot† test strip	Protein error indication	orange → green	instant
Pro-tect® (Biotrace Ltd)	self-contained* swab-based device	Biuret reaction (Section 3.2.6.2.2)	green → purple	10 min
Swab & Check Professional Hygiene Monitoring Kit (Ruskinn Data Systems, Leeds, UK)	swab-based multi-shot† system	Biuret reaction	green → purple	10 min

* self-contained: test requires few, if any, additional manipulations (i.e. reagents are contained within device)

† multi-shot: test requires multiple manipulations (e.g. transfer of swab and/or manual addition of reagent)

3.2.6.2.1. Protein error indication

Both Check-It and Check Pro are based upon diagnostic test methods used to detect the presence of protein in body fluids, particularly in urine. Such methods usually comprise test papers, which have been impregnated with both a buffer solution and a protein error indicator. Protein error indicators, for example tetrabromophenol blue, are pH indicators, the pK value of which is displaced in the presence of protein causing a colour change (Rittersdorf *et al.* 1977). The buffer, therefore, generally has a pH value, which lies below this change region, thus, in the absence of protein the indicator is present in its acidic form.

In the presence of protein, the nitrogen bases react with the indicator causing it to change, ideally, into a more strongly coloured form, the intensity of this colour change corresponding to the amount of protein present.

3.2.6.2.2. The biuret reaction

Both Pro-tect and the Swab & Check Professional Hygiene Monitoring Kit are based upon a biuret reaction, which incorporates bicinchoninic acid (BCA), a highly sensitive, stable and specific reagent for copper I (Cu^{+}). Under alkaline conditions, the peptide bonds of proteins form a complex with the copper II (Cu^{2+}) of the biuret reagent, reducing the latter to Cu^{+}. BCA reacts with the reduced copper, resulting in the formation of an intense purple colour that can then be assessed visually.

Stage 1: $\text{Protein} + Cu^{2+} \xrightarrow{OH^{-}} Cu^{+}$

Stage 2: $Cu^{+} + \text{BCA} \longrightarrow \text{BCA-}Cu^{+}\text{ complex}$

Although these tests primarily detect protein residues they are also able to detect the presence of other substances capable of reducing Cu^{2+} to Cu^{+}, for example reducing sugars, such as glucose and fructose.

3.2.6.3. Detection of multiple component residues

The VERIcleen™ Food Residue Surface Test (Charm Sciences Inc) detects the presence of both carbohydrate and phosphate residues. Organic debris is drawn along a test strip, via capillary action, toward a test indicator, which in the presence of detectable food residues turns purple. To ensure that such food residues reach the test indicator, it is essential that

the volume of liquid present on the surface is sufficient to assure effective capillary action. Thus, this test method incorporates a wetting indicator, which turns dark grey once the strip has become sufficiently wet. It became apparent during the current study that the original 0.1 ml inoculum was not sufficient to adequately moisten the test strip and, therefore, the evaluation of VERIcleen™ was conducted using surfaces that had been inoculated with 0.5 ml of a comparable sample dilution. Again the test strip was used in accordance with the manufacturer's instructions and the formation of a purple colour within 1 min indicated the presence of food residues.

3.2.7. Interpretation of results

The cleaning protocol used during this investigation ensured that, prior to inoculation, all traces of residual organic debris were removed from the test surface. After inoculation, therefore, if the presence of residual organic debris was detected, then the surface would be presumed unclean. This was the case if average ATP readings were > 100 RLU (Clean-Trace™ / Uni-Lite®) or > 0 RLU (PocketSwab Plus / Firefly®), or if the colour of the instrument-free residue tests differed from that of clean as indicated by the manufacturer. The presence of microbial contaminants was presumed, if the average number of microorganisms recovered from the surface was > 1 cfu 100 cm^{-2} (hygiene swabs) or > 1 cfu 25 cm^{-2} (dipslides).

3.3. Results

During this investigation, the limits of detection of the different cleanliness assessment methods for nine different food residues (Appendix II) were determined under controlled laboratory conditions. However, the results for just four of these food types are presented. These foods have been selected on the basis of their microbial (Section 3.2.3.1) and protein content (Holland *et al.* 1991) and their association with foodborne disease (Table 3.2). Those nutritional values associated with the component residues detected by the various test methods and, thus, those having relevance to the following discussion are presented in Table 3.3.

Table 3.2. Significance of selected food samples

Food Type	Microbial level*	Protein content†	Examples of associated outbreaks	Reference
Poultry (e.g. chicken)	high	high	196 cases of salmonella	Palmer *et al.* (1990)
			1987-1991 UK; 128 outbreaks (3500 cases)	Anon (1991)
Pasteurised whole milk	low	high	1983 Massachusetts; 49 cases of listeriosis	Fleming *et al.* (1985)
			1996 NW England; 12 cases of *E. coli* O157	Clark *et al.* (1997)
			2000 Japan; 13,809 cases of *Staph. aureus*	Asao (2003)
Fresh produce (vegetables; ready-to-eat salads)	high (unwashed)	low	1981 Canada 41 cases of listeriosis	Prazak *et al.* (2002)
	low (washed)		1990 USA; 176 cases of salmonella	Hedberg *et al.* (1999)
			1997 Michigan; 60 cases of *E. coli* O157	Anon (1997)

	high	low
* **microbial level (g^{-1} or ml^{-1})**	$> 10^5$ cfu	$< 10^3$ cfu
† **protein content (g^{-1} or ml^{-1})**	> 25 mg	< 10 mg

Table 3.3. Composition of selected food samples (Holland *et al.* 1991)

	Composition of Foods (g 100 g^{-1})										
	Protein	**Fat**	**Fatty Acids**			**Carbohydrate**	**Starch**	**Sugars**	**Fibre**	**Phosphorus***	**APC†**
			Saturated	***Monounsat***	***Polyunsat***						
chicken breast (raw)	21.8 *(11.2)*	3.2	1.0	1.3	0.6	0	0	0	0	250	$> 10^7$
whole milk (pasteurised)	3.2	3.9	2.4	1.1	0.1	4.8	0	4.8	0	92	230
carrot (raw, unwashed)	0.6 *(0.009)*	0.3	0.1	Trace	0.2	7.9	0.3	7.4	2.6	15	7×10^5
tomato (raw, washed)	0.7 *(0.012)*	0.3	0.1	0.1	0.2	3.1	Trace	3.1	1.3	24	0
white rice (boiled)	2.6 *(0.014)*	1.3	0.3	0.3	0.5	30.9	30.9	Trace	1.0	54	0

* Phosphorus (mg 100 g^{-1})

† Aerobic plate count (cfu g^{-1} or ml^{-1} of sample) (Section 3.2.3.1)

(x) protein present within the homogenised food sample as determined experimentally by means of the biuret or Lowry procedure (Section 3.2.3.2)

The bars associated with Figures 3.1 to 3.4 indicate those levels of contamination that were detected by the various test methods and this, in turn, can be related to test sensitivity. As the level of contamination detected by a specific test decreases (i.e. as the detectable food sample becomes more dilute), the sensitivity of the test method increases. Thus, conversely, as detection limit increases, sensitivity decreases.

3.3.1. Detection of Microorganisms *or* Protein Residues (Figure 3.1)

3.3.1.1. Detection of bacteria on stainless steel surfaces

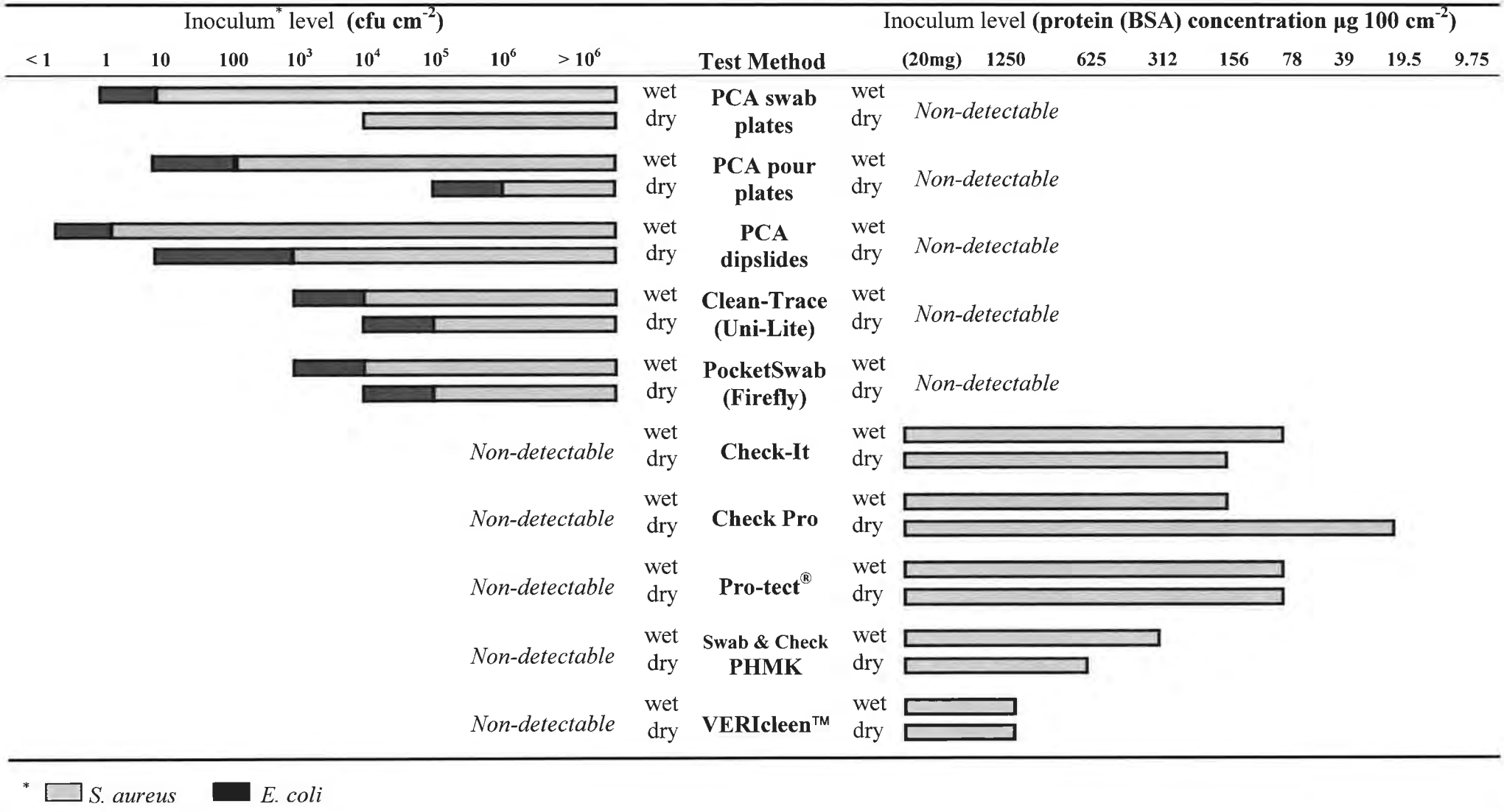
The minimum detection limit of those test methods capable of detecting the presence of microorganisms on a surface was, in general, lower for *E. coli* than that for *S. aureus*. In both cases the use of dipslides was the most sensitive means to indicate the presence of bacterial contaminants, detecting an inoculum level of < 1 and 10 *E. coli* colonies cm^{-2} and 1 and 10^3 *S. aureus* colonies cm^{-2}, on a wet and dry surface respectively. When used to sample a wet surface, both ATP bioluminescence systems were less sensitive than all three agar-based microbiological methods. However, when a dry surface was sampled, although still less sensitive than the dipslides, the use of ATP bioluminescence was a more sensitive means to detect bacterial contamination than the traditionally used pour plate methodology. Neither, the VERIcleen™ Food Residue Surface Test nor any of the four protein detection systems were able to detect the presence of even very high levels of bacteria (10^6 cfu cm^{-2}).

3.3.1.2. Detection of bovine serum albumen (BSA) on stainless steel surfaces

In contrast to the aforementioned bacterial cultures, commercial bovine serum albumen (BSA) is high in protein (20g / dl), does not contain actively metabolizing cells and has a low microbial count. Thus, BSA was not detectable on surfaces using either ATP bioluminescence or traditional microbiological methods. However, despite their sensitivities differing, all four protein detection systems indicated that the test surfaces were unclean.

The Swab and Check Professional Hygiene Monitoring Kit (PHMK) was the least sensitive of the four protein systems in detecting the presence of residual protein on either a wet or dry surface. The other three tests were, within the limits of the experimental protocol, comparable and when used to sample a wet surface, were capable of detecting between 78 and 156 μg protein 100 cm^{-2}. When a dry surface was sampled, the sensitivity of Check-It and Pro-tect® were again comparable, whilst Check Pro was capable of detecting just 19.5 μg protein 100 cm^{-2}. VERIcleen™ detected the presence of 1.25 mg protein 100 cm^{-2} on both a wet and dry stainless steel surface, thus, although primarily detecting the presence of carbohydrate and phosphate residues, VERIcleen™ also appears capable of detecting the presence of relatively high levels of protein.

Figure 3.1. Detection of either bacteria or protein (BSA) from a wet and dry stainless steel surface using a range of different test methods



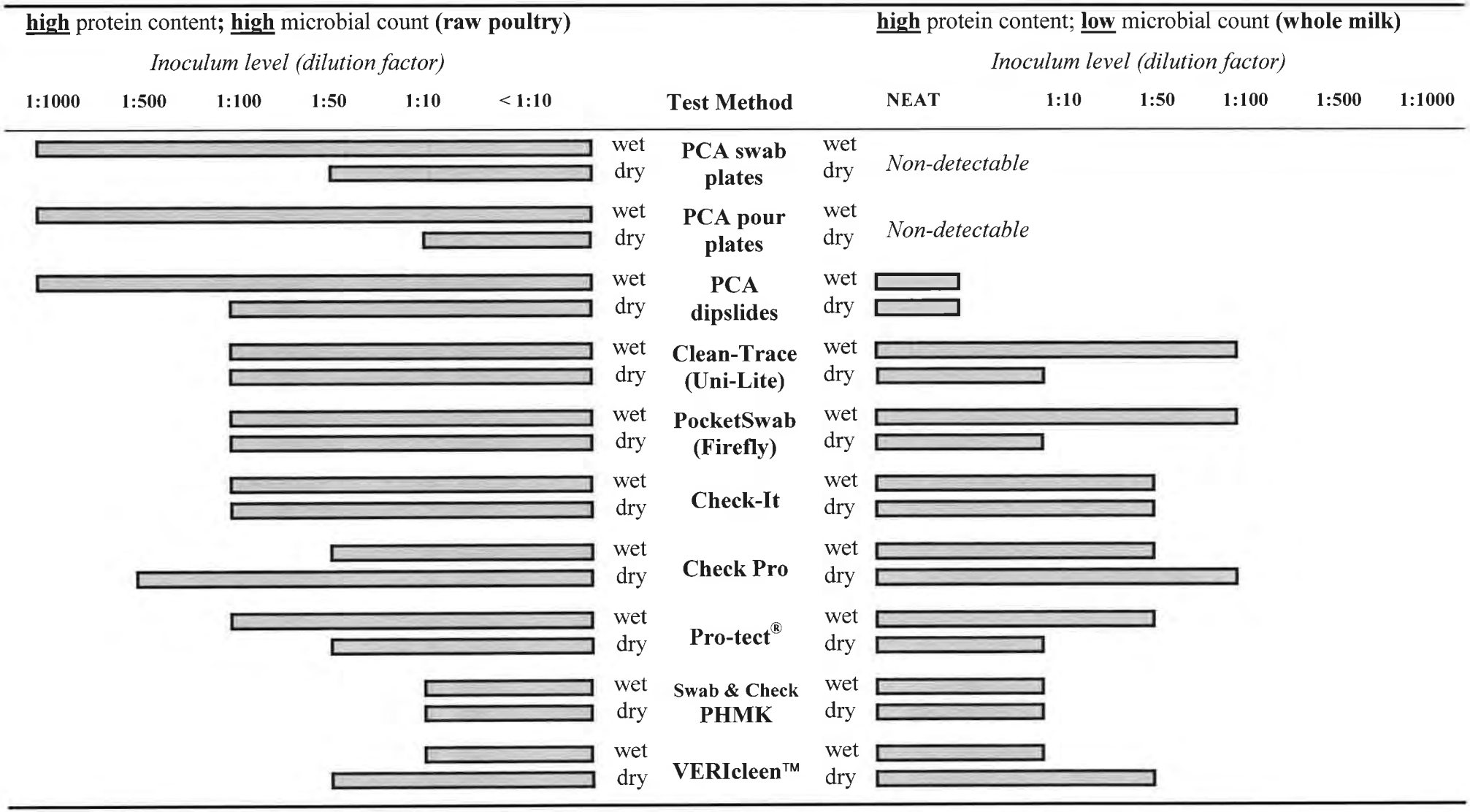
3.3.2. Detection of High-Protein Product Residues (Figure 3.2)

3.3.2.1. Detection of residual food debris with a high protein content (> 100 mg g^{-1}) and a high microbial count (> 10^7 cfu g^{-1}) (e.g. raw poultry)

Traditional microbiology was the most sensitive means of detecting the presence of raw poultry residues on a wet surface and these methods were capable of detecting an inoculum that had been diluted 1000-fold. However, when a dry surface was sampled, the detection limits of these test methods were observed to markedly increase.

Unlike the agar-based techniques, surface dryness did not appear to adversely affect the performance of ATP bioluminescence. Thus, although less sensitive than the microbiological methods when used to sample a wet surface, ATP bioluminescence was a more effective means of detecting the presence of this type of organic debris from a dry surface. Similarly, although the ability of the protein detection systems to detect these food residues varied by type, in general, their use also appeared more suitable than traditional hygiene swabs when the surface to be sampled was dry. When used to sample a wet surface, the most sensitive of the protein tests (Check-It and Pro-tect®) were capable of detecting the presence of homogenized raw chicken, which had been diluted 100-fold. In comparison VERIcleen™ was only capable of detecting the presence of a sample that had been diluted 10-fold. Nevertheless, when these residues were allowed to dry, the minimum detection limit of this particular test method was observed to fall, equating to a 5-fold increase in sensitivity.

Figure 3.2. Detection of high-protein food residues from a wet and dry stainless steel surface using a range of different test methods



3.3.2.2. Detection of residual food debris with a relatively high protein content (> 25 mg ml^{-1}) and a relatively low microbial count (< 10^3 cfu ml^{-1}) (e.g. pasteurised whole milk)

The number of microorganisms present within the high-protein milk residues was, in comparison to the raw poultry, relatively low and in this case neither the use of swab nor pour plates indicated the presence of bacteria and, consequently, these methods passed as 'clean' all the surfaces tested. The dipslides did detect microbial contaminants but only on those coupons that had been inoculated with undiluted milk – at this concentration these surfaces were also visually dirty.

The minimum detection limits of the non-microbiological test methods were also, in general, higher for milk than that for raw chicken residues. Both ATP bioluminescence systems were able to detect milk that had been diluted 100-fold, but only from a wet surface. When used to sample a dry surface, ATP bioluminescence was only capable of detecting milk that had been diluted 10-fold and, thus, was not as sensitive in detecting this type of organic debris as the two non-swab based protein detection methods (Check-It, Check Pro). All four protein detection tests indicated that the surfaces were unclean but, again, their sensitivities varied. As with raw poultry residues, when a wet surface was sampled, the PHMK was the least sensitive system, whilst the other three tests were, within the limits of the experimental protocol, comparable, detecting the presence of milk that had been diluted 50-fold. When a dry surface was sampled, the sensitivity of the Pro-tect® was again reduced whilst that of Check Pro again increased, the latter able to detect a 100-fold dilution of milk that had been allowed to dry on to the surface.

When used to detect the presence of milk residues on a wet surface, VERIcleen™ was, in general, the least sensitive of the non-microbiological test methods. However, when a dry

surface was sampled, its sensitivity, as with that of the Check Pro, was observed to increase. When used to detect the presence of chicken residues, a similar increase in sensitivity made no real difference to the comparative performance of VERIcleen™. However, when detecting the presence of milk residues, this increase, in combination with the observed reduction in sensitivity of the majority of the other methods, resulted in VERIcleen™ detecting the presence of dried milk residues on surfaces that had been passed as 'clean', not only by traditional microbiological techniques but also by ATP bioluminescence and the two, swab-based, protein detection methods (Pro-tect®, PHMK).

3.3.3. Detection of Residual Food Debris with High Levels of Microbial Contamination (Figure 3.3)

3.3.3.1. Detection of residual food debris with a low protein content (< 10 mg g^{-1}) and a relatively high microbial count (> 10^5 cfu g^{-1}) (e.g. raw, unwashed vegetables)

Although the protein content of the raw carrot homogenate was, in comparison to that of the raw chicken, very low, the number of microorganisms present was still relatively high. In this case, when used to sample a wet surface, both the swab plates and dipslides detected the presence of bacteria on surfaces that had been inoculated with a sample of homogenized carrot, which had been diluted 1000-fold. As was also observed when the surfaces were inoculated with the raw chicken residues, although the sensitivity of these microbiological methods decreased markedly when they were used to sample a dry surface, the performance of both ATP bioluminescence systems remained unaffected. Furthermore, the minimum detection limit of ATP bioluminescence for this type of raw vegetable extract was lower than that for raw chicken residue, despite the latter containing a comparatively higher number of microorganisms.

Although the microbiological methods indicated the presence of large numbers of bacteria, none of the four protein detection tests suggested that any of the surfaces sampled would be unacceptable for food production. However, as with ATP bioluminescence, VERIcleen™ was capable of detecting the presence, on either a wet or dry surface, of a raw carrot homogenate that had been diluted 1000-fold.

3.3.4. Detection of Residual Food Debris with a Low Protein Content and a Low Microbial Count

When drained, boiled rice was homogenized and inoculated onto the surface (results not presented), none of the cleanliness assessment methods deemed any of the wet surfaces unclean. Check Pro did detect contamination on surfaces that had been allowed to air-dry, but only on those that had been inoculated with the initial 10-fold dilution.

However, when the residual food debris also had a high ATP content, as was the case with a raw, washed tomato homogenate (Figure 3.4), then the ATP bioluminescence technique was capable of detecting the presence, on a wet and dry surface, of a sample that had been diluted 10,000- and 1,000-fold respectively. In this case, Check Pro and Pro-tect® were also able to detect the presence of homogenized raw tomatoes but only from dry surfaces that had initially been inoculated with a 50-fold and a 10-fold dilution respectively. In contrast, VERIcleen™ was capable of detecting the presence, on both a wet and dry surface, of a sample that had been diluted 1000-fold and, thus, in comparison to protein detection, appeared a much more effective means of detecting food residues of this type.

Figure 3.3. Detection of food residues, comprising high levels of microbial contamination, from a wet and dry stainless steel surface using a range of different test methods

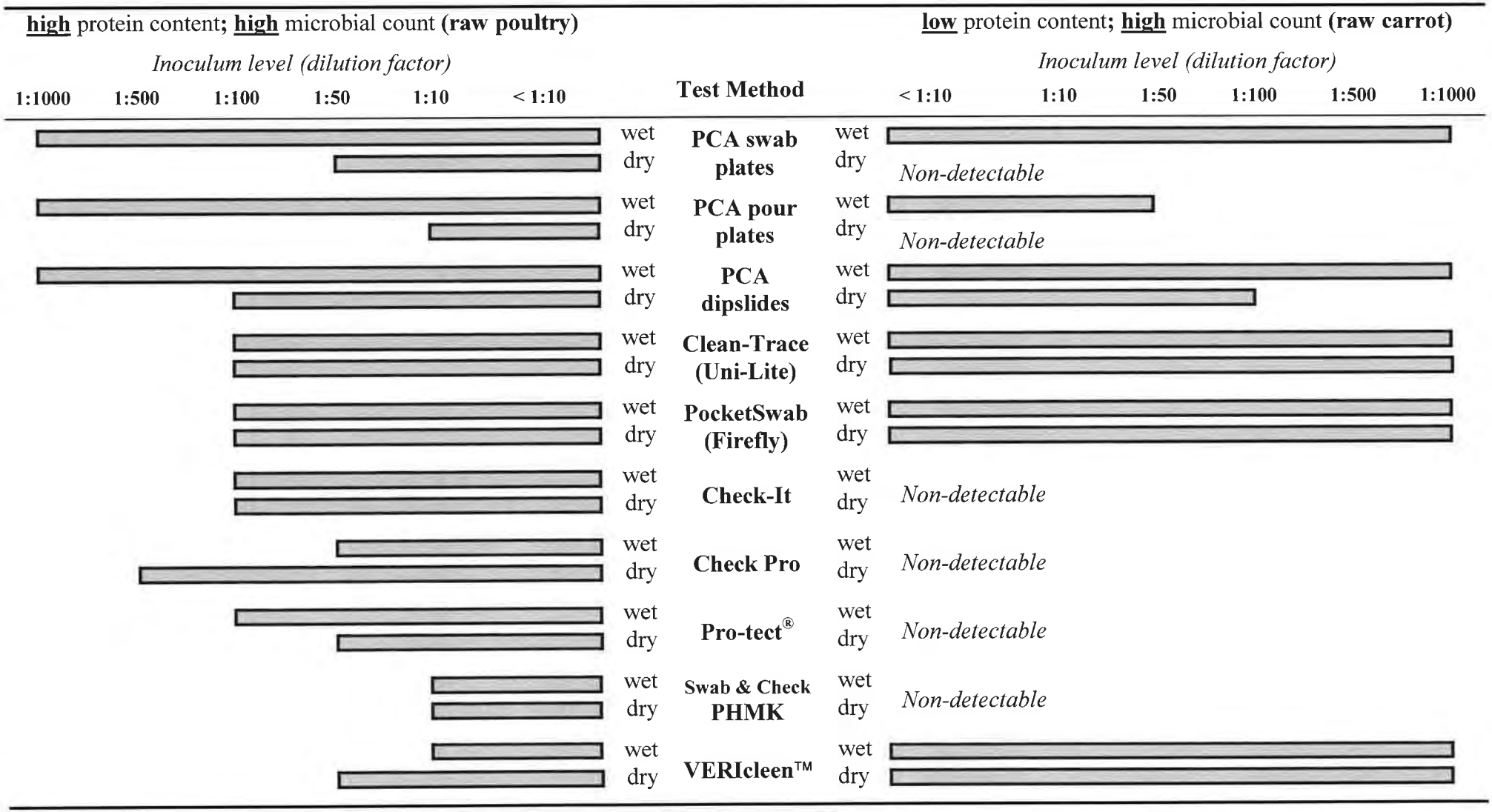
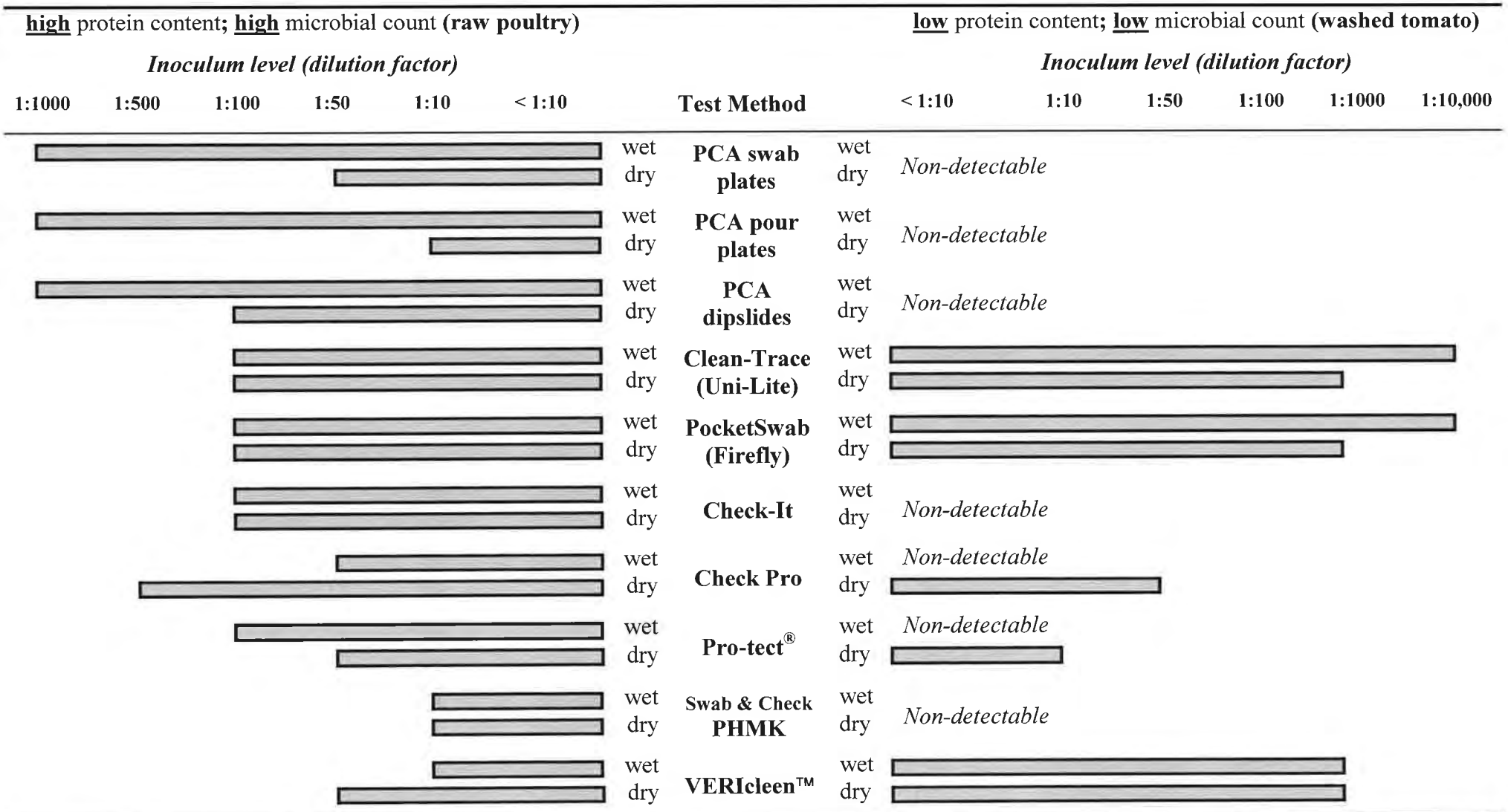


Figure 3.4. Detection of high- and low-protein product residues comprising a high- and low level of microbial contamination respectively from a wet and dry stainless steel surface using a range of different test methods



3.4. Discussion

In many countries, increasing food hygiene legislation has led to a heightened awareness within the food industry of the need to rapidly assess the efficacy of sanitation procedures. It is important, therefore, that food companies are provided with accurate information with regard to the performance of the ever-increasing range of test methods becoming available to them. Are, for example, these methods readily interchangeable? Can they be used within any processing environment? – After all, any test method, regardless of how quickly results can be obtained, would be of little value to a food business if it were unable to detect the type of residues likely to be present.

Numerous investigations have compared traditional microbiological techniques with ATP bioluminescence and/or protein detection methods (Tebbutt and Midwood, 1990; Kyriakides *et al.* 1991; Bautista *et al.* 1993; Ogden, 1993; Poulis *et al.* 1993; Bell *et al.* 1994; Seeger and Griffiths, 1994; Wirtanen *et al.* 1996; Davidson *et al.* 1999; Tebbutt, 1999; Illsley *et al.* 2000; Miettinen *et al.* 2001; te Giffel *et al.* 2001; Páez *et al*, 2003). However, the majority of these studies were conducted *in situ* and it has been acknowledged that the comparative performance of the different test methods may have been influenced by both uncertainty and variation in the type and level of contamination present (Greene and Herman, 1961; Tebbutt, 1999).

There are, therefore, advantages in conducting some comparison studies under controlled conditions. During the current investigation for example, not only could the surface be thoroughly cleaned before each experiment, ensuring that all traces of residual organic debris were removed, but it was also possible to subsequently inoculate the surface, in a uniform manner, with known types and levels of food residues. Consequently, in the presence of varying levels and combinations of microbial and food debris, the relative

sensitivities of both traditional and the more recently developed methods for assessing surface cleanliness could be determined more accurately than they could in the field. Thus, as shall subsequently be discussed, the results obtained from this study provide the food industry with important information regarding the performance of the various test methods, including their advantages, drawbacks and limitations.

3.4.1. Microbiological Methods for Assessing Surface Cleanliness

It is now widely accepted that agar-based microbiological methods have only limited value in terms of the routine monitoring of surface hygiene. Nonetheless, traditional techniques can be used to determine the types of organisms present and, thus, provide a microbial profile of the processing environment, which, in turn can lead to the development and application of an appropriate sanitation programme (Illsley *et al.* 2000). In addition the data provided can be used in fault analysis and to identify and predict problems relating to microbial quality (Griffith *et al.* 1994). The importance of microbiological methods must not, therefore, be underestimated yet a survey of 500 food manufacturing businesses in the UK showed that 48% of respondents used hygiene swabs in order to assess surface cleanliness (Davidson *et al.* 1999).

The efficiency of the swabbing technique is, in general, very poor (Chapter 2). Thus, although swab-based microbiological methods are capable of detecting the presence, on a wet surface, of relatively low levels of bacteria (Figures 3.1 and 3.3), the number of colonies isolated should not be considered an accurate reflection of the level of contamination present. This is particularly true when the surface to be sampled is dry. Under these circumstances, the use of hygiene swabs can result in the surface appearing free from microbial contaminants, despite the use of dipslides suggesting the presence of

relatively high levels of bacteria (Figure 3.3). These results substantiate conclusions drawn in Chapter 2 and suggest that bacteria initially inoculated onto a surface can survive the drying process and that a loss in microbial viability is not the only contributing factor in reducing the sensitivity of the swabbing technique.

As many of the problems associated with the swabbing technique can be attributed to some aspect of swab design (Chapter 2), poor bacterial recovery and the risk of underestimating the numbers of bacteria present, are issues that should concern any individual food business and, thus, should be appreciated by the food industry in general. The results of the current investigation support the findings of previous studies (Wirtanen *et al.* 2000) and suggest that for flat surfaces, dipslides rather than swabs should be used to detect the presence of microbial contaminants. However, when using dipslides, the lack of pressure involved during sample collection means that clumps of food residues are not broken up and organisms present within the debris are not released (Scheusner, 1982). Thus, the sensitivity of this microbiological test method will depend not only on the level of bacteria initially present on a surface but also the aggregation of microorganisms within different types of contaminating food debris, suggesting, therefore, its performance may be influenced by the type of processing environment sampled. Indeed, during the current investigation, when they were used to sample a dry surface, the detection limit of the dipslides was observed to vary with residue type.

The level of bacteria present within the original 100-fold dilution of the raw chicken and carrot homogenate (i.e. the minimum detection limit of the dipslides) equated to approximately 10^2 and 7 cfu cm^{-2} respectively (Figure 3.3). Thus, whilst both raw chicken and raw, unwashed vegetables can be contaminated with very high levels of microorganisms (Table 3.3), dipslides were relatively less effective in detecting residues associated with the former. Raw chicken and vegetables differ significantly with regard to

protein and fat content (Table 3.3) and this may have influenced the degree of microbial aggregation within the different homogenates. However, in addition, such food macromolecules adsorb readily onto stainless steel forming a conditioning film onto which microorganisms can become attached (Section 1.1.1). Results from the previous chapter (Section 2.3.1.2) illustrate that bacteria can become more difficult to remove from a protein-soiled surface over time. Thus, with the use of dipslides generating little or no mechanical energy, stronger microbial-surface associations caused, for example, by the increased adhesion of microorganisms to high-protein food debris, may reduce the efficacy of this sampling technique and result in the number of bacteria present on the surface again being underestimated. These results imply that to have confidence in even the most sensitive of microbiological techniques, the absence of non-microbiological surface contamination should also be assured.

3.4.2. Non-microbiological Methods for Assessing Surface Cleanliness

Unlike the agar-based methods, surface dryness appeared to have little effect upon the performance of the ATP bioluminescence and protein/carbohydrate detection techniques. However, other factors, particularly those associated with the chemical composition of the organic residues, did influence the results obtained using these non-microbiological test methods. These included the universality of the component residue being tested for and its intrinsic level within the food debris itself. Additionally, both these factors worked in combination with the sensitivity of the chemistry associated with each of the test methods.

3.4.2.1. ATP bioluminescence

When a new method for assessing surface cleanliness becomes available to the food industry, its performance is likely to be compared to that of the methodology currently in use, which in most cases, despite its unreliability, will be microbial enumeration (Griffith *et al.* 1997). However, Worsfold and Griffith (2001) state that attempting to correlate surface ATP bioluminescence results to bacterial counts has limited value and that industry takes the more pragmatic view that if a surface has a high ATP level, whether of microbial or non-microbial origin, it is soiled. Whilst this may be true, it is important that food businesses appreciate that discrepancies between the results of these different test methods can occur (Tebbutt and Midwood, 1990; Poulis *et al.* 1993; Bell *et al.* 1994; Miettinen *et al.* 2001) and, in addition, in order to recognise the limitations of ATP bioluminescence, when and why such disagreements exist.

On average bacteria contain approximately 1 fg (10^{-15} g) of ATP (Vanne *et al.* 1996). Nevertheless, intracellular levels have been shown to vary with organism type (Andrews *et al.* 2001) and this could lead to inconsistencies with regard to the ability of the ATP bioluminescence technique to detect microbial contaminants (Bautista *et al.* 1993). In the current investigation, as with previous studies (Corbitt *et al.* 2000), in comparison to the number of *E. coli* cells required, a higher number of *S. aureus* cells were necessary to produce a positive ATP result (Figure 3.1). However, under practical conditions, the sensitivity of the luciferin-luciferase chemistry is approximately 1000 fg (Vanne *et al.* 1996), thus, the minimum detection limit of ATP bioluminescence for bacteria, regardless of type is >1000 cells. Consequently, as clearly illustrated in Figure 3.1, traditional microbiological methods are capable of detecting the presence of far fewer bacterial contaminants on a wet surface than ATP bioluminescence. Thus, in the absence of food debris, should moderate numbers of bacteria ($< 10^3$) be present, then surfaces may be

passed as 'clean' using ATP bioluminescence but deemed unacceptable for food production by means of traditional microbiology.

The inoculation of a 1000-fold dilution of raw chicken homogenate (Figure 3.2; Section 3.3.2.1) reflects this situation and could arise should the terminal disinfection stage of a cleaning protocol be ineffective or if surfaces become re-contaminated with microorganisms after cleaning. Additionally, there are areas within the production environment, which can harbour high levels of bacteria despite the presence of little or no residual food debris. Tebbutt and Midwood (1990) demonstrated that whilst ATP levels associated with washbasins were either low or not detectable, 30% of those surfaces sampled were contaminated with high numbers of microorganisms. Such a disagreement has particular significance within 'high-risk' production areas, where contaminated surfaces, such as these, could serve as reservoirs of bacteria and viruses that could easily be transferred via direct contact or aerosolisation to hands or work tools (Rahkio and Korkeala, 1997; Griffith *et al.* 1999; Rusin *et al.* 2002). Thus, a surface deemed acceptable for food production using ATP bioluminescence cannot be guaranteed to be free from microbial contaminants, implying, that ideally the technique should be used in conjunction with traditional microbiological methods.

On those food contact surfaces where microorganisms are absent (Figure 3.4; Section 3.3.4) or at levels proportionally much lower than that of the food debris (Figure 3.2; Section 3.3.2.2), surfaces acceptable for food production by means of microbiological methods can be deemed 'unclean' by the ATP bioluminescence technique. Many surfaces, specifically those associated with the 'high-risk', post-process areas of a food production environment, should have only minimal levels of microbial contamination and, therefore, are more likely to 'fail' ATP but 'pass' microbiological analysis. Nevertheless, it is

perhaps more accurate to state that this disparity will occur when the levels of microbial ATP are proportionally much lower than that of non-microbial ATP.

The minimum detection limit of ATP bioluminescence for raw carrot residues was lower than that for raw chicken (Figure 3.3), despite the latter containing a comparatively higher number of microorganisms (Table 3.3). Consequently, when the surfaces were sampled after they had been allowed to air-dry, the ATP bioluminescence technique proved 1000-times more sensitive than the traditional pour plate procedure when sampling the raw carrot, but only 10-times more sensitive when sampling raw chicken residues. These two food types differ significantly with regard to the level of ATP derived from their original tissue (i.e. the intrinsic ATP) (Sharpe *et al.* 1970). Chicken breast (i.e. the flight muscle) is, as a muscle, fairly redundant and, thus, it contains few mitochondria and generates low levels of ATP (Coultate, 1989). In comparison, vegetables, such as carrots, comprise many actively metabolising cells. Similarly, although pasteurisation destroys over 99% of those bacteria present, milk can contain high numbers of somatic cells (Heggum, 2001) and, thus, has been shown to contain ATP at levels comparable to those of raw poultry (Corbitt *et al.* 2000). Thus, a high ATP reading does not necessarily indicate high levels of microorganisms and should a surface 'fail' using ATP bioluminescence, without the use of a detection method capable of detecting the presence of food residues only, there is no way of rapidly determining whether the cleaning or disinfection stage of the sanitation protocol has been ineffective.

Finally, in the majority of cases, ATP bioluminescence appeared unaffected by surface dryness, nevertheless, the sensitivity of this technique was observed to fall when it was used to sample surfaces contaminated with dried milk residues (Figure 3.2). It has been suggested that fatty material present in other dairy-based emulsions may inhibit the extraction of ATP (Corbitt *et al.* 2000). Alternatively, milk may adhere more firmly to a

surface than non emulsion-based product residues or adsorb more firmly to the swab bud, which could, depending on test format/protocol, result in lower levels of ATP being released into the reagents (Carrick *et al.* 2001). Thus, it is possible that those factors influencing the recovery of microorganisms using hygiene swabs may also have an effect upon the sensitivity of the ATP bioluminescence technique (Lundin, 1999).

3.4.2.2. Protein detection

Many of the residue tests currently available to the food industry detect the amount of protein present on food contact surfaces. Very little comparison work has been conducted on these protein detection methods, probably because of their relatively recent introduction. During the current investigation, therefore, their limits of detection were initially established by inoculating the surface with a protein standard (Figure 3.1; Section 3.3.1.2).

The ability of any test to remove organic debris will, to a certain extent, be influenced by the swabbing or sampling procedure used (Davidson *et al.* 1999). The degree of pressure applied to any such test method is very difficult to quantify and, with regard to the protein tests used during this study, equally difficult to standardise. Differences in their design dictated the pressure that could be applied to each test and this, it is believed, led to differences in the amount of protein removed from the surface and, consequently, the apparent differences in test sensitivity.

Although, the Professional Hygiene Monitoring Kit (PHMK) and Pro-tect® are both swab-based biuret reactions, the latter is a more sensitive test (Figure 3.1). This may be due to the larger bud associated with Pro-tect® and, thus, its potential capability of picking up

greater amounts of bioburden from a larger area. However, the swab itself is longer and more flexible than that of the PHMK and although the performance of the protein detection methods appeared, in general, to be unaffected by surface dryness, a reduction in mechanical energy may have been the reason for the observed reduction in the sensitivity of Pro-tect®, when it was used to sample dry, raw chicken or milk residues (Figure 3.2). In contrast, the design of Check-It and Check Pro enabled a relatively high amount of pressure to be applied to the surface during sampling. Nevertheless, despite similarities in the sampling method, when the surface was wet, the performance of Check-It was superior to that of Check Pro, whilst when the surface was dry, the opposite was true. Unlike Check-It, Check Pro requires pre-moistening before use and this, as with hygiene swabs (Section 2.4.1.2) appears to improve the removal of organic debris from a dry surface.

This pattern of sensitivity was also seen when the tests were used to detect realistic food debris (Figure 3.2). Their detection limits for the protein standard appeared to correlate well with those for milk; the most sensitive methods being capable of detecting the presence of milk residues that equated to 64 μg protein 100 cm^{-2}. However, unlike liquid samples, the protein associated with foods such as meat or poultry, forms an integral part of the tissues and muscles and, thus, may remain bound within the matrix of the food debris and be inaccessible to protein detection methods. The sensitivity of these methods will, therefore, also depend upon the type of food debris present and the degree to which it has become solubilised. During the current study, homogenising the raw chicken within a stomacher is unlikely to have released a high proportion of the protein into the diluent, hence, the marked difference between the amount of protein present in raw chicken and that of the homogenate, as determined by the biuret procedure (Table 3.3). However, although the limits of detection of the protein methods for raw chicken were observed to be higher than that for milk, when used to detect either of these residue types, the most sensitive protein tests were superior or comparable to ATP bioluminescence. It can be

concluded, therefore, that protein detection may be of use to those businesses involved in the production of high-protein foods but who are unable to afford to utilise the ATP bioluminescence technique.

Nevertheless, a fundamental difference between ATP bioluminescence and protein detection is the inability of the latter to detect the presence of even very high levels of bacteria (Figure 3.1; Section 3.3.1.1). Thus, in food processing environments where any residual food debris is likely to be low in protein, surfaces may have thousands of bacteria on them but still 'pass' the protein test and be considered acceptable for food production. This situation is illustrated in Figure 3.3 and has also been described by Tebbutt (1999) during a study assessing the risk of bacterial cross contamination from cutting boards and emphasises not only the importance of interpreting the results of these test methods with caution but also the need to combine their use with some form of microbiological assessment.

In comparison to ATP bioluminescence and traditional microbiology, where wide variations in ATP values and bacterial counts (Griffith *et al.* 1997) make single estimates difficult to interpret, protein tests involve a simple colour change and are, therefore, more robust and less subject to error (Tebbutt, 1999). However, although the results can, in general, be considered very repeatable, the subjectivity involved in their interpretation raises issues regarding the reproducibility of these test methods. Intermediate levels can, for example, be identified and can manifest as either differences in the amount or intensity of the colour (Check-It and Check Pro) or as a mixture of two colours (PHMK and Pro-tect®). Interpretation was found to be particularly awkward when the surfaces were deemed 'marginally unclean' (i.e. when residues were present at levels nearing the minimum detection limit of the test methods).

3.4.2.3. Detection of multiple chemical residues

Similar difficulties were encountered when the VERIcleen™ Food Residue Surface Test was used to sample the surface. The manufacturer's of this test state that the formation of a purple colour within 1 min indicates the presence of food residues but warn that even a 'clean' surface will turn the test purple in 5-10 min. During the current investigation, 65 clean surfaces were sampled (i.e. those inoculated simply with sterile, de-ionised water) and although the mean time for the VERIcleen™ test strips to turn purple was recorded as being 5 ½ min, this time was extremely variable and was observed to range from 2 ½ to 10 min. To avoid false impressions being made with regard to the hygienic status of surfaces, it is, therefore, important that readings are taken within the 1 min recommended reaction time. As with protein detection, the intensity and speed of the colour change corresponded to the levels of detectable food residues, which again varied with food type. However, unlike protein detection, VERIcleen™ has the ability to detect multiple chemical residues and this, in turn, increased the range of organic debris that could be detected.

Fruits and vegetables comprise high levels of carbohydrate, largely in the form of polysaccharides (Table 3.3). Starch, for example, is the major carbohydrate/energy reserve in tissues, such as seeds and tubers (e.g. carrots) and is entirely composed of amylose and amylopectin, both of which are made up of thousands of glucose molecules. Other important plant polysaccharides include cellulose, an essential component of all plant cell walls and the pectins which, comprise a substantial proportion of the structural material of soft tissues, such as the parenchyma of fleshy roots and soft fruits (e.g. tomatoes) (Coultate, 1989). Thus, when used to sample surfaces contaminated with low-protein product residues, such as the raw carrot and tomato homogenates, surfaces passed as 'clean' using protein detection were deemed unacceptable for food production using VERIcleen™, the performance of which, despite its inability to detect microorganisms, was

comparable to that of ATP bioluminescence (Figures 3.3 and 3.4). It can, therefore, be assumed that VERIcleen™ would also be capable of detecting residues such as apple pulp and citrus peel and, consequently, its use could prove useful in those processing environments involved in the production of fruit juices, where contamination of unpasteurised product is a continual problem (Pao and Davis, 2001). Conversely, despite rice comprising a high proportion of starch, the results presented in Section 3.3.4, suggest that VERIcleen™ is unable to detect food residues of this type. However, it is acknowledged, that during the current investigation, the rice was boiled and drained and, therefore, any starch that leached from the rice during boiling was discarded prior to the inoculation of the surface!

The performance of VERIcleen™, when used to detect the presence of high-protein raw chicken or milk residues was, despite its limited ability to detect protein (Figure 3.1), comparable to that of the least sensitive protein detection methods (Figure 3.2). Despite comprising no carbohydrate, chicken does contain high levels of phosphorous (Table 3.3). Interestingly, VERIcleen™ appeared twice as effective in detecting the presence of ready-to-eat cooked chicken than raw chicken residues (Appendix II) and this may have resulted from the level of phosphates in the former having been artificially increased prior to cooking. Meats, particularly chicken may be injected with a brine of NaCl and phosphates as a means to retain water and to reduce weight losses during the cooking process (Varnam and Sutherland, 1995).

Milk also contains phosphorous. Casein proteins comprise approximately 80% of the total proteins present within cows' milk and 64% of these (the α- and β-caseins) have, at their polar ends, a number of phosphoserine residues (Coultate, 1989). In addition, 5% of cows' milk is lactose, a reducing sugar and carbohydrate (Coultate, 1989), yet, on a wet surface, the ability of VERIcleen™ to detect three different chemical residues did not appear to

improve its performance in comparison to that of protein detection. One reason for this may have been the high proportion of saturated fatty acids (Table 3.3) inhibiting the capillary action of the milk emulsion and preventing components of the aqueous phase, including the proteins and carbohydrates, from reaching the test indicator. Nevertheless, after the surface had been allowed to air-dry, VERIcleen™ was a more effective means of detecting residues of this type than either traditional microbiology, ATP bioluminescence or the two swab-based protein detection methods. For optimum test performance, it is important to ensure that a sufficient volume of liquid is present on the surface to be sampled. When the surface to be sampled is dry, therefore, approximately 0.5 ml of a wetting solution (supplied by the manufacturer) must be sprayed on to the surface. Although, the main reason for this is to facilitate effective capillary action, it may also help in detaching dried-on product residues, so enhancing the performance of this particular test method. However, it must again be recognised, that regardless of the range of food residues detected, the use of VERIcleen™ will not provide any indication as to the levels of microbial contamination present.

Although many food manufacturing plants are associated with a specific product type, other sectors of the food industry are involved in the production of a much wider range of food stuffs. Companies within the retail and food service industries could, for example, find VERIcleen™ particularly beneficial, especially as the majority of those surfaces sampled are likely to be dry (Griffith *et al.* 2001). However, within such establishments, sanitation procedures usually incorporate cleaning solutions, which do not need to be rinsed from the surface. Cleansers and sanitizers have been shown to affect the sensitivity of the ATP bioluminescence technique (Section 1.4.2.2.1) and, thus, to investigate the effect of residual sanitizer upon the sensitivity of VERIcleen™, the inoculated surfaces were also sampled in the presence of a combined detergent/disinfectant sanitizer (QAC-based). Although, in general, the ability of VERIcleen™ to detect the presence of residual

food debris was not adversely affected by the presence of QAC residues (results not presented), it is recognised that phosphates are often incorporated within other types of cleaning solution – trisodium phosphate in particular, is a good emulsifier with dispersive properties (Chapter 1; Table 1.3), and such solutions *may* affect the performance of VERIcleen™. Further work is, therefore, required in order to assess the affect of a range of cleaning solutions upon the sensitivity of this and all recently developed test methods.

3.5. Conclusion

The relative sensitivities of a range of methods for assessing surface cleanliness have been determined under controlled laboratory conditions. Microbiological testing, although capable of detecting the presence of low levels of microorganisms, detects only the microbial component of any residual contamination. ATP bioluminescence provides an indication of total surface contamination within minutes, but is currently unable to distinguish between the microbial and non-microbial components. Instrument-based systems are evolving into inexpensive, instrument-free test kits that are capable of detecting specific component residues. However, the use of protein detection for example, can only indicate that a surface is free of residues relatively high in protein. VERIcleen™ is likely to be the first of many single test protocols capable of detecting a variety of food components and could be used to rapidly assess the cleanliness of food production areas within which, a wide range of different food types are prepared. If however, rather than being assumed from the results of such non-microbiological sampling methods, the microbiological status of a surface is to be assured, assessment must also involve some form of microbiological testing.

The results of this laboratory-based investigation confirm, therefore, that given the variability in food debris and surface contamination, no one method is ideal for assessing cleanliness and strongly suggest that in order to obtain an accurate depiction of the hygienic status of a surface, rather than being interchangeable, test methods should be used in combination. In addition, the method(s) used must depend on the microbial load, the type and level of organic soil and the general state (i.e. dryness) of the bioburden likely to be present. Factory trials are, therefore, recommended prior to developing an assessment strategy and the information obtained from this laboratory-based study can be used to select the most appropriate method(s) for evaluation. However, it must be recognised that in factory conditions it is difficult to standardise the level of bioburden present. When, how and what is sampled within the factory environment is extremely variable and, therefore, it is possible that differences in the relative performance of these test methods may occur *in situ*. This shall be investigated in Chapter 4.

Chapter 4

A Field Comparison of Traditional and Recently Developed Methods for Assessing Surface Cleanliness within the Food Industry

4.1. Introduction

Food residues that are allowed to accumulate on any food contact or environmental surface can act as a continuous contamination source in which microorganisms can reside and multiply (Tuompo *et al.* 1999). Consequently, the hygiene of the process and processing environment is an important factor in both assuring food quality and protecting the consumer from pathogens (Miettinen *et al.* 2001).

Currently, the most effective way to reduce microbial contamination and microbial growth in foods is to establish in-house food safety and quality management programmes (Eisel *et al.* 1997). Good Manufacturing Practice emphasises sanitary effectiveness and hygienic practices during the processing of foods and, thus, cleaning either as part of general hygiene or specified as a control measure within a HACCP plan, is of great importance to caterers, retailers, manufacturers and processors alike and should be treated as an integral part of the production process itself (Adams and Moss, 1995). Nevertheless, the inadequacy of cleaning and disinfection procedures is a frequently cited cause for food product contamination and resulting outbreak (Salvat *et al.* 1995; Roels *et al.* 1997; Gill *et al.* 1999a; Samelis and Metaxopoulos, 1999; Corry *et al.* 2002; de Sousa *et al.* 2002; Lundén *et al.* 2002; Midelet and Carpentier, 2002).

It is acknowledged that 'acceptable cleanliness' is a relative concept – what is acceptable in one situation may be unacceptable in another (Section 1.4). Nevertheless, UK Enforcement officers assess the cleanliness of premises and wherever there is a risk to the food 'dirt' simply needs to exist for an offence to have been committed (Dillon and Griffith, 1999). Thus, although the structure of sanitation programmes can vary, depending upon industry sector, food premises and surface location, ideally *all* food companies require a simple and rapid method for assessing the hygienic status of food preparation areas and the efficacy of the cleaning procedures used.

When selecting the most appropriate method for use within any given processing environment, a company must decide what their priorities are and what specific attributes relating to cleanliness assessment are needed in relation to their own operation (Griffith *et al.* 1997). Those most commonly cited are the ease of use, speed and cost of the test method together with the need for accurate and reliable results (Griffith *et al.* 1997). However, the latter depends upon the ability of the test to detect the type of residues likely to be present (Chapter 3) and, thus, there is no one ideal method and no one 'best buy' for all companies.

In an attempt to help food businesses make an informed decision with regard to test selection, previous studies have been conducted in a variety of food processing environments comparing ATP bioluminescence to traditional microbiological methods (Bautista *et al.* 1993; Kyriakides *et al.* 1991; Ogden, 1993; Poulis *et al.* 1993; Illsley *et al.* 2000; Miettinen *et al.* 2001). However, these methods assess different parameters (Chapter 3) and, thus, it is speculated that such studies, by simply correlating those surfaces that each test method 'passed' or 'failed', may actually provide the food industry with limited and/or misleading information. In addition, there is relatively little published information regarding the comparative performance of the more modern instrument-free

cleanliness assessment methods. Chapter 3 discussed how a range of recently developed test methods performed under controlled laboratory conditions. Laboratory studies allow consistency with regard to surface type, cleanliness and condition, inoculum level and residue type and, in addition, the time these residues are allowed to dry prior to the surface being sampled. However, despite providing valuable information regarding test sensitivity, repeatability and reproducibility, laboratory studies do not necessarily replicate 'in-use' conditions, where surface material, residue type and moisture levels can all vary (Michaels *et al.* 2001a).

The aims of this chapter are, therefore, to:

- Evaluate a recently developed, instrument-free test method as a means of assessing surface cleanliness within a variety of food processing environments.
- Compare the performance characteristics of this new test method to those of both ATP bioluminescence and traditional microbiological methods.
- Determine and make recommendations as to how food businesses should evaluate new test methods and perform in-house comparison trials.

Objectives

- Select, on the basis of the results obtained in Chapter 3, the most appropriate test methods to use within the current study.
- Recruit food companies willing to take part in the investigation.
- Identify, on the basis of set criteria, those surfaces to be sampled within each food processing environment.
- Use the selected test methods to sample each surface both before cleaning and again after normal cleaning procedures have been carried out.

- Determine the level of agreement between the results obtained using the different test methods.

4.2. Materials and Methods

4.2.1. Premises

Businesses, within a 50-mile radius of UWIC, were recruited using an opportunity sample.

The study was conducted within four categories of food processing environment: a cooked meat processor, a cheese manufacturer, a bakery and a frozen ready-meal production plant. In all cases, visits were arranged with the agreement of the technical manager, who was made aware of the precise details of the study. However, to minimise observer bias, this information was not disclosed to production staff or those who carried out the cleaning.

4.2.2. Surface Samples

It has been acknowledged, that during previous studies, the differences observed between the results of different test methods, may have been due to possible variations in the level of contamination present (Section 3.4). During this investigation, therefore, in an attempt to minimise the error associated with being unable to standardise the level of bioburden present, the surfaces sampled had to fulfil a number of criteria. In addition to appearing in good repair and condition, each also had to be flat and large enough to allow three adjacent surface areas to be sampled using traditional microbiology, ATP bioluminescence and

protein detection. The cleanliness of each surface was also assessed visually. Information regarding the location of the sample site and whether the surface was wet or dry at the time of sampling was also recorded as were details regarding the cleaning agents and sanitation procedures used.

Forty-five different surfaces were sampled, including surfaces in direct contact with the product (e.g. tables, bins and conveyer belts), in indirect contact (e.g. control panels and door handles) and environmental surfaces (e.g. walls). Each surface was sampled after production had finished, both before cleaning and again after normal cleaning procedures had been carried out, thus, in total, 90 surfaces were sampled using each of the different test methods. All the surfaces sampled were within 'high risk' areas of the different production plants and as a result all were associated with cooked/post-processed products.

4.2.3. Microbiological Sampling of the Surfaces

When used to sample flat surfaces, dipslides are capable of detecting the presence of fewer bacteria than traditional hygiene swabs (Chapter 3). However, dilution of the sample is not possible and, therefore, if the surface is heavily contaminated with a mixed bacterial population, it becomes very difficult to detect the presence of specific microorganisms (Tebbutt and Midwood, 1990). Although, dipslides are available which comprise selective agars, it has been reported that the selective agents in the medium may inhibit the recovery, from cleaned surfaces, of detergent stressed cells (Miettinen *et al.* 2001). Nevertheless, the number of aerobic bacteria recovered from a surface can also be used to assess its cleanliness and an aerobic colony count (ACC), by providing an estimate of the overall bacterial population, can be considered indicative for the quality of the sanitation procedures used (Linton *et al.* 1997).

Both sides of a Plate Count agar dipslide (PC2; Dimanco Ltd) were pressed firmly onto the surface so as to sample a 25 cm^2 surface area. The dipslides were then incubated at 30°C for 48 h. After incubation, the slide was compared to the growth chart provided by the manufacturer, with the number of colonies isolated, signifying the level of viable microorganisms present on the surface.

4.2.4. Non-microbiological Sampling of the Surfaces

4.2.4.1. ATP measurement

ATP bioluminescence primarily detects the presence of food residues, yet is also capable of detecting microorganisms and, thus, gives an indication of total surface contamination (Section 1.4.2.2.1). During this investigation, ATP measurements were performed by sampling the surfaces, (approximately 100 cm^2), using the Clean-Trace™ Rapid Cleanliness Test (UXL 100; Biotrace Ltd). The device was activated in accordance with the manufacturer's instructions and readings were taken using the Biotrace Uni-Lite® luminometer. The reading in relative light units (RLU) was recorded.

4.2.4.2. Protein detection

The performance of four different protein detection systems, each only capable of detecting the presence of food residues, was previously evaluated under controlled laboratory conditions (Chapter 3). Although the sensitivity and repeatability of the swab-based Pro-tect® device (Biotrace) was comparable to the other commercially available protein detection kits (Section 3.4.2.2), this device was considered the most user-friendly

and, in addition, is also capable of detecting the presence of reducing sugars and other reducing agents (Section 3.2.6.2.2). Thus, Pro-tect® was chosen to represent this particular method of assessing surface cleanliness.

An area measuring approximately 100 cm^2 was sampled in accordance with the manufacturer's instructions. The device was activated and left for up to 10 minutes at room temperature to allow the colorimetric reaction to occur.

4.2.5. Interpretation of Results

The cleaning protocol used throughout the laboratory-based study (Section 3.2.4.1) was capable of reducing residual surface contamination to such a level, that prior to inoculation, microbial counts and bioluminescence readings taken from the surface were at an absolute minimum. Consequently, it was possible to set relatively strict 'pass' 'fail' specifications (Section 3.2.7). It would be impractical to suppose that a similar level of surface cleanliness could be achieved within the factory environment and, thus, it was necessary to increase the specifications for both microbial and bioluminescence background levels.

However, there are no standards for food surface cleaning and setting a level at which a test 'fails' a surface has proved difficult (Tebbutt, 1999). Nevertheless, a general microbial target value of < 2.5 cfu cm^{-2} after disinfection has been suggested (Table 1.5) and has been found to be attainable for a range of surfaces (Griffith *et al.* 2000). Best cleaning and disinfection practice has also indicated that when using the Biotrace system, although it is possible, through the implementation of a good, validated sanitation protocol to

consistently achieve lower bioluminescence readings, an ATP value of 500 RLU for a clean surface is a realistic upper critical limit (Griffith *et al.* 2000).

During this investigation, therefore, a surface would 'fail' and be presumed unclean if the number of colonies recovered from the surface was > 2.5 cfu cm^{-2} or if ATP values > 500 RLU. The colour reaction of the protein test was compared to the test card provided by the manufacturer and the surface would fail if the colour matched that of level 3 or 4 on the card. Level 2 indicated a caution result.

The χ^2 test was used to determine whether there was a correlation between the results obtained using the three different test methods, whilst the agreement between two different test methods was calculated using equation 1 (Illsley *et al.* 2000).

$$\frac{\text{No. of surfaces 'failed' by both methods + No. of surfaces 'passed' by both methods}}{\text{Number of surfaces sampled}} \times 100 \quad (1)$$

4.3. Results

A comparison of the results that were obtained after traditional microbiology, ATP bioluminescence and protein detection were used to sample the food contact and environmental surfaces is illustrated in Figures 4.1 and 4.2 and Tables 4.1 to 4.4. The percentage agreement signifies the number of times that the different test methods agreed on the hygienic status of the surfaces (Illsley *et al.* 2000).

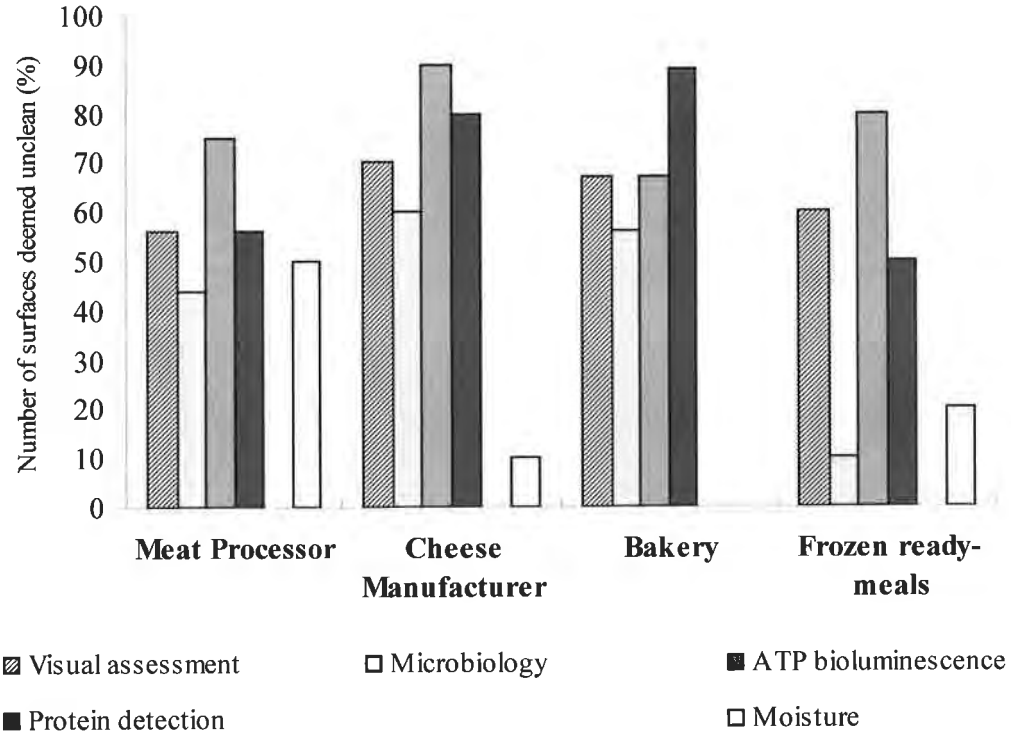
To confirm the reliability of the results, one of the premises was visited on two different occasions. Although, the precise values obtained varied slightly, the overall pattern with regard to the comparative performance of the different test methods was consistent on both occasions (Appendix III).

4.3.1. Sampling the Surfaces Prior to Cleaning

Figure 4.1 illustrates the percentage of surfaces that were deemed unclean using traditional microbiology, ATP bioluminescence and protein detection, before each of the different processing facilities had carried out their normal cleaning procedures. The percentage of surfaces relating to those appearing visually dirty and those that were sampled when wet is also presented. Taken collectively, these results provide a profile as to the type and level of contamination initially present on the surface. In addition, they also confirm that should the surfaces be inadequately cleaned and/or disinfected, the residues likely to be present are of a type that can be detected by one or more of the different test methods.

Prior to cleaning, the majority of the surfaces sampled were visibly dry and, depending upon the production unit sampled, between 56% and 70% of these surfaces appeared visually dirty. In addition, between 67% and 90% and between 50% and 89% of the surfaces 'failed' using ATP bioluminescence and protein detection respectively, implying that regardless of processing environment, prior to cleaning, many of the surfaces sampled were contaminated with relatively high levels of detectable food debris.

Figure 4.1. Percentage of surfaces sampled prior to cleaning that were deemed 'unclean' using visual assessment, traditional microbiology, ATP bioluminescence and protein detection.



In all four cases, a comparison of the results obtained using protein detection and visual assessment resulted in a relatively high level of agreement, ranging from 67% within the bakery to 90% within the cheese manufacturing unit (Table 4.1), suggesting that much of the gross food debris comprised high levels of protein and/or reducing agents. In most cases, the results of the protein detection method also correlated well with those obtained via traditional microbiology (Table 4.1), implying that many of the microorganisms present, particularly within the high-protein processing environments, were associated with the product residues and, therefore, had been transferred to the surface with the food. Cheese residues for example, can be expected to contain large numbers ($> 10^8$ cfu g^{-1}) of lactic acid bacteria – those microorganisms responsible for the fermentation process (Richter *et al.* 1992; Harrigan, 1998).

Table 4.1. Percentage agreement between traditional microbiology, ATP bioluminescence, protein detection and visual assessment after each had been used to assess surface cleanliness prior to normal cleaning procedures being carried out.

Production facility	**Percentage agreement (before cleaning)**			
	Micro / ATP	***ATP / Protein***	***Protein / Micro***	***Protein / Visual***
Meat processor	63	75	81	81
Cheese manufacturer	50	70	80	90
Bakery	33	67	44	67
Frozen ready-meals	10	50	60	70

However, the number of surfaces deemed unclean using traditional microbiology ranged from just 10% within the frozen ready-meal environment to 60% within the cheese production unit (Figure 4.1), suggesting that whilst microorganisms were present, they were, in many cases, at levels proportionally much lower than that of the food debris. This is reflected in the results presented in Table 4.2, which illustrates, most notably, that 20 of the 45 surfaces sampled (44%) were deemed unclean by way of ATP bioluminescence but adjudged as being clean using traditional microbiology. Similarly, 12 of the 45 surfaces (27%) 'failed' using protein detection but were deemed clean using dipslides. Overall, therefore, despite some correlation, there was a statistically significant difference between the results obtained using ATP bioluminescence, protein detection and traditional microbiology ($\chi^2 = 17.63$; $p < 0.05$).

Table 4.2. Comparison of results, according to set pass and fail values, that were obtained after traditional microbiology, ATP bioluminescence and protein detection were used to sample 45 different food contact and environmental surfaces prior to them being cleaned.

Before cleaning		*Traditional Microbiology (Dipslides)*			*Protein detection*		
		Pass	**Caution**	**Fail**	**Pass**	**Caution**	**Fail**
ATP bioluminescence	**Pass** [1]	6	0	4	5	1	4
	Caution [2]	0	0	0	0	0	0
	Fail [3]	20	0	15	8	1	26
Protein detection	**Pass**	13	0	0			
	Caution	1	0	1			
	Fail	12	0	18			

Percentage agreement		
Micro / ATP	***ATP / Protein***	***Protein / Micro***
47	69	69

[1] Pass: number of colonies recovered from the surface < 2.5 cfu cm^{-2}, ATP value < 500 RLU and protein colour = level 1

[2] Caution: protein colour = level 2

[3] Fail: number of colonies recovered from the surface > 2.5 cfu cm^{-2}, ATP value > 500 RLU and protein colour = level 3 or 4

Table 4.3. Comparison of results, according to set pass and fail values, that were obtained after traditional microbiology, ATP bioluminescence and protein detection were used to sample 45 different food contact and environmental surfaces after they had been cleaned.

After cleaning		*Traditional Microbiology (Dipslides)*			*Protein detection*			
		Pass	**Caution**	**Fail**	**Pass**	**Caution**	**Fail**	**No result**
ATP bioluminescence	**Pass** [1]	22	0	5	17	3	4	3
	Caution [2]	0	0	0	0	0	0	0
	Fail [3]	12	0	6	11	0	6	1
Protein detection	**Pass**	23	0	5				
	Caution	1	0	2				
	Fail	6	0	4				
	No result [4]	4	0	0				

Percentage agreement		
Micro / ATP	***ATP / Protein***	***Protein / Micro***
66	51	60

[1] Pass: number of colonies recovered from the surface < 2.5 cfu cm^{-2}, ATP value < 500 RLU and protein colour = level 1

[2] Caution: protein colour = level 2

[3] Fail: number of colonies recovered from the surface > 2.5 cfu cm^{-2}, ATP value > 500 RLU and protein colour = level 3 or 4

[4] No result: unusual colour formation made interpretation impossible

4.3.2. Sampling the Surfaces After Normal Cleaning Procedures had been Conducted

In general the number of surfaces that were 'passed' as clean using traditional microbiology, ATP bioluminescence and protein detection increased after the different production facilities had carried out their normal cleaning procedures (Table 4.3). Nonetheless, there was again a significant difference between the results obtained using these three different test methods ($\chi^2 = 17.90$; $p < 0.05$).

Although the level of agreement between ATP bioluminescence and traditional microbiology had improved, 12 of the 45 surfaces sampled (27%) were still deemed unclean using ATP bioluminescence despite being 'passed' as clean by way of the dipslides (Table 4.3). Similarly, 24% (11/45) and 11% (5/45) of those surfaces that were adjudged as being clean using protein detection, were 'failed' and deemed unacceptable for food production using ATP bioluminescence and traditional microbiology respectively.

However, whilst prior to cleaning, the percentage agreement between two test methods helped to characterise the nature of the surface contamination, comparing the relative performance of the two methods after cleaning can, it appears, provide misleading information. Within the frozen ready-meal facility, all the surfaces that were sampled after cleaning were 'passed' as clean using both the protein detection method and visual assessment, resulting in a level of agreement between these two test methods of 100% (Table 4.4). However, 80% of these surfaces were deemed unacceptable for food production using ATP bioluminescence (Figure 4.2). Thus, within any given processing environment, a high level of agreement between test methods may result from neither being able to detect the residues actually present and, therefore, neither should be used to assess surface cleanliness.

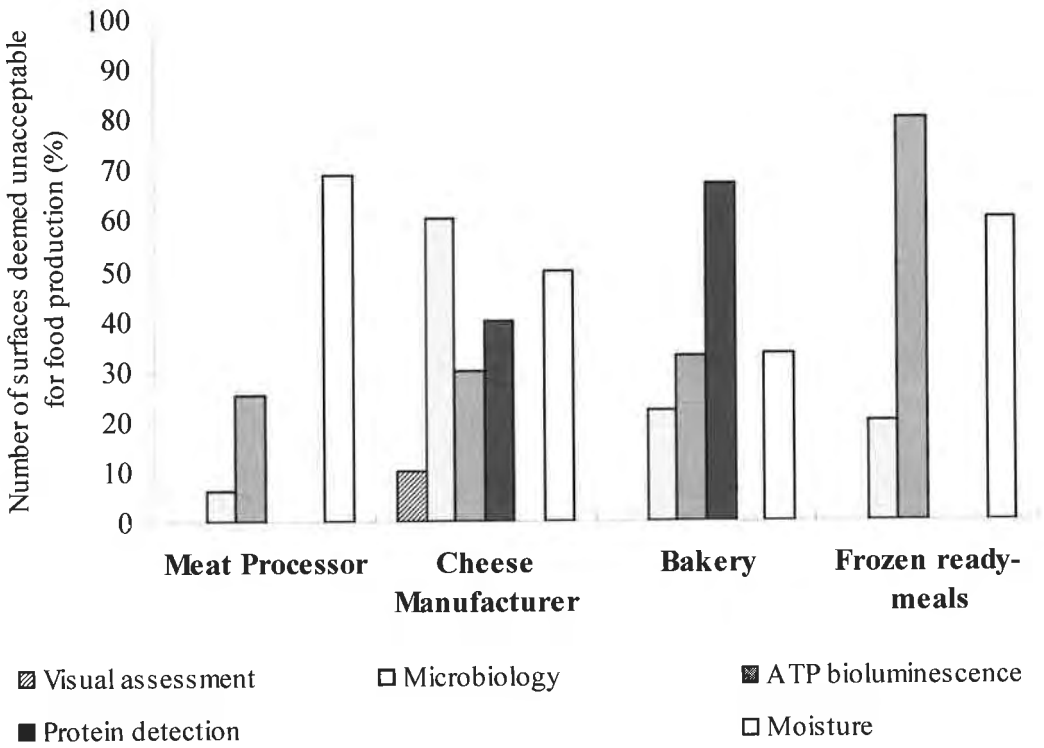
Table 4.4. Percentage agreement between traditional microbiology, ATP bioluminescence, protein detection and visual assessment after they had been used to assess surface cleanliness after normal cleaning procedures had been carried out.

Production facility	Percentage agreement (after cleaning)			
	Micro / ATP	*ATP / Protein*	*Protein / Micro*	*Protein / Visual*
Meat processor	75	56	75	81
Cheese manufacturer	40	70	30	40
Bakery	89	56	44	22
Frozen ready-meals	20	10	80	100

Figure 4.2 illustrates the number of 'cleaned' surfaces that were deemed unacceptable for food production by each of the different test methods. When taken collectively, these results again provide an impression of the types of contamination present and, thus, as shall subsequently be discussed, when compared to the results obtained prior to cleaning, they help highlight inadequacies within the various sanitation procedures. However, in addition, these 'failure' rates also identify the most appropriate test method(s) for use within the different processing environments.

In general, the use of visual assessment appeared to be a poor indicator of cleaning efficacy. Although 90% of the surfaces sampled within the cheese production unit appeared visually clean, the use of traditional microbiology revealed that 60% of these surfaces were contaminated with bacteria at levels of > 2.5 cfu cm^{-2}. Furthermore, all the surfaces sampled within the baking facility and the frozen ready-meal production plant appeared visually clean. However, 67% of the surfaces sampled within the bakery 'failed' using protein detection and, as already mentioned, 80% of the surfaces within the frozen ready-meal plant were deemed unacceptable for food production using the ATP bioluminescence technique (Figure 4.2).

Figure 4.2. Percentage of surfaces sampled after cleaning that were deemed unacceptable for food production using visual assessment, traditional microbiology, ATP bioluminescence and protein detection.



The results presented clearly illustrate that when choosing the most appropriate means of assessing surface cleanliness, the primary concern for a food company should be to ensure that the test method(s) is able to detect the food residues likely to be present and, thus, consideration must be given to the composition of the final product. However, observations made during the current investigation also highlight the need to consider the possible effect, upon test results, of extraneous substances.

The majority of the surfaces sampled after cleaning were wet (Figure 4.2) either with rinse water and/or residual cleaning chemicals. Although overall, surface dryness had no significant effect ($p < 0.05$) upon the number of surfaces each test method 'passed' or 'failed', the presence of cleaning agents did, in some cases, have an adverse effect upon the colorimetric reaction of the protein detection method.

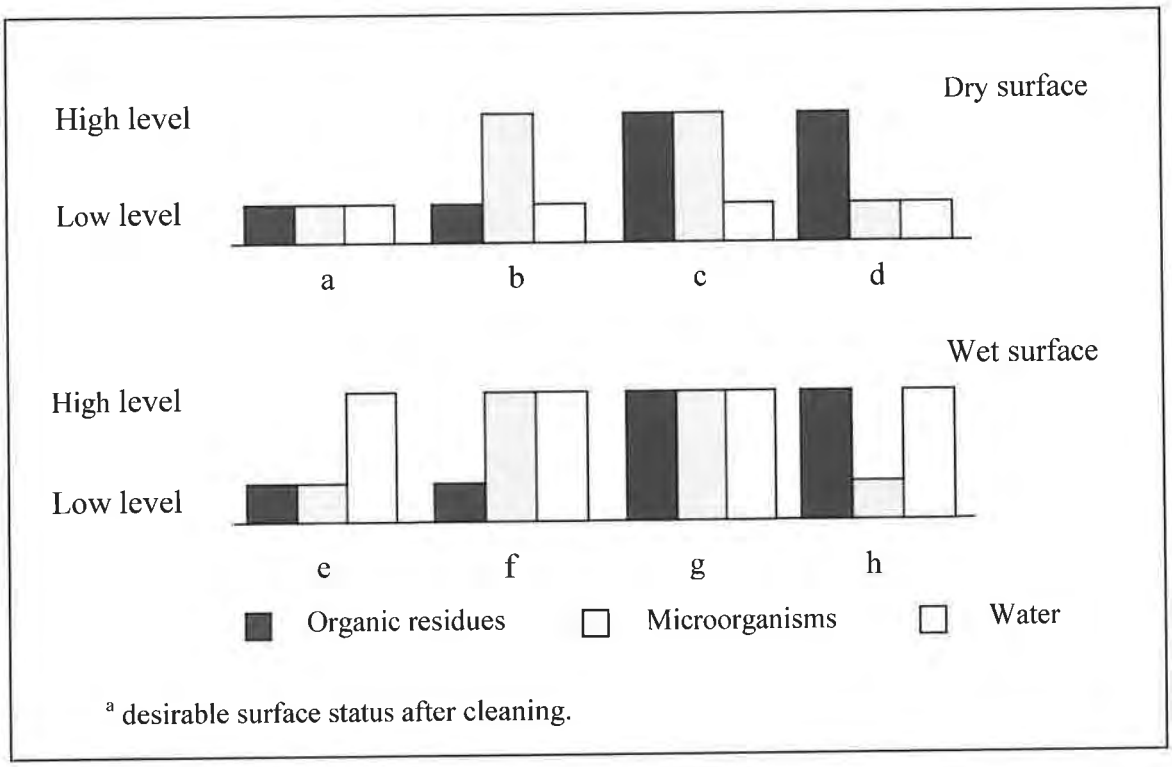
Within the meat processing and cheese manufacturing plants, the cleaning solutions (as stated by the manufacturer) did not need to be rinsed from the surfaces. As a result, sampling the surfaces before they had been allowed to dry may have led to an unusually large volume of cleaning chemical being incorporated within the test. This subsequently appeared to affect the activity of the reagents and led to an unusual colour formation, which resulted in the inability to interpret the results of 9% (4/45) of the protein detection tests used (Table 4.3).

Within the UK, it is currently unclear as to whether a surface should be rinsed after disinfection. Whilst some companies require surfaces to be rinsed with potable water, others advocate the use of disinfectants which are of low taint and low toxicity and which can be left on a food contact surface without the need for rinsing. The final choice of whether or not to rinse lies, therefore, with the individual company but it is recommended that a risk analysis is carried out to determine the risks associated with small quantities of cleaning chemical reaching the final product (Rigarlsford, 2002). The results of the current study suggest that trials also need to be conducted in order to determine whether the cleaning chemicals will have an effect upon the methods used to assess surface cleanliness and, thus, highlight the need to consider cleaning and cleaning assessment as a whole.

4.4. Discussion

Soiling is a natural process that occurs in all food plants (Chapter 1). However, the composition of the organic soil and the identity, numbers and physiological condition of the microorganisms likely to be present will depend upon the production environment, the nature of the food and the process to which it is being subjected (Poulis *et al.* 1993; Verran *et al.* 2001a). Figure 4.3 illustrates the different microbial and organic soil mixtures that could contribute to the contamination present on a surface. Any of these combinations could result from either a single soiling event or a number of soiling events separated by inadequate cleaning and/or disinfection (Verran *et al.* 2001a).

Figure 4.3. Organic and microbial soil mixtures that could contribute to the contamination present on a surface before and after cleaning.



[a] desirable surface status after cleaning.

Different test methods assess surface cleanliness by detecting the presence of different components associated with any residual surface contamination (Chapter 3). Consequently, when conducting in-house comparison trials, a 100% agreement between

the results obtained using different test methods should not necessarily be either expected or considered ideal (Section 4.3.2). However, regardless of how the performance of microbiological and non-microbiological methods may compare, under normal circumstances, it is relatively pointless to use a rapid cleanliness test on a visually unclean surface (Dillon and Griffith, 1999). The visual assessment of surfaces can, and did, reveal the presence of gross food debris (Figure 4.1) and previous studies have shown that routine visual inspection of food premises can be important for predicting the risk of foodborne disease outbreaks (Irwin *et al.* 1989; Kassa *et al.* 2001). However, after each of the four food processing environments had carried out their normal cleaning procedures, the number of surfaces that were deemed unacceptable for food production using either ATP bioluminescence, protein detection or traditional microbiology far outnumbered those that were failed using visual assessment (Figure 4.2). These results concur with the findings of Tebbutt (1986) and Worsfold and Griffith (2001), who concluded that visual inspection underestimates the extent of soiling and as a result is a poor indicator of cleaning efficacy.

The results obtained from sampling a small number of sites can be used to indicate the general hygienic status of an entire production area (Ogden, 1993). The number of surfaces that were 'passed' as clean using ATP bioluminescence, protein detection and traditional microbiology generally increased after the different production facilities had carried out their normal cleaning procedures (Figures 4.1 and 4.2). Nevertheless, although not the primary aim of this investigation, the results, when taken collectively, did highlight areas of potential concern within each of the different sanitation programmes. However, there was a significant difference ($\chi^2 = 17.90$; $p < 0.05$) between the results obtained using the three different test methods (Tables 4.3 and 4.4) and, therefore, depending upon which was used, different conclusions could be drawn regarding the hygienic status of the different production plants.

4.4.1. Assessing the Cleanliness of Production Surfaces which, Prior to Cleaning, are Likely to be Contaminated with Relatively Low Levels of Microorganisms

During the production of the cooked meats and ready-meals, despite the presence of visible food debris, the use of traditional microbiology did not detect the presence of microbial contaminants on many of the food contact surfaces (Figure 4.1). Thus, prior to cleaning, the surface contamination within these particular processing environments most likely resembled that depicted in Figure 4.3d and h. However, it is important to emphasise that the surfaces sampled were within the 'high-risk', post-process areas of these two different production plants. It is predicted that should sampling be conducted within a 'low-risk' environment such as an abattoir, a poultry processing plant or any area associated with raw, pre-processed meats or vegetables, the surfaces would more likely be contaminated with high numbers of microorganisms as well as high levels of organic debris (Figure 4.3c and g) and, thus, the number of surfaces 'failed' by means of traditional microbiology would be significantly higher (Chapter 3). As shall subsequently be discussed, a similar situation can occur within 'high-risk' environments, particularly if the product in contact with the surfaces is of a type likely to comprise high levels of microorganisms (Section 4.4.2). However, freshly prepared cooked, uncured meats normally contain < 100 cfu g^{-1} (Johnston and Tompkin, 1992) and, thus, the post-process areas associated with the production of cooked meats *should* have only minimal levels of microbial contaminants and, therefore, any surface contamination *should* consist predominantly of organic debris.

4.4.1.1. Assessing surface cleanliness within a meat processing plant

After the meat processing plant had carried out its normal cleaning procedure, the agreement between traditional microbiology and both ATP bioluminescence and protein

detection was relatively high (Table 4.4), with relatively few surfaces being deemed unacceptable for food production using any of these test methods (Figure 4.2).

The cleaning procedure used within this particular processing plant, involved the application of a combined detergent/disinfectant, by means of a high pressure, foam trigger-spray, resulting in a single stage clean and disinfect. The results suggest that this method was relatively effective in removing both organic residues and microorganisms (Figure 4.3a and e). Nevertheless, 25% of the surfaces sampled did give bioluminescence readings of > 500 RLU, suggesting that the mechanical energy provided by the trigger spray may not have been sufficient to remove all the soil from all of the surfaces. This concurs with previous studies, which have implied that whilst spraying may be an efficient physical means of cleaning a surface, microbial cells, perhaps because of their larger size, do appear to be removed more readily by the force of the spray than residual food debris (Verran *et al.* 2001a). Furthermore, should the food debris comprise high levels of fats and/or proteins, then this is thought to aid the formation of a coagulum and, thus, promote aggressive surface attachment; meat residues in particular have been demonstrated as becoming progressively more difficult to remove over time (Michaels *et al.* 1999). However, the polyurethane foam swab associated with the Pro-tect® enabled a relatively high amount of mechanical energy to be applied to the surface during sampling (Section 2.4.2.1) and as all the surfaces sampled were 'passed' as clean using this particular test method (Figure 4.2), it could be concluded that those organic residues that remained on the surfaces were of a type likely to be low in protein.

During the current investigation, the surfaces were sampled immediately after cleaning. However, it has been reported that the use of high pressure cleaning systems can create aerosols of viable microorganisms, which can re-contaminate the surfaces several hours after cleaning has been completed (Section 1.3.3.4). Should food debris also be present

then this can facilitate the survival and subsequent growth of such organisms. Thus, whilst ATP bioluminescence and/or protein detection should be used within 'high-risk' environments to rapidly assess the efficacy of the sanitation procedures employed, to ensure the surfaces are not becoming re-contaminated, it is recommended that ATP bioluminescence and/or microbiological analysis is also conducted immediately prior to production at the start of each working day. Nevertheless, it is acknowledged that, prior to production, food contact and environmental surfaces are likely to be dry and, thus, careful consideration must be given to the microbiological sampling method used. Previous chapters have demonstrated that the sensitivity of traditional hygiene swabs is severely compromised when the surface sampled is dry (Chapters 2 and 3). In contrast, results have suggested that surface dryness has less of an effect upon the overall performance of dipslides (Chapter 3 and Figure 4.1).

4.4.1.2. Assessing surface cleanliness within a frozen ready-meal production plant

It is imperative that an effective cleaning protocol should remove all organic residues, thereby depriving those bacteria that are present, together with any potential microbial contaminants, of an available source of nutrients (Worsfold and Griffith, 2001). Soil and bacteria are typically retained in surface imperfections, particularly pits and crevices. It is thought that such defects and, thus, the retention of contaminants is exacerbated by the abrasion caused by the forces associated with spray-washing (Stevens and Holah, 1993). Thus, although stainless steel is relatively resistant to surface change (Section 1.3.2), the potential damage to other materials used within the food industry has prompted some authors to conclude that wiping is a more effective means of reducing surface contamination (Stevens and Holah, 1993).

The frozen ready-meal production plant, visited during this investigation, employed a "clean-as-you-go" system, whereby dedicated personnel cleaned surfaces and equipment as production runs were completed. In the majority of cases, re-usable cloths were used for this purpose. The use of dipslides revealed that after normal cleaning procedures had been carried out, 20% of the surfaces sampled were contaminated with microorganisms at levels > 2.5 cfu cm^{-2}. However, 80% of these surfaces were deemed unacceptable for food production using ATP bioluminescence (Figure 4.2). Thus, within this particular processing environment, had only traditional microbiology been employed to assess surface cleanliness, its use, despite assuring the presence of minimal microbial contamination, would not have revealed the high levels of residual food debris and, thus, the inadequacies that likely exist within the cleaning protocol.

The ability of re-usable cloths to act as a vehicle for cross contamination has been highlighted in previous studies (Tebbutt, 1988; Scott and Bloomfield, 1990; Tebbutt and Southwell, 1997; Hilton and Austin, 2000; Gorman *et al.* 2002; Sagoo *et al.* 2003). When using a cloth, its relatively smooth surface structure means that it has a large surface area in contact with a surface at any one time and, therefore, its use carries a risk of transferring food debris and/or microorganisms maintained upon it to any subsequently wiped food preparation surface (Hilton and Austin, 2000). Although rinsing can dislodge potential contaminants, previous studies have implied that, in general, re-usable cloths are cleaned and/or disinfected infrequently (Tebbutt, 1988; Sagoo *et al.* 2003).

During this particular study, neither the water nor the cloths used to clean the surfaces were tested either microbiologically or chemically. Nevertheless, there is a strong possibility that the transfer of food debris to the surfaces via a dirty cloth may have contributed to the contamination detected by the ATP bioluminescence technique. However, the number of surfaces that were 'passed' as clean using the protein detection system (Figure 4.2)

indicates that the high protein food residues that were initially present (Figure 4.1) had been effectively removed. This could either imply that when wiping a surface, as was the case when a more forceful cleaning protocol was applied (Section 4.4.1.1), debris comprising low levels of protein may be more difficult to remove or, alternatively, that low protein organic residues are transferred more readily from one surface to another by means of a dirty cloth.

4.4.2. Assessing the Cleanliness of Production Surfaces which, Prior to Cleaning, are Likely to be Contaminated with Relatively High Levels of Microorganisms

The highest levels of microbial contamination were detected within the bakery and the cheese production unit (Figure 4.1), where due to the use of yeasts and starter cultures, the presence of microorganisms prior to cleaning cannot be unexpected (Section 4.3.1). During production, the majority of the surfaces sampled were dry and thus, prior to cleaning, the residual surface contamination present within these two processing environments most likely resembled that depicted in Figure 4.3c.

4.4.2.1. Assessing surface cleanliness within a bakery

In the majority of cases, food manufacturing plants are involved in the production of a specific product type. It was hypothesised, therefore, that although beneficial, such processing establishments have no real need for a test capable of detecting a wide range of food residues. Hence, for the purposes of this industry trial, protein detection, as opposed to a multi-residue detection method, was chosen to represent the instrument-free, non-microbiological methods of assessing surface cleanliness. Nevertheless, the ability of any

cleanliness assessment method to detect multiple chemical residues increases the range of organic debris that can be detected (Section 3.4.2.3).

Bakeries are not normally associated with the production of high-protein foods, consequently, since the residual surface contamination would likely be of a type low in protein, protein detection may not be considered an effective means of assessing surface cleanliness (Chapter 3). Additionally, previous reports have suggested that the use of ATP bioluminescence may also be inappropriate within these particular processing environments, specifically within those areas where the presence of dry, fine soil such as flour, advocates the use of dry cleaning and where, due to food residues never being entirely eliminated, high background ATP values are often obtained (Griffiths, 1996; Illsley *et al.* 2000).

Situations can exist, therefore, where the detection of neither ATP nor protein residues will give an accurate indication as to the hygienic status of food contact surfaces. However, during the current investigation, 67% of those surfaces sampled after the baking facility had carried out its normal cleaning procedures, 'failed' using the protein detection method (Figure 4.2). These results did not correlate with those obtained using ATP bioluminescence (Table 4.4) and suggest that much of the residual food debris may also have contained a low level of intrinsic ATP (Chapter 3). Thus, the ability of the Pro-tect® surface hygiene test to detect the presence of both protein and reducing sugars could prove particularly beneficial, especially within production plants, such as bakeries, where residues such as jam are likely to be present on surfaces that have been inadequately cleaned. Indeed, with cloths again being used to clean the surfaces, only the routine use of Pro-tect® would have given an indication that those issues discussed in Section 4.4.1.2 may also have been affecting the efficiency of the cleaning procedure employed by this particular processing plant. However, despite not being evaluated during this industry

trial, it is suspected, on the basis of its performance during the laboratory-based study (Chapter 3), that the VERIcleen™ Food Residue Surface Test may prove an even more appropriate means to assess surface cleanliness within this particular baking facility.

4.4.2.2. Assessing surface cleanliness within a cheese production unit

In the production of ready-to-eat foods, the use of non-microbiological test methods is considered essential in order to rapidly identify any problems that may exist within the sanitation procedures employed. However, the results obtained within the cheese production unit, highlight how the sensitivity of the chemistry associated with the ATP bioluminescence technique can affect the performance of the test.

Protein detection can be used to indicate that a surface is free from high protein product residues and, with regard to Pro-tect® that a surface is also free from residual reducing sugars. However, protein detection alone cannot reveal the presence of even very high levels of microbial contaminants (Chapter 3). Although ATP bioluminescence can detect the presence of microbial contamination, in the absence of food debris, this technique is only capable of detecting the presence of 10^3 cfu cm^{-2}. In comparison, traditional microbiological methods are able to detect the presence, on a wet surface, of < 10 cfu cm^{-2} (Chapter 3). Within the cheese production unit, the number of surfaces that 'failed' by means of ATP bioluminescence and protein detection dropped from 90% and 80% to 30% and 40% respectively (Figures 4.1 and 4.2). Had either of these rapid tests been used in isolation, therefore, the results would imply that the cleaning procedure, although not perfect, had been relatively effective. However, the number of surfaces deemed unacceptable for food production using traditional microbiology remained at 60% (Figures

4.1 and 4.2). Thus, although significant levels of food debris had been removed from the surfaces, the disinfection procedure appears to have been ineffective (Figure 4.3b and f).

A number of contributory factors can result in ineffective disinfection (Section 1.3.3.3). It is known for example, that in the presence of dairy soils, disinfectants, either by being absorbed by, or reacting with, the organic matter, become less active (Lambert and Johnston, 2001). However, although ensuring that food debris has been effectively removed may prevent microorganisms from being protected from the direct action of sanitisers and disinfectants, in order to accurately assess the efficacy of disinfection procedures, some form of microbiological testing will also be required.

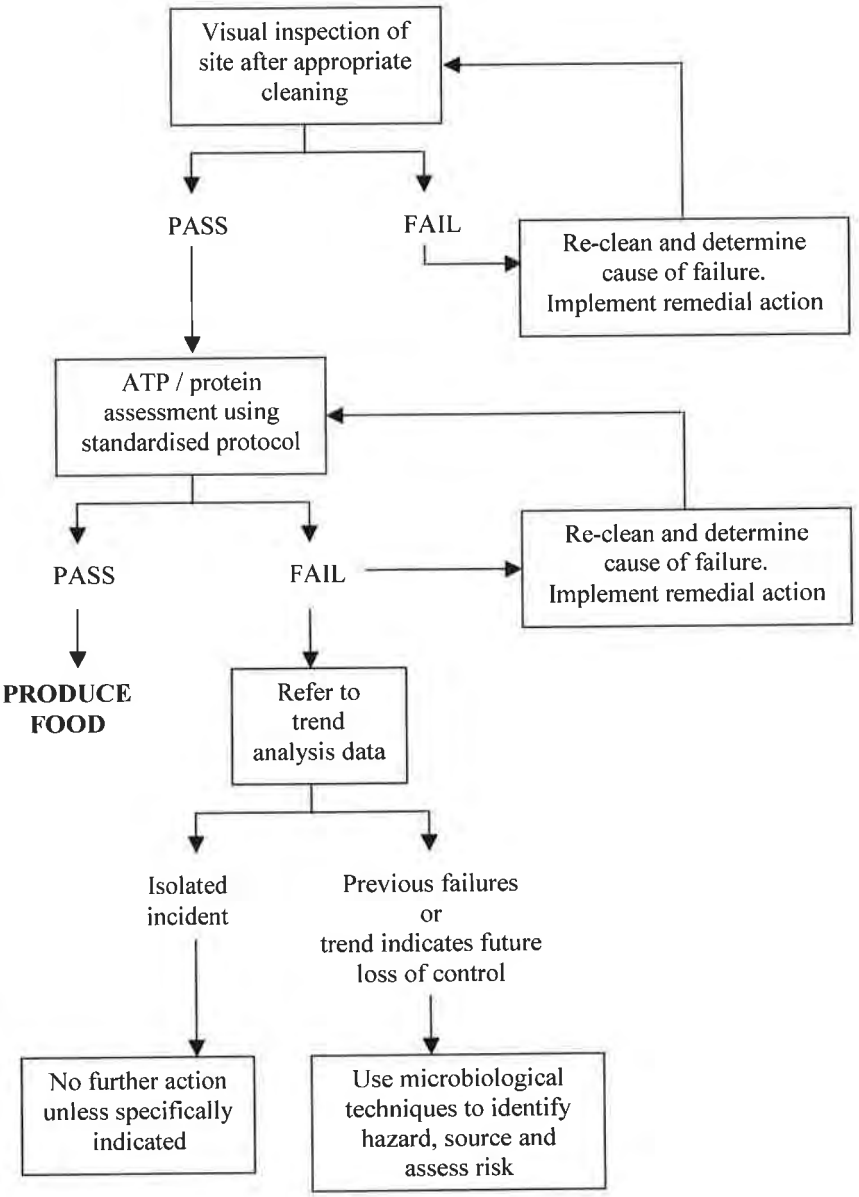
4.5. Conclusion

There is a need for food manufacturers to objectively test their cleaning programmes, chemicals and protocols in order to establish regimes that can be shown to be successful and economic (Griffith *et al.* 1997). Hygiene assessment, therefore, is an important part of the QC system and most food manufacturing facilities will initiate environmental sampling as a means to meet HACCP pre-requisites and/or to validate and verify the cleaning process (Salo and Wirtanen, 1999; Slade, 2002).

However, the degree of confidence that can be placed upon the sample result is dependent upon the sampling plan employed (Kvenberg and Schwalm, 2000). The results of this investigation support the conclusions of Griffith *et al.* (1997), who postulated, that for maximum benefit, visual, non-microbiological and microbiological methods should be

combined, resulting in the production of an integrated cleaning assessment strategy (Figure 4.4).

Figure 4.4. Stages in an integrated cleaning assessment strategy (Griffith *et al.* 1997)



Within such a strategy, should a surface appear visually dirty, it would be unnecessary to use any other assessment method and the surface should simply be re-cleaned and the reasons for failure investigated. However, within for example, a high protein food processing environment, a visually clean surface could be tested using both ATP bioluminescence and protein detection. A positive protein result would suggest that the cleaning component of the sanitation procedure had been inadequately carried out, whereas

a high bioluminescence reading coupled with a negative protein result could indicate ineffective disinfection. In both cases, microbiological testing could be of value, not only to detect the presence of low levels of bacteria but also for the purposes of validating and verifying HACCP plans and to identify specific microbial hazards.

Such an approach will naturally require the purchase of additional test methods. However, these costs need to be considered in relation to the costs of failing to assess surface cleanliness effectively. Cleaning costs the food industry many millions of pounds per year and ineffective cleaning wastes time, money and energy. The use of such an integrated cleaning assessment strategy should ensure that if a surface is inadequately cleaned, the problem can be identified quickly and the correct remedial action implemented, before such a surface becomes a hazard and a risk to the safety and quality of the product.

However, the results of both this industry trail and the previously discussed laboratory-based study confirm that when selecting any method to assess surface cleanliness, it is imperative that the method chosen is appropriate to the processing environment. If the levels of organic debris and/or microorganisms likely to be present are below the detection limit of the analytical method used, or, if the residues are of a type that are simply not detected by a particular test method, it will give the illusion that the surface is free from the residue(s) in question. Should such invalid sampling techniques be used and data from these samples interpreted without appreciation of the drawbacks and limitations of the test method utilised, misunderstandings could result with regard to the effects of the cleaning process and the microbial condition of the final product (Greene and Herman, 1961; Brown *et al.* 2000). Thus, although the proposed assessment strategy could be utilised within a variety of food processing plants, it is by no means definitive and in-house comparison trials must be conducted to determine the most appropriate test method(s) to use within any given processing environment.

During an in-house comparison trial it is, therefore, essential to ensure that any new test method is capable of detecting the residues likely to be present, thus, it should initially be used to sample the surfaces prior to cleaning. Its performance, after cleaning, can then be compared to the company's reference method but, rather than emphasis being placed upon how well the results of the two test methods correlate, the number of surfaces each test 'fails' should be considered indicative as to which method is the most appropriate for use within a particular processing environment. However, the results from this and the previous investigation (Chapter 3) have demonstrated that when comparing the performance of different test methods and when interpreting the results obtained, there are a range of factors that should also be taken into consideration. Consequently a checklist that could be of use to a food business when conducting an in-house comparison trial has been devised and is presented in Figure 4.5.

Results from both the laboratory-based study (Chapter 3) and this industry trial provide important and new information relating to the comparative performance of the range of cleanliness assessment methods that are now available to the food industry and this can be used to up-date, expand and improve upon the previously devised strategy (Figure 4.4). However, prior to this, for some key sectors of the food industry, appropriate methods for assessing surface cleanliness are currently lacking. Results from previous chapters, suggest that problems may exist regarding the detection of important food groups such as fats. Furthermore, the importance and relevance of microbial sample data should not be underestimated yet the inability to rapidly detect surface contaminants prevents microbiological sampling being incorporated within a HACCP plan and, thus, a routine hygiene monitoring strategy. The design, development and evaluation of methods that could be used for such purposes will be the subject of Chapters 5 – 7.

Figure 4.5. Factors to be considered when determining which cleanliness assessment method is best suited to a production environment

Surface	Site	Low-risk ☐	
		High risk ☐	**Consider** microbiological specification of final product. If low, surfaces should be more likely to be contaminated with organic debris and less likely to 'fail' using traditional microbiological techniques.
	State at time of sampling	Wet ☐	
		Dry ☐	**WARNING:** performance of traditional swabbing technique can be severely affected
Cleaning protocol	Area requires 'dry-cleaning'	Yes ☐	**WARNING:** presence of residual debris can lead to high background ATP levels; ATP bioluminescence may be unsuited to processing environment
		No ☐	
	Surfaces rinsed post-disinfect	Yes ☐	
		No ☐	**WARNING:** presence of residual cleaning chemicals can affect the performance of non-microbiological assessment methods
Composition of food	Protein content	High ☐	***protein detection suited to processing environment;*** **(Caution**: provides no indication as to level of microbial contamination present)
		Low ☐	**WARNING:** protein detection unsuited to processing environment
	Carbohydrate content	High ☐	***glucose detection (e.g. VERIcleen™) suited to processing environment*** **(Caution**: provides no indication as to level of microbial contamination present)
		Low ☐	**WARNING:** glucose detection unsuited to processing environment
	Fat content	High ☐	**WARNING:** performance of ATP bioluminescence technique may be affected
		Low ☐	

Chapter 5

The Development of a Non-Microbiological Test Method for Assessing Surface Cleanliness within a High-Fat Processing Environment

5.1. Introduction

The adhesion of product residues to food contact surfaces facilitates microbial survival and growth by changing the physiochemical properties of the surface, so aiding microbial attachment, by providing a nutrient source for adsorbed microorganisms and by protecting them from the direct action of disinfectants (Chapter 1). In addition, the build up of food residues can attract pests, increase maintenance costs, reduce the efficiency and life span of equipment and increase product wastage (Dillon and Griffith, 1999). Thus, the effective removal of food debris not only contributes to product safety and quality but, to any individual food business, is also of considerable economic importance.

The results from previous chapters illustrate quite clearly that no ideal method exists for determining the efficacy of the cleaning and disinfection procedures applied and suggest, that for maximum benefit, visual, non-microbiological and microbiological methods should be combined to form an integrated cleaning assessment strategy. Nevertheless, for such an approach to succeed it must consistently provide reliable, relevant and meaningful information. It is imperative, therefore, that in any given processing environment, the methods incorporated within a cleaning assessment strategy are chosen via informed decisions made on the basis of the type and level of food residues that are likely to be present. A range of test methods are available that are capable of detecting the presence, on an inadequately cleaned and/or disinfected surface, of ATP, proteins and/or

carbohydrates (Chapters 3 and 4). However, although present within a wide variety of foodstuffs, there are key sectors of the food industry involved in the production of foods that are unlikely to predominantly contain any of these chemical groupings.

Yellow fat spreads (e.g. butters and margarines) are composed primarily of oil or fat and water and although they may also contain minor ingredients such as milk and milk products, preservatives and salt, they exist, as do most high-fat foods, as water-in-oil emulsions - colloidal systems comprising small water droplets dispersed throughout an oil phase (Fox and Cameron, 1995; Delamarre and Batt, 1999).

The ability of microorganisms to grow within an emulsion largely depends upon the size of the droplets associated with the aqueous phase. Most microorganisms are confined to the water droplets and, thus, the finer the emulsion, the more limited the area available for microbial growth and the lower the level of available nutrients (Delamarre and Batt, 1999). Furthermore, the total salt concentration of an emulsion is contained within its aqueous phase and, therefore, in a product comprising 80% fat, a salt content of 2% effectively results in a salt concentration within each water droplet of around 10% - a concentration inhibitory to many microorganisms (Hocking, 1994).

However, despite the relatively inhospitable environment, several strains of the yeast *Candida lipolytica* have been isolated from salted butters (Delamarre and Batt, 1999) and moulds, by virtue of mycelial growth, are not necessarily confined to the water droplets and can spread throughout the oil phase and, thus, the product (Hocking, 1994). Additionally, despite the generally low water content making butters and margarines more susceptible to fungal spoilage, the production of extracellular compounds such as lipases and surfactants by lypolytic bacteria such as *Pseudomonas*, *Micrococci* and *Bacillus* spp.

can also cause the breakdown of an emulsion leading to putridity and rancidity, particularly of low-salt butters (Jay, 2000).

Microbial spoilage and its prevention remain, therefore, important concerns during the production of high-fat foods. However, studies have confirmed that, with the exception of lypolytic organisms, traditional non-dairy spreads do not, in general, support bacterial growth, particularly that associated with potential pathogens such as *Salmonella*, *E. coli* and *L. monocytogenes* (ter Steeg *et al.* 1995; Cirigliano and Keller, 2001; Holliday and Beuchat, 2003; Holliday *et al.* 2003). Consequently, such products are not considered "potentially hazardous foods" (Holliday and Beuchat, 2003). Nevertheless, despite the lack of confirmed cases of foodborne disease associated with yellow fat spreads, an outbreak of listeriosis in Finland has been attributed to the consumption of contaminated butter (Lyytikäinen *et al.* 2000).

As few microorganisms survive pasteurisation, the microbiological safety and quality of butter primarily depends upon the hygienic conditions during subsequent processing. Nevertheless, in the presence of fats, there is a general increase in the heat resistance of some microorganisms (Adams and Moss, 1995) and it has been reported that *L. monocytogenes* can survive the butter-making process (Holliday and Beuchat, 2003). Furthermore, during its subsequent refrigerated storage, the number of contaminants present within the butter can increase by several orders of magnitude (Olsen *et al.* 1988). The butter surface, if subjected to incidental post-process contamination is also capable of supporting the growth of *L. monocytogenes*, particularly if prior to storage, refrigerated or otherwise, the product is also subjected to temperature abuse (Holliday *et al.* 2003).

The dose (cfu) of *L. monocytogenes* required to cause illness in 90% of the population has been approximated as being 10^9 and 10^7 for normal and susceptible individuals

respectively (Farber *et al.* 1996). However, not only is the level of *L. monocytogenes* present in the food important but also the amount of food consumed. Thus, although it is conceivable that a high-fat food such as butter, could be contaminated to such an extent that a single portion could provide the necessary dose to cause illness, particularly in a susceptible individual, the prolonged consumption of a product contaminated with a much lower level of *L. monocytogenes* could prove equally as hazardous (Maijala *et al*, 2001). This is particularly relevant considering the suggestion that fats may have a protective effect for pathogens during passage in the gastro-intestinal tract (Kapperud *et al.* 1990) and the increasing popularity, within hospitals, catering establishments and homes, of large multiple-use containers of butter and margarine-type spreads (Holliday *et al.* 2003).

Increasing health concerns regarding the consumption of excess salt and full-fat foods have led to the development of reduced-salt and/or reduced- (< 60%) and low-fat (< 30%) butters and margarines. However, a reduction in salt concentration makes the aqueous phase of the emulsion less inhibitory to microorganisms (Hocking, 1994) and by lowering the fat content and, thus, increasing the water droplet size, cells are provided with more space for growth and more water soluble nutrients from added ingredients (Delamarre and Batt, 1999). The need, therefore, to prevent both spoilage and potentially pathogenic organisms from directly or indirectly contaminating, particularly dairy-based spreads, has, in recent times, become even more important. The implementation of Good Manufacturing Practice and HACCP during the production of butters, margarines and low-fat spreads can ensure their microbiological stability (Klapwijk, 1992), yet, an appropriate means to assess the cleanliness of such processing environments is currently lacking.

The aim of this chapter is, therefore, to:

- Develop an appropriate non-microbiological surface sampling method, capable of detecting the presence of fat residues on food contact surfaces, for use within an integrated cleaning assessment strategy.

Objectives

- Investigate appropriate assay chemistry.
- Assess the ability of the proposed assay to detect the presence of fats.
- Develop the assay into a method capable of detecting the presence of fat residues on food contact surfaces.
- Refine the test method to optimise assay sensitivity.
- Compare assay performance to that of other cleanliness assessment methods already available to the food industry.

5.2. Materials and Methods

5.2.1. Preparation of Fat Samples

Each solid fat sample (Table 5.1) was placed in a beaker and immersed in a water-bath at 45°C. Once melted, each of the samples, together with the already liquid olive oil sample, was serially diluted 2-fold, using 0.1% bacteriological peptone (Oxoid) with 0.1% bacteriological agar (Oxoid); a diluent, commonly used during the cultural examination of

butter as a means of stabilising the emulsion (Harrigan, 1998). To prevent the diluted samples from solidifying, the serial dilutions were kept in the water bath until needed.

Table 5.1. Composition of fat samples

	Composition of Foods (g 100 g^{-1})					
	Protein	**Fat**	**Fatty Acids**			**Carbohydrate**
			Saturated	*Monounsat*	*Polyunsat*	
dripping (animal fat)	0	100	57	35	3	0
vegetable fat	0	100	33	43	11	0
olive oil	0	100	14	74	12	0
butter	0.5	81.7	54	19.8	2.6	trace

5.2.2. Preparation of Test Surface

The test surface, a food-grade stainless steel table marked with eighty-four 10 cm x 10 cm squares, was cleaned and disinfected as described in Section 3.2.4.1.

5.3. Assay Development

Using the instrument-free test kits already available to the food industry as examples (Chapter 3), it was decided that the simplest and, thus, the most acceptable to operatives and the most appropriate format for the proposed fat residue test to take, would be that of a swab-based, presence/absence detection method. It was envisaged that a swab, after being used to sample a surface, would be introduced directly into a receptacle containing an

appropriate reagent. Ideally, this would then react with any fat present on the swab bud and result in a noticeable colour change.

5.3.1. Assay Reagents

Light microscopy is increasingly being used to study the influence of ingredients and processing conditions on food structure. The technique, by providing the ability to visualise the distribution and physical state of specific food components, particularly starches and fats, can for example, provide an explanation as to why foods of similar chemical constitution can have markedly different textures (Flint, 1994).

A food specimen is prepared and, in order to visualise its fat component, stained using a lysochrome (a fat-soluble dye) such as, Sudan IV, Oil Red O or Sudan Black B. These dyes are more soluble in liquid fats than in the aqueous solvent used as a staining medium and, thus, they colour fats by means of a partition mechanism (Flint, 1994). Microscopic examination was not anticipated to play a part in the proposed fat residue test. However, it was assumed that the addition of fat to a liquid medium incorporating a lysochrome would result in a similar reaction. The developmental process began, therefore, by testing this hypothesis.

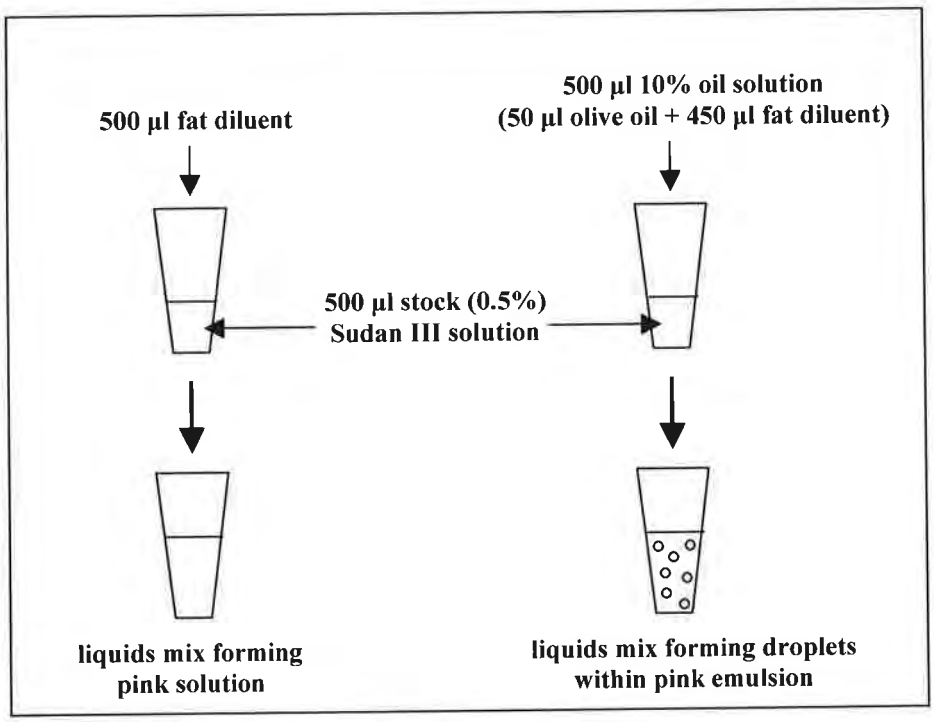
5.3.1.1. Sudan III

The lysochrome used during the current investigation was Sudan III (CI 26100), which was readily available within the laboratory at the time the work was conducted. A stock solution was prepared by adding 0.5 g (Flint, 1994) of the powdered dye (Fisher Scientific)

to 100 ml 70% isopropanol; a solvent incorporated within many commercially prepared, ready-made Sudan stains.

To simulate the introduction of fat via a contaminated swab, varying volumes and concentrations of olive oil were added to aliquots of the stock Sudan III solution. An example of the reaction that occurred between the two liquids is illustrated in Figure 5.1.

Figure 5.1. The effect of adding olive oil to a Sudan III solution



The presence of fat within the 0.5% Sudan III solution could only be determined via the occurance of pink fat droplets within an already pink emulsion (Figure 5.1). Although the size of these droplets gave an indication as to the concentration of oil present, it was thought that if associated with a swab bud, such droplets would be indistinguishable against the surrounding medium. In microscopy, to prevent the specimen from appearing invisible, it is essential it has a refractive index different from that of the mountant (Flint, 1994). By analogy, in order to detect the presence on a swab of any fat picked up from a

surface, it was deemed necessary to alter the colour of the assay medium to one which would contrast well with the red/pink of the Sudan III.

5.3.1.2. Methylene blue

Methylene blue (CI 52015), unlike Sudan III and the other lysochromes, is insoluble in fat and very soluble in water and, as the name suggests, its addition to an aqueous solution results in the liquid turning blue, the intensity of the colour depending upon the concentration of dye used. It was hoped, therefore, that the addition of methylene blue to the assay medium would aid in differentiating the presence of fats, whilst having no effect upon any coloration of the residues themselves. Additionally, should proteins and other non-fat constituents also be picked up from the surface, the presence of water within the assay solution would, by ensuring they remain hydrated, prevent them from being stained (Flint, 1994).

5.3.1.3. Optimisation of assay solution

When deciding upon the proportions in which the two dyes should be present within the assay medium, two major factors were taken into consideration:

i) the concentration of the methylene blue solution, whilst being high enough to contrast well against any bound and stained fat, would have to be low enough so as not to obscure the swab from view.

ii) the proportion of Sudan III, whilst being high enough to effectively stain any fat residues picked up from the surface, would have to be low enough so as not to discolour the methylene blue and, thus, reduce the colour contrast between the different constituents of the assay mix.

It was anticipated, that the level of Sudan III that would be required within the assay medium would be higher than that of methylene blue. With this theory as a starting point, dilution series of both solutions were prepared and incorporated together in a variety of combinations to form a range of potential assay solutions. Olive oil was again used to directly inoculate 1 ml aliquots (i.e. the volume needed within the cuvette to cover a swab bud) of each of these assay mixes.

Under these circumstances, the addition of a 0.045% Sudan III suspension (in 70% isopropanol) to a 0.0005% methylene blue solution resulted in an assay medium that, in the absence of fat appeared as a clear, pale blue liquid but once oil was introduced stained the fat forming a bright red band, clearly visible against the otherwise blue solution. It was encouraging to note that 20 µl of oil (equivalent to swab saturation) could easily be differentiated within 10 min of its addition to the assay medium. However, it is acknowledged that it was simply the immiscibility of the directly inoculated oil and the methylene blue solution that led to the formation of the distinct band of colour illustrated in Figure 5.2 and, consequently, a similar partition phenomenon was unlikely to be observed should fat be introduced bound to a swab bud. Thus, before continuing with the investigation, it was necessary to incorporate a fat-contaminated swab within the proposed assay solution to ensure that adequate and appreciable staining would occur (Table 5.2).

Figure 5.2. The effect of adding olive oil to a methylene blue-Sudan III solution

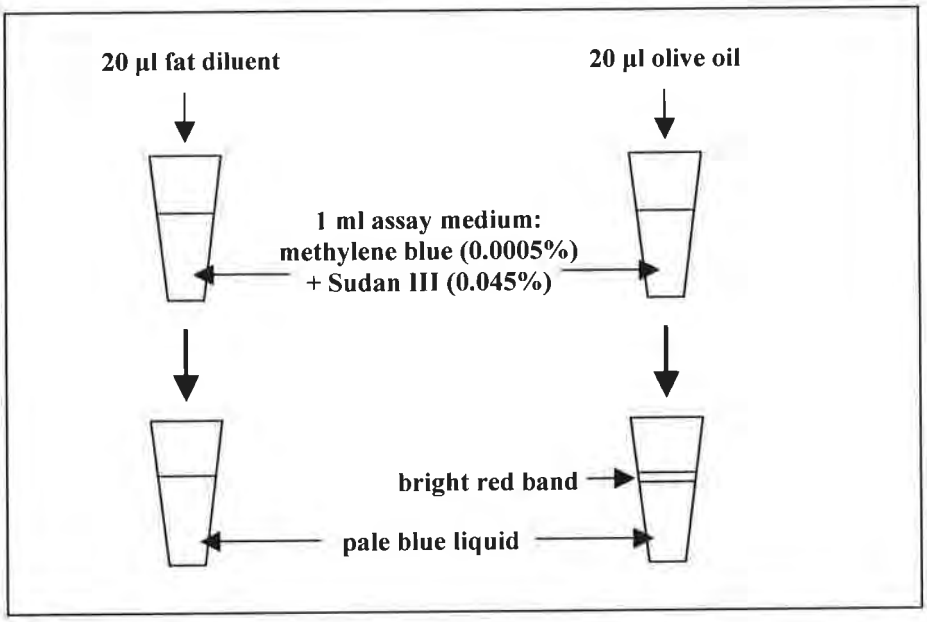


Table 5.2. The effect that a range of potential assay solutions had upon the coloration of a directly inoculated swab bud

Method	*Composition of Assay solution (µl)*			*Observed coloration*		*Comments*
	water	**methylene blue** *	**Sudan III** †	**solution**	**swab bud**	
dacron swab dipped in olive oil	900	0	100	pale pink	small pink spot on base of bud	*Coloration of swab bud more difficult to distinguish against a non-blue background*
	800	100	100	pale blue	small pink spot on base of swab	
	750	50	200	blue / grey	pink spot on base of bud	
	700	100	200	pale blue	large pink spot on base of bud	
1 ml assay solution	600	100	300	pink/grey	pink spot on base of bud	*Contrast between swab and media reduced*

* methylene blue stock solution (0.005%)

† Sudan III stock solution (0.045%)

On the basis of the results typified by those presented in Table 5.2, it was concluded that the assay solution would comprise 700 µl sterile deionised water, 100 µl stock (0.005%) methylene blue solution and 200 µl stock (0.045%) Sudan III suspension. Under these assay conditions, those areas of the swab bud to which the fat residues had adsorbed were stained pink/red and were clearly visible within the surrounding medium, which again had remained pale blue in colour.

It had been established, therefore, that the methylene blue-Sudan III assay mix was capable of indicating the presence of fats on a swab bud and, thus, the next stage of the investigation was to determine the sensitivity of the assay.

5.3.2. Initial Assay Sensitivity

The bud of a sterile dacron swab was coated with a thin layer of fat by being dipped into a cuvette containing the appropriate sample dilution (Section 5.2.1). Control assays were performed by dipping the swab into fat diluent alone. In both cases, any excess liquid was removed from the bud by wiping the swab around the inner walls of the cuvette. The swab was then snapped into a second cuvette containing the assay medium (Section 5.3.1.3) and incubated at room temperature. The pattern and intensity of any changes in coloration occurring on the bud and/or within the surrounding medium were monitored over time.

The minimum detection limit of the assay was identified as being the lowest inoculum tested that resulted in the swab bud visibly changing in colour and differing from that of the control swabs. Observations were based on triplicate samples and are typified by those presented in Tables 5.3 to 5.5.

Table 5.3. Observations and associated minimum detection limit (% fat) of the proposed fat residue assay when used to indicate the presence of dripping (animal fat) on a directly inoculated swab bud

Dilution	*Concentration of fat present*	*Time*	*Observations*
Neat	100%	< 1 min	A pink "spot" (approximately 1 cm in diameter) appears almost instantly on the tip of the swab bud.
			The colour of this "spot" increases in intensity over time.
		> 5 min	The pink coloration associated with the sub bud starts to spread around the base of the swab bud.
		15 min	Specks of colour are evident on all parts of the swab bud.
1:2	50%		As Neat
1:4	25%	< 1 min	A pink "spot", smaller than that observed when the swab was inoculated with a higher level of fat, appears almost instantly.
			The colour of this "spot" increases in intensity over time.
		15 min	Specks of colour are evident on all parts of the swab bud.
1:8	12.5%	< 1 min	A pink "spot", smaller and paler than that observed when the swab was inoculated with a higher level of fat, appears almost instantly.
		15 min	Specks of colour are evident on all parts of the swab bud.
1:16	6.25%	< 1 min	A very pale pink "spot" appears almost instantly on the tip of the swab bud.
		15 min	Coloration of swab bud visibly different from control swabs. Unlike those swabs inoculated with higher levels of fat, coloration appears restricted to bud base.
1:32	**3.12%**		As 1:16 dilution **MINIMUM DETECTION LIMIT**
1:64	1.06%	20 min	No real colour change
Control	0%	20 min	No real colour change

Table 5.4. Observations and associated minimum detection limit (% fat) of the proposed fat residue assay when used to indicate the presence of vegetable fat on a directly inoculated swab bud

Dilution	*Concentration of fat present*	*Time*	*Observations*
Neat	100%	< 1 min	A pink "spot" (approximately 1 cm in diameter) appears almost instantly on the tip of the swab bud.
		5 min	"Spot" very bright pink in colour.
		10 min	"Pinkness" increasing in intensity over time and now appearing on all parts of the swab bud.
		15 min	Swab bud bright pink in colour.
1:2	50%		As Neat
1:4	25%	< 1 min	A pink "spot", paler than that observed when the swab was inoculated with a higher level of fat, appears almost instantly.
		2 min	"Spot" has become much brighter in colour.
		15 min	Base of swab bright pink in colour – less colouring associated with rest of bud.
1:8	12.5%	< 1 min	A very pale pink "spot" appears almost instantly on the tip of the swab bud.
		5 min	"Spot" has become much brighter in colour.
		15 min	"Pinkness" has increased in intensity over time but coloration is restricted to bud base.
1:16	6.25%		As 1:8 dilution (although "pinkness" visibly paler)
1:32	3.12%	3 min	A very pale pink "spot" appears at base of bud.
		5 min	"Spot" has become slightly brighter in colour.
		15 min	Coloration of swab bud visibly different from control swabs. **MINIMUM DETECTION LIMIT**
1:64	1.06%	90 min	No real colour change
Control	0%	90 min	No real colour change

Table 5.5. Observations and associated minimum detection limit (% fat) of the proposed fat residue assay when used to indicate the presence of butter on a directly inoculated swab bud

Dilution	*Concentration of fat present*	*Time*	*Observations*
Neat	81%	1 min	A pink "spot" appears almost instantly on the tip of the swab bud.
		5 min	"Spot" has become much brighter in colour – although paler in comparison to the coloration associated with those swabs dipped in the dripping or vegetable fat.
		15 min	Base of swab relatively bright pink in colour – no colouring evident elsewhere on the bud.
1:2	40.5%		As Neat
1:4	20.25%	15 min	As was observed when the swabs were dipped in the 1:2 dilution except coloration comparatively paler. Slight pink hue to assay solution.
1:8	10.12%	1 min	Small, pale pink "spot" at base of swab bud.
		5 min	Coloration of swab bud visibly different from control swabs.
		15 min	"Pinkness" has increased in intensity over time - coloration remains restricted to bud base.
1:16	**5%**	15 min	"Pinkness" has increased in intensity over time but not to the same extent as when the swab was inoculated with higher concentrations of fat. Coloration of swab bud visibly different from control swabs. **MINIMUM DETECTION LIMIT**
1:32	2.5%	30 min	No real colour change
Control	0%	30 min	No real colour change

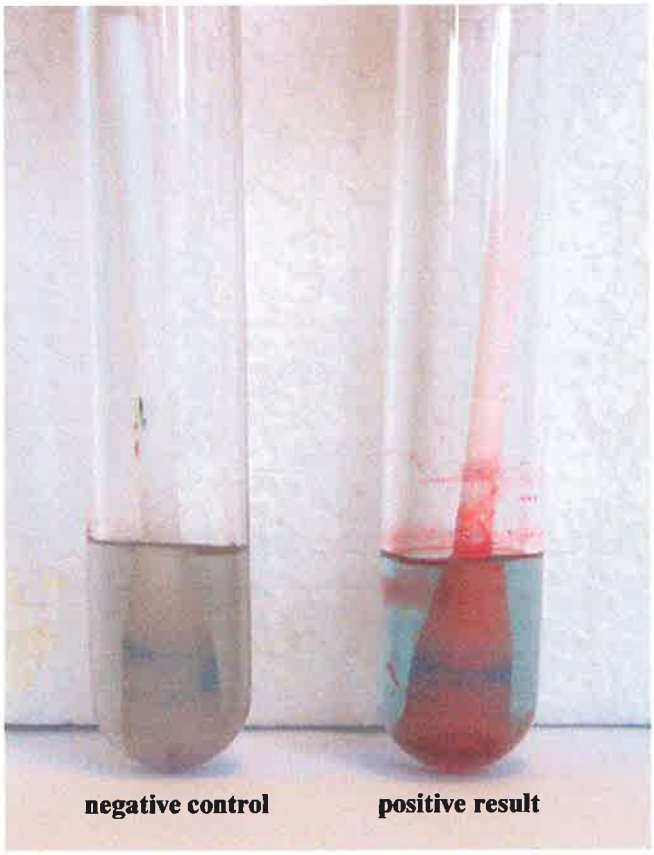
The results initially obtained suggested that the proposed assay would be capable of indicating the presence on a swab of residues comprising between 3% and 5% fat. Nevertheless, there were some subtle differences, depending upon the type of fat present, regarding the pattern of staining that occurred. For example, the staining associated with those swabs dipped into molten animal fat was observed, over time, to extend over all parts of the bud. In comparison although the coloration of the swabs dipped into molten vegetable fat appeared visibly brighter, the staining seemed to be restricted to the bud base (Tables 5.3 and 5.4).

All natural fats contain both saturated and unsaturated fatty acids. However, the greater the proportion of the later, the lower the melting point of the fat, thus, those high in unsaturates are liquid at room temperature, whilst those rich in saturates are solid (Fox and Cameron, 1995). Animal fats comprise a much higher proportion of saturated fatty acids than vegetable fats (Table 5.1). It is conceivable, therefore, that after dipping the swabs in the molten dripping, the fat solidified and adsorbed to the bud more readily than did the vegetable fat, which having a lower melting point, may have remained in a more liquid state and run down the sides of the bud, accumulating at the base. Nevertheless, if this were the case, then it would appear that the Sudan III was still capable of staining the dripping, despite lysochromes only being soluble in liquid fats. Solid fats, however, comprise a network of minute crystals surrounded by a smaller quantity of liquid triglycerides (Fox and Cameron, 1995) and, thus, are 'stained' by virtue of the liquid fats associated with them. This implies, therefore, that the higher the proportion of unsaturated fatty acids, the greater and more intense the staining of the fat by Sudan III, hence, the visibly brighter coloration of the swab contaminated with vegetable fat.

The observations presented in Tables 5.3 to 5.5 were made over a 20 min period, a relatively short reaction time but one fairly comparable to that associated with protein

detection (Chapter 3) and, thus, it can be presumed, one food businesses are likely to consider acceptable when rapidly assessing surface cleanliness. However, a factor common to all the fat types tested was that if present on the swab at levels above the identified minimum detection limit, then incubating the assay at room temperature for hours, as opposed to minutes, appreciably enhanced the coloration of the fat associated with the bud. Indeed, as illustrated by Figure 5.3, leaving the assay overnight (18 h) led to the development of what, in terms of the current investigation, could be described as being the "ideal positive result".

Figure 5.3. The appearance of the assay tubes after being incubated at room temperature overnight (18 h) and incorporating a swab directly inoculated with either a fat diluent (negative control) or olive oil (positive)



Nevertheless, such a noticeable colour change, although encouraging, resulted from an assay procedure that did not require the swab to initially remove the fat residues from a

surface. The next stage of the investigation, therefore, was to ensure that a swab could, in fact, pick up detectable levels of fat.

5.3.3. Detection of Fat Residues from a Stainless Steel Surface

Each test surface (100 cm^2; Section 5.2.2) was inoculated with 0.1 ml of appropriate fat dilution. The sample was spread evenly over the test area, before the surface was sampled using a sterile dacron swab. Control assays were performed by sampling a surface that had been inoculated with fat diluent only and, in both cases, the swab was snapped into a cuvette containing 1 ml of the assay solution (Section 5.3.1.3). The minimum detection limit of the assay was again identified as being the lowest inoculum tested that resulted in the bud of the swab appearing pink in colour and visually differing from that of the control swabs. The results that were obtained are illustrated in Table 5.6 and clearly show that, in comparison to when the swabs were directly inoculated, when used to detect the presence of fat residues on a stainless steel surface, the sensitivity of the proposed assay was considerably reduced.

Table 5.6. The minimum detection limit (% fat) of the proposed fat residue assay

	minimum detection limit (20 min reaction time)	
	swab bud directly inoculated	***swab used to sample surface***
animal fat (100% fat)	**3% fat** (1:32 dilution)	**25% fat** (1:4 dilution)
vegetable fat (100% fat)	**3% fat** (1:32 dilution)	**12.5% fat** (1:8 dilution)
butter (81.7% fat)	**5% fat** (1:16 dilution)	**20% fat** (1:4 dilution)

Surface sampling and sanitation are fundamentally linked, for either to be successful, residual food debris and/or microorganisms must be effectively removed from the surface. The importance of applying ample mechanical energy during cleaning (Section 1.3.3.1) and when assessing surface cleanliness, both microbiologically (Section 2.4.2.1) and non-microbiologically (Section 3.4.2.2), has been discussed. Similarly, many of the previously described swabbing solutions (Section 2.2.4.1; Table 2.1) contain surfactants, which, by providing the solution with detergent-like properties, help the swab lift microbial contaminants away from the surface. In an attempt, therefore, to improve the overall performance of the proposed fat-residue test, experiments were conducted that investigated appropriate mechanical and chemical energies and their effect upon assay sensitivity.

5.3.3.1. Mechanical energy and the effect of swab type

The flexibility of the dacron swab used during the current investigation allowed only limited pressure to be applied to the surface during sampling (Section 2.4.2.1). To increase the level of mechanical energy, sampling sponges (further discussed in Chapter 7) are sometimes used in preference to swabs when detecting microbial contamination and many non-microbiological residue detection kits incorporate a simple test strip (Section 3.2.6), both of which can be pressed, and wiped, firmly over the surface to be sampled.

Sterile sampling sponges that were available and to hand within the laboratory, were cut into finger-width strips and used to sample the inoculated test surfaces before being placed in universal bottles containing 5 ml of assay solution. Unfortunately, and perhaps typically, the sponges used were either blue or orange in colour and, thus, did not contrast well with the methylene blue solution or the Sudan III staining respectively. Test strips, taken from the Check Pro protein detection kit (Section 3.2.6.2) were also used to sample

the surface, but rather than adding the protein reagents to the test pad, 0.1 ml of the current assay solution was added instead. However, despite a large amount of pressure being applied to the surface, fat residues were not detected. The test strip associated with this particular detection method comprises a relatively small, non-absorbent test pad, thus, it was suspected that only a small proportion of the fat sample was actually removed from the surface and that the assay solution was, under these circumstances, simply not sensitive enough to detect its presence.

The natural absorbency of cotton swabs leads to them removing a significant proportion of the microbial contaminants present on a surface. However, as also discussed, the preferred sites for the entry of water and similar reagents are the spaces within the lamellae, formed as the cotton fibre undergoes limited swelling (Section 2.4.1.2). During the current investigation, therefore, when a cotton swab was used to sample the surface, regardless of the proportion of the fat sample removed, when it was placed in the assay medium a high percentage of the solution volume was absorbed and lost within the swab bud. This not only resulted in the cotton bud effectively being dyed blue, but may also have led to a reduction in the level of Sudan III available within the assay medium for staining any fat residues that had adsorbed to the exterior of the swab.

Foam swabs are not as absorbent as cotton swabs and, in addition, are made of a much rougher material, thus, their use can generate a relatively high level of mechanical energy. However, despite the potential of this swab type to increase the amount of fat removed from the surface, such residues did not appear to adsorb particularly well to the swab bud. The staining, rather than remaining closely associated with the swab, was observed to accumulate at the base of the cuvette and whilst this improved the contrast and, as a consequence, the visualisation of high levels of fat, when such residues were present in low

concentrations (i.e. those close to the minimum detection limit of the assay), the opposite was true and, thus, assay sensitivity was reduced.

Overall, therefore, in comparison to the other methods investigated, the use of dacron swabs appeared the most appropriate means of detecting fat residues from stainless steel surfaces and, thus, as assay sensitivity had not yet been improved, attention turned to potential swab-wetting solutions.

5.3.3.2. Chemical energy and the effect of swabbing solution

A major problem within the milk industry is the fouling of ultrafiltration membranes via the precipitation of microorganisms, proteins, fats and minerals. Mohammadi *et al.* (2002) concluded, that the most effective means of removing such debris is the use of a cleaning solution comprising 0.2 M sodium hydroxide and, as a surfactant, 0.5% (w/v) sodium dodecyl sulphate (SDS). A description of how these two chemicals may attack and remove such a grease layer has previously been provided (Chapter 1; Table 1.3 and Figure 1.3) and considering the important similarities between cleaning and surface sampling, it was decided that the ability of the NaOH-SDS mix to act as an effective swab-wetting solution should be evaluated.

The effective removal of fats and greasy stains poses a similar problem to the textile industry and during the dry-cleaning process, where such issues are particularly relevant, solvents have been used successfully for many years (McCall *et al.* 1998). In the analysis of foods, the most commonly used fat solvent is petroleum ether, yet, although less selective for triglycerides, ethyl ether is considered the better general solvent as it extracts both triglycerides and non-triglyceride lipids. However, the latter must first be made

anhydrous otherwise sugars and other non-polar substances will also be extracted and, consequently, a combination (1:1) of petroleum ether and diethyl ether is often recommended (Kirk and Sawyer, 1991).

Prior to sampling the inoculated test surfaces, the dacron swabs were pre-moistened with either the NaOH-SDS solution or the petroleum ether-diethyl ether solvent. However, use of neither solution had any effect upon overall assay sensitivity and, in addition, the incorporation of the NaOH-SDS solution within the assay mix was observed to cause significant coloration of the control swabs – a situation that could potentially lead to a number of false positive results and which, may have been caused by the Sudan III reacting with the SDS. It was also hypothesized that due to the low absorbency of the dacron swab, the volume of swab-wetting solution that came into contact with the surface may have been insufficient to increase the amount of fat removed. An inadequate contact time between solvent and surface may also have led to assay sensitivity remaining unaffected.

To investigate these possibilities, the SDS was first removed from the cleaning solution before either the solvent or the NaOH alone was sprayed onto the test surfaces, which were then swabbed immediately or left for 1, 2 or 5 min before being sampled. Subsequent observations gave the impression that as contact time between NaOH and the surface increased, the intensity of the coloration associated with the swab bud also increased, implying, that given time, a higher proportion of stainable fats could be removed from the surface. However, the control swabs again showed faint signs of colouring. Although false positive results appeared to be less of an issue when the swabs were used to sample surfaces wetted with the solvent, the low boiling point of both petroleum ether and diethyl ether resulted in them evaporating from the surface with 1 min of the solvent being applied. Nevertheless, when the surface was sampled immediately, the additional volume

of solvent present during the swabbing procedure did appear to increase assay sensitivity. A further set of experiments was conducted in order to substantiate this finding.

5.3.4. Assay Sensitivity

Each surface area was inoculated with the appropriate fat dilution. Immediately prior to sampling, the petroleum ether-diethyl ether solvent was applied to the surface via 6 pumps (approximately 0.5 ml) of a spray diffuser. A sterile dacron swab, held approximately half-way down the stick in order to maximise the amount of mechanical energy generated, was then used to sample the surface (Section 2.2.4.2) before being snapped into a cuvette containing 1 ml of assay medium. The changes in bud coloration were monitored over time with the minimum detection limit of the assay again identified as being the lowest inoculum tested that resulted in the swab bud visibly turning pink and differing from that of the control swabs. Observations were based on duplicate samples with the experiment being repeated on three different occasions to validate the endpoints. The results are typified by those illustrated in Figures 5.4 to 5.7.

Figure 5.4. The appearance of the proposed fat residue assay when used to detect the presence of dripping (animal fat) from a stainless steel surface

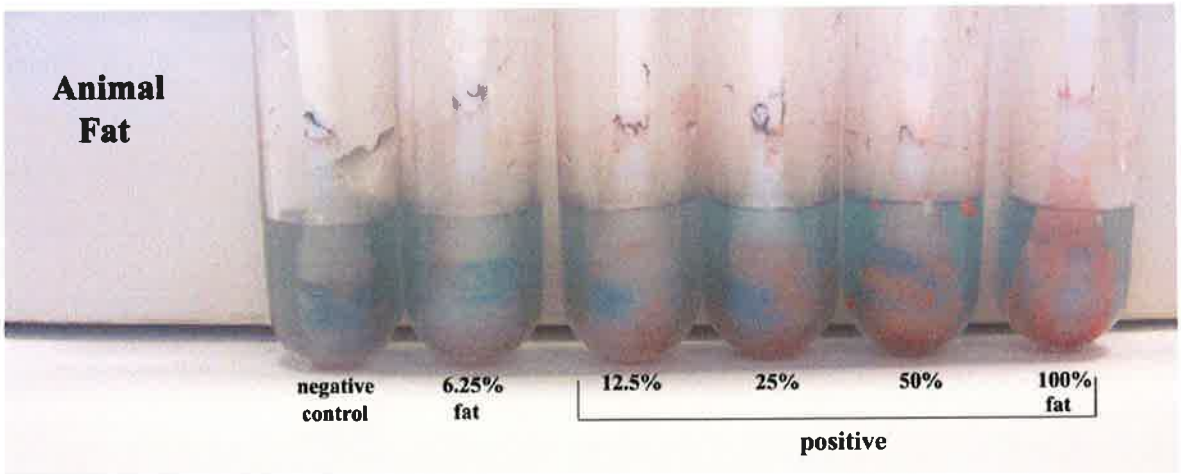


Figure 5.5. The appearance of the proposed fat residue assay when used to detect the presence of vegetable fat from a stainless steel surface

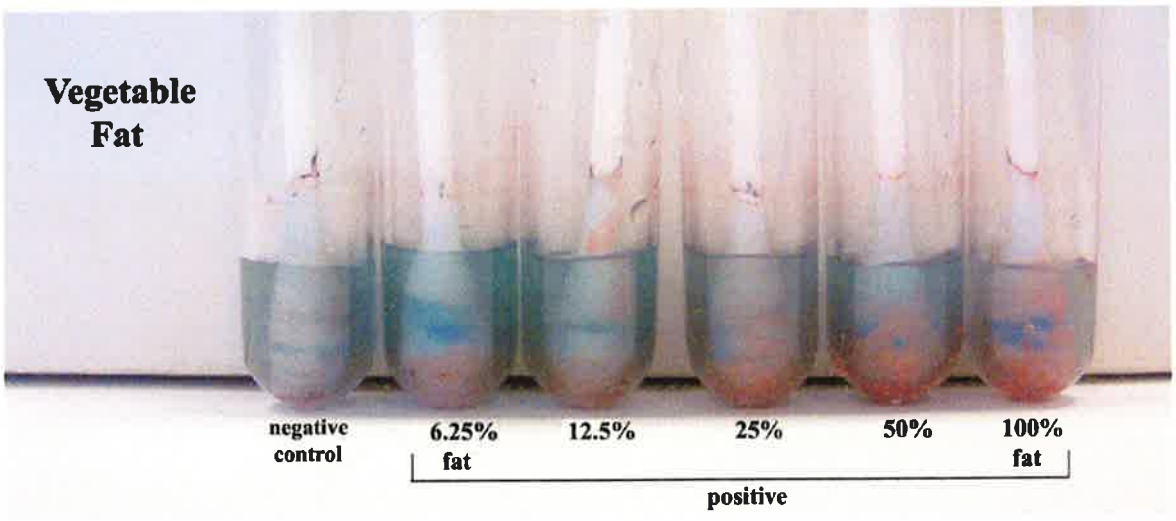
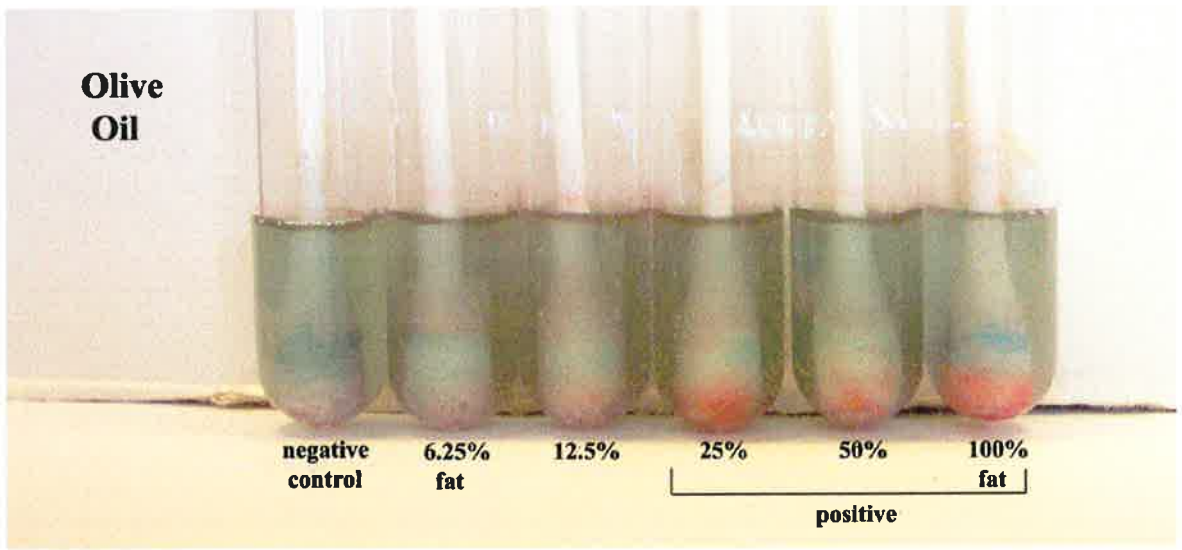


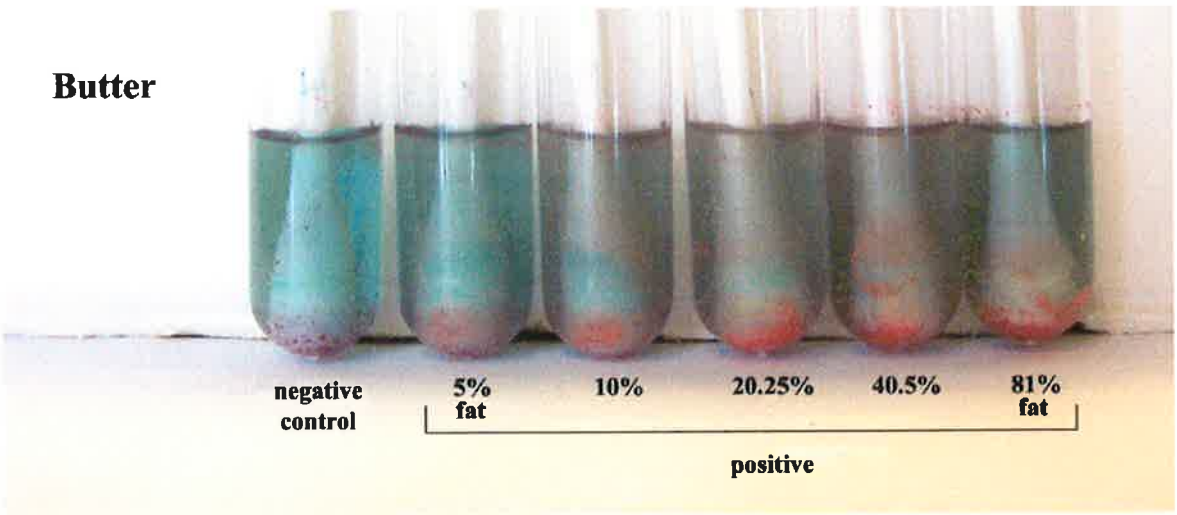
Figure 5.6. The appearance of the proposed fat residue assay when used to detect the presence of olive oil from a stainless steel surface



Depending upon the type of fat present on the surface, the swab-based assay was capable of detecting the presence of residual food debris comprising between 5% and 25% fat. As was observed when the swabs were directly inoculated, the test appeared more sensitive when detecting the presence of vegetable as opposed to animal fats (Section 5.3.2). However, interestingly the assay was the least effective when used to detect olive oil. It was suspected, that whilst those fats which had solidified on the surface had readily

adsorbed to the bud, the low absorbency of the dacron material had limited the amount of liquid oil removed from the surface. The results also imply that the emulsion-like butter samples were the easiest to remove. However, whilst the swabs used to detect dripping and vegetable fat were observed to change colour within minutes, those used to detect the butter residues had to remain within the assay solution for a number of hours.

Figure 5.7. The appearance of the proposed fat residue assay when used to detect the presence of butter from a stainless steel surface



Although in many cases, results could be obtained rapidly (< 30 min), Figures 5.4 to 5.7 typify the appearance of the assay tubes after an 18 h incubation period – a reaction time considered, on the basis of all the observations made during the current investigation, to be more appropriate. The reasoning behind this was two-fold. Firstly, the time required before results were obtainable depended not only on type but also the concentration of fat present and, in addition, even when results were provided within minutes, in the majority of cases, assay sensitivity could be increased two-fold by incubating the swab overnight. Secondly, when the swab, solvent and residues were initially introduced to the assay medium, the solution became disturbed. However, as the Sudan III dissolved within the fat present on the swab bud, it was drawn out of the medium. Over time, therefore, as the bud became pinker, the solution became bluer and, thus, colour contrast visibly improved. As

can be seen in Figure 5.7, in the absence of fat (i.e. the negative control), the Sudan III was sometimes observed to precipitate out of the assay solution, appearing as pink granules at the base of the cuvette. Dye precipitation has also been observed during the microscopic examination of foodstuffs, where the phenomenon has subsequently been improved via the addition of 1% dextrin to the staining medium (Catalano and Lillie, 1975; Flint, 1994).

Obtaining results in 18 h is not ideal in terms of assessing surface cleanliness within a HACCP system (Chapter 3). Nevertheless, the question remained as to how the proposed fat residue assay would compare to those test methods already available to the food industry.

5.3.5. The Comparative Performance of the Proposed Fat-Residue Test

Each surface area was inoculated with the appropriate fat dilution and either assessed visually or sampled using the fat-residue assay (Section 5.3.4), the Clean-Trace ™ ATP bioluminescence system (Section 3.2.6.1) or the Pro-tect® protein detection method (Section 3.2.6.2) immediately after inoculation or after it had been allowed to air-dry for 1 h under ambient conditions. The minimum detection limit of the two colorimetric test methods was identified as being the lowest inoculum tested that resulted in the colour of the swab bud and/or test medium differing from that of the control samples. However, the diluent within the fat samples contained high levels of ATP and, consequently, the minimum detection limit of the bioluminescence technique was identified as being the lowest inoculum tested that resulted in a light signal over twice the average RLU reading of the control samples (Corbitt *et al.* 2000). Observations/measurements were based on duplicate samples and each experiment was repeated to validate the endpoint.

Figure 5.8. Detection of fat residues from a wet and dry stainless steel surface using different detection methods

Fat type	Detection method	Reaction time	Inoculum detected (% fat) *(wet and dry surface)* 100% 50 25 12.5 6.25 3.12
dripping	*fat detection*	20 min	
		18 h	
	protein detection	10 min	Non-detectable
	ATP bioluminescence	instant	Non-detectable
	visual assessment	instant	
vegetable fat	*fat detection*	20 min	
		18 h	
	protein detection	10 min	Non-detectable
	ATP bioluminescence	instant	Non-detectable
	visual assessment	instant	
olive oil	*fat detection*	30 min	
		18 h	
	protein detection	10 min	Non-detectable
	ATP bioluminescence	instant	Non-detectable
	visual assessment	instant	
butter	*fat detection*	2 h	
		18 h	
	protein detection	10 min	Non-detectable
	ATP bioluminescence	instant	Non-detectable
	visual assessment	instant	

 inoculum level detected

The results presented in Figure 5.8 clearly illustrate that neither protein detection nor ATP bioluminescence would be appropriate for use within high-fat production plants. However, it is acknowledged that the test surfaces were inoculated with fat residues only and should microorganisms and/or other food components also be present then ATP bioluminescence in particular, may be of use. Nevertheless, it has been suggested that the efficacy of such

test methods may be reduced even when the food debris comprises relatively low levels of fat (Section 3.4.2.1). In contrast, even those surfaces inoculated with relatively dilute fat samples appeared visually unclean. However, the surfaces sampled during this investigation were flat, accessible and, thus, easily assessed by eye. It is anticipated that swab-based methodology would be a more appropriate means of sampling the difficult-to-clean nooks and crannies that are associated with production equipment and machinery. Within processing environments where the residual organic soil is likely to be composed primarily of fat, the use of a cleaning assessment strategy incorporating the proposed fat residue test could, therefore, prove beneficial and a viable alternative to any of the methods currently available to the food industry.

5.4. Conclusion

The basis of a test method, capable of fulfilling the cleaning assessment requirements of those businesses involved in the production of high-fat foods, has been successfully developed. The proposed colorimetric assay has been demonstrated to be a more effective means of detecting the presence of fat residues than those test methods already available to the food industry and, in addition, can, depending upon the type of fat present, provide this information within minutes. Although requiring more time to detect the presence of emulsions such as butter, the minimum detection limit of the assay implies, that the proposed test method would also be capable of detecting the presence, on an inadequately cleaned surface, of residues associated with reduced- or low-fat spreads. Furthermore, with starch dextrins being widely used as thickeners in low-fat products (Flint, 1994), the incorporation of such residues within the assay medium may in fact improve assay performance (Section 5.3.4).

The main aim of the current investigation was to develop a test method, which, under controlled laboratory conditions, would successfully detect the presence of fats. Nevertheless, some consideration was given as to how the assay could be appropriately marketed. The test could for example, be supplied either in 'kit-form' (Figure 5.9), very similar to that of the original protein detection methods (e.g. Swab & Check Professional Hygiene Monitoring Kit; Section 3.2.6.2) or, as with the more modern swab-based protein tests (e.g. Pro-tect®), as a 'single-shot' device (Figure 5.10).

Figure 5.9. Possible 'kit' format for the proposed fat residue assay

Small, clear, capped assay tube containing 800 µl methylene blue solution (0.0005%)

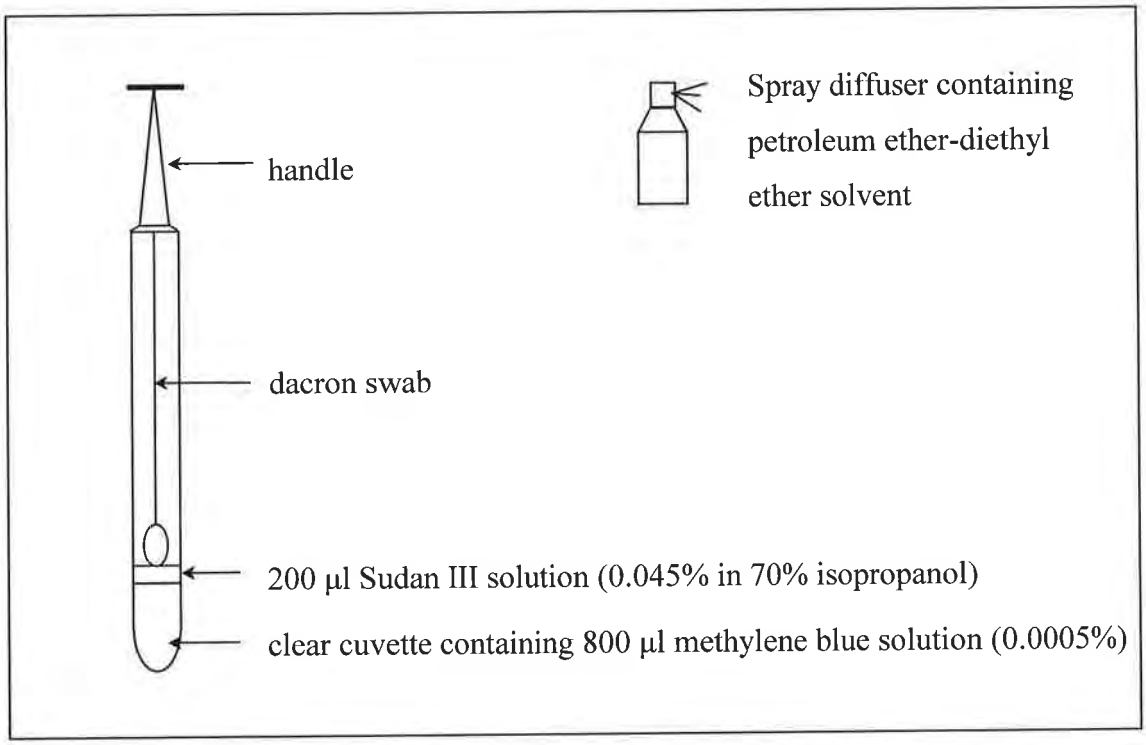
Dropper bottle* containing Sudan III (0.045% in 70% isopropanol). Prior to sampling, 4 drops to be added to assay tube

Spray diffuser containing petroleum ether-diethyl ether solvent. Immediately prior to sampling, 6 pumps to be applied to surface.

Dacron swab, short enough so as to fit into the assay tube after sampling; the reduced length would also maximise the level of mechanical energy generated during sampling

* Volume of each drop approximately 50 µl

Figure 5.10. Possible 'single-shot' format for the proposed fat residue assay



However, prior to the assembly of either test format, further research is required in order to refine and improve the assay procedure itself.

The petroleum ether-diethyl ether solvent, despite proving the most effective of the sampling solutions evaluated and having the added benefit of evaporating from the surface, presumably leaving little or no chemical residue, is flammable and, thus, could prove a potential fire/safety hazard to personnel. The ability of alternative solutions to remove fat residues from the surface should, therefore, be assessed.

The results of any colorimetric test method become worthless should known negative samples consistently cause the colour change indicative of a positive result. Many of the residue detection methods already available to the food industry involve a reaction, which although occurring faster when the residue in question is present will, given an appropriate period of time, which in some cases may only be a matter of minutes, also occur in the absence of detectable food residues (Section 3.4.2.3). Confusion can arise and misleading

information be provided, therefore, should the results of such test methods be obtained outside the reaction times specified by the manufacturer. Although time did not appear to adversely affect the interpretation of the proposed fat residue test – negative samples were identified as being 'negative' even after 18 h (Figures 5.4. to 5.7), the results suggested that false positive reactions could be caused by the presence of extraneous substances within the assay solution, again highlighting the importance of conducting *in situ* evaluation trials (Chapter 4). There is a need, therefore, to assess the performance of the fat-residue test both before and, considering the assay seemed particularly affected by the presence of a surfactant (SDS), after an appropriate processing environment has carried out its normal cleaning procedures. However, uncertainty regarding the type and level of residues present and the absence of an appropriate reference method (Figure 5.8) would make interpretation of the data difficult and, thus, further laboratory work is required.

Finally, the interpretation of all colorimetric test methods can be very subjective, particularly if the residues tested for are present at levels close to the minimum detection limit of the assay (Section 3.4.2.2). The mixing of Sudan stains reportedly results in the production of a saturated solution comprising more colour than that of single Sudan stains (Kay and Whitehead, 1941) and the incorporation of such a solution within the proposed fat residue assay could help in differentiating an otherwise uncertain positive from a definite negative result.

However, regardless of initial fat concentration and the potential for improving colour intensity, in contrast to many non-microbiological detection methods, the mechanism of the proposed assay is such, that results become easier to interpret over time. The production of high-fat foods is a relatively low-risk process and, thus, the need to assess surface cleanliness is more likely to be based upon quality rather then safety concerns. Consequently, a test method providing reliable and relevant information, albeit within

18 h, can be considered a worthwhile development and, particularly as a viable alternative is currently unavailable, an appropriate means of assessing the efficacy of the cleaning procedures employed within high-fat processing environments. However, the proposed assay *is* capable of detecting the presence of fat, on either a wet or dry surface, within minutes. In addition the results obtained are repeatable and the test easy-to-use and cheap. The proposed assay does, therefore, possess many of the characteristics required of an ideal, non-microbiological test method (Griffith *et al.* 1997) and, thus, despite its simplicity, could form part of an integrated cleaning assessment strategy for use within an appropriate HACCP system.

Colorimetric reactions form the basis of a variety of test methods, each capable of rapidly detecting the presence of food macromolecules such as proteins (Section 3.4.2.2), carbohydrates (section 3.4.2.3) and fats. However, despite such methods enabling the detection of relatively low levels of residual food debris, for colorimetry to detect the presence of microbial contaminants, rather than a means of enhancing assay appearance (as is the case with the proposed fat residue assay), a lengthy incubation time, is, as will subsequently be discussed, essential for assay performance. Consequently, to detect microorganisms within a time period considered appropriate for use within HACCP, alternative methodology must be investigated and this shall be the subject of Chapter 6.

Chapter 6

The Design and Development of a Chemiluminescent, Swab-based Assay for the Rapid Detection of Coliforms on Food Contact Surfaces.

6.1 Introduction

Previous chapters have discussed how the presence of food debris on production surfaces, equipment and machinery can facilitate microbial survival and growth and as a consequence, how the detection of food residues such as ATP, protein and carbohydrates has become an important means of rapidly assessing the efficacy of a company's sanitation programme. However, despite the acknowledged limitations of traditional microbiological sampling, its use coupled with the use of non-microbiological residue detection methods is, at present, the only way for a food business to *fully* characterise the type of contamination present on a surface and, thus, the only way they can obtain an accurate indication as to the efficacy of *both* the cleaning and disinfection procedures applied (Chapters 3 and 4).

The routine acquisition of microbiological data can provide historical microbial profiles of both food contact and environmental surfaces, which can be used to determine or verify that the microorganisms of concern are being controlled (Buchanan, 2000). In addition, microbiological test methods can identify new and/or previously unrecognised microbial hazards and this, in turn, will help the development of strategies and criteria for assuring the microbiological safety of the final product (Buchanan, 2000). Both types of microbiological information can, therefore, be considered essential to effective food safety and quality management systems.

However, the great number and diversity of microorganisms makes it difficult to test for every organism of concern (Erdmann *et al.* 2002). Furthermore, the detection of specific pathogens, which may be present in very low but significant numbers, often requires quite elaborate isolation procedures (Frank *et al.*1990). Thus, an alternative is to look for an associated organism or group of microorganisms, present in much larger numbers and indicative for the possible presence of pathogens (Frank *et al.* 1990; Ingham *et al.* 2000; Nack *et al.* 2002) – a concept originally developed as a means to indicate the presence, in water, of pathogens spread by the faecal-oral route (Adams and Moss, 1995). The presence of *Escherichia coli* in the environment, or in foods, generally implies some history of faecal contamination (Geissler, *et al.* 2000). However, testing for *E. coli* can itself be relatively involved and a number of simpler alternatives are often used.

Coliforms, including *E. coli,* are members of the *Enterobacteriaceae* and make up approximately 10% of the intestinal microorganisms of humans and other animals (Prescott *et al.* 1993). The general ease with which the coliform bacteria, as a whole, can be cultivated and differentiated has resulted in their widespread use as indicator organisms (Jay, 2000) and their detection has traditionally been used, particularly within the dairy industry as an indication of unsanitary conditions or inadequate processing (Cooke *et al.* 1985; Birollo *et al.* 2001; Silbernagel and Lindberg, 2002). However, the coliform bacteria also include organisms such as *Citrobacter* and *Enterobacter* spp., which are not predominantly of faecal origin. Thus, the detection of coliforms is less specific than that of *E. coli* and their presence does not necessarily point to the presence of faecal-associated pathogens (Mossel *et al.* 1995).

Nevertheless, detecting the presence and/or assessing the levels of indicator organisms is an important step in both Good Manufacturing Practice and HACCP programmes (Eisel *et al.* 1997) and the detection and enumeration of coliforms *is* widely used within the food

industry to reflect the overall effectiveness of sanitation procedures (Jay, 2000; Eisel *et al.* 1997; Brown *et al.* 2000; Robach, 2001; Slade, 2002). Furthermore, if the absence of coliforms can be repeatedly verified, then the probability that a food contact surface is ever dangerously contaminated with enteric pathogens is virtually nil (Mossel *et al.* 1995). The methodology used, therefore, should permit fully reliable detection even when the indicators are present in low numbers (Jay, 2000). Thus, the food industry has an obvious need for a faster (Chapter 3) and more reliable (Chapter 2) means of determining the presence and/or levels of coliform bacteria on both food contact and environmental surfaces (Manafi *et al.* 1991; George *et al.* 2000).

In an attempt to provide more accurate and more rapid microbiological information, the emphasis is changing from visual, manual detection methods to detection based on other features of the target organism (Bolton, 1998). Methods based on the chemical composition of cells or the activity of specific cell components, such as lipids or enzymes, can be organism specific (Nyrén and Edwin, 1994) and, in addition, may be performed using the primary isolation media, thus, by-passing the need for time consuming isolation procedures, prior to identification (Manafi *et al.* 1991).

The indicative property of coliforms is the ability to ferment lactose and was used by MacConkey as early as 1908 to differentiate between the lactose fermenting, generally non-pathogenic bacteria of the gut flora and the pathogenic, non-lactose fermenting *Salmonella* and *Shigella* spp. (Bascomb, 1987). β-galactosidase, the first enzyme in the catabolism of lactose, has been studied extensively ever since.

$$\text{Lactose} \xrightarrow{\beta\text{-galactosidase}} \text{Glucose} + \text{Galactose}$$

The ability of many enzymes to act on more than one substrate allows the use of synthetic substrates for the detection and measurement of specific enzyme activities (Bascomb, 1987). A variety of substrates for the detection of β-galactosidase have been developed and given that the detection of β-galactosidase in cultured bacteria detects, indirectly, the presence of coliforms (Masuda-Nishimura *et al.* 2000), these have since been incorporated into a variety of media and test kits used to detect coliform bacteria in drinking water (e.g. Colilert, Colibag, Colicheck), freshwater, seawater and sewage (Davies and Apte, 2000; Geissler *et al.* 2000; George *et al.* 2000).

Many of these detection methods are based on the hydrolysis of chromogenic substrates for β-galactosidase, such as *o*-nitrophenyl-β-D-galactopyranoside (ONPG), *p*-nitrophenyl-β-D-galactopyranoside (PNPGAL) and 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-GAL) (Manafi *et al.* 1991). The incorporation of such chromogenic substrates into a primary isolation medium enables the differentiation and enumeration of coliform bacteria directly on the isolation plate. Alternatively, qualitative (presence/absence) enzyme assays can be performed on suspensions of non-proliferating bacterial cells in which case, assay speed and sensitivity will be determined by the method used to detect the enzymatic activity (Manafi *et al.* 1991).

The presence of β-galactosidase can be detected subjectively by observing a colour change within the bacteria-substrate mix. However, under these circumstances, the assay affords limited sensitivity. Although, this may be of little consequence during a confirmatory ONPG test, when the high number of available cells will permit the rapid cleavage of substrate, to detect low levels of coliform bacteria, approximately 24 h is required for sufficient bacterial propagation and enzymatic hydrolysis to occur and for the yellow colour of the nitrophenol to become visually detectable (Van Poucke and Nelis, 1995).

Shortening the observation time has been a major subject of research and has focussed mainly on the utilisation of instrumental rather than visual endpoint detection. The use of a spectrophotometer has been shown to increase the sensitivity of chromogenic-based assays (Van Poucke and Nelis, 1997a) and many studies have investigated a sensitive fluorogenic substrate, 4-methyl-umbelliferyl-β-D-galactopyranoside (Mu-Gal) (Robison, 1984; Fiksdal and Tryland, 1999; Davies and Apte, 2000), the use of which has enabled the presence of 1 faecal coliform in 100 ml of water to be detected within 7 h (Berg and Fiksdal, 1988). As a consequence of continual research and development, chromogenic and fluorogenic substrates have, particularly within the field of water microbiology, become a powerful tool, utilising the β-galactosidase of coliform bacteria either in addition to or instead of traditional methods (Manafi *et al.* 1991). However, the β-galactosidase test has found limited applications in food microbiology, except for identification purposes.

Nevertheless, portable luminometers, by virtue of the wide use of ATP bioluminescence, are becoming commonplace within the food industry. Luminescence-based assays are finding increased use in water microbiology and medical diagnostic and molecular biology research, and bio- and chemiluminogenic substrates for β-galactosidase, used either alone, or in conjunction with gene reporter assays have been demonstrated to offer a greater sensitivity and more rapid detection than both colorimetry and fluorimetry (Beale *et al.* 1992; Van Poucke and Nelis, 1995; Masuda-Nishimura *et al.* 2000). Thus, it is postulated that a luminescence-based, β-galactosidase assay could form the basis of a novel test method, capable of detecting the presence of coliforms on food contact and environmental surfaces with greater speed, sensitivity and accuracy than those methods currently available to the food industry.

The aim of this chapter is, therefore, to:

- Design and develop a 'user-friendly', luminescence-based surface sampling method, capable of rapidly, accurately and reliably detecting the presence of coliforms on food contact surfaces.

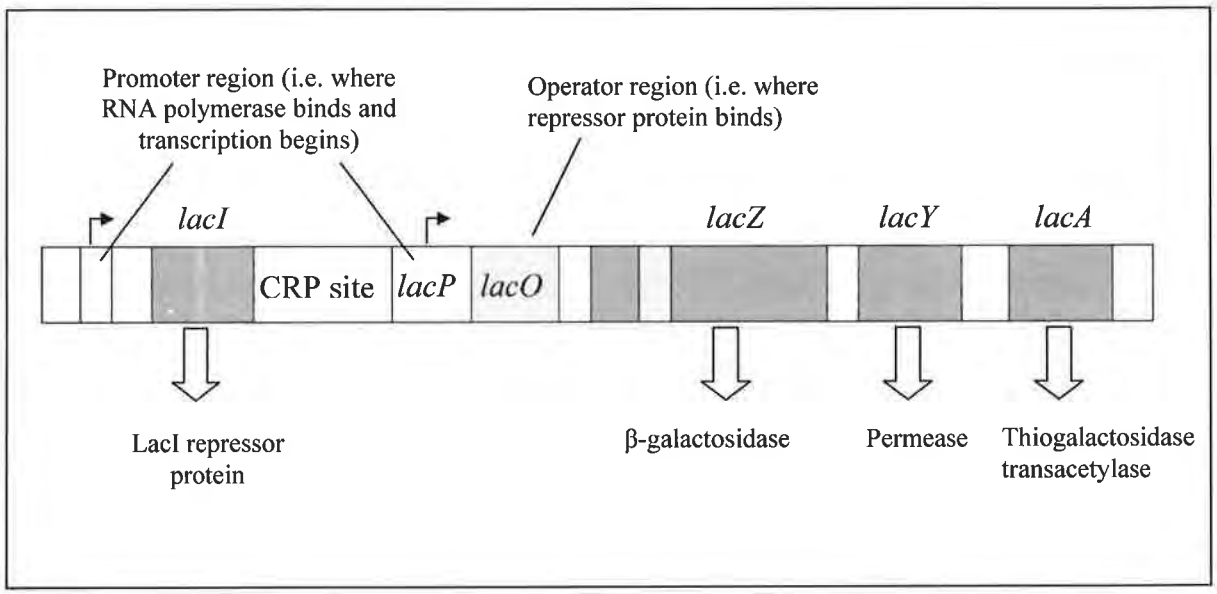
Objectives

- Investigate appropriate assay chemistry.
- Assess the ability of the proposed assay to detect the presence of coliforms.
- Assess the specificity of the proposed assay.
- Develop the assay into a method capable of detecting the presence of coliforms on food contact surfaces.
- Evaluate appropriate swab-wetting solutions.
- Investigate appropriate test format(s).
- Incorporate the assay within a test format appropriate for use within the food industry.
- Assess the repeatability of the test method.
- Compare assay performance to that of traditional swab-based methodology.
- Refine the test method to optimise assay sensitivity.

6.2 Induction of the *lac* Operon

β-galactosidase is the product of the *lacZ* gene, one of a number of genes, which make up the *lac* operon (Figure 6.1)

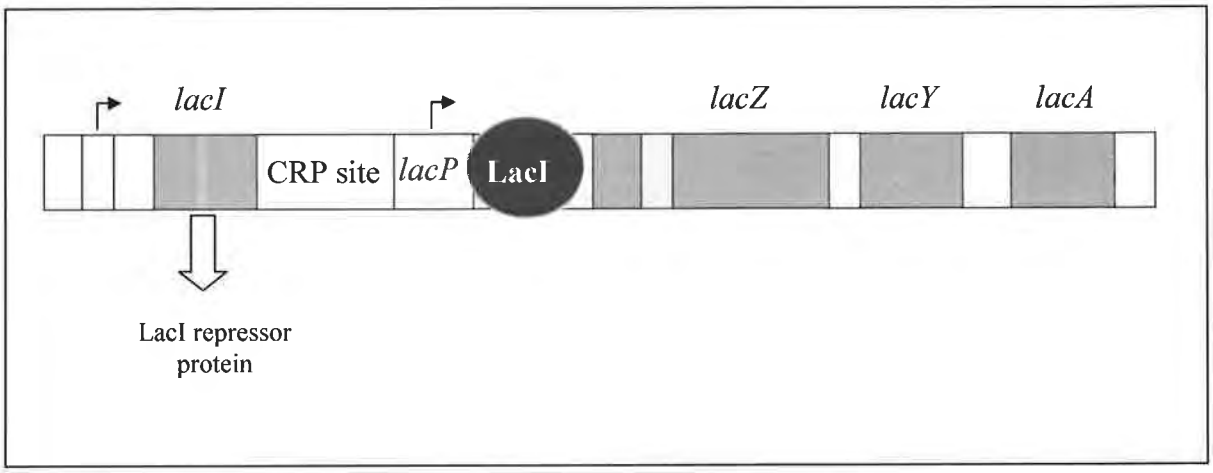
Figure 6.1. Genetic organisation and products of the *lac* operon (Moat *et al.* 2002)



The *lac* operon is responsible for the utilisation of lactose as a carbon source and, under normal circumstances, is an inducible system governed by a negative control regulator - a repressor protein, encoded for by the *lacI* gene. LacI binds to an operator region (*lacO*), which lies between the promoter region *lacP* (i.e. the site where RNA polymerase attaches and transcription begins) and the *lacZ* gene (Figure 6.2).

It is thought that the presence of the repressor protein inhibits transcription of *lacZ* and other associated genes by either physically blocking the movement of RNA polymerase into the structural genes or competing with RNA polymerase for binding within the promoter/operator region (Moat *et al.* 2002).

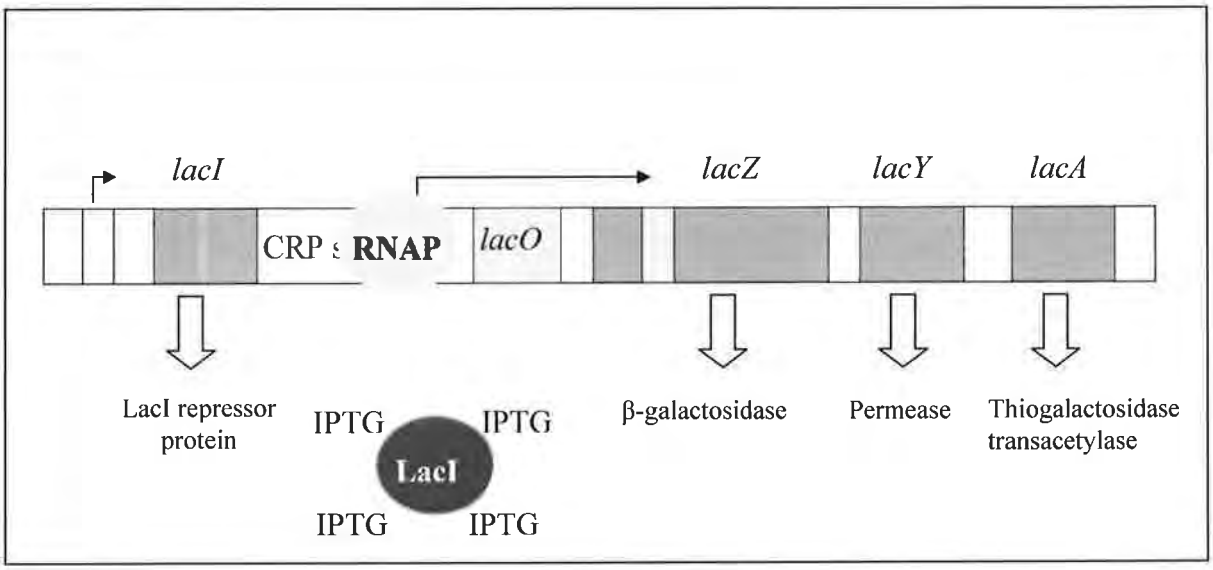
Figure 6.2. Repression of the *lac* operon



Induction of the *lac* operon occurs when a secondary molecule (an inducer) interacts with LacI and, in doing so, allosterically alters the protein, lowering its affinity for *lacO* DNA. Once the repressor is removed from *lacO*, transcription of *lacZYA* can proceed.

Negatively controlled inducible operons are, therefore, normally 'turned off' - in the case of the *lac* operon, this prevents the cell wasting energy synthesizing lactose-utilising enzymes when an ample supply of glucose is available. For the same reason, the *lac* operon also possesses an additional positive regulatory control system (Moat *et al.* 2002). In the presence of lactose, induction of the *lac* operon occurs when allolactose interacts with LacI. However, in this case the inducer molecule is also a substrate for β-galactosidase and experiments have demonstrated that the addition of glucose to an induced *E. coli* culture results in the cessation of β-galactosidase synthesis; a phenomenon known as catabolite repression (Moat *et* al. 2002). Experiments designed to examine the induction of the *lac* operon usually, therefore, require the incorporation of an inducer molecule, which without being a substrate for β-galactosidase itself, will bind to the repressor protein and inactivate it. Commonly used for this purpose is the chemical isopropyl-β-thiogalactopyranoside (IPTG) (Figure 6.3).

Figure 6.3. Induction of the *lac* operon



6.3 Chemistry of the β-galactosidase Assay

Prior to conducting any experiments, a comprehensive literature review was performed in order to identify appropriate assay chemistry.

The β-galactosidase assay that was ultimately chosen to form the basis of the proposed test method was a modification of a protocol described by Van Poucke and Nelis (1995). These authors demonstrated that cleavage, by β-galactosidase, of a 1,2-dioxetane substrate, resulted in a chemiluminescent reaction that was 4- and 1000-times more sensitive and provided results 1- and 6-h earlier than those involving fluorogenic Mu-Gal and chromogenic ONPG respectively.

6.3.1 Reaction Buffer

6.3.1.1 Substrate

The chemiluminogenic substrate used throughout the current investigation was a 1,2-dioxetane substrate, named Galacton-*Star*® (10mM concentrate; Tropix (PE Biosystems), Warrington, UK). The cleavage of Galacton-*Star*® by β-galactosidase, leads to the formation of an unstable anion (Figure 6.4) which, readily decomposes and, in doing so, emits visible light that builds to a steady glow (Tropix, 1998). This light output, which is stable for a few hours, can then be measured using a luminometer. The luminometer used during the current study was the Biotrace Uni-Lite®, which reads light at a wavelength of 540nm.

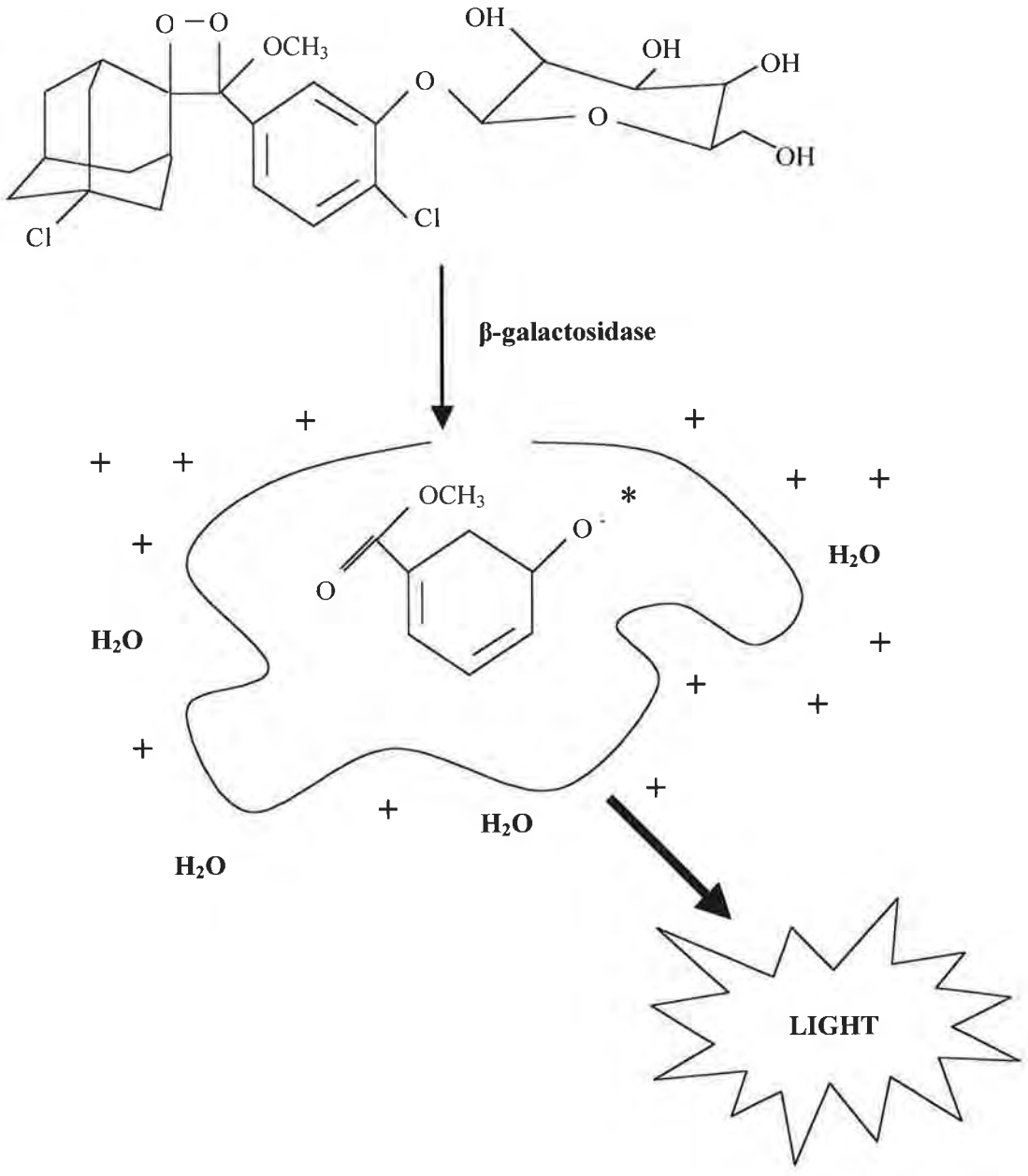
6.3.1.2 Diluent

The reaction buffer diluent comprised a sodium phosphate buffer (0.1M, pH 7.5; Sigma-Aldrich, Poole, Dorset, UK), magnesium chloride (1mM; Sigma-Aldrich) ($MgCl_2 \cdot 6H_2O$) as a co-factor for β-galactosidase (Van Poucke and Nelis, 1995) and Emerald-II™ (5% (v/v); Tropix), a macromolecular signal enhancer.

Although, the presence of $MgCl_2 \cdot 6H_2O$ in the assay mix was essential for the reaction to occur, the addition of the enhancer was optional. However, its inclusion did have major advantages with regard to assay performance. Aqueous environments reportedly reduce the intensity of the chemiluminescent light signal via water-induced quenching. The addition of an enhancer provides a hydrophobic microenvironment for the unstable anion, thereby preventing its protonation to a stable, non-light producing form (Figure 6.4). Thus,

enhancers increase the efficiency of light production; Emerald-II for example, shifts the maximum light emission from 475nm to 542nm (Tropix, 1998). During this investigation, therefore, the use of Emerald-II not only provided maximum signal intensity, but also allowed for optimum light collection by the luminometer.

Figure 6.4. Mechanism of light production using the 1, 2-dioxetane substrate Galacton-*Star*® and the signal enhancer Emerald-II™.



As directed by the manufacturer, the reaction buffer was prepared by diluting the Galacton-*Star*® (1:50) using the buffer diluent.

6.3.2 Membrane Permeabilisation

Entrance of lactose into the cell requires the *lac* permease, the product of the *lacY* gene (Figure 6.1). Similarly, it is not possible for synthetic substrates for β-galactosidase to rapidly pass through the membrane of a living cell unaided. Thus, it is necessary to treat the cells with a detergent and/or organic solvent in order to destroy or increase the permeability of the cell membrane (Pommepuy *et al.* 1996; Fiksdal and Tryland, 1999).

Van Poucke and Nelis (1995) demonstrated that when serial dilutions of *E. coli* were treated with a dioxetane substrate, alone, then in combination with a permeabiliser the detection limit of the chemiluminescent assay decreased from approximately 10^4 cfu ml^{-1} to 10^2 cfu ml^{-1} respectively.

6.4 Evaluation and Optimisation of Assay Chemistry

The main requirements for any good microbiological test method are reliability, sensitivity, selectivity and economy (Vanne *et al.* 1996). Thus, the developmental process began by conducting a preliminary set of experiments, the aims of which were to:

i. evaluate a range of extractants and membrane permeabilisers
ii. confirm the sensitivity of the chemiluminescent assay
iii. assess the specificity of the assay

6.4.1 Microorganisms

Gram-negative, lactose-fermenting rods were isolated from a variety of environmental sources and identified using biochemical test strips (API 20E; bioMérieux) as being *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter amnigenus*, *Enterobacter cloacae*, *Escherichia coli* and *Serratia liquefaciens*. Bacterial cultures were prepared and maintained as described in Section 2.2.2.

6.4.2 Growth Medium

Each overnight culture was serially diluted using a low-nutrient growth medium (LNM), originally developed by Van Poucke and Nelis (1995) as a means to minimise the effects of luminescent background and light quenching. This medium consisted of a potassium chloride buffer (0.05M, pH 7.3), containing sodium chloride (5g l^{-1}) (NaCl), tryptone (1g l^{-1}; Oxoid), sodium dodecyl sulfate (0.05g l^{-1}; Sigma-Aldrich) (SDS), and IPTG (0.01g l^{-1}; Section 6.2).

6.4.3 Assay Procedure

It has previously been demonstrated, that for maximum sensitivity, a propagation phase is required prior to the actual enzyme assay and, therefore, pre-incubation of the samples was carried out at 37^0C for 4 h (Van Poucke and Nelis, 1995).

After incubation, the initial assay procedure, as recommended by the manufacturer of the reagents, involved the addition of 100 µl of microbial dilution to 100 µl of extractant. The

suspension was left for 1 min at room temperature to allow cell permeabilisation to occur. After this short incubation period, 20 µl of the solution was transferred to a cuvette containing 300 µl of the reaction buffer (Section 6.3) and left, again at room temperature, for 1 h. The cuvette was then attached to a Biotrace Hold-Tite™ and placed in the Uni-Lite® luminometer. Readings, in relative light units (RLU), were recorded. Control samples were conducted by substituting the microbial dilution for 100 µl of un-inoculated growth medium (Section 6.4.2). A sample was assumed positive for β-galactosidase and, thus, coliforms if the assay resulted in an RLU reading over twice the average RLU reading of the control samples (Corbitt *et al.* 2000).

6.4.4 Assay Sensitivity

Five different coliform strains were treated with a range of extractants and permeabilisers.

Extractants A, B and C (Biotrace Ltd) were all associated with ATP bioluminescence and have been used as a means of extracting ATP from both microbial and somatic cells. The antibiotic, polymyxin B, is known to bind to the lipopolysaccharide contained within the outer membrane of gram-negative bacteria and, in doing so, disrupt its structure and permeability properties (Vaara, 1992). Chlorpromazine has been shown to affect membrane fluidity in *E. coli* (Tanji *et al.* 1992).

The effect that these extractants and membrane permeabilisers had upon the chemiluminometric response is exemplified by the results presented in Table 6.1. Although the RLU values associated with each of the coliform strains differed, the overall pattern of sensitivity was the same.

Table 6.1. The effect of a range of different extractants and membrane permeabilisers upon the chemiluminometric response

Inoculum[†] (CFU)	Chemiluminescent light signal (RLU)				
	Extractant			*Permeabiliser*	
	A	**B**	**C**	**Chlorpromazine[‡]**	**Polymyxin B**
0 (Control)	153	256	615	254	429
10^1	1,380[*]	465	941	980[*]	4,563[*]
10^2	7,130	1,206[*]	4,574[*]	3,481	27,714
10^3	47,829	7,724	35,316	45,324	208,326
10^4	>500,000	84,938	>500,000	>500,000	>500,000

[†] cfu (*Ent. amnigenus*) theoretically present in the original 100 µl sample (i.e. prior to pre-incubation)

[‡] solution consisted of a HEPES buffer (25mM, pH 7.75), chlorpromazine (1mM; Sigma-Aldrich) and EDTA (2mM; Sigma-Aldrich); (W. Simpson; personal communication)

[*] minimum detection limit of assay (i.e. the lowest dilution assayed, which resulted in an RLU value more than twice that of the control (background) value)

In comparison to when either Extractant B or C was incorporated into the assay mix, the use of chlorpromazine increased the sensitivity of the assay 10-fold (Table 6.1). Thus, on the basis of these results, Extractants B and C were omitted from further investigation. However, for practical reasons, not least its extreme toxicity, the use of chlorpromazine was also considered inappropriate. Nevertheless, the use of either Extractant A or polymyxin B (100 µg ml^{-1}) also resulted in an assay that, in just 5 h, was capable of detecting the presence, within the original 100 µl sample, of approximately 10 cfu (i.e. 10^2 cfu ml^{-1}). Furthermore, those RLU values obtained after assaying this level of bacteria were approximately 10-times higher than those associated with the control samples, suggesting that the detection limit of the chemiluminescence-based assay was in fact lower, thus, supporting the findings of Van Poucke and Nelis (1995) who reported that

dioxetane-based chemiluminometry is capable of detecting as little as 2 fg of β-galactosidase; the equivalent of just 1 induced *E. coli* cell.

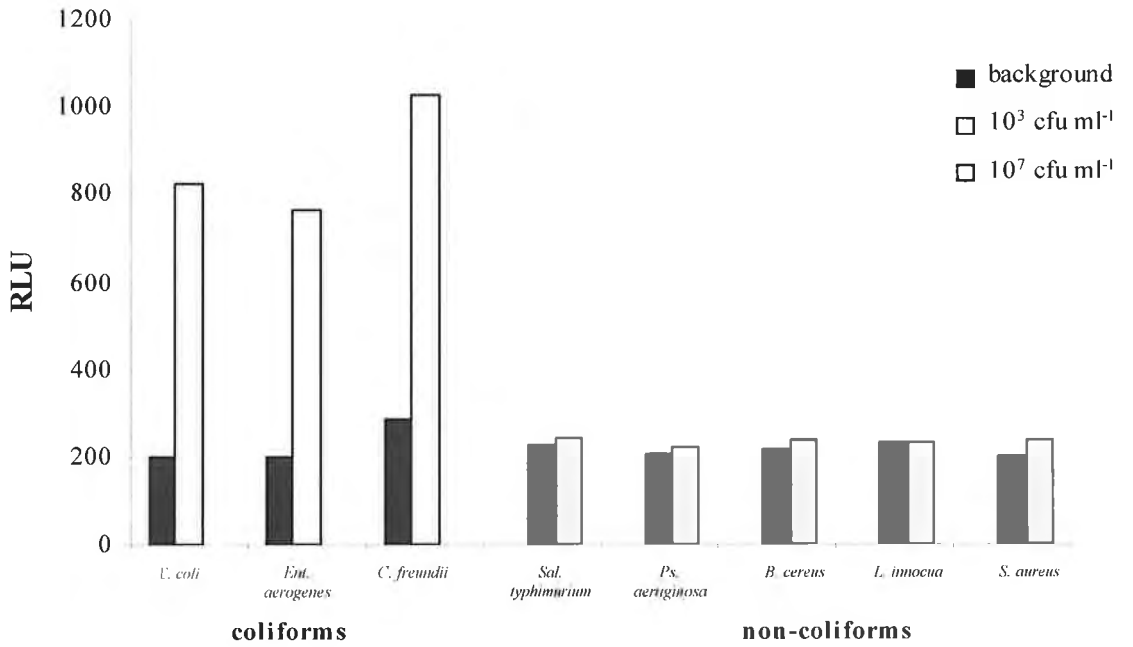
However, whilst these initial findings confirmed that the proposed chemiluminescence-based assay was indeed a rapid and highly sensitive means of detecting the presence of coliforms, its specificity still needed to be assured.

6.4.5 Assay Specificity

Previous studies have concluded that the sensitivity of dioxetane-based chemiluminometry is such, that β-galactosidase activity of non-coliform bacterial strains is also detected. This can, it has been reported, lead to a high number of false positive reactions, the majority of which are thought to be caused by gram-positive organisms such as *Bacillus* and *Staphylococcus* spp. (Van Poucke and Nelis, 1997a). In an attempt to inhibit the growth of such bacteria, Masuda-Nishimura *et al.* (2000) incorporated sodium deoxycholate into a bioluminescence-based assay medium. However, on addition of the culture fluid, these workers observed that the medium components induced a non-specific luminescence peak. Similarly, previous studies have indicated that common medium ingredients may also lead to high background readings and/or quench the chemiluminescent light signal (Van Poucke and Nelis 1997b). However, Tryland and Fiksdal (1998) demonstrated that the β-galactosidase of non-target microorganisms showed no, or only slight, induction by IPTG. Consequently, these authors stated, in contrast to the conclusions of Van Poucke and Nelis (1997a), that non-coliform bacteria must be present in much higher concentrations than target bacteria in order to interfere with their detection.

During the current study it was hypothesised that should Extractant A, which lyses a wide range of microbial cells, be added to the assay mix, then some form of selective agent would need to be added to the growth medium, which may affect the light output and ultimately the sensitivity of the assay. However, polymyxin B has a narrow spectrum of activity and is effective against gram-negative organisms only. Thus, it was postulated that its use as a selective permeabiliser coupled with the use of IPTG as the inducer molecule, would allow the detection of target β-galactosidase only.

Figure 6.5. The detection of target and non-target bacteria using dioxetane-based chemiluminometry



The results presented in Figure 6.5 illustrate the amount of light that was produced after a range of organisms, prior to the addition of the reaction buffer, were treated with polymyxin B (100 μg ml^{-1}). The graph indicates that the presence of coliforms at levels equivalent to 10^3 cfu ml^{-1} resulted in a light output significantly higher than that associated with the control samples. Conversely, when non-coliform bacteria, including *Bacillus cereus* and *Staphylococcus aureus* were present even at levels that exceeded 10^7 cfu ml^{-1},

the RLU values obtained did not significantly differ from those associated with the background luminescence.

Taken collectively, the results of the initial cuvette-based assays demonstrate quite clearly that chemiluminometry is a rapid, sensitive and specific means of detecting coliform bacteria. However, as with previous chemiluminescence-based studies (Van Poucke and Nelis, 1995; D'Haese *et al.* 1997; Van Poucke and Nelis, 1997a, Van Poucke and Nelis, 1997b), these preliminary experiments involved liquid test samples. The next stage of the investigation, therefore, was to adapt the assay for use within a swab-based detection system.

Nevertheless, before continuing with the development of the assay, it was necessary to ensure that chemiluminometry rather than bioluminometry should form that basis of the proposed test method. Bioluminogenic substrates for β-galactosidase have been developed (Ugarova *et al.* 1991; Geiger *et al.* 1992) and the use of one such substrate, D-luciferin-O-β-galactopyranoside (Lu-Gal) has been investigated as a means of rapidly detecting the presence of coliforms (Masuda-Nishimura *et al.* 2000; Taksumi and Fukuda, 2002). The ability, therefore, of the chemiluminescence-based assay, described in Section 6.4.4, to detect coliform bacteria was compared to that of a similar assay procedure, which incorporated Lu-Gal. The results obtained indicated that the background luminescence associated with the Lu-Gal reaction was more than 70-times higher than that associated with dioxetane-based chemiluminometry. This not only resulted in the bioluminescence-based assay appearing less sensitive but also implied that the Lu-Gal was unstable and possibly breaking down without the action of β-galactosidase. For these reasons together with the high cost of the associated reagents, a bioluminescence-based assay was considered inappropriate and, consequently, work continued on the development of a novel, chemiluminescent, swab-based coliform detection method.

6.5 Development of a Swab-based β-galactosidase Assay

6.5.1 Adaptation of Assay Procedure

It has been concluded that when sampling a stainless steel surface, the most important contributory factor with regard to the efficiency of the swabbing technique is the effective release of bacteria from the swab bud (Chapter 2). Similarly, the results presented in Table 6.1 clearly indicate that an increase in the number of coliforms within the original 100 µl sample leads to an increase in the amount of light produced. It was assumed, therefore, that whilst the sensitivity of the proposed swab-based assay would ultimately depend upon the extent of coliform growth and enzyme induction that occurred over the 4 h pre-incubation phase, it would initially depend upon sufficient numbers of coliform bacteria being released into the low nutrient medium. Thus, on the basis of the results presented in Chapter 2, pre-moistened dacron swabs (ULH 100S; Biotrace) were used to sample the stainless steel surfaces.

It was also hypothesised that a further increase in RLU could be achieved, post-propagation, by increasing the volume of the microbial/permeabiliser suspension incorporated within the final assay mix. However, the design of the Uni-Lite® luminometer dictated that for optimum light absorption, the final assay volume should lie between 300 µl and 400 µl, thus, any increase in suspension volume would need to be accompanied by a reduction in the volume of the reaction buffer. The results presented in Figure 6.6 confirmed that this would have little effect upon overall assay sensitivity. Nevertheless, the results of a subsequent experiment demonstrated that sufficient substrate had to be available for cleavage; otherwise, as illustrated in Figure 6.7, light output would fall.

Figure 6.6. The effect that an increasing volume of microbial suspension together with a corresponding decrease in reaction buffer volume, had upon the chemiluminometric response

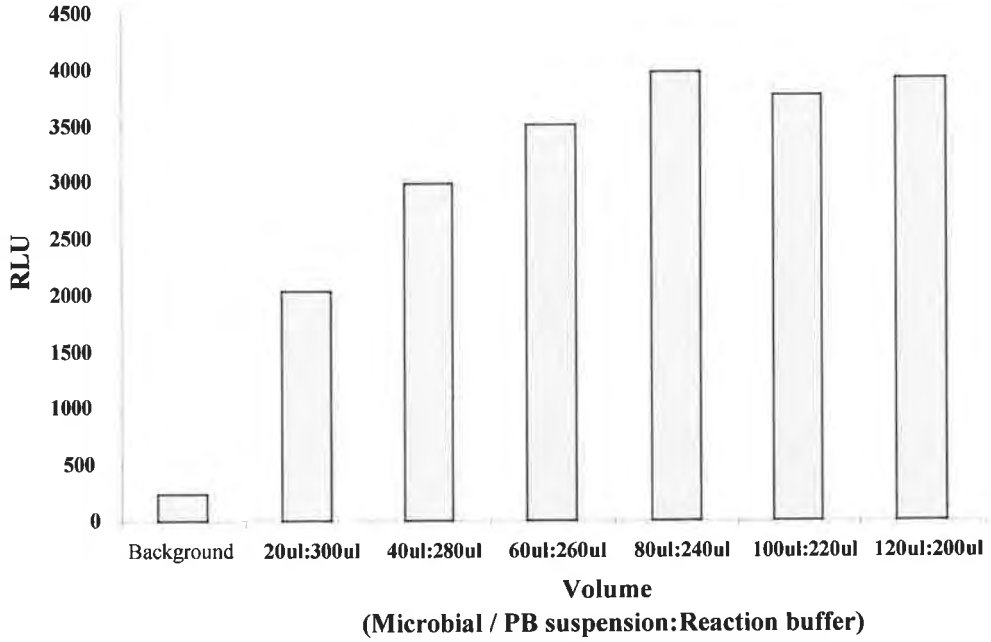
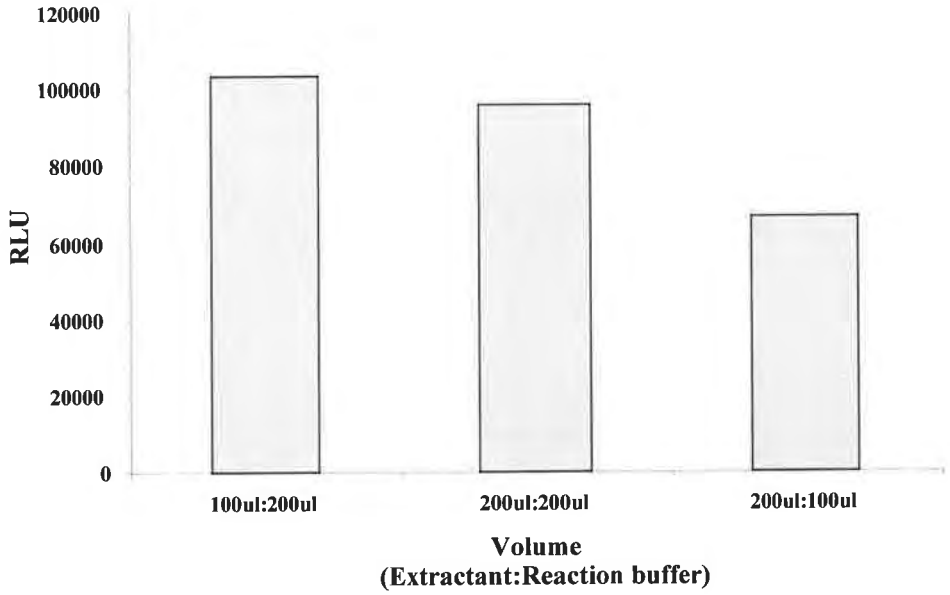
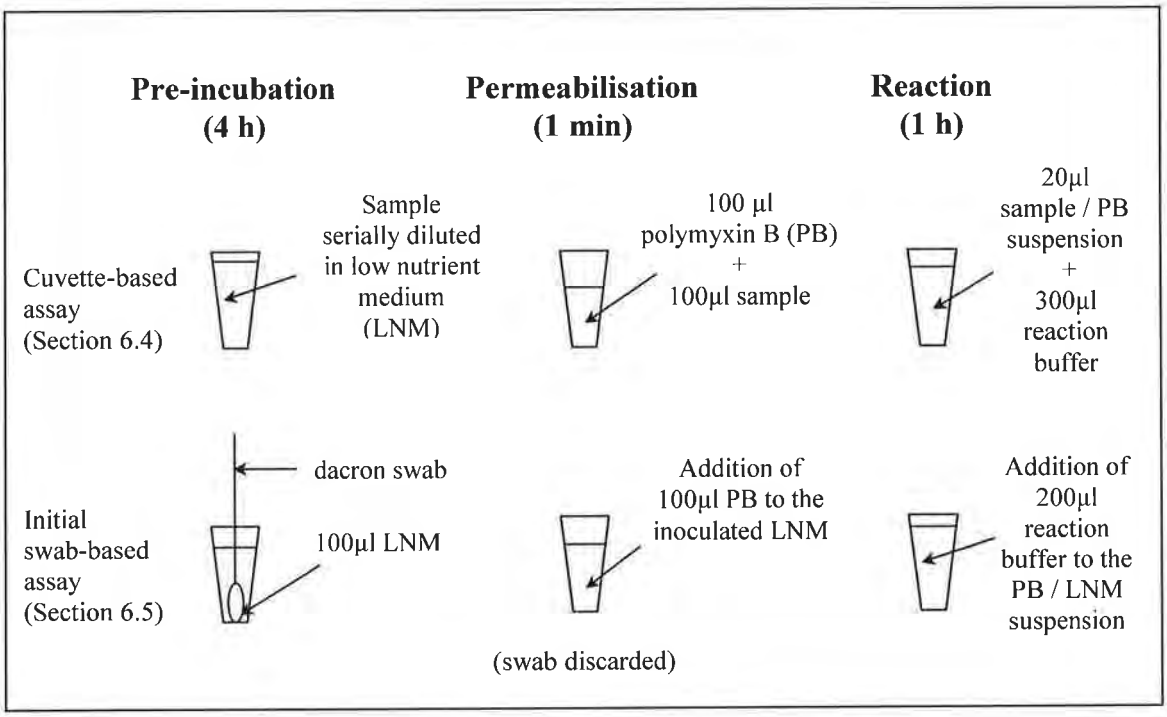


Figure 6.7. The effect, upon the chemiluminometric response, of reducing the volume of reaction buffer present within the final assay mix



These observations, the need to minimise the cost of the assay and the overall aim of ultimately incorporating the reagents within a single-shot device (see Section 6.6), resulted in the decision being made to add 200 µl of reaction buffer to the entire 200 µl of coliform/polymyxin B suspension. Figure 6.8, therefore, illustrates how the cuvette-based assay described in Section 6.4 was initially adapted in order to detect the presence of coliform bacteria on food contact surfaces.

Figure 6.8. The cuvette- and initial swab-based, β-galactosidase assay procedures



6.5.2 Sensitivity of Initial Swab-based Assay

Sterile dacron swabs were pre-moistened with LNM. The swabs were then either directly inoculated with 20 µl (equivalent to swab saturation) of a known serial dilution (Section 6.4.2) or used to sample a stainless steel surface that had been prepared and inoculated as described in Section 3.2.4. The surface was sampled, using the swabbing protocol described in Section 2.2.4.2, immediately after inoculation, whilst it was still wet, or after

it had been allowed to air-dry for 1 h, after which time no visible liquid remained on the surface. The swabs were then snapped off into a cuvette containing 100 µl LNM and incubated at 37°C for 4 h. The subsequent assay procedure followed that described in Figure 6.8. In both cases, to obtain the level of background luminescence, control assays were conducted by substituting the microbial dilution for un-inoculated LNM. Experiments were repeated to verify the limits of detection, which are typified by those illustrated in Tables 6.2 and 6.3.

Table 6.2. The minimum detection limit (cfu swab^{-1}) of the chemiluminescent swab-based assay

Test condition		**Minimum detection limit (cfu)***
Direct swab inoculation	*Escherichia coli*	69
	Enterobacter aerogenes	110
	Enterobacter amnigenus	8
	Citrobacter freundii	64
	Serratia liquefaciens	1.09×10^5

* minimum detection limit of assay (i.e. the lowest inoculum (cfu) assayed, which resulted in an RLU value more than twice that of the control (background) value)

In the majority of cases, the minimum detection limit of the chemiluminescence-based assay, when used to detect the presence of coliforms on a directly inoculated dacron swab, ranged, depending on coliform strain, from <10 cfu to approximately 100 cfu swab^{-1} (Table 6.2). Thus, despite very few bacteria initially being present on the bud, after permeabilisation the β-galactosidase levels within the medium were such, that a positive chemiluminescent response was produced, confirming that a sufficient proportion of the bacteria had been released from the swab bud and had grown, over the 4 h propagation phase, to detectable levels within the medium (Section 6.5.1). However, whilst bacterial release and growth can be considered the most important contributory factors, coliform type may also have a significant effect upon assay sensitivity.

In order to detect the presence of *Serratia liquefaciens*, it was necessary to initially inoculate the swab bud with approximately 10^5 colonies (Table 6.2). Despite producing β-galactosidase, *Serratia* spp. are known to attack lactose either slowly or not at all. It has been speculated that the reason for this, is the inability of this group of bacteria to transport lactose into the cell (Mossel *et al.* 1995). However, during the current investigation the cells were treated with polymyxin B, which, it was presumed, would facilitate the entry of the β-galactosidase substrate (i.e. Galacton-*Star*®). Nevertheless, post-propagation, in relation to the number of cells that were present within the growth medium, only low levels of β-galactosidase were detected, suggesting that minimum induction of the *lacZYA* genes had occurred. Thus, the absence of *lac* permease may not be the only reason for the differences, regarding lactose fermentation, observed between *Serratia* spp. and other coliform bacteria. A mutation for example, in the *lacI* locus is known to exist, which results in an uninducible *lac* operon. Under these circumstances, $lacI^S$ produces a super-repressor with increased operator binding and/or diminished inducer (IPTG)-binding properties (Moat *et al.* 2002).

Nevertheless, in general, the minimum detection limits presented in Table 6.2 are similar to those of the cuvette-based assays (Table 6.1), within which is was not necessary to release bacteria from a swab. This suggests that the poor bacterial release alluded to in Chapter 2 may not have the same effect upon the overall sensitivity of the chemiluminescent swab-based assay as it appears to have upon the traditional swabbing technique. Not only is this likely to be due to the 4 h propagation phase, but also, unlike the traditional swabbing protocol, the chemiluminescent swab-based assay incorporates the entire volume of media into which the bacteria have been released. However, although encouraging, it is acknowledged that these initial swab-based assays involved the direct inoculation of the bud and, thus, did not take into consideration the necessity to initially remove the bacteria from the surface.

Table 6.3 illustrates the minimum detection limit of the swab-based assay when it was used to detect the presence of coliforms on a wet and dry stainless steel surface. In this case, and all subsequent surface-associated assays, the minimum detection limits have been converted to cfu cm^{-2} using equation 1.

$$MDL = \frac{N \times d \times v}{A} \quad (1)$$

Where:

MDL = minimum detection limit (cfu cm^{-2})
N = number of cfu ml^{-1} (determined via conventional cultivation of the bacterial culture)
d = dilution factor
v = volume of sample
A = the surface area inoculated

Table 6.3. The minimum detection limit (cfu cm^{-2}) of the proposed chemiluminescent swab-based assay when used to detect the presence of coliforms on a wet and dry stainless steel surface

Test condition		Minimum detection level (cfu cm^{-2})*
Wet surface	***Escherichia coli***	< 1
	Citrobacter freundii	< 1
Dry surface	***Escherichia coli***	500
	Citrobacter freundii	5.5×10^3

* minimum detection limit of assay (i.e. the lowest inoculum (cfu cm^{-2}) assayed, which resulted in an RLU value more than twice that of the control (background) value)

When a wet surface was sampled, the swab-based assay was, after just 5 h, capable of detecting < 1 coliform colony cm^{-2}. However, although unsurprising given the results presented in Chapters 2 and 3, the sampling of a dry surface resulted in a marked reduction in assay sensitivity; its minimum detection limit increasing, depending on coliform strain,

to between 5 x 10^2 and 5 x 10^3 cfu cm^{-2}. As discussed in Chapter 2, a loss in microbial viability may have contributed to this reduction, as could an increase in bacterial adhesion and/or, particularly as dacron swabs were used, a reduction in the number of bacteria that were initially removed from the surface.

The addition of various substances to a swabbing solution, can not only improve the detachment of bacteria from a surface (Chapter 2), but can also neutralise the effects of any residual cleaning chemicals. However, up until this point in the investigation, the swabs had been moistened with the low nutrient growth medium (Section 6.4.2), which contained neither a surfactant to aid pick-up, nor a recognised neutralising agent. In an attempt, therefore, to improve the sensitivity of the assay and to optimise assay performance, a range of different swab wetting solutions were investigated.

6.5.3 Swab-wetting Solution

6.5.3.1 The evaluation of a variety of swab-wetting solutions

One of the conclusions drawn from the study discussed in Chapter 2, was that, particularly when sampling a dry surface, pre-moistening a swab with a solution containing Tween 80 appears to improve bacterial release and, thus, overall swabbing efficiency. Since Tween 80 can neutralise the effects of quaternary ammonium compounds (QACs) (Russell, 1981), the advantages of its incorporation within a swab-wetting solution may be two-fold.

The ability of swabs pre-moistened with the MES buffer-based solution (Section 2.2.4.1) to detect the presence of coliforms on a wet and dry stainless steel surface was assessed, as was the effect of the solution upon the chemiluminometric response. Its performance was

compared to that of the swab-wetting solution used to date (LNM) and to a recovery medium described by Barnes *et al.* (1996), which contained Tween 80 at a level of 3%. This solution was used in preference to the 3% Tween solution used throughout the investigation discussed in Chapter 2. The latter (Section 2.2.4.1) also contained lecithin, which appeared to contribute to the yellowness of the solution and it was felt that this coloration could adversely affect the chemiluminometric response. The assays were performed as described in Section 6.5.2 and the results are presented in Table 6.4.

Table 6.4. Effect of three different swab-wetting solutions upon the chemiluminometric response

Test condition	Inoculum† (CFU)	Mean RLU value (n=2)		
		LNM	MES buffer	SRM‡
Direct swab inoculation	**Background**	157	118	38
	14	171	153	22
	140	524*	406*	26
	1.4 x 10^3	3577	2982	449*
Wet surface	**Background**	640	1257	190
	20	2224*	5897*	629*
Dry surface	**Background**	471	531	205
	200	515	675	199
	2.0 x 10^3	714	1078*	255
	2.0 x 10^4	3059*	19413	327

† cfu (*Ent. aerogenes*) theoretically inoculated onto either the bud (i.e. cfu 20 μl^{-1}) or surface (i.e. cfu cm^{-2})

‡ Swab Recovery Medium (pH 7.1), containing peptone (1g l^{-1}; Oxoid), NaCl (8.5g l^{-1}); sodium thiosulphate (3g l^{-1}; Fisher Scientific) and Tween 80 (30ml l^{-1}; BDH Laboratory Supplies, Poole, Dorset, UK)

* minimum detection limit of assay (i.e. the lowest dilution assayed, which resulted in an RLU value more than twice that of the control (background) value)

When swabs, prior to their direct inoculation, were moistened with the Swab Recovery Medium (SRM), the sensitivity of the β-galactosidase assay was, in comparison to when

the other swab-wetting solutions were used, reduced 10-fold. A comparison of the associated background luminescence light readings suggested that the observed reduction in sensitivity might have been caused by components of the SRM quenching the light signal.

A reduction in light signal appeared to have less of an effect upon the sensitivity of the assay when it was used to detect the presence of coliforms on a wet surface – assays incorporating swabs that had been pre-moistened with any of the three different solutions were capable of detecting 20 cfu cm^{-2}. However, the difference between the RLU values obtained when assaying this level of bacteria and those of the control samples, suggest that the minimum detection limit of assays incorporating swabs pre-moistened with either LNM or the MES buffer-based solution, may in fact be lower.

This was indeed the case when the assay was used to detect the presence of coliforms on a dry surface. Under these conditions, when the swabs were pre-moistened with LNM, the β-galactosidase assay was capable of detecting an original inoculum of approximately 2 x 10^4 cfu cm^{-2}. However, when the assay incorporated swabs pre-moistened with SRM, this level of bacteria remained undetected. In comparison, use of the MES buffer-based solution resulted in the detection of approximately 2 x 10^3 cfu cm^{-2} (Table 6.4).

These results suggest that whilst relatively high levels of Tween 80 may quench the chemiluminescent light signal, the incorporation of lower levels can, perhaps by improving bacterial pick-up and/or release, improve assay sensitivity. Nevertheless, although high concentrations of Tween can neutralise the effects of QACs, low concentrations can, it has been reported, potentiate their action (Allwood, 1973) and the use of cyclodextrins has been proposed as an alternative (Simpson, 1992).

In order to assess the effect that β-cyclodextrin, chosen for its inactivation of a wide range of QACs (Simpson, 1992), would have upon the chemiluminometric response, a cuvette-based assay, similar to that described in Section 6.4.3, was performed. β-cyclodextrin (10mM (Simpson, 1992); Sigma-Aldrich) was added to the MES buffer-based solution, 100 µl of which was then transferred to a cuvette containing 20 µl of pre-incubated microbial sample. Following permeabilisation with polymyxin B, 200 µl of reaction buffer was added to the cuvette and the light output, after 1 h, was recorded. The results are presented in Table 6.5 and are compared to those that were obtained when β-cyclodextrin was added to 'Solution 2' - a neutralising solution based upon one used in the manufacture of dipslides. In both cases, the chemiluminometric response is compared to that observed when β-cyclodextrin was omitted from the final assay mix.

Table 6.5. The effect of β-cyclodextrin (10mM) upon the chemiluminometric response

	Inoculum (cfu 20µl^{-1})	**Mean RLU value (n=2)**			
		MES buffer	**MES buffer + β-CD**	**Solution 2‡**	**Solution 2‡ + β-CD**
Ent. cloacae	**Background**	404	95	150	90
	1.6 x 10^2	472	100	159	131
	1.6 x 10^3	2104*	129	372*	116
	1.6 x 10^4	21332	411*	2949	320*
C. freundii	**Background**	346	85	109	83
	1.0 x 10^3	755*	95	175	87
	1.0 x 10^4	4928	124	696*	108
	1.0 x 10^5	55399	833*	7571	617*

‡ Neutralising solution comprising: MES buffer (0.01M; pH 7.0), sodium thiosulphate (0.8g l^{-1}), Tween 80 (3 ml l^{-1}) and histidine (1.4g l^{-1}).

* minimum detection limit of assay (i.e. the lowest dilution assayed, which resulted in an RLU value more than twice that of the control (background) value)

The addition of β-cyclodextrin (10mM) to either neutralising solution appeared to quench the light signal and thus, regardless of coliform strain, reduced the sensitivity of the β-galactosidase assay. Likewise, in the absence of β-cyclodextrin, the only significant difference between the MES buffer-based solution and Solution 2, was the inclusion in the latter of histidine and its presence also appeared to cause a considerable reduction in light output. When used to detect the presence of *Ent. cloacae* this made no difference in overall assay sensitivity. However, when used to sample for *C. freundii*, those assays incorporating swabs pre-moistened with the MES buffer-based solution were capable of detecting 10-times fewer colonies than those incorporating Solution 2. Thus, the MES buffer-based solution was again used to compare the effects of a range of β-cyclodextrin concentrations upon the chemiluminometric response.

Table 6.6. Effect of a range of β-cyclodextrin concentrations upon the chemiluminometric response

Inoculum (cfu 20µl^{-1})	Mean RLU values (n=2)							
	MES buffer		MES buffer + 5mM β-CD		MES buffer + 1mM β-CD		MES buffer + 0.5mM β-CD	
	Cuvette†	*Swab*†	*Cuvette*†	*Swab*‡	*Cuvette*†	*Swab*‡	*Cuvette*†	*Swab*‡
Background	69	568	65	251	83	530	57	382
10^1	78	-	71	-	83	-	64	-
10^2	255*	861	99	330	140	547	162*	641
10^3	1646	1937*	386*	745*	804*	1153*	983	1438*
10^4	-	76754	-	1639	-	6202	-	10141

† cuvette-based assay (Section 6.4.3)

‡ direct swab inoculation (Section 6.5.2)

* minimum detection limit of assay (i.e. the lowest dilution assayed, which resulted in an RLU value more than twice that of the control (background) value)

As was observed with increasing Tween concentration, the level of β-cyclodextrin incorporated within the assay mix also influenced the extent to which the associated

chemiluminescent light signal was quenched (i.e. as the concentration of β-cyclodextrin was increased, the amount of light produced was observed to decrease). Consequently, in comparison to when higher concentrations were used, the incorporation within the cuvette-based assay of 0.5mM β-cyclodextrin resulted in a 10-fold increase in assay sensitivity (Table 6.6). Interestingly, the RLU readings relating to the background luminescence associated with the cuvette-based assay were observed to be much lower than those of previous experiments (Tables 6.1 and 6.4). These results, although incidental to the developmental process as a whole, highlight one of the difficulties that arose throughout this investigation.

6.5.3.1.1 Difficulties associated with the experimental protocol

Throughout the current study, different formulations of the same solution or reagent were continually being assessed and, as a result, relatively small volumes of each were prepared on a day-to-day basis. Although this avoided excessive wastage, there was the risk that any inaccuracies that occurred during the weighing and/or measuring of component parts would be magnified and have a significant effect upon assay performance. It was hypothesised for example, that the unusually low RLU readings presented in Table 6.6 had been caused by the incorporation, within the MES buffer based solution, of a concentration of Tween 80 greater than the required 0.3%. An assay comparing the effect that previous batches of the MES buffer-based solution had upon the chemiluminometric response, supported this theory. Nevertheless, it was interesting to note, that this 'Tween-associated light reduction' appeared to have a much lesser effect upon the swab-based assay and it was assumed that this was due to a relatively high proportion of the Tween adhering to the swab bud and, thus, being removed from the final assay mix.

6.5.3.2 The neutralisation of a quaternary ammonium compound

The purpose of a swab-wetting solution is not only to aid bacterial pick-up and release but also to neutralise the bactericidal effects of residual cleaning chemicals, which in addition, have been shown to reduce the sensitivity of the ATP bioluminescence technique by quenching the light signal (Section 1.4.2.2.1). The results of the previous set of experiments (Tables 6.4 - 6.6) have suggested that for maximum sensitivity, the swabs associated with the proposed β-galactosidase assay should be pre-moistened with the MES buffer-based solution, with or without the addition of low concentrations of β-cyclodextrin. Thus, to investigate the effect of residual sanitizer upon the sensitivity of the proposed assay, its ability to detect coliforms in the presence of "Bioscan" (Henkel Hygiene Ltd, Swindon, UK), a quaternary ammonium compound was assessed.

To simulate a situation where inadequate rinsing of a surface had occurred, "Bioscan", diluted to the manufacturer's recommended in-use concentration (1:80) was sprayed over a clean stainless steel surface. Pre-moistened swabs were then used to sample a 100 cm^2 surface area, before being directly inoculated with 20 µl of microbial dilution. The swabs were placed in a cuvette containing 100 µl LNM and incubated for 4 h at 37°C. The subsequent assay procedure followed that described in Figure 6.8 and the results, presented in Table 6.7, show the effect that the presence of the QAC had upon the chemiluminometric response.

It was assumed that if effective neutralisation occurred, bacterial numbers would increase over the 4 h propagation phase and the sensitivity of the β-galactosidase assay would, in comparison to previous experiments, remain unaffected. Conversely, if the biocide remained active, bacterial numbers and, thus, β-galactosidase activity would be reduced.

The results presented in Table 6.7 illustrate that a further reduction in β-cyclodextrin concentration again increased the amount of light produced. However, they also suggest that β-cyclodextrin at levels low enough to prevent the quenching of the chemiluminescent light signal may not have been sufficient to ensure the complete neutralisation of residual QACs. However, the low level of Tween 80 incorporated within the original MES buffer-based solution, rather than enhancing the action of the biocide, did appear capable of its inactivation.

Table 6.7. The neutralisation of a quaternary ammonium compound by a MES buffer-based solution containing three different levels of β-cyclodextrin

Test condition	Inoculum[†] (cfu 20µl^{-1})	Mean RLU (n=2)		
		MES buffer	MES buffer + 0.5mM β-CD	MES buffer + 0.25mM β-CD
Direct swab inoculation	Background	512	381	475
	4	531	417	514
	40	1343[*]	606	910
	400	7996	4360[*]	5261[*]

[†] *C. freundii*

[*] minimum detection limit of assay (i.e. the lowest dilution assayed, which resulted in an RLU value more than twice that of the control (background) value)

On the basis of these results, it was concluded that the MES buffer-based solution was the optimum swab-wetting solution for use within the proposed swab-based β-galactosidase assay. The developmental process would continue, therefore, by attempting to transform the swab-based assay, discussed throughout Section 6.5, into a more-user friendly means of detecting coliforms from food contact surfaces. However, before doing so, it was necessary to address the polymyxin concentration used to permeabilise the outer membrane of the target cells.

6.5.4 Membrane Permeabiliser

Up until this point in the investigation, polymyxin B had been used at a concentration of 100 μg ml^{-1}. However, its toxicity at this level resulted in a need to assess the ability of a range of polymyxin concentrations to permeabilise the coliform outer membrane. A swab-based assay, based on that described in Figure 6.8, was performed and the results are presented in Table 6.8.

Table 6.8. The effect of polymyxin B concentration upon the chemiluminometric response

Test condition	Inoculum† (cfu 20 μl^{-1})	Mean RLU (n=2) *Polymyxin B concentration*				
		100ug ml^{-1}	10ug ml^{-1}	5ug ml^{-1}	2ug ml^{-1}	1ug ml^{-1}
Direct swab inoculation	**Background**	555	475	439	500	610
	6	580	628	532	582	640
	60	1308*	1249*	1751*	1337*	1426*
	600	9119	11148	6595	8325	7467

† *E. coli*

* minimum detection limit of assay (i.e. the lowest dilution assayed, which resulted in an RLU value more than twice that of the control (background) value)

The results illustrate that the sensitivity of the β-galactosidase assay remained unaffected over a wide range of polymyxin B concentrations and suggest that polymyxin B at a level of 1 μg ml^{-1} is as effective in facilitating the entry of Galacton-*Star*® into the cell as a level of 100 μg ml^{-1}. The minimum concentration of polymyxin B required to permeabilise the outer membrane has been observed to range from 0.3 μg to 1 μg ml^{-1} (Vaara, 1992). However, magnesium, present within the buffer diluent, has been documented to reduce the effect of polymyxins (Bolton, 1998). For this reason and the need, as discussed in Section 6.5.3.1.1, to prevent batch variation, it was concluded that polymyxin B would subsequently be used at a level of 2 μg ml^{-1}.

6.6 Development of a User-friendly Assay Format

6.6.1 Single- or Multi-shot?

It has been established that the swab-based assay described in Section 6.5 is capable of detecting the presence of low levels of coliform bacteria on either a wet or dry surface in only 5 h. However, the protocol involves multiple manipulations, which although acceptable during the laboratory-based developmental process, is in reality completely impractical and would be of little use to the food industry. The next stage of the investigation, therefore, was to incorporate the assay within a user-friendly test format and two options existed:

i. the test could be supplied in a multi-shot format (i.e. the assay would still involve a number of steps but reagents and solutions would be provided ready-to-use)
ii. a single-shot device could be developed (i.e. the test would be self-contained and the user would have to conduct very few, if any, additional manipulations)

In order to investigate these options, several assay procedures were evaluated. The protocols are detailed in Figure 6.9 and the results are presented in Table 6.9.

6.6.2 Evaluation and Sensitivity of Possible Test Formats

The aim of Assay 2 (Figure 6.9), was to simulate a multi-shot procedure, which would require the appropriate volume of LNM, polymyxin B and reaction buffer to be supplied pre-measured, in three sealed cuvette-like containers. The user would then simply transfer a swab from container to container after the time appropriate for each stage of the test had

elapsed. Although, this method was capable of detecting an original inoculum of approximately 4 x 10^2 cfu bud^{-1} (Table 6.9) and appeared as sensitive as the swab-based assay used to date (Assay 1), the problems associated with this multi-shot concept were two-fold.

Firstly, this assay format supposes that the reaction buffer can be supplied ready-to-use. However, the manufacturer of the reagents does not recommend the long–term storage of solutions comprising both Galacton-*Star*® and the buffer diluent and stability trials conducted throughout the current investigation confirmed that this solution should be made up daily as needed (results not presented). Thus, despite appearing user-friendly, this assay format would still require the user to combine the substrate and buffer diluent prior to conducting the assay, removing the possibility of the three cuvettes being supplied ready-to-use.

Secondly, the transfer of the swab from cuvette to cuvette meant that the bacteria assayed and, thus, those ultimately detected, were those that were present on the bud itself, not those remaining in the growth medium. It was hypothesized that this, potentially large, reduction in coliform numbers would lower the overall sensitivity of the assay. This theory was confirmed via the assay of the LNM remaining in cuvette 1 (Figure 6.9; Table 6.9), strongly suggesting, therefore, that for optimum assay performance, the original growth medium should be included in the final assay mix.

Figure 6.9. Various assay protocols used to establish an ideal test format

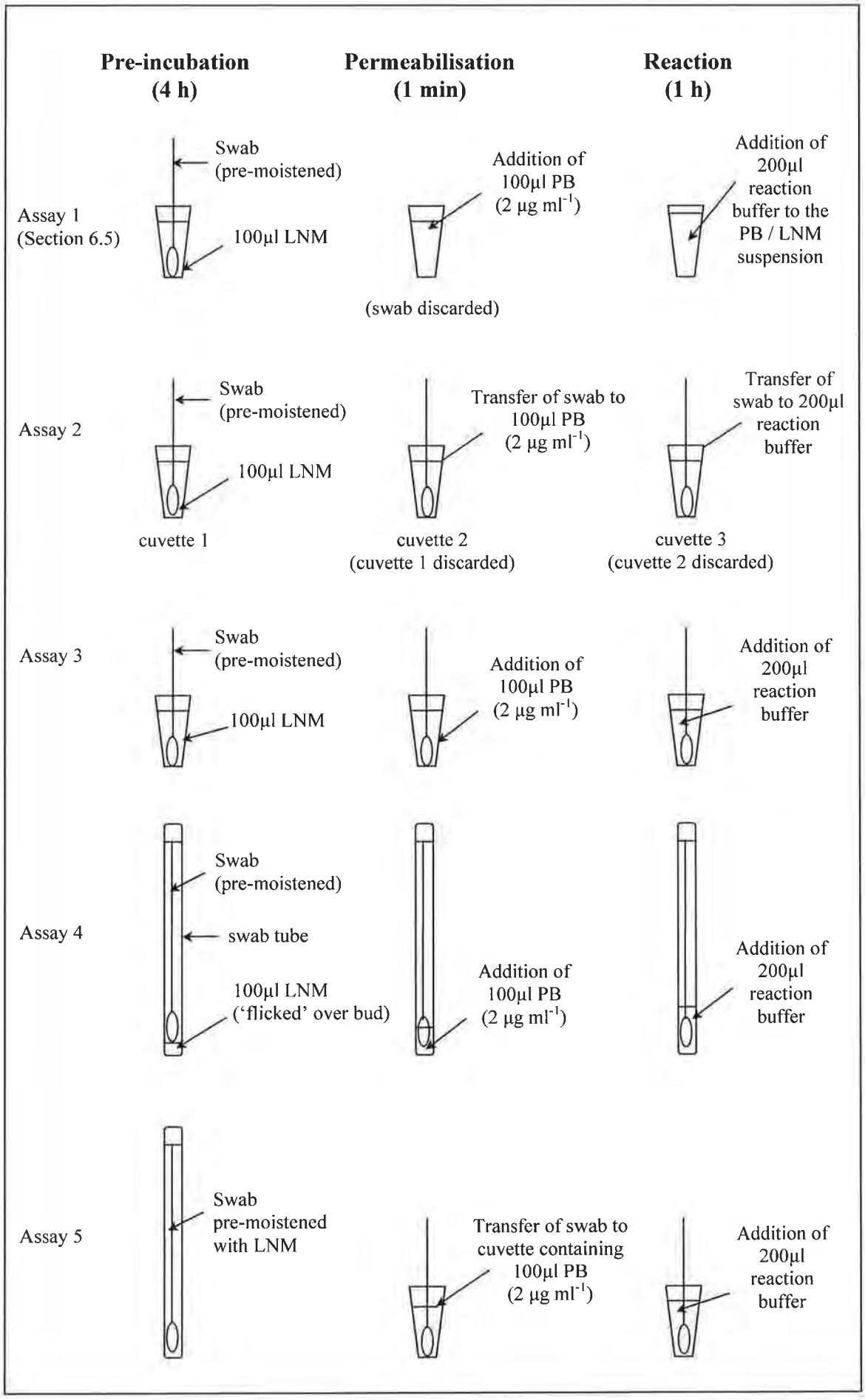


Table 6.9. The minimum detection limit of a variety of different chemiluminescent, swab-based assays

Test condition	CFU[†]	Mean RLU (n = 3)								
		Assay 1	Assay 2			Assay 3		Assay 4		Assay 5	
			swab in[a]	*swab out*[b]	*cuvette 1*	*swab in*[a]	*swab out*[b]	*swab in*[a]	*swab out*[b]	*swab in*[a]	*swab out*[b]
Direct swab inoculation	**Background**	1001	842	615	1163	580	455	-	-	-	-
	4	1047	773	562	1371	640	478	-	-	-	-
	40	1678	1129	834	2343*	1382*	1095*	-	-	-	-
	400	8557*	2655*	1709*	10128	8924	7185	-	-	-	-
Wet surface	**Background**	683	-	-	-	-	-	324	267	534	370
	< 1	844	-	-	-	-	-	462	370	605	428
	4	2906*	-	-	-	-	-	1127*	857*	1996*	1389*
	40	15258	-	-	-	-	-	13083	10418	19670	14800
Dry surface	**Background**	719	-	-	-	-	-	365	281	567	406
	40	875	-	-	-	-	-	480	391	678	502
	400	1935*	-	-	-	-	-	1570*	1223*	1071	685
	4 x 10^3	13881	-	-	-	-	-	6266	4968	7475*	4326*

[†] cfu (*C. freundii*) theoretically inoculated onto either the bud (i.e. cfu 20 μl^{-1}) or surface (i.e. cfu cm^{-2})

[a] light readings taken with swab remaining in cuvette/tube [b] swab removed from cuvette/tube prior to light readings being taken

* minimum detection limit of assay (i.e. the lowest dilution assayed, which resulted in an RLU value more than twice that of the control (background) value)

During Assay 3, the swab was allowed to remain in the same cuvette throughout the pre-incubation, permeabilisation and reaction stages. This appeared to reduce the background luminescence and consequently, in comparison to Assay 1, where the swab was removed prior to the addition of the reaction buffer, the use of Assay 3 increased the sensitivity of the β-galactosidase assay 10-fold. Furthermore, the sensitivity of this assay procedure remained unaffected, regardless of whether the swab was allowed to remain in the cuvette or was removed prior to the measurement of the light signal (Table 6.9). Thus, despite this format still requiring all the reagents to be added by the user, it does form the basis of a single-shot device. Consequently, this procedure was elaborated upon in Assay 4, where after being used to sample a surface, rather than the assay taking place in a cuvette, the swab was instead returned to its tube (Figure 6.9).

Prior to the re-insertion of the swab, 100 µl of LNM was pipetted into the base of the tube (Figure 6.9). However, the design of the swab was such, that the swab bud did not reach the tube base. Consequently, the tube required a 'flick' in order to coat the bud with the growth medium. The subsequent assay procedure was then very similar to Assay 3, the exception being that all the reagents were ultimately contained within the swab tube, which in turn was placed directly into the luminometer. This assay procedure was capable of detecting the presence on a wet and dry surface of approximately 4 cfu cm^{-2} and 400 cfu cm^{-2} respectively and appeared equally as sensitive as the swab-based assay used to date (Assay 1). Furthermore, although allowing the swab to remain in the tube enhanced the background chemiluminescent light signal, this increase was not sufficient to reduce the overall sensitivity of the assay (Table 6.9). These results implied that the swab could be contained and subsequently discarded within the tube – an advantage should a single-shot device be successfully developed. Nevertheless, this assay procedure does carry the risk that the small volume of extractant may, when added to the tube, attach to the inner walls and, consequently, not reach the swab bud.

Assay 5, therefore, supposes that the user has been provided with a pre-measured volume of polymyxin B in a cuvette-like container and whilst Assay 4 requires the user to initially moisten the swab and add the 100 µl of growth medium to the tube, these two steps have been combined in Assay 5 by simply pre-moistening the swab with LNM (Figure 6.9). In this case, any bacteria removed from the surface, rather than being incubated within a volume of growth medium, will grow up on the swab bud itself. In contrast to Assay 2, therefore, this lack of additional growth medium should, in theory, result in minimal bacterial losses during the transfer of the swab to the extractant. Nevertheless, despite appearing as sensitive in detecting coliforms from a wet surface, in comparison to Assays 1 and 4, the sensitivity of this assay procedure, when used to sample a dry surface, was reduced 10-fold.

As discussed in Section 6.5.3.1, this reduction in sensitivity may have been the result of fewer bacteria being removed from the surface, which in turn may have been due to a lack of Tween 80 in the swab-wetting solution. However, if, as proposed in Assay 5, swabs are supplied pre-moistened with no additional medium in the tube, then the wetting solution must contain nutrients and most importantly IPTG. To fulfil both these requirements, the LNM was combined with the MES buffer-based neutralising solution and its effect upon the sensitivity of the assay and the chemiluminometric response was assessed and is illustrated in Table 6.10.

When used to sample a dry surface for *Ent. aerogenes* the minimum detection limit of both Assay 1 and Assay 5 (Figure 6.9), was approximately 10^4 cfu cm^{-2} (Table 6.10). Thus, the sensitivity of the β-galactosidase assay was, regardless of format, lower than when it was used to detect *C. freundii* (Table 6.9), suggesting that some coliform strains may be more able to tolerate drying conditions than others. Nevertheless, the results suggested that LNM and the MES buffer-based solution could be combined without adversely affecting

assay sensitivity (Table 6.10). Not only would a combined solution make the production and use of a multi-shot assay more economic and user-friendly respectively, but it would also avoid the potential difficulties involved in incorporating a small volume of growth medium within a single-shot device.

Table 6.10. The integration of the low nutrient growth medium with the MES buffer-based neutralising solution and its effect, when used to sample a dry surface, upon the chemiluminometric response.

Inoculum (cfu cm^{-2})	**Mean RLU (n = 3)**					
	LNM		**MES buffer-based solution**		**LNM + MES-based solution**[†]	
	Assay procedure[‡]					
	Assay 1	***Assay 5***	***Assay 1***	***Assay 5***	***Assay 1***	***Assay 5***
Background	1027	681	767	473	781	484
2.3 x 10^2	804	936	853	469	700	463
2.3 x 10^3	1234	1072	1234	773	1346	814
2.3 x 10^4	4294*	3128*	5224*	3037*	6201*	1937*

[†] MES buffer (0.01M; pH 6.8), NaCl (5g l^{-1}), tryptone (1g l^{-1}), Tween 80 (0.3g l^{-1}), sodium thiosulphate (0.25g l^{-1}), SDS (0.05g l^{-1}), IPTG (0.01g l^{-1})

[‡] see Figure 6.9

* minimum detection limit of assay (i.e. the lowest dilution assayed, which resulted in an RLU value more than twice that of the control (background) value)

However, regardless of swab wetting solution, the light signal associated with Assay 5, although offering the same sensitivity, was consistently lower than that obtained when Assay 1 was used to sample the surface (Tables 6.9 and 6.10). It was hypothesised, therefore, that the 100 µl of LNM, lacking in Assay 5, but incorporated within Assays 1-4, may, in fact, be required either for bacterial growth during the pre-incubation stage or to provide the optimum volume within the cuvette to be measured by the Uni-Lite®.

The results of a subsequent experiment (Table 6.11) confirmed that the additional 100 µl of LNM, although not necessarily providing conditions more conducive to bacterial growth, did appear to have a beneficial effect upon the light output of the assay by increasing the overall volume of the test. Despite the implications of these results, the magnitude of the RLU readings suggested that any reduction in assay sensitivity caused by a reduction in overall assay volume may in fact be minimal and, consequently, should assay format dictate, the additional 100 µl of LNM could be omitted.

Table 6.11. The effect of the additional 100 µl growth medium upon the chemiluminometric response.

Test condition	**Inoculum (cfu $20\mu l^{-1}$)**	**Mean RLU (n = 5)**		
		100µl of LNM absent during 4 h pre-incubation phase. Tests hypothesis that its addition is required to increase the volume of the assay mix		
		Final assay volume = 400µl. Tests hypothesis that the additional LNM is required for bacterial growth during the 4 h pre-incubation phase		
		100µl LNM added prior to pre-incubation	**100µl LNM added prior to permeabilisation**	**100µl LNM absent**
Direct swab inoculation	**Background**	343	367	399
	70	616	1007*	693
	700	2519*	6818	3629*
	7.0×10^3	52802	86765	31126

* minimum detection limit of assay (i.e. the lowest dilution assayed, which resulted in an RLU value more than twice that of the control (background) value)

However, it is acknowledged that the results presented in Table 6.11 were associated with direct swab inoculation and, particularly when sampling a dry surface, that the presence of additional growth medium could aid the recovery of sub-lethally injured bacteria. It was

decided, therefore, that until such a time that it became unnecessary, the additional LNM would continue to be incorporated within the final assay mix. Nevertheless, the results of a further experiment (results not presented) revealed that this additional growth medium could, without affecting overall assay sensitivity, also be present in the form of the LNM/MES buffer-based solution (as Table 6.10). As already discussed, the use of this one solution (subsequently referred to as the growth medium) would reduce the materials and consumables used during production and, thus, the overall cost of the test.

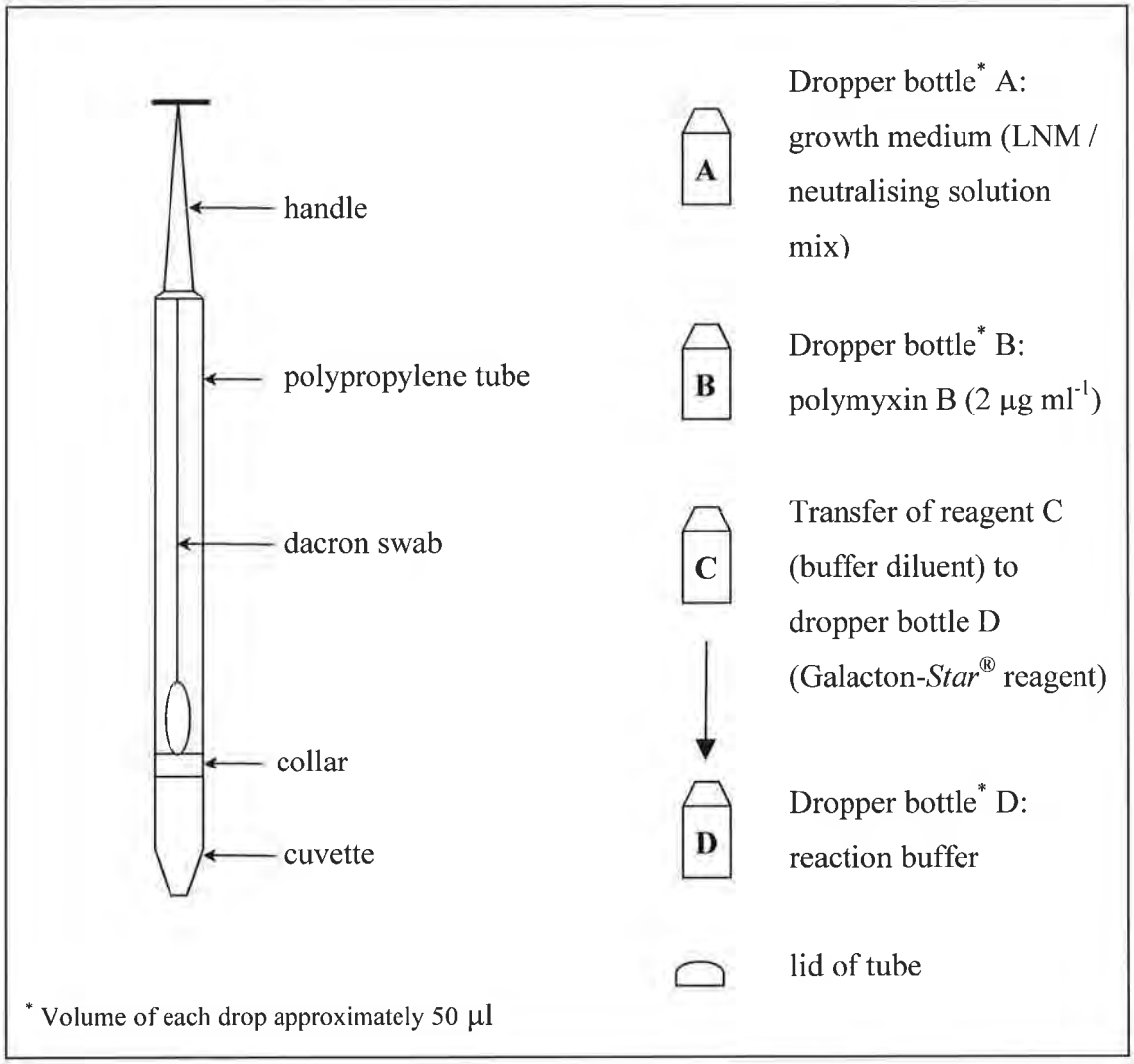
The results presented in Tables 6.9 to 6.11, highlight the fact that the chemiluminescent, swab-based assay clearly lends itself to a multi-shot test format (Assay 4). However, they also allude to the distinct possibility of incorporating the assay within a single-shot device. The decision was made, therefore, to continue the development of the ultimately more marketable, single-shot test method. However, in case unsuccessful, an optimum multi-shot format would first be assembled and its sensitivity and repeatability validated.

6.7 Multi-shot Format

6.7.1 Proposed Protocol

The current investigation was conducted with the cooperation of Biotrace Ltd (Bridgend, UK) and to ensure that the multi-shot format resembled the rest of their product range, the swab and tube combination depicted in Figure 6.9 was substituted for those used to construct the Clean-*Trace*™ Rapid Cleanliness Test (Figure 6.10).

Figure 6.10. The components of the proposed multi-shot test format



There were concerns that some aspects of the new swab design would adversely affect the sensitivity of the assay; more specifically, the tapering of the cuvette, which could prevent effective mixing of the reagents and/or its slight opaqueness, which could affect the amount of light reaching the luminometer. However, assays conducted within these new cuvettes proved just as sensitive as when the original, clear, rounded cuvettes were used. Nevertheless, a reduction in assay sensitivity was observed when the swab was allowed to remain in the device during light measurement.

Thus, the proposed assay procedure, based upon Assay 4 (Figure 6.9), was as follows:

1. Detach tube from collar. Using bottle A, add 2 drops of growth medium to the cuvette. Replace tube.
2. Remove swab. Using bottle A, moisten the swab bud using 2 drops of growth medium. Sample surface.
3. Replace swab and activate device (i.e. push handle of swab down into the tube, so that the swab bud is in contact with the growth medium).
4. Incubate device (in an upright position) for 4 h at 37°C.
5. During this 4 h pre-incubation phase, transfer the contents of bottle C to bottle D and mix gently.
6. After incubation, remove the swab from the tube and discard. Detach tube from collar and, using bottle B, add two drops of membrane permeabiliser to the cuvette. Leave for 1 min in an upright position.
7. Using bottle D, add 4 drops of assay reagent to the cuvette. Replace tube and attach lid. Incubate device (in an upright position) for 1 h at room temperature.
8. Place device in the Biotrace Uni-Lite® luminometer and record light output (RLU).
9. *Control sample*: To obtain the level of background luminescence, repeat steps 1-8, omitting surface sampling.
10. A sample can be assumed positive for β-galactosidase and, thus, coliforms if the test assay results in an RLU reading over twice the RLU reading of the control sample.

6.7.2 Sensitivity and Repeatability of the Multi-shot Format

The most important attribute of any microbiological assay system is its ability to detect the organism of interest with both accuracy and precision (Fung, 2000). Whilst, accuracy

represents how close the results produced by the assay are to the actual value, precision is a measure of how consistent these results are (Griffith *et al.* 1997). Thus, not only was it important to determine the sensitivity and repeatability of the results obtained using the proposed multi-shot assay, but also to compare its performance to traditionally used reference methods.

The results presented in Table 6.12 illustrate the mean RLU readings that were obtained after the swabs had been directly inoculated with a range of microbial dilutions. The precision of these results is represented by the coefficient of variation (cv), which was calculated using equation 2. The lower the cv, the more repeatable and, thus, more precise the test method (Griffith *et al.* 1994).

$$cv = \frac{\text{Standard deviation}}{\text{Mean}} \times 100 \qquad (2)$$

The proposed multi-shot assay was capable of detecting the presence on a directly inoculated swab bud of, depending on coliform strain, between 6 and 60 colony forming units. Furthermore, the precision of these results was observed to range from just 5% to 44%; in comparison, the cv values associated with the traditional swabbing technique have been documented as ranging from 84% to 300% (Griffith *et al.* 1997) implying, therefore, that the multi-shot, β-galactosidase assay could be relied upon to consistently detect low levels of coliform bacteria. Nevertheless, in all cases, a slight reduction in assay repeatability was observed to occur with increasing inoculum level. This may have been due to the higher bacterial numbers resulting in an increased level of cellular aggregation and, consequently, greater variation in the numbers of bacteria inoculated onto each of the swabs. This in turn, may have led to differences in the numbers of bacteria present within the cuvettes at the end of the 4 h propagation phase.

Table 6.12. The repeatability and minimum detection limit of the multi-shot, chemiluminescent-based, coliform detection system

Test condition	Inoculum (cfu 20µl^{-1})	Mean RLU (n = 10)			
		E. coli	*C. freundii*	*Ent. amnigenus*	*Ent. cloacae*
Direct swab inoculation	**background**	112 cv = 12%	171 cv = 10%	81 cv = 5%	95 cv = 8%
	3	131 cv = 14%	200 cv = 16%	136 cv = 23%	97 cv = 11%
	6	148 cv = 13%	223 cv = 17%	176* cv = 18%	109 cv = 15%
	30	354* cv = 13%	555* cv = 38%	604 cv = 20%	-
	60	626 cv = 19%	982 cv = 20%	1120 cv = 20%	266* cv = 26%
	300	2624 cv = 25%	5317 cv = 44%	6344 cv = 26%	1222 cv = 28%

* minimum detection limit of assay (i.e. the lowest dilution assayed, which resulted in an RLU value more than twice that of the control (background) value)

6.7.3 Comparison of traditional methods

Despite the poor precision and the other acknowledged limitations associated with cotton-tipped hygiene swabs (Chapter 2), traditional cultivation-based methodology remains the most common means of detecting microorganisms from food contact surfaces. There is no unanimity with regard to methods for the examination of environmental surfaces. However, in the UK, when testing for coliforms it is both an international standard (ISO 4832:1991) and considered Good Laboratory Practice to use violet red bile lactose agar (VRBA) (Harrigan, 1998). The ability, therefore, of the multi-shot assay to detect coliforms from food contact surfaces, under controlled laboratory conditions and *in situ*, was assessed and was compared to that of VRBA (Oxoid; 38.5 g l^{-1}) swab and pour plates.

Table 6.13. The minimum bacterial detection limits (cfu cm^{-2}) of a range of different coliform detection methods

Test condition (n = 5)		Test method	Minimum detection limit*	Time before results could be obtained
Wet surface	***C. freundii***	**multi-shot assay**	< 1	5 h
		swab plate	< 1	24 h
		pour plate	4	24 h
	Ent. amnigenus	**multi-shot assay**	< 1	5 h
		swab plate	< 1	24 h
		pour plate	3.5	24 h
Direct swab inoculation	***E. coli***	**MacConkey broth**	3	24 h
			3.5×10^5	6 h
		multi-shot assay	20	5 h
			2.0×10^5	1 h

* minimum detection limit of assay (i.e. the lowest inoculum (cfu) assayed, which resulted in:
multi-shot assay: an RLU value more than twice that of the control (background) value
VRBA swab and pour plates: an average count of > 1 CFU, with growth being evident on each of the replicate plates
MacConkey broth: the media associated with each of the replicate samples changing from purple to yellow

When a wet surface, inoculated with known levels of coliform bacteria, was sampled, the multi-shot assay was capable of detecting the presence of < 1 cfu cm^{-2}, and was as effective in indicating the presence of coliforms as were hygiene swabs when their use was coupled with swab plate methodology (Table 6.13). Similarly, when used to sample the salad and vegetable compartments of four different domestic fridges, there were no significant differences ($\chi^2 = 0.026$, $p > 0.05$) between the number of sites designated positive for coliforms by either of these test methods (Table 6.14a).

Table 6.14. A comparison of the number of surfaces designated positive for coliform bacteria, by means of the multi-shot, β-galactosidase assay and the traditional (a) swab plate and (b) pour plate procedures

a)	Multi-shot assay	VRBA spread plate
Coliforms detected	13	14
Coliforms not detected	6	5

b)	Multi-shot assay	VRBA pour plate
Coliforms detected	15	9
Coliforms not detected	5	11

The results provided by the β-galactosidase assay are semi-quantitative (i.e. it can be assumed that the higher the RLU reading, the greater the level of bacteria initially present on the surface). In comparison, those obtained using more traditional swab-based methodologies are considered fully quantitative in nature. Nevertheless, as an enumeration technique the swab plate is fairly inaccurate and vortexing the swab in a diluent is a more effective means of breaking up bacteria. However, this extra dilution factor increases the minimum detection limit of the pour plate technique (Table 6.13). Thus, in comparison to pour plate methodology, the multi-shot, β-galactosidase assay appeared a more sensitive means of detecting coliforms from food contact surfaces and this was confirmed when these two methods were used to sample domestic fridges (Table 6.14b).

A paired Chi squared test of association (McNemar's test) with Yates' correction (Hassard, 1991) revealed that a significant difference existed between the results obtained using the multi-shot and pour plate procedures ($\chi^2 = 8.167$, $p < 0.01$). More specifically, it could be concluded that if both methods were used within an environmental sampling plan, the multi-shot assay would be more likely to detect the presence of coliforms. However, the biggest advantage that the multi-shot, β-galactosidase assay has over this traditionally used cultivation-based technique is its ability to provide these results in just 5 h.

6.7.4 Feasibility of Shortening the Duration of the Chemiluminescent-based Assay

When hygiene swabs are used to inoculate an agar medium, at least 18 h is normally required before the resulting colonies have grown large enough to facilitate enumeration. It is possible that qualitative results, a colour change for example, can indicate the presence of the target organism within a much shorter period of time. As illustrated in Table 6.13, when hygiene swabs were used to inoculate MacConkey broth (purple) (40g l^{-1}; Oxoid), the purple → yellow colour change, indicative for coliform presence, was observed to occur in 6 h. However, the minimum number of colonies required for the reaction to take place this rapidly was approximately 3.5×10^5. In comparison, the multi-shot assay was capable of detecting this level of inoculum within just 1 h (i.e. β-galactosidase was at a detectable level without the bacteria having to undergo a pre-incubation phase).

Despite these results alluding to the possibility of shortening the pre-incubation and/or reaction stages of the multi-shot procedure and, in turn, reducing the overall duration of test, the results presented in Table 6.15, suggest that any reduction would likely be at the expense of assay sensitivity. Depending on coliform strain, reducing the pre-incubation period from, for example, 4 h to 3 h resulted in a 5- to 10-fold increase in the minimum detection limit of the assay. Allowing 30 min as opposed to 1 h for the Galacton-*Star*® to react with the inoculum, resulted in an approximate 50% reduction in light output, which could, although not in this case, also affect overall assay sensitivity.

Table 6.15. The effect of shortening the duration of the multi-shot assay upon the chemiluminometric response

Test condition		Inoculum	Mean RLU			
			Pre-incubation time			
			3 h		4 h	
			Reaction time			
			30 min	1 h	30 min	1 h
Direct swab inoculation (n = 5)	***E. coli***	**Background**	72	79	74	78
		4	78	88	90	114
		20	97	128	178*	268*
		40	111	150	255	421
		200	255*	450*	1182	2064
	Ent. cloacae	**Background**	80	87	77	83
		3	80	91	87	99
		14	81	95	111	157
		30	92	118	170*	273*
		140	197*	365*	528	1126
Wet surface (n = 7)	***E. coli***	**background**	-	-	88 (cv = 10%)	95 (cv = 8%)
		< 1	-	-	275* (cv = 24%)	450* (cv = 29%)
		2	-	-	964 (cv = 22%)	1722 (cv = 21%)
	Ent. cloacae	**background**	-	-	98 (cv = 12%)	127 (cv = 17%)
		< 1	-	-	125 (cv = 10%)	199 (cv = 19%)
		4	-	-	285* (cv = 12%)	589* (cv = 10%)

* minimum detection limit of assay (i.e. the lowest inoculum (cfu) assayed, which resulted in an RLU value more than twice that of the control (background) value)

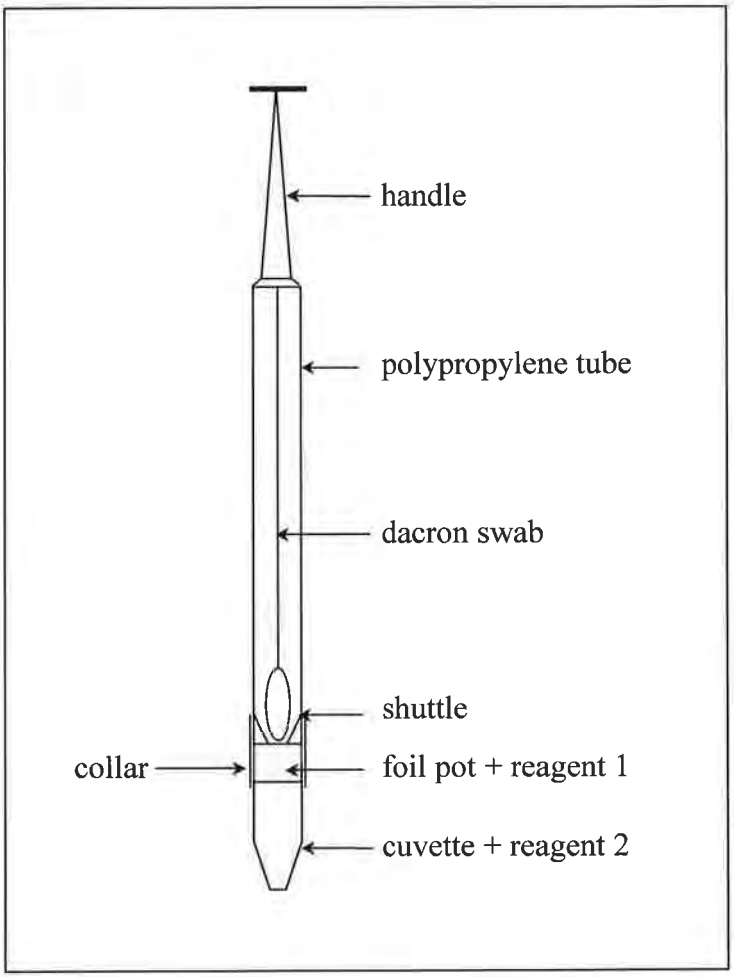
Nonetheless, the β-galactosidase assay in its multi-shot format is capable of providing results within the 6 h time frame usually available to industry – given the shift pattern in many food processing plants – to regain control of a process. This, together with its sensitivity and reliability, in comparison to traditionally used methods suggests that this chemiluminescence-based assay could be successfully applied as a means of detecting coliforms from food contact surfaces and play an important role within food safety management systems, such as HACCP.

However, whilst the main objective of the current investigation had been achieved, the ultimate goal was to develop the *most* marketable test method and, in doing so, provide the food industry with the *most* practicable means of detecting surface-associated coliform bacteria.

6.8 Single-shot Format

The major problem encountered when converting the multi-shot to a single shot assay format was the instability of the reaction buffer (Section 6.6.2) and, consequently, the necessity to incorporate the buffer diluent and the reagent (Galacton-*Star*®) separately within the device. It was hypothesised, that one way in which this could be achieved would be by compartmentalising the swab device already associated with the multi-shot assay and, therefore, with the design of the Clean-*Trace*™ again being used as a model, the addition of a foil pot was proposed as a means of isolating the reagents (Figure 6.11).

Figure 6.11. The proposed design of the single-shot device



Two options were available for consideration:

i. The Galacton-*Star*® could be stored in the pot and the buffer diluent and polymyxin B could be combined within the cuvette
ii. The buffer diluent could be stored in the pot and the Galacton-*Star*® and polymyxin B could be combined within the cuvette

In either case, on activation, the swab should force the shuttle down into the cuvette, and in doing so pierce the foil pot resulting in the mixing of the reagents.

When evaluating, which of the two options would be the most appropriate, it was important that the reagents within the assay were at concentrations equivalent to those used throughout the investigation. Thus, it was necessary to ensure that 200 µl of reaction buffer was present within the final assay mix, with the Galacton-*Star*® having been diluted (1:50) within the buffer diluent.

However, this would mean that should 200 µl of a diluent/polymyxin B mix be present within the cuvette (as would be the case in option 1), just 4.08 µl of Galacton-*Star*® would be contained within the pot. It would be unlikely that such a small volume would reach the cuvette and, consequently, insufficient reagent would be available for cleavage by β-galactosidase. Thus, the possibility of incorporating a larger, more dilute form of the reagent was investigated.

6.8.1 Assay Format One: *Galacton-Star*® *stored in pot with the buffer diluent and polymyxin B combined within the cuvette*

Four hundred microlitres of Galacton-*Star*® was added to 7.6 ml of either a sodium phosphate buffer (Section 6.3.1.2) or a 1:1 mix of isopropyl alcohol and water (Tropix, 1998). One millilitre of the diluted reagent (theoretically containing 50 µl of Galacton-*Star*®) was then added to 2.45 ml of buffer diluent, thus diluting the Galacton-*Star*® 50-fold. A cuvette-based assay (Assay 1, Figure 6.9) was then used to assess the effect that these different solutions had upon the chemiluminometric response.

The results implied, that if necessary, a larger volume of diluted Galacton-*Star*® could be incorporated within a single-shot device without adversely affecting assay sensitivity and that if the storage of the reagent within the foil pot *was* demonstrated to be the most

suitable assay format then, for maximum light output, the Galacton-*Star*® should be diluted using the sodium phosphate buffer (Table 6.16).

Table 6.16. The effect of diluting the Galacton-*Star*® upon the chemiluminometric response

Inoculum (cfu 20µl^{-1})	Mean RLU (n = 3)		
	Control †	Reagent diluted with sodium phosphate buffer	Reagent diluted with isopropyl alcohol
Background	306	201	221
2	543	447	304
10	1979*	1108*	1007*
100	15591	14362	9203

† reagent prepared as normal (i.e. 100 µl Galacton-*Star*® added to 4.9 ml buffer diluent)

* minimum detection limit of assay (i.e. the lowest dilution assayed, which resulted in an RLU value more than twice that of the control (background) value)

6.8.2 Assay Format Two: *Buffer diluent stored in pot with Galacton-Star® and polymyxin B combined within the cuvette*

Should the foil pot contain 200 µl of buffer diluent then the cuvette would be required to contain 100 µl of a Galacton-*Star*®/polymyxin B mix. Thus:

per test: 4.08 µl of Galacton-*Star*® would need to be mixed with 95.92 µl of polymyxin B (2 µg ml^{-1})

per 25 tests: 50 µl of a stock solution of polymyxin B (100 µg ml^{-1}) was added to 2.35 ml of sterile deionised water and mixed with 100 µl of Galacton-*Star*®

The multi-shot assay was then used to compare the effect, upon the chemiluminometric response, of adding:

i. 100 µl of polymyxin B (2 µg ml^{-1}) then 200 µl of Galacton-*Star*®/buffer diluent mix (i.e. the reaction buffer) as a two-step process (i.e. Figure 6.10)
ii. 100 µl of polymyxin B/Galacton-*Star*® mix together with 200 µl of buffer diluent as a single-step process
iii. 100 µl of pre-incubated polymyxin B/Galacton-*Star*® mix together with 200 µl of pre-incubated buffer diluent as a single-step process (i.e. the reagents had been allowed to incubate along with the swab for 4 h at 37°C)

Table 6.17. The effect upon the chemiluminometric response of adding the reagents to the microbial sample in a single step

Test condition	**Inoculum (cfu $10\mu l^{-1}$)**	**Mean RLU (n = 3)**		
			Buffer diluent in pot with Galacton-Star and polymyxin B combined within cuvette	
		Multi-shot assay format (Figure 6.10)	**Reagents added as a single step**	**Reagents pre-incubated prior to their addition as a single step**
Direct swab inoculation	**Background**	315	313	270
	20	446	456	575*
	100	1913*	1630*	1905
	200	3211	3299	2701
	1000	14150	15409	16624

* minimum detection limit of assay (i.e. the lowest dilution assayed, which resulted in an RLU value more than twice that of the control (background) value)

As illustrated by the results presented in Table 6.17, neither combining the polymyxin B and the Galacton-*Star*®, nor adding the reagents in a single step, appeared to adversely affect the sensitivity of the assay. Furthermore, although based only on triplicate samples, the results also implied that, prior to their addition, the pre-incubation of the reagents could enhance assay sensitivity. Thus, it would appear both feasible and beneficial to incorporate the reagents as part of a single-shot device.

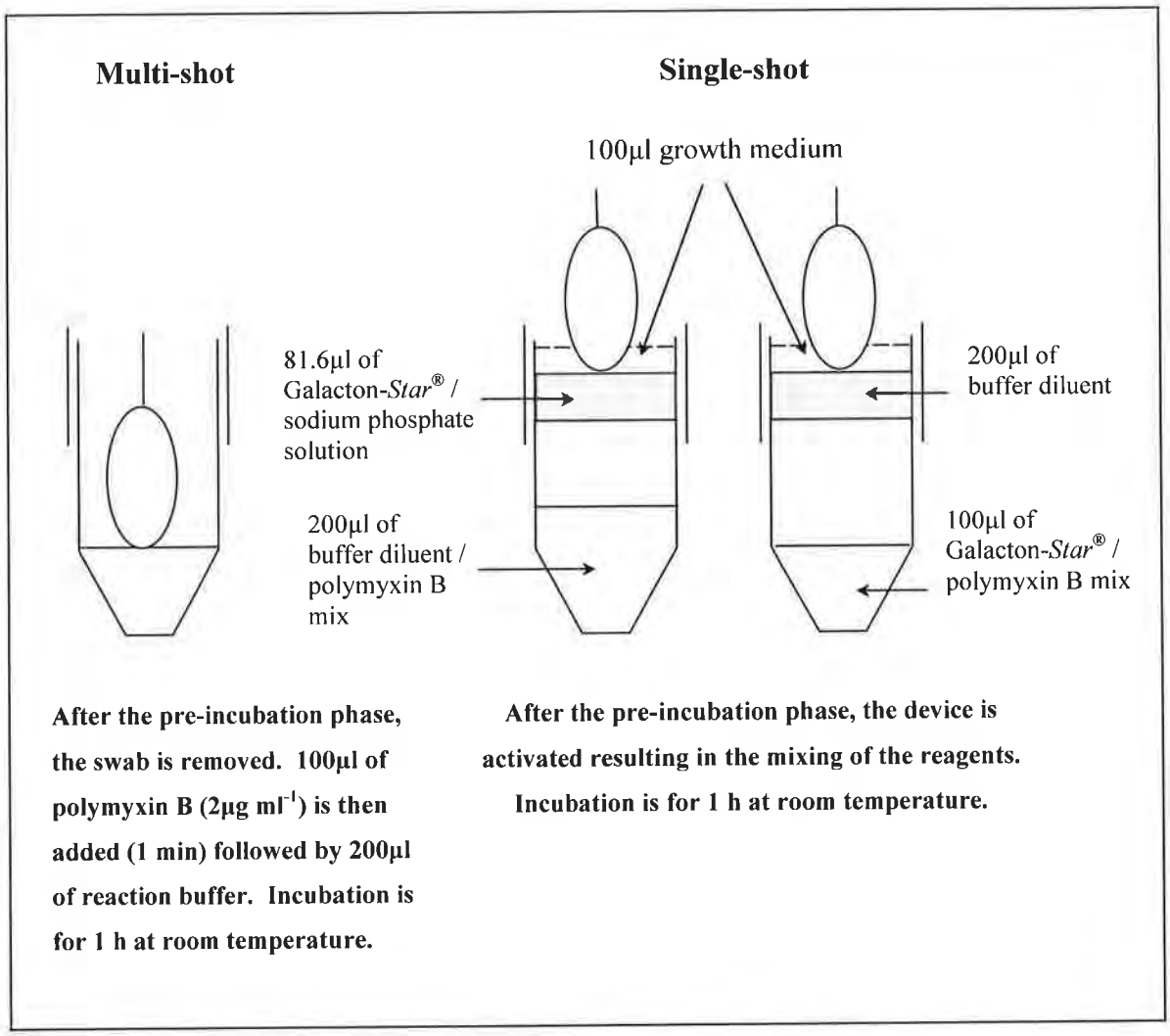
However, during the multi-shot procedure (Section 6.7.1), the swab is removed prior to the addition of the reagents, in the proposed single-shot format, the swab would remain within the device throughout all stages of the assay process. Previous experiments (Table 6.10) have suggested that when the swab forms an integral part of the final assay, the RLU readings are lower than when the swab is removed - this could ultimately affect the sensitivity of the proposed single-shot assay.

6.8.3 Comparison of the Two Different Single-shot Assay formats

To assess the effect that a reduction in light output may have upon overall assay sensitivity, the two different single-shot devices were constructed (Figure 6.12) and their performance was compared to that of the multi-shot assay.

Prior to the pre-incubation of the single-shot devices, 100 µl of additional growth medium was pipetted on top of each of the foil pots. Thus, the final volume in each of the different assay formats was approximately 400 µl. The swabs associated with each of the test devices were inoculated with a known bacterial dilution and the assays were performed as depicted in Figure 6.12.

Figure 6.12. The incorporation, of reagents within the multi- and single-shot assay formats



The results confirmed that the RLU values obtained when swabs were included in the final assay were, regardless of single-shot format, approximately 10-times lower than those resulting from the multi-shot assay procedure (Table 6.18). However, the results also implied that this reduction in light output equated to just a five-fold reduction in assay sensitivity.

The performance of the two different single-shot formats appeared fairly comparable (Table 6.18). However, when the Galacton-*Star*® was stored in the foil pot, the light output was observed to be slightly lower than when the substrate was combined with the polymyxin B within the cuvette. Furthermore, the need for an additional buffer solution in

order to dilute the Galacton-*Star*® could increase production time and costs and, therefore, it was decided that the ideal single-shot format would incorporate the buffer diluent within the foil pot.

Table 6.18. The minimum detection limits (cfu bud^{-1}) of the different chemiluminescent, swab-based coliform detection methods

Test condition	Inoculum (cfu $10\mu l^{-1}$)	Mean RLU (n = 3)		
			Single-shot format	
		Multi-shot format (Figure 6.10)	Galacton-Star® in pot (Section 6.8.1)	Buffer diluent in pot (Section 6.8.2)
Direct swab inoculation	**Background**	154	66	63
	6	260	74	88
	12	348*	105	118
	60	1195	178*	465*
	120	1834	323	397
	600	16673	1827	2174

* minimum detection limit of assay (i.e. the lowest dilution assayed, which resulted in an RLU value more than twice that of the control (background) value)

6.8.4 Sensitivity and Repeatability of the Single-shot Format

The RLU readings obtained using the preferred single-shot format (Section 6.8.3) appeared, in general, slightly more variable than those from the multi-shot assay (Table 6.19). This may have been due to an inconsistent mixing of the buffer diluent with the substrate/extractant mix during swab activation. Nevertheless, when used to sample a wet surface, the single-shot, β-galactosidase assay was capable of detecting the presence, regardless of strain, of < 2 coliform colonies cm^{-2}.

Table 6.19. The minimum detection limit (cfu cm^{-2}) of the optimum multi- and single-shot, chemiluminescence-based, coliform detection method

Test condition		Inoculum (cfu cm^{-2})	Mean RLU (n = 5)	
			Multi-shot	Single-shot
Wet surface	***E. coli***	**Background**	97 cv = 9%	79 cv = 4.5%
		< 1 (0.1)	492* cv = 13%	152 cv = 38%
		< 1 (0.3)	652 cv = 62%	282* cv = 25%
		1.5	3760 cv = 34%	767 cv = 46%
	E. cloacae	**Background**	109 cv = 5%	77 cv = 8%
		< 1	220* cv = 14%	131 cv = 15%
		1.7	549 cv = 20%	213* cv = 26%
		3.4	1786 cv = 9%	556 cv = 60%

* minimum detection limit of assay

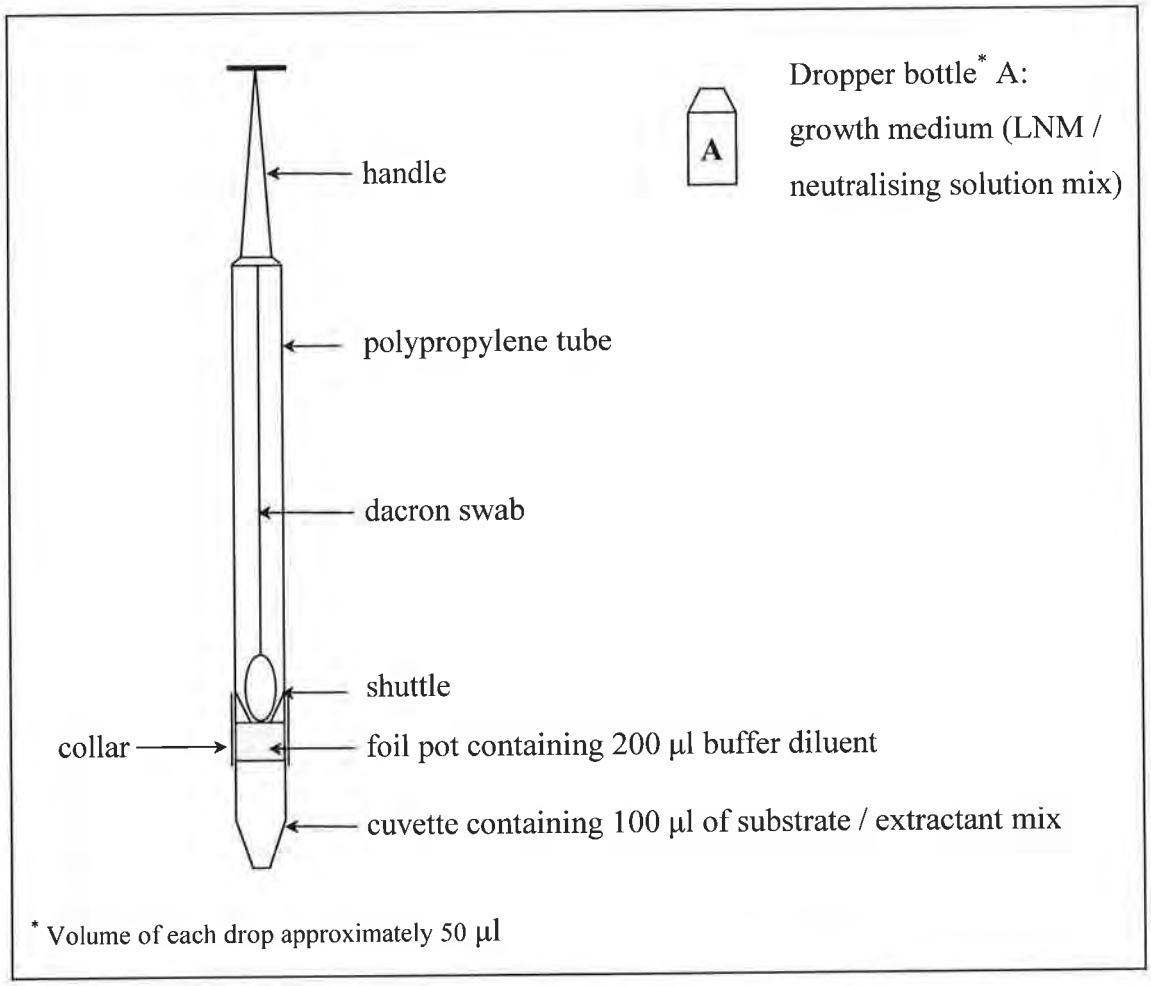
6.8.5 Optimum Single-shot Format

A rapid, single-shot, swab-based assay capable, within 5 h of sampling a wet surface, of detecting the presence of < 1 cfu cm^{-2}, has been successfully developed (Figure 6.13).

To avoid the risk of the swabs drying out during storage, it was decided that a dropper bottle containing the growth medium should also be supplied so as the user could moisten the swabs prior to sampling the surface. This would also enable the 100 µl of additional growth medium to be added to the device prior to pre-incubation. Nevertheless, should

customer feedback indicate that this extra step is considered too laborious, then the results from Section 6.6.2, suggest that it could be omitted from the assay protocol. If this was the case, it could be possible to supply the growth medium in a single-use, cuvette-like container, which after being used to saturate the swab bud could be discarded, so preventing the potential contamination of subsequent devices. However, further work would have to be conducted to ensure assay sensitivity would not be greatly affected.

Figure 6.13. Components of the proposed single-shot, chemiluminescence-based coliform detection method



The proposed single-shot assay procedure is, therefore, as follows:

1. Remove swab. Using bottle A, moisten the swab bud using 2 drops of growth medium. Sample surface.
2. Using bottle A, add 2 drops of growth medium to the tube.
3. Replace swab taking care not to push the handle of the swab down into the tube.
4. Incubate device (in an upright position) for 4 h at 37°C.
5. After incubation, activate swab (i.e. firmly push the handle of the swab down into the tube, so piercing the foil pot and mixing the reagents).
6. Incubate device (in an upright position) for 1 h at room temperature.
7. Place device in the Biotrace Uni-Lite® luminometer and record light output (RLU).
8. *Control sample*: To obtain the level of background luminescence, repeat steps 1-7, omitting surface sampling.
9. A sample can be assumed positive for β-galactosidase and, thus, coliforms if the test assay results in an RLU reading over twice the RLU reading of the control sample.

6.8.6 Stability

To ensure that assay sensitivity would not diminish due to the long-term storage of the test devices, it was important to establish whether any degradation of the assay reagents or solutions occurred over time. Thus, stability trials were conducted at monthly intervals throughout the investigation (Appendix IV) and by the conclusion of the project, both the buffer diluent and the growth medium (i.e. the LNM/MES buffer-based neutralising solution) had proven to be stable for 12 months when stored at < 5°C. Similarly, the Galacton-*Star*®/polymyxin B mix, when stored at < 5°C, showed no signs of deterioration over a 9 month period. In all cases, there was no indication of microbial contamination

and growth. Although this suggests a potentially lengthy shelf-life, ideally, a stability study of the single-shot device itself should be conducted (i.e. to ensure the stability of the extractant/substrate mix and the diluent when incorporated within the cuvette and the foil pot respectively).

Despite the stability of the reagents and solutions, the intensity of the background luminescence associated with the β-galactosidase assay was observed to vary greatly with differing batches of Galacton-*Star*® (results not presented). This could be a potentially serious problem, not only with regard to assay sensitivity – higher background readings could mask subtle changes in light output caused by the presence of low levels of coliform bacteria – but also its repeatability and reproducibility. The user of any hygiene/microbiological test method must be confident that the assay will provide consistent results wherever and whenever the samples are taken. Nevertheless, this was a problem that occurred during the production of the reagent itself and, consequently, after being informed of the batch variation, the manufacturer of the Galacton-*Star*® planned to introduce a QC step to highlight 'problem' batches prior to shipment.

6.9 Concluding Discussion

The coliform bacteria are widely used to indicate sanitary effectiveness and, as part of this thesis, a test method has been developed that is capable of their detection in just 5 h. This chapter has discussed its evolution from liquid chemistry (Sections 6.3 and 6.4) to a cuvette-based assay capable of detecting low levels of coliform bacteria on a swab bud (Section 6.5). This assay was subsequently incorporated within a test kit, which although, more appropriate for use within the food industry still required the reagents to be added by

the user (Sections 6.6 and 6.7) and finally transformed into a more user-friendly single-shot device requiring very few additional manipulations (Section 6.8). However, despite the apparent success of this investigation in achieving its main aims and objectives, to meet the expectations of users, new and/or alternative microbiological methods must fulfil certain requirements (van der Zee and Huis in't Veld, 1997).

- **Rapidity:** A test method can be considered 'rapid' if, by exchanging and/or excluding a time-consuming work or process step and, thus, usually via the implementation of a different measuring principle, the results are obtained in the shortest possible time, or, alternatively, if the results are obtained faster, despite basic measuring principles being retained, simply because the test can be carried out with higher speed (Matissek, 1990).

  Traditional microbiological methods can be laborious and time-consuming. Thus, the ability to shorten the time between sampling a surface and obtaining results would, it can be assumed, be a major reason for a food company to employ an alternative 'rapid' microbiological test method. The reaction that forms the basis of the proposed assay is the cleavage by β-galactosidase, of a 1, 2-dioxetane substrate – such enzymatic reactions can, in comparison to traditional cultivation-based techniques, occur in a very short period of time (Henniger, 1990), in this case, low levels of coliforms can be detected in just 5 h. In addition, once the surface has been sampled, the single-shot format enables the reagents to be added quickly and easily. Thus, the proposed assay would enable a food business to detect coliforms within a short period of time *and* with relatively high speed.

- **Sensitivity:** When testing for coliforms, the sensitivity required by any individual food business will most likely depend upon the type of food produced and the

process to which it is being subjected. A company involved in the production of raw meats may for example, assess surface cleanliness as part of GMP, in which case, the sensitivity required may be far less than that needed within a 'high-risk' processing environment where the cleanliness of surfaces is likely to be designated critical to food safety (Griffith *et al.* 1997). The aim of the current investigation was, therefore, to develop a test method with a detection limit low enough to cater for all industry requirements.

The assay is, therefore, incorporated within a swab-based device – despite their limitations, swabs can be used to sample any size or shape of surface, including the difficult-to-clean nooks and crannies of equipment and machinery. To optimise assay performance, it is proposed that the swabs, prior to sampling, are moistened with a solution comprising sodium thiosulphate to neutralise hypochlorite-based cleaning chemicals and Tween 80 which, neutralises residual QACs and has also been shown to aid the removal of bacteria from a surface (Chapter 2). Previous chapters have also alluded to Tween 80 improving the release of bacteria from a swab bud and, in doing so, improving overall bacterial recovery. However, in contrast to the traditional swabbing technique, the swab associated with the proposed coliform test method forms an integral part of the assay and as such, ineffective bacterial release is unlikely to be the major contributory factor with regard to assay sensitivity. These issues, coupled with the sensitivity of the assay chemistry itself, has resulted in a test method capable of detecting the presence on a wet surface of < 1 coliform colony cm^{-2}.

- **Accuracy and precision of results:** Preliminary studies have suggested a good correlation between the results of the proposed coliform assay and those of conventional standard methods – this will, however, be investigated further in

Chapter 7. Furthermore, in comparison to traditional swab-based techniques, the coliform test method has also been shown to provide very precise and, thus, very repeatable results and, consequently, could be relied upon to consistently detect the presence of low levels of coliform bacteria.

- **Specificity:** Only specific methods give accurate results. If a test method lacks specificity then false positive reactions can occur, which in turn can provide misleading information regarding the hygienic status of the surfaces sampled. Enzymes, by reacting with their associated substrate only, can form the basis of highly specific test methods. However, during the current study, it was still necessary to prevent *non-target* β-galactosidase from reacting with the chemiluminogenic substrate. This has been achieved by incorporating IPTG within the growth medium, to induce the production of β-galactosidase within target cells, coupled with the selective permeabilisation of gram-negative bacteria via the use of polymyxin B. As a consequence, the assay has been demonstrated to be coliform specific (Section 6.4.5).

- **Ease of Use:** Many microbiological methods require highly skilled personnel, which may not always be present in quality control laboratories within the food industry (van der Zee and Huis in't Veld, 1997). The proposed single-shot assay requires very few additional manipulations and as a result is very easy to use. In addition, since the presence of coliforms is determined via the use of a luminometer, no colony enumeration is required. A comparison of the light readings associated with the test and a control sample can indicate the presence of coliforms with the magnitude of the light signal relating to the levels of coliform bacteria likely to be present.

- **Cost:** Alternative methods are often more expensive than conventional methods. At the conclusion of this project, the cost of the reagents required to assemble just one single-shot device was estimated as being approximately 40 pence and, in addition, the test method requires the use of the Biotrace Uni-Lite®. Although, it is anticipated that potential customers would already utilise a Biotrace ATP bioluminescence system, it is acknowledged that the perceived cost of utilising this detection method could be quite high. Nevertheless, as is the case with ATP bioluminescence, this additional expenditure should be weighed against the benefits of lower detection limits and the reduced costs associated with labour, time and materials.

So, is there a market for this newly developed coliform detection method? As will be illustrated in Chapter 7, the range of coliform tests that are already available to the food industry is testament to the fact that the detection of coliforms is widely used and has found a niche, particularly within the European market. However, the continual debate that surrounds the coliform bacteria and their suitability as microbial indicators cannot be ignored.

The presence of those members of the *Enterobacteriaceae*, such as *Salmonella* and *Yersinia* spp., which are unable to ferment lactose, will be missed using this and any standard coliform detection method. Furthermore, previous studies have reported that there is little correlation between the prevalence of such pathogens and the presence of the lactose fermenting *Enterobacteriaceae* – the coliform bacteria (Jiménez *et al.* 2002). Consequently, it has been suggested that rather than the detection and/or enumeration of coliforms, that of the *Enterobacteriaceae* as a whole – a wider, better-defined group of organisms, would provide a more thorough assessment of surface contamination (Silbernagel and Lindberg, 2002).

However, it is argued that a relationship does not necessarily have to exist between indicator and pathogen numbers. It can generally be assumed that the latter will be outnumbered by the organism indicative for it and that any process leading to a reduction in the number of indicator organisms will also result in pathogen levels being similarly reduced (Brown *et al.* 2000). Thus, although the detection of coliforms perhaps cannot be used to *predict* the presence of pathogens, such an indirect approach does assist in assessing *possible* pathogen presence and, as a result, is considered particularly useful in the evaluation of PRPs (Robach, 2001). The use of the coliform bacteria as microbial indicators can, therefore, lead to improved sanitation and product quality and, thus, their accurate and reliable detection remains an important component of many microbiological sampling plans.

Finally, when new test methods are developed the results of these techniques have to be reliable as the traditionally used reference methods. Furthermore, in order to have a chance of being implemented they must offer additional advantages or take away the drawbacks of the reference method (Mackintosh, 1990). The ability of the novel, chemiluminescent swab based assay, developed during this investigation, to reliably indicate the presence of low levels of coliforms within 5 h, is certainly an advantage. However, how its performance, when used to detect coliforms, particularly from a dry surface, compares to those detection methods already available to the food industry, still needs to be ascertained. This will be the subject of Chapter 7.

Chapter 7

A Comparison of Surface Sampling Methods for Detecting Coliforms on Food Contact Surfaces

7.1. Introduction

The potential for incurring massive financial losses through microbial spoilage, the increased awareness of the consumer with regard to the issue of food safety, and the more stringent governmental regulation of the food industry, has resulted in an increase in the number of different microorganisms that are being controlled on a routine basis (Priego *et al.* 2000). Previous chapters have highlighted the important role that the effective cleaning and disinfection of food processing surfaces, equipment and machinery plays in the production of safe and wholesome food. Also discussed has been the need to continually assess the efficacy of the sanitation procedures employed and the relevance of using non-microbiological test methods to detect residual food debris, particularly if results can be obtained rapidly (Chapters 3 – 5). However, these studies have also demonstrated why microbiological analysis, despite the time required to obtain results, should not be abandoned completely and how, for maximum benefit, its use should be combined with visual and non-microbiological methodology to form an integrated cleaning assessment strategy (Chapters 3 and 4). Nevertheless, to test for every microorganism of concern would require a large amount of time, money and resources and, thus, the detection of associated indicator organisms, such as the coliform bacteria, could provide a viable alternative (Chapter 6).

A range of factors, related to individual company needs and requirements, will determine those methods for coliform detection that are selected and used. A simple presence/absence test, where a surface 'fails' should coliforms be detected, may be adequate for some companies, whilst other companies may require numerical data for trend analysis. However, for most companies, whether qualitative or quantitative data are required, two key features are likely to be sensitivity (i.e. limits of detection) and speed.

The use of indicator organisms to evaluate sanitation efficacy implies that the cleaning and disinfection procedures applied will progressively reduce their numbers to the lowest level possible (Brown *et al.* 2000). When setting coliform criteria and standards, therefore, a company must determine both appropriate target values (i.e. the number of coliforms regularly obtained after a validated cleaning programme has been fully and correctly performed) and associated critical limits, denoting upper acceptable coliform levels (i.e. coliform numbers above which a surface should be considered a significant risk factor for contamination) (Dillon and Griffith, 1999; Jay, 2000). Both these values are likely to depend upon environment, product and process, yet, although general microbial target values of < 2.5 cfu cm^{-2} have been suggested (Section 1.4.2.1) and the Meat (HACCP) Regulations (2002) require *Enterobacteriaceae* to be present at levels of < 1 cfu cm^{-2}, no surface specifications for coliforms, after disinfection, are commonly available. Nevertheless, the sensitivity of any coliform detection method must be adequate to provide any individual food business with the confidence that the test is capable of detecting sufficiently low levels of microbial contamination and that the sanitation procedures have been effective.

Should coliforms be consistently detected at levels higher than the specified critical limit, then the role of microbiological testing within an integrated approach should expand to include the identification of hazards, their likely source, causes of failure and the risk

associated with the organisms of concern as well as the identification of remedial action. Nevertheless, given the shift patterns in many food processing plants, the time available to regain control of a product or process may be no more than 6 h. Thus, any measures that can be taken to reduce the time required to obtain final coliform counts, without disrupting the integrity of the pre-requisite or HACCP programme, would enable earlier identification of microbiological problems relating to both product quality and safety (Linton *et al.* 1997; Priego *et al.* 2000).

The importance of microbiological sampling plans means that detection methods must be both efficient and practical (Cooke *et al.* 1985). Chapter 6 described and discussed the development of a novel, chemiluminescence-based assay, which is capable of detecting low levels of coliform bacteria within 5 h of sampling a stainless steel surface, yet, how its performance compares to that of more traditional techniques has yet to be determined.

The aim of this chapter is, therefore, to:

- Compare the performance characteristics of the chemiluminescence-based assay to those of traditional microbiological techniques and other recently developed swab-based coliform detection methods.

Objectives

- Determine, under controlled laboratory conditions, the limits of detection of a range of traditional and recently developed, coliform detection methods.
- Assess the overall performance of the chemiluminescence-based assay when used to sample an appropriate production environment.

7.2. Materials and Methods

7.2.1. Microorganisms

Gram-negative, lactose-fermenting rods were isolated from a food environment and identified using biochemical test strips (API 20E; bioMérieux) as being *Enterobacter cloacae* and *Escherichia coli*.

To survive and persist within any food processing environment, bacteria must often adapt to a range of environmental stresses and tolerance of one damaging environment may enhance survival in another (Humphrey *et al.* 1995). To investigate possible isolate-to-isolate differences with regard to the ability of coliforms to survive on food contact surfaces, the detectability of an *Escherichia coli* type culture (ATCC 25922) was also investigated.

Bacterial cultures were maintained and prepared as described in Section 2.2.2.

7.2.2. Preparation and Inoculation of Test Surface

The test surface, a food-grade stainless steel table marked with eighty-four 10 cm x 10 cm squares, was sanitized and inoculated as described in Section 3.2.4.

Control assays were performed by inoculating the surface with 0.1 ml of sterile, un-inoculated, ¼ strength Ringer solution.

7.2.3. Microbiological Sampling of the Surface

The surface was sampled immediately after inoculation, whilst it was still wet, or after it had been allowed to air-dry for 1 h, after which time, no visible liquid remained on the surface.

7.2.3.1. Conventional microbiological methods

7.2.3.1.1. Traditional hygiene swabs

Sterile cotton swabs, pre-moistened with sterile ¼ strength Ringer solution, were used to sample the surface using the swabbing protocol described in Section 2.2.4.2. The swabs were then either streaked directly onto the surface of pre-poured TSA plates or snapped off into 10 ml ¼ strength Ringer solution and 1 ml VRBA pour plates prepared (Section 2.2.6).

When using violet red bile agars it is standard practice to overlay a pour plate with a thin layer of the same media. The agar overlay ensures anaerobic conditions, which suppresses the growth of non-fermentative gram-negative bacteria and improves the specificity of the medium (Bridson, 1998). However, whilst selective agars enable the isolation of fully viable target microorganisms, they can contain agents that may inhibit the repair and growth of damaged cells (Section 2.4). Previous studies have attempted to improve the recovery of sublethally injured organisms and overlaying a pre-poured TSA plate with a thin layer of VRBA, has been shown to aid the recovery of environmentally stressed coliform bacteria, whilst still allowing for coliform specificity (Hartman, *et al.* 1975).

All plates were incubated at 37°C for 24 h.

7.2.3.1.2. Petrifilm™

Petrifilm™ plates are sample-ready films. A paper surface, coated with a dehydrated medium, usually optimised for the growth of a specific organism type, is covered by a plastic film, which contains a cold-water soluble gelling agent. Diluted samples are added to the surface and pressure applied to the upper film spreads the sample over a growth area of approximately 20 cm^2. When using Petrifilm™, therefore, no media preparation is required, incubator and storage space is reduced and in addition, the inclusion within the medium of both a pH indicator, and a dye to enhance the visualisation of growth, facilitates the enumeration of colonies. Consequently, the use of Petrifilm™ may be considered a more practical alternative to traditional hygiene swabs (Linton *et al.* 1997; Silbernagel and Lindberg, 2002).

The surface was sampled following the procedure described for hygiene swabbing. However, in this case, after the swabs had been vortexed, 1 ml of the suspension was pipetted onto one Petrifilm™ Coliform Count Plate (3M Healthcare Ltd, Loughborough, UK). The inoculum was spread evenly over the surface and the gelling agent allowed to solidify before the Petrifilm™ plates were incubated at 37°C for 24 h.

7.2.3.1.3. Dipslides

Both sides of a VRBA ready-made dipslide (VRBL; Dimanco Ltd) were pressed firmly onto the surface to be sampled. The dipslide was returned to its accompanying tube before being incubated at 37°C for 48 h.

7.2.3.1.4. Sterile sampling sponge

An envelope containing a compressed sterile sponge and glove (TS15-B; Technical Service Consultants Limited) was opened and the glove aseptically removed and shaken so as to become unfurled. Care was taken not to contaminate the outside of the glove or the inside of the envelope. The glove was placed on the hand and used to check, by feel, that the sponge had remained moist. The sponge was then carefully removed from the envelope and held, dimpled side uppermost and bent slightly between the thumb and three forefingers. Using moderate pressure, the sponge was pressed onto the surface and using three up and down movements the entire 100 cm^2 surface area was sampled. The sponge was then held at right angles to the first movement and the process repeated. The sponge was then returned to the envelope.

An appropriate volume of Maximum Recovery Diluent (Oxoid; 9.5 g l^{-1}) was added to the envelope in order to dilute the sponge 10-fold (w/v). The sponge was then repeatedly compressed for 30 s using a Stomacher 400 laboratory blender before 1 ml VRBA pour plates were prepared (Section 7.2.3.1.1) which, once set were incubated at 37°C for 24 h.

7.2.3.2. Newly developed swab-based microbiological methods

7.2.3.2.1. Self-contained media-based hygiene swabs

The Coliform SwabCheck (Biopath Inc, West Palm Beach, Florida) was aseptically removed from its sterile pouch, pre-moistened with ¼ strength Ringer solution and used to sample the surface. The swab was then inserted into the accompanying culture tube and pushed firmly into the semi-solid selective agar.

The Coliform Path-Chek (Microgen Bioproducts Limited, Camberley, UK) was pre-moistened with the Path-Chek Wetting Agent (Microgen) and after being used to sample the surface was activated in accordance with the manufacturer's instructions.

In both cases, the surfaces were sampled using the swabbing protocol described in Section 2.2.4.2. and the swabs incubated at 37°C for 18 h.

7.2.3.2.2. Self-contained swab-based chemiluminescence assay

The single-shot chemiluminescence-based coliform detection method (Chapter 6) was used to sample the surface using the procedure described in Section 6.9.1.

7.2.4. Determination of Minimum Detection Limits

Each experiment was carried out using five replicates and then repeated, using five-fold serial dilutions, to validate the end points.

For those methods which required bacterial enumeration, the minimum detection limit was identified as the lowest inoculum level tested, which resulted in an average count of > 1 cfu 100 cm^{-2} (swabs) or > 1 cfu 25 cm^{-2} (dipslides), with growth being evident on each of the five replicate plates/slides. For the coliform SwabCheck and Path-Chek, the minimum detection limit was recorded as the lowest inoculum level tested, which resulted in the media associated with each of the replicate samples differing in colour from that of the control samples and corresponding to that of positive as indicated by the manufacturer. The minimum detection limit of the chemiluminescence-based assay was identified as

being the lowest inoculum level tested, which resulted in an RLU reading of > 2 x the average RLU reading of the control samples.

The minimum detection limits were converted to cfu cm^{-2} using the formula cited in Section 6.5.2.

7.2.5. Field Trial

7.2.5.1. Premises and surface sampling

The study, arranged with the agreement of the technical manager, was conducted within a seafood processing plant. Twenty-three different surfaces were sampled, which included surfaces in direct contact with the product, in indirect contact and environmental surfaces. Each surface was sampled using pre-moistened, cotton-tipped hygiene swabs in combination with the pour plate procedure (Section 7.2.3.1.1), the Clean-Trace™ Rapid Cleanliness Test (Section 4.2.4.1) and the single-shot, chemiluminescence-based assay (Section 7.2.3.2.2).

7.2.5.2. Interpretation of results

A surface would 'fail' if the number of coliforms recovered from the surface was > 2.5 cfu cm^{-2}, if the ATP values were > 500 RLU (Section 4.2.5) or if the chemiluminescence-based assay resulted in an RLU reading of > 2 x the average RLU reading of the control samples. The χ^2 test and regression analysis were used to analyse the results obtained.

7.3. Results

7.3.1. Controlled Laboratory Study

The minimum bacterial detection limits of traditional and recently developed surface sampling methods, when used to detect the presence of coliforms on a wet and dry stainless steel surface, are illustrated in Table 7.1.

When a wet surface was sampled, hygiene swabs coupled with the swab plate methodology, dipslides and the self-contained swab-based methods, were the most effective means of indicating the presence of coliforms, and were capable of detecting the presence of < 1 *E. coli* colony cm^{-2}. Those methods, that prior to cultivation, involved vortexing a hygiene swab in 10 ml of diluent (i.e. the traditional pour plate and Petrifilm™ procedures) were, in comparison, slightly less sensitive, detecting between 2 and 3 cfu cm^{-2}. The sterile sampling sponge was 100-times less sensitive than the other microbiological techniques in detecting the presence of coliforms on a wet surface.

The sampling of a dry surface resulted in an increase in the minimum detection limit of all the surface sampling methods evaluated, and in some cases, particularly when hygiene swabs were used, this increase, and the associated reduction in sensitivity, was observed as being between 4 and 5 log values. The reduction in sensitivity of the dipslides and the self-contained, media-based swabs was less and, consequently, these methods were the most effective means to indicate the presence, on a dry surface, of the environmentally isolated coliforms, detecting an original inoculum level of approximately 10^2 and 10^3 cfu cm^{-2} respectively.

Table 7.1. The minimum bacterial detection limits (cfu cm^{-2}) from a wet and dry stainless steel surface using different methods for coliform detection.

		minimum detection limit (cfu cm^{-2})							
		Conventional microbiological methods					*Self-contained swab-based methods*		
		cotton hygiene swab-based methods					*media-based swabs*		
		TSA / VRBA swab plate *	**VRBA pour plate** *	**Coliform Petrifilm™** *	**Sampling sponge** *	**VRBA dipslides** †	**Coliform SwabCheck** ‡	**Coliform Path-Chek** ‡	**chemiluminescent assay** #
***Escherichia coli* (environmental isolate)**	***Wet surface***	< 1	2.05	2.05	2.05×10^2	< 1	< 1	< 1	< 1
	Dry surface	1.2×10^4	3.3×10^5	3.3×10^5	1.5×10^6	3.3×10^2	7.0×10^3	1.1×10^3	1.4×10^4
***Enterobacter cloacae* (environmental isolate)**	***Wet surface***	2.75	3.10	3.10	3.1×10^2	< 1	< 1	< 1	3.10
	Dry surface	2.75×10^4	1.9×10^5	4.2×10^4	4.2×10^5	2.2×10^3	2.3×10^3	2.2×10^3	2.6×10^4
***Escherichia coli* (ATCC 25922)**	***Wet surface***	< 1	2.90	2.15	8.7×10^2	< 1	< 1	< 1	< 1
	Dry surface	$> 9.2 \times 10^4$	$> 9.2 \times 10^4$	$> 9.2 \times 10^4$	$> 2.9 \times 10^5$	9.87×10^4	$> 2.9 \times 10^4$	$> 2.9 \times 10^4$	not tested

* colonies visible after 24 h

† colonies visible after 24 h but easier to count after 48 h

‡ results attainable after 18 h; positive result indicated by colour change (SwabCheck: green/brown → yellow; Path-Chek: green → yellow)

results attainable after 5 h; positive result indicated by RLU reading > 2 x average RLU reading of control samples

7.3.2. Field Trial

Traditional microbiology (hygiene swabs), the chemiluminescence-based coliform assay and ATP bioluminescence were used to sample surfaces within both the low- and high-risk areas of the seafood processing plant. The results obtained are presented in Table 7.2.

Only one of the 23 surfaces tested was passed as 'clean' using the ATP bioluminescence technique, indicating relatively high levels of widespread surface contamination. Although, coliforms were detected on 22% and 64% of the surfaces sampled within the high- and low-risk areas respectively, there was no correlation between ATP measurement and the level of coliform contamination detected.

When coliform colonies were isolated via the use of traditional hygiene swabs, the results of the chemiluminescence-based assay also indicated the presence of coliform bacteria. Furthermore, the magnitude of the chemiluminescent light signal appeared to be influenced by the number of colonies isolated ($r = 0.78$, 21 df, $p < 0.01$, $r^2 = 0.61$) (Figure 7.1). On no occasion did the isolation of colonies correspond to a 'negative' chemiluminescent light signal. However, on five occasions, despite microbiological analysis suggesting that the surfaces were free from coliforms, use of the chemiluminescence-based assay indicated that these surfaces were in fact contaminated with, in some cases, relatively high levels of coliform bacteria. Nevertheless, only 23 surfaces were sampled and, overall, there was no significant difference between the number of surfaces the two different coliform detection methods 'passed' or 'failed' ($\chi^2 = 1.43$; $p > 0.05$).

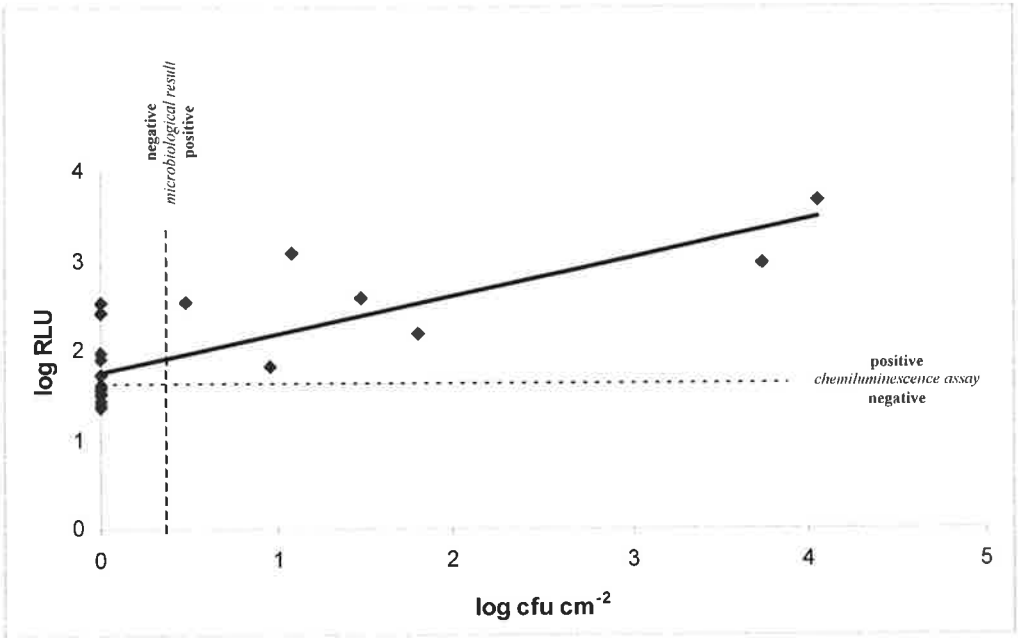
Table 7.2. Comparison of the results that were obtained after traditional microbiology, the chemiluminescence-based coliform assay and ATP bioluminescence were used to sample 23 different surfaces within a seafood processing plant.

		Traditional microbiology (cfu cm^{-2})	Chemiluminescent assay (RLU) *	ATP bioluminescence (RLU)
Low risk	**Surfaces in direct contact with product**	**12**	**1228**	108,767
		< 1	**96**	36,301
		< 1	**53**	4,302
		3	**345**	4,097
		5.56×10^3	**943**	3,980
		30	**393**	3,323
		< 1	**265**	1,979
		1	32	37
	Surfaces in indirect contact with product	< 1	**650**	60,217
		< 1	41	7,491
		< 1	37	2,674
		< 1	25	633
	Environmental surfaces	**63**	**148**	15,873
		< 1	**81**	5,661
High risk	**Surfaces in direct contact with product**	< 1	39	> 500,000
		< 1	33	> 500,000
		< 1	33	> 500,000
		< 1	40	327,944
		1	27	23,222
		< 1	24	12,417
		9	**66**	1,954
	Surfaces in indirect contact with product	**1.16×10^4**	**4645**	> 500,000
		< 1	28	18,140

* The average control value (obtained via the direct activation of the device (i.e. omitting surface sampling)) was 21.5 RLU. Therefore, an RLU value > 43 RLU was taken as an indication of coliform presence.

The figures in **bold text** highlight those microbiological results and chemiluminescent readings that were considered indicative for coliform presence (Section 7.2.5.2).

Figure 7.1. Relationship between the number of coliform colonies isolated (cfu) and the corresponding chemiluminescent light signal (RLU).



7.4. Discussion

In recent years, the importance of obtaining microbiological information rapidly has increased, as has the apparent willingness to adopt 'non-traditional' microbiological techniques (Betts, 1999). Both these factors prompted the design and development of the rapid, chemiluminescence-based coliform detection method discussed throughout Chapter 6. However, for this or any new method to be of value to the food industry, evidence must be provided that demonstrates the efficacy of the methodology when tested under defined conditions and, in addition, gives potential users confidence in the results obtained (Betts, 1999).

During the current investigation, the controlled laboratory study was conducted in order to determine how the sensitivity of the chemiluminescence-based assay compares to that of those coliform detection methods already available to the food industry. The advantages of conducting some comparison trials under controlled conditions have previously been alluded to (Chapter 3) and the results that were obtained (Table 7.1) are, in terms of this thesis, of great interest. Not only do they demonstrate that the sensitivity of the chemiluminescence-based assay is comparable, and in some cases, superior to that of traditional swab-based methodology, but they also support many of the hypotheses previously put forward with regard to the factors that may influence the recovery of microorganisms from stainless steel surfaces (Chapter 2).

The removal of bacteria from a surface is a fundamental part of each of the surface sampling techniques evaluated. As discussed, swab design, its flexibility for example, can prevent adequate pressure being applied to the swab during sampling and, thus, can restrict the number of bacteria picked up from the surface (Chapter 2). In an attempt to overcome such limitations, the use of sterile sampling sponges has been suggested as an alternative (Silliker and Gabis, 1975). However, subsequent poor performances have prompted speculation that some sponges may contain inhibitory compounds that can significantly compromise the accuracy and sensitivity of sampling programmes (Daley *et al.* 1995). Indeed, during the current study, when used to detect the presence of coliforms, the sampling sponge technique was, regardless of surface dryness at the time of sampling, the least sensitive of all the methods evaluated (Table 7.1). Nevertheless, the sponge was shown, by means of a procedure described by Daley *et al.* (1995), to exhibit no inhibitory properties against any of the coliform strains used (results not presented).

The ability of a sampling sponge to absorb a large volume of liquid is considered by some its major advantage over the traditional hygiene swab (Llabrès and Rose, 1989). However,

considering the facts presented in Chapter 2, it is surmised that whilst any bacteria that become dislodged from the surface during sampling are likely to be absorbed with the liquid, they are also likely to become trapped within the matrix of the sponge. Furthermore, the repeated compression of the sponge within a diluent probably only exacerbates this problem, as those bacteria that do become detached from the sponge are likely to simply become reabsorbed. Prior to plating, squeezing the sponge to release the diluent did not significantly improve bacterial recovery ($p > 0.05$), confirming that bacterial retention was not simply due to the absorption and retention of liquid (results not presented). Thus, it is postulated that rather than the sponge having an inhibitory effect upon the bacteria, it is the adsorption and entrapment within the fibres and the ineffective release of these bacteria that is the major reason for the comparative poor performance of the sampling sponge technique. In terms of surface sampling this is likely to result in the levels of microbial contamination being considerably underestimated. However, ineffective bacterial release could have greater consequences within a domestic or catering setting.

During a study conducted by Josephson *et al.* (1997), sponges used for washing dishes and wiping off surfaces were demonstrated to be consistently contaminated with a variety of microorganisms, including faecal coliforms. Rinsing sponges prior to use has been shown, perhaps unsurprisingly, to have little effect upon bacterial numbers (Hilton and Austin, 2000) and consequently, Josephson *et al.* (1997) concluded that their use has high potential for transferring bacterial contaminants to other food contact surfaces. Conversely, however, Hilton and Austin (2000) hypothesise that once microorganisms become entrapped within the sponge matrix, they are then held away from contact surfaces and thus, cross contamination may actually be prevented. Interestingly, therefore, and in contrast to the themes discussed in Section 5.3.3, those properties of a sponge that may

prove advantageous during cleaning are the same properties that make the sponge an inefficient means of assessing surface cleanliness.

During the current study, the performance of the sampling sponge technique was comparatively poor. In contrast, when Silliker and Gabis (1975) introduced sponges directly into an enrichment medium, a high proportion of the samples were demonstrated as being positive for *Salmonella*, despite the use of cotton hygiene swabs failing to detect this organism from any of the surfaces sampled. These results imply that the sensitivity of the sampling sponge technique can be significantly improved if the release step of the protocol is omitted. Likewise, the swab associated with the SwabCheck, Path-Chek and the chemiluminescence-based assay also forms an integral part of the cultivation stage of the test method. Consequently, rather than having to be transferred from the bud to either a diluent or solid medium, any coliform bacteria present on the swab comes directly in contact with the growth media and/or reagents. Thus, bacterial release becomes unnecessary and again by omitting this stage from the sampling procedure, the sensitivity of a media and swab-based surface sampling method is increased (Table 7.1).

Three different coliforms were used during the laboratory-based study and, in the majority of cases, when a wet surface was sampled, coliform type did not affect minimum detection limits (Table 7.1). However, interestingly, the chemiluminescence-based assay did appear slightly less sensitive when used to detect *Enterobacter cloacae*, implying that different coliform strains may differ significantly in terms of intracellular levels of β-galactosidase. All test methods were less effective in detecting the presence of coliforms on a dry surface, with the type culture being the most difficult to recover, suggesting it may have been less able than the environmentally isolated coliforms to tolerate drying conditions. Nevertheless, in all three cases, the reduction in dipslide sensitivity was not to the same extent as that associated with swab-based methodology. These results support the

hypotheses (Chapter 2) that whilst losses in microbial viability do occur, substantial numbers of bacteria can survive the drying process and, in addition, when sampling a dry surface, whilst test sensitivity can be increased by omitting the release step of a sampling procedure, preventing shear stress and, in doing so, reducing the risk of bacterial injury can further increase the sensitivity of a surface sampling technique.

On the basis of the results obtained from the controlled laboratory study, it was concluded, that overall the chemiluminescence-based assay compares very favourably to traditional cultivation-based methodology - there remained, however, an important need to assess its performance within an appropriate production environment (Chapter 4).

Choice of indicator organism is generally product and process specific (Swanson and Anderson, 2000). Cattle produce a "staggering" 23.6 kg of faeces each day with each gram reportedly containing 2.3×10^5 *E. coli* cells (Falconer, 2003). Faecal contamination can readily be transferred from hide to carcass to equipment and machinery (Sheridan, 1998; Gill *et al.* 1999b). Grinders in particular have been demonstrated to not only distribute microbial contaminants but also to act as a reservoir for pathogens such as *E. coli* O157 and *Salmonella* spp. (Farrell *et al.* 1998; Flores and Tamplin, 2002) and, as a consequence, many of the illnesses caused by these organisms are associated with the consumption of undercooked, contaminated ground beef (Gonthier *et al.* 2001; Flores and Tamplin, 2002). Similarly, in addition to endogenous microflora, shellfish, particularly when caught from polluted waters, are also likely to be heavily contaminated with bacteria, either from the mud trawled up with them or from the water itself (Adams and Moss, 1995). Preventing the transfer of such contaminants, particularly if faecal in origin, from processing surfaces, equipment and machinery to the final product, is again of considerable importance, especially considering shellfish are often eaten raw or only partially cooked. Consequently, within both the meat and seafood industries, coliforms are often used as

indicators of sanitary effectiveness (Matches and Abeyta, 1983; Tompkin, 1983) and, thus, the ability of the chemiluminescence-based assay to detect the presence of coliform bacteria within a seafood processing plant was assessed.

The sole aim of the field study was to compare the performance of the chemiluminescence-based assay to that of traditional swab-based methodology *not* to assess the cleanliness of the processing environment. In fact, in order for the trial to provide meaningful data, it was necessary to ensure that those surfaces sampled *were* likely to be contaminated with coliform bacteria and it was anticipated that unless sampling occurred during production, any coliforms present on the surfaces would be removed via the plant's normal cleaning and disinfection procedures. Many of the surfaces sampled were within the 'low-risk' area of production where although surfaces were likely to be contaminated with high numbers of microorganisms, high levels of organic debris were also present. ATP analysis was, therefore, included mainly for interest – would, for example, the highest number of coliforms be isolated from those areas associated with the highest levels of overall contamination? However, no such relationship existed (Table 7.2).

Coliforms were detected within both the 'low'- and 'high-risk' areas of the production plant and, in terms of the number of surfaces each test method 'passed' or 'failed', statistical analysis inferred that the performance of the chemiluminescence-based assay was comparable to that of traditional swab-based methodology (Section 7.3.2). Nevertheless, the magnitude of the chemiluminescent light signal (RLU) obtained after 6 of the 23 surfaces were sampled, implied that coliforms were present on these surfaces at levels much higher than those suggested by the number of colonies isolated via traditional microbiology (Table 7.2; Figure 7.1). The influence of ineffective bacterial release upon the efficiency of the swabbing technique has already been discussed. However, there are

other factors that could contribute to the apparent, if not statistically significant, superiority of the chemiluminescence-based assay.

Previous studies have demonstrated that coliform bacteria, when exposed to a range of environmental stresses, can become non-culturable (Rigsbee *et al.* 1997; Fiksdal and Tryland, 1999; Kolling and Matthews, 2001). Such cells, unlike those that are metabolically injured, will not, once plated on a non-selective, non-inhibitive medium, grow and repair (Jay, 2000), yet, despite losing their culturability, these cells reportedly remain metabolically viable and still retain assayable β-galactosidase activity (Davies *et al.* 1995; Pommepuy *et al.* 1996; Fiksdal and Tryland, 1999; George *et al.* 2000). It is postulated, therefore, that the presence of coliforms in such a viable but non-culturable state, despite being missed using standard plate count methods, would be detected by means of the chemiluminescence-based assay.

Additionally, the results of the laboratory-based study suggest that whilst fully viable coliform strains are unlikely to differ with regard to their culturability (Table 7.1) they may contain quite different levels of β-galactosidase. It is virtually impossible, using colony enumeration alone, to distinguish any metabolic differences that may exist between colonies of similar morphological appearance and, as a result, it is highly likely that such differences could contribute to discrepancies between colony counts and RLU readings.

Alternatively, and a potential limitation of the chemiluminescence-based assay within this particular type of processing environment, is the contribution to the light signal by non-target bacteria. Although the specificity of the assay has been evaluated, the range of bacteria used for this purpose was fairly limited (Section 6.4.5). There have been reports that bacteria, likely to be present in sewage and contaminated waters, such as marine vibrios and *Aeromonas* spp., can interfere in β-galactosidase assays and result in false-

positive readings (Fiksdal *et al.* 1997; Van Poucke and Nelis, 1997a; Tryland and Fiksdal, 1998).

All three of these possibilities warrant further investigation.

7.5. Conclusion

An integral component of food safety and quality management systems is the verification that both HACCP and pre-requisite programmes are being implemented correctly (Chapter 1). Such procedures can involve the periodic microbial assessment of food contact and environmental surfaces (Linton *et al.* 1997) and those that incorporate tests for indicator organisms as well as tests for the plant's unique microflora, will permit a more accurate assessment of the microbiological contamination and, thus, the hygienic status of equipment, machinery and the plant in general, than pathogen testing alone (Sveum *et al.* 1992).

The general ease with which coliforms can be cultivated and differentiated makes them nearly ideal as indicators (Jay, 2000). However, just as choice of non-microbiological cleanliness assessment method must depend upon the type and level of food residues likely to be present, testing for coliforms will only provide relevant information within those processing environments where detectable levels of coliforms are likely to be present on an inadequately cleaned and/or disinfected surface. Within such environments, the isolation of coliforms can provide useful information with regard to the general microbiological risks associated with a site and, thus, can be of great value in terms of ensuring the safety and quality of the final product (Sveum *et al.* 1992; Jay, 2000). Nevertheless, the use of

coliforms as indicator organisms is still subject to criticism (Section 6.9), yet, as with any sampling procedure, their use would not be needed at all if all aspects of food production including cleaning and disinfection were carried out correctly and human error could be eliminated (Reinbold, 1983). As this is unlikely to happen, it is imperative that those food businesses for which the detection of coliforms *is* appropriate can do so with accuracy, reliability and with confidence. However, there are advantages and disadvantages to each of the coliform detection methods evaluated during the current investigation confirming that the method chosen must depend upon a company's priorities and needs.

Overall, although only providing semi-quantitative data, dipslides are the most effective means to detect the presence of coliforms, particularly from a dry surface. Nevertheless, dipslides can only be used to sample flat surfaces, whereas swabs can be used to sample difficult to clean, uneven and irregular surfaces. However, traditional hygiene swabbing can considerably underestimate the number of indicator organisms present. Although, the self-contained, media-based swabs proved more sensitive than the traditional swabbing technique, they provide qualitative data only, indicating that coliform bacteria are present but giving no idea as to what level. It has been suggested that a simple presence/absence statement gives only limited information regarding process performance (Brown *et al.* 2000).

With the exception of the sampling sponge technique, all the media and cultivation-based methods were able to detect the presence of coliforms, on a wet surface, at levels of < 2.5 cfu cm^{-2}, yet, in all cases, the time required to obtain these results was at least 18 h. However, the chemiluminescence-based assay was capable of providing semi-quantitative results that were comparable, if not superior to those obtainable by traditional swab-based methodology, in only 5 h (Tables 7.1 and 7.2). Rapid results enable a company to take remedial action immediately trend analysis indicates a loss, or future loss, of control and

results from this study strongly suggest that during the hygienic assessment of food contact surfaces, when testing for coliforms, detectability need not be at the expense of speed. Although none of the sampling methods evaluated would be appropriate for mid-shift sampling, any of the newly developed, self-contained media-based swabs, including the chemiluminescence-based assay, could prove a valuable tool for use within an integrated cleaning assessment strategy. In addition, the ability of the chemiluminescence-based assay to provide results within the 6 h timeframe usually available to industry to regain control of a process, suggests it could also play an important role within food safety management systems such as HACCP.

Chapter 8

Conclusions and Recommendations

8.1. Synoptic Discussion

As recently as the mid-1970's, the general feeling within the UK was that "food as eaten was generally safe, and that, if there were risks, these were so low as to be negligible" (Cooter and Fulton, 2001). The past 25 years has, however, seen a rise in reported food poisoning cases and these and the ever-increasing media and press coverage have heightened consumer awareness of both the quality and, perhaps more importantly, the microbiological safety of food. As issues of food safety have become more publicised they have inevitably become more politicised (Cooter and Fulton, 2001). Consequently, there is at present, more UK legislation concerning food than at any other time and than any other commodity (Griffith, 1997) and, thus, it is perhaps ironic that today over 60% of people do not trust food companies to produce safe food (Leach *et al.* 2001).

However, whilst the food industry has a legal and moral obligation to supply a safe, fresh and organoleptically acceptable product, the responsibility for reducing foodborne disease must be shared by industry, government and consumer alike. The British Medical Association has recently acknowledged the role it must play in "helping the public to get a better understanding of the very real dangers posed by poor food hygiene" (Leach *et al.* 2001). Likewise, the UK Food Standards Agency (FSA) intends to raise standards in the home through the implementation of a national food safety education campaign (Redmond, 2002). However, whilst such measures may help the FSA to reach its target of reducing

foodborne disease by 20% by 2006, it *must* be appreciated that the food industry itself also needs guidance.

During processing, food may be contaminated directly with spoilage and/or potentially pathogenic organisms from the raw material, or indirectly, at any point during the production process, by those that first contaminate surfaces, equipment or machinery (Notermans *et al.* 1991). Whilst ingredients should be purchased from a reputable supplier, the 'surface route' of food product contamination can be controlled on a day-to-day basis via the implementation of effective cleaning and disinfection programmes. However, despite a basic requirement in the production of safe and wholesome food, there is evidence to suggest that 'cleaning' is, in general, poorly carried out (Elvers, 1999; Gibson *et al.* 1999; Griffith *et al.* 2001). The reasons for ineffective cleaning and/or disinfection are numerous and varied (Chapter 1). Consequently, there is a need, common to all sectors of the food industry, to continually assess surface cleanliness in order that any problems associated with the 'cleaning' procedure can be identified and rectified rapidly.

The aim of applied food safety research is to provide the food industry with answers and/or guidance concerning specific problems or issues that may prevent them from producing safe and wholesome food. The development of methods for assessing surface cleanliness is a particularly dynamic area of research, yet, the ever-increasing number and variety of test methods becoming available to the food industry has only added to the already widespread confusion regarding why, when and how cleanliness assessment should be carried out. This thesis, however, epitomises applied food safety research. It not only provides the food industry with relevant data regarding the factors that affect the efficacy of a range of different test methods but also, when taken collectively, this information can and has been used to form a strategy that will help any individual food business identify the most appropriate test method(s) to use within their specific processing environment.

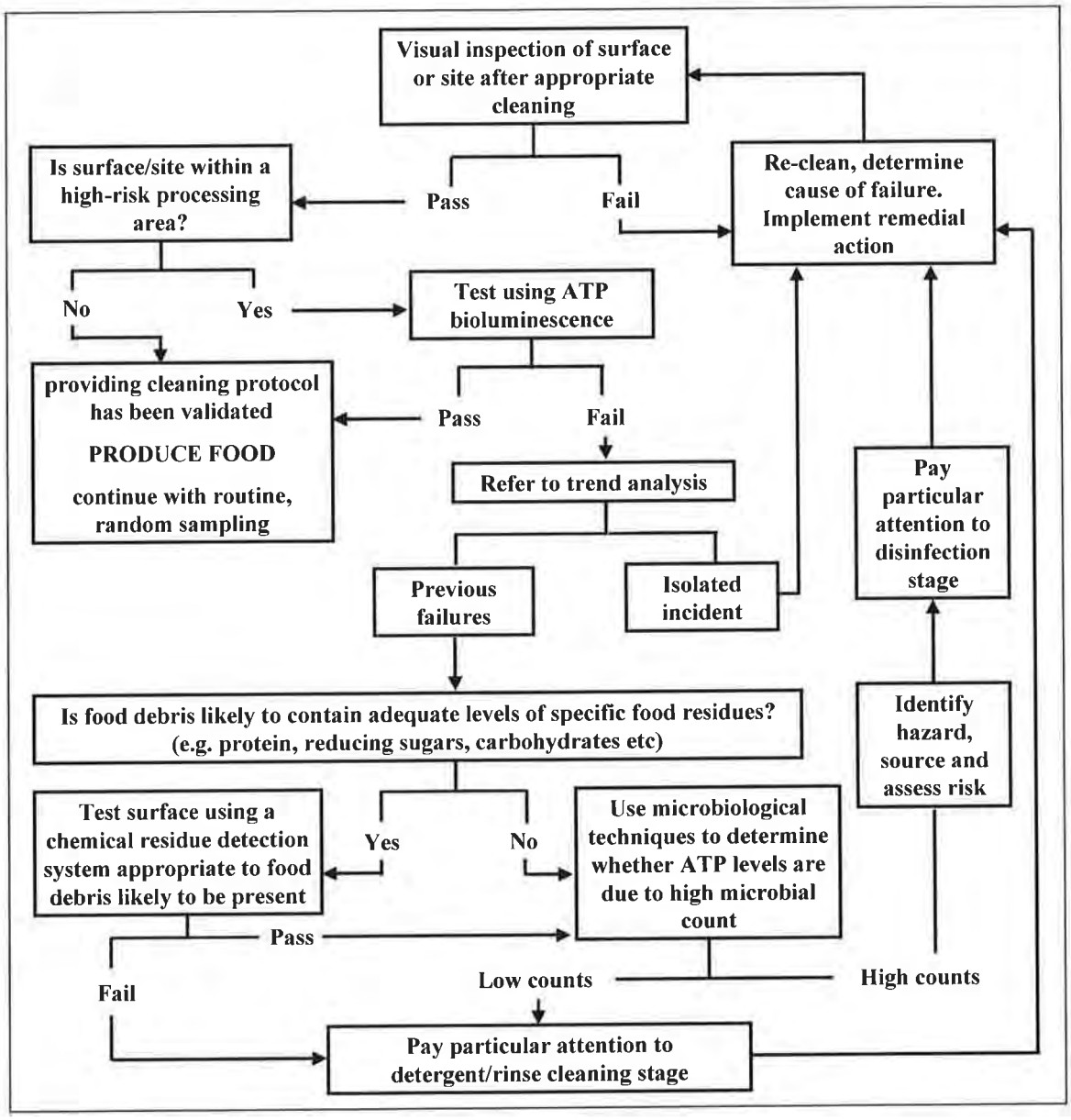
The general lack of information currently available to the food industry together with the commercial self-interest of the various test manufacturers being, perhaps, at the expense of them being totally open in terms of test efficacy, means there is, at a present, a danger that food businesses may consider the numerous test methods to be readily interchangeable. However, it is quite clear that many of these methods assess different parameters (Chapter 3) and, thus, no one assay procedure can completely characterise the contamination on a surface (Chapter 4). Instead, therefore, the different methods should be seen to complement each other and the results obtained throughout this study clearly support the implementation of an integrated cleaning assessment strategy.

Such a strategy has previously been advocated (Griffith *et al.* 1997). However, it was devised during a time when ATP bioluminescence was considered the only alternative to microbiological testing. Consequently, no references are made to the type of food produced or indeed to the risk associated with the environment itself and, in addition, no means of differentiating failures in cleaning from failures in disinfection (Chapter 4; Figure 4.4). Thus, although forming the basis of this thesis, the information this original strategy would provide would, in reality, be of limited value to the food industry.

However, the strategy presented in Figure 8.1 incorporates current thinking and current test methods and has been designed for use primarily within large manufacturing plants involved in the production of 'ready-to-eat-foods'. It takes into consideration the need to ensure both the safety and quality of the product and, thus, the need to monitor and assess surface cleanliness within a HACCP and PRP system respectively. Furthermore, by combining ATP bioluminescence, residue detection methods – the choice of which, being dictated by the type of food debris likely to be present, and microbiological techniques, its use will allow any food business to effectively monitor and verify both their cleaning *and* disinfection procedures.

The way the strategy has been structured is subject to a clear rationale that will subsequently be discussed. However, whilst it is believed the food industry would benefit greatly from implementing such an integrated approach, it is essential, to prevent erroneous conclusions from being drawn, that they are also familiar with the limitations of the assessment methods used.

Figure 8.1. Stages in an integrated cleaning assessment strategy for use, primarily, within large food manufacturing plants (or medium sized businesses with a strong commitment to cleaning) involved in the production of 'ready-to-eat' foods.



To successfully implement the proposed strategy a company will be required to purchase additional test methods. Whilst it is important that this additional expenditure is considered in relation to the costs of failing to assess surface cleanliness effectively, unnecessary implementation costs can be avoided by minimising the use of needless and/or inappropriate test methods. It is for example, relatively pointless to employ any commercially produced cleanliness assessment technique on a visually unclean surface, hence, despite proving a poor indicator of cleaning efficacy (Chapter 4), visual assessment still plays an important and potentially money-saving role within the proposed strategy. Economic consideration has also been given to the relevance of sampling visually clean surfaces sited within a 'low-risk' production environment. Under these circumstances, provided the cleaning protocols employed have been properly validated, sampling need only be conducted periodically, as part of Good Manufacturing Practice, on a routine and random basis. However, if the surfaces are within a 'high-risk' processing environment, then their cleanliness is likely to be considered critical to food safety and, thus, their effective cleaning and disinfection will be designated a control measure that will require rapid and regular monitoring.

The ability of ATP bioluminescence to provide, in real-time, an indication of total surface contamination means that it plays a pivotal role in the proposed cleaning assessment strategy. If a surface is passed as 'clean' using this technique then the universality of ATP is such that, in many cases, the surface can effectively be considered free from contaminating debris and acceptable for food production (Figure 8.1). However, it must be remembered, that the sensitivity of the ATP bioluminescence technique is such, that in the absence of detectable food residues, the presence of relatively low levels of microorganisms ($< 10^3$ cfu cm^{-2}) may be overlooked (Chapters 3 and 4). Thus, it is imperative that ATP bioluminescence is not considered an alternative to traditional microbiological assessment and that, even if surfaces are deemed 'clean' using ATP bioluminescence, the latter, albeit

on a routine and random basis, is continually employed in order to verify the efficacy of the disinfection procedures applied (Figure 8.1).

It is important to recognise that environments exist and/or situations may arise where the use of ATP bioluminescence may be inappropriate and/or provide misleading information. Thus, whilst the ultimate goal of the proposed strategy is to aid the food industry in general to assess surface cleanliness, it is appreciated that it must also be flexible in terms of meeting the specific needs of any individual food business.

Although present in a wide variety of foodstuffs, there are those and, thus, processing environments, in which ATP is either absent or present in very low levels. Consequently, any residual surface contamination will remain undetected unless the method used is more appropriate to the food debris likely to be present. If, therefore, as would be the case within a high-fat processing environment, ATP bioluminescence would provide limited information regarding surface cleanliness (Chapter 5), its use would not only be irrelevant but also an unnecessary expenditure and as a result could quite legitimately be omitted from a surface assessment programme.

Several companies now produce ATP bioluminescence equipment and the increased competition has resulted in the development and launch of cheaper instruments and reagents. Two different ATP bioluminescence systems, one of which incorporated a low-cost luminometer, were used during the study discussed in Chapter 3 and the results obtained were, within the limits of the experimental protocol, comparable. Nevertheless, despite the success of some companies in reducing the overall cost of the technique without, it appears, compromising on assay sensitivity, it is recognised that ATP bioluminescence is still perceived by many smaller businesses as being too costly to implement. Depending upon the food residues present, both protein detection and the VERIcleen™ Food Residue Surface

Test – a multiple residue detection method, have been demonstrated to be as sensitive, if not more so, than ATP bioluminescence, particularly when used to detect high-protein and/or high-sugar foods (Chapters 3 and 4). It is feasible, therefore, that smaller manufacturing plants involved in the production of such food types, or larger catering establishments within which, food contact surfaces are likely to be contaminated with a wide variety of foodstuffs, could also exclude the more expensive ATP bioluminescence technique and, provided appropriate instrument-free test methods are employed, still obtain relevant and meaningful information regarding surface cleanliness.

Nevertheless, these latter test types are qualitative in nature and although the speed of the reaction can often give some idea, in general, the results provide no indication as to the level of contamination present. In contrast, should a surface 'fail' by means of ATP bioluminescence, the magnitude of the light signal (i.e. the RLU value) can provide an indication as to how 'badly' the surface has 'failed' and, in addition, can be used in trend analysis enabling management to determine whether the 'failure' was an isolated incident, the result of a sudden, catastrophic loss of control, such as the incorrect formulation and/or application of the cleaning solutions or, alternatively, if the sanitation programme had, in fact, been losing its effectiveness over time (Buchanan, 2000).

The incorporation of ATP bioluminescence within the proposed cleaning assessment strategy is not necessarily essential. However, its ability to provide numerical data coupled with the speed and ease with which non-technically trained staff can detect a wide range of contaminating residues, does make the technique nearly ideal in terms of initial cleanliness assessment and, consequently, its use *is* recommended in the majority of food processing environments. Nevertheless, ATP bioluminescence is unable to distinguish microbial from non-microbial ATP. To ensure that correct remedial action is implemented, therefore, the technique should not be used in isolation. Thus, in order to determine if the surface has

'failed' due to the presence of food debris and/or microorganisms and, consequently, whether the cleaning and/or disinfection stage of the sanitation programme has proved ineffective, the proposed strategy also involves the detection of specific food residues.

One of the major conclusions to be drawn from the work presented in this thesis, is that with the ever-increasing number of test methods becoming available to the food industry, choosing the correct method and, thus, the ultimate success of any surface sampling programme will depend upon proper consideration being given to the composition of the food produced and, therefore, the residues likely to be present on an inadequately cleaned surface. If the test used is unsuited to the production environment and unable to detect the food debris present, then the results obtained will give the impression that the surface is free from the residue(s) in question (Chapter 4). This could have particular significance if associated food allergens also remain undetected – it has been reported that allergen risk management is critical to at least 1% of the UK population (Gowland, 2002). However, if appropriate test methodology does detect the presence of food residues, it can be concluded that the cleaning phase of the sanitation programme has been ineffective and, perhaps, requires modification.

When comparing the performance of different test methods, laboratory trials allow consistency with regard to surface type, cleanliness and condition, inoculum level and residue type. Consequently, on the basis of observations made during the comparison study discussed in Chapter 3, it could be concluded that in contrast to quantitative test methods, tests involving a simple colour change provide a far more repeatable and reproducible means of assessing surface cleanliness, probably because of their comparative inability to detect subtle changes in residue level. Nevertheless, by their very nature colorimetric residue detection methods are very subjective and, thus, a surface deemed 'clean' by one operator may require re-cleaning in the eyes of another. Clarifying the end-point and preventing false

positive reactions are two ways in which test manufacturers could provide the food industry with a more user-friendly means of detecting residual food debris. However, at present, although non-technically trained staff can carry out rapid cleanliness assessment, it is important that adequate training *is* given with regard to the use and interpretation of such test methods. It is also imperative that the food industry appreciates that the results obtained using residue detection methods do not give any indication as to the level of microbial contamination present (Chapters 3 and 4). This cannot be over emphasised and is of even greater importance if, for reasons previously discussed, ATP bioluminescence does not form part of a company's cleaning assessment strategy. If a surface is passed as 'clean' using any residue detection method, it cannot be guaranteed to be free from microbial contaminants, hence, the important need to continue with microbiological assessment.

In any given processing environment those target values/critical limits considered indicative of a clean surface (Chapter 7) are likely to be influenced by the sensitivity of the detection method used. Thus, if the methodology becomes too sensitive, the target values may effectively be set too low and the company may find itself cleaning unnecessarily, wasting time, money and energy – the very thing an integrated cleaning assessment strategy aims to avoid. It is anticipated that if the detection method provides fully or semi-quantitative results, such a situation could be avoided by the company simply re-validating its cleaning and disinfection procedures and adjusting their target values and/or critical limits accordingly. However, problems are foreseen should the sensitivity of colorimetric residue detection methods significantly increase, particularly, as is often the case, if it is this sensitivity and, thus, the manufacturer of the test that dictates the level of residues that should 'pass' or 'fail'.

The residue detection methods currently available to the food industry are capable of detecting the presence of relatively low levels of food components (Chapter 3). Thus, rather

than increasing their sensitivity, the manufacturers of such test methods should, perhaps, work to increase the range of detectable food residues. There is for example, no test method currently commercially available that could be used successfully within a high-fat processing environment – although an assay capable of rapidly detecting the presence of fat residues has, during the current study, been developed (Chapter 5). In the event that an appropriate residue test method is lacking, the proposed strategy again recommends the use of microbial analysis which, under these circumstances will provide an indication as to whether a high ATP value is the result of ineffective disinfection, or, if low microbial counts are obtained, ineffective cleaning (Figure 8.1).

The sensitivity of any microbiological test method is important not only when assessing the efficacy of the disinfection stage of the sanitation programme but also during the subsequent identification of hazards, the risks associated with the organism(s) of concern and the effectiveness of the remedial action. How and when a food company tests for microorganisms will, therefore, most likely depend upon the type of information they require and the speed with which results can be obtained (Chapter 7). However, Chapter 2 discussed an innovative investigation into all aspects of the swabbing procedure and clearly demonstrates that other factors should also be taken into consideration.

Hygiene swabs are most effective when used to sample wet surfaces. Nonetheless, bacterial recovery is poor and swabs should not be relied upon to give an accurate indication as to the level of microorganisms present. This need not be considered detrimental to a sampling plan; it has been suggested that the exact measure of the microbial population is of little importance and relevance should only be placed on whether microbial levels lie above or below a pre-determined critical limit (Bautista *et al.* 1995). However, it is imperative, that if swabs are to be used, those levels that represent the acceptable safety and quality standard, are set low enough to offset the poor performance of the swabbing technique – too high and

the number of colonies isolated may be considered acceptable, despite, in reality, many more being present on the surface.

The mechanical energy generated during sampling, the absorbency of the bud material and the swab-wetting solution used, all contribute to the efficacy of the traditional swabbing procedure (Chapter 2). Overall, however, it is the ease with which the bacteria are released from the swab bud that has the greatest effect and omitting this step via the use of dipslides or the more recently developed self-contained swab devices, can increase the sensitivity of microbiological assessment (Chapter 7). This is particularly true when sampling a dry surface, which, despite traditional thinking, must also be considered capable of harbouring microorganisms (Chapters 2, 3 and 7). Whilst dipslides have been shown to be the most effective means of detecting microbial contaminants, they are only able to sample flat surfaces and, thus, those that are the easiest to clean. It could be argued, therefore, that their use actually defeats the object of cleaning assessment. Although flexible agar contact plates have recently been developed, they still appear unsuited for sampling the difficult-to-clean nooks and crannies of machinery and equipment. Thus, the production of a swab-dipslide hybrid could, perhaps be investigated. However, work should also continue on increasing both the sensitivity and speed of more traditional swab-based techniques.

The material of the swab bud clearly plays an important role with regard to the sensitivity of traditional cultivation-based methodologies (Chapter 2). It is felt, therefore, particularly considering the similarities between cleaning and cleaning assessment, that there is scope for collaboration between swab manufacturers and, for example, those companies involved in the production of cleaning cloths, in order that a material can be developed that is capable of removing a high proportion of surface contamination and, more importantly, once rinsed/vortexed effectively releasing it. Furthermore, detectability need not be at the expense of speed. The detection of pathogens such as *Salmonella* and *Listeria* spp. can, if

traditional enrichment techniques are used, take up to 5 d, yet self-contained media-based swabs are available that can detect the presence of these pathogens within 18 h. Although, the performance of these specific tests has not been evaluated in depth and specificity still needs to be assured, preliminary investigations (not presented) suggest that test sensitivity is comparable to that of traditional methods.

Nevertheless, it is appreciated that although useful and perhaps essential for validation and verification purposes, traditional cultivation can have no place in a HACCP system. However, a novel, swab-based, enzymatic method capable of detecting the presence of coliforms in just 5 h has, as part of this thesis, been developed (Chapter 6). Enzymes have been described as "the most specific reagents known" and, thus, enzymatic methods, in addition to being both sensitive and rapid, can also be considered the most accurate (Henniger, 1990). This thesis also demonstrates, therefore, the possibilities for alternative microbiological techniques within the food industry, enabling for example, microbial levels and, thus, disinfection procedures effectively to be monitored.

According to the World Health Organisation, food bio-terrorism is a new and global threat, yet, even without such concerns, with new European legislation expected to take effect in 2005, food manufacturers face challenging new controls regarding food hygiene (Pendrous, 2003). This thesis provides the food industry with new and valuable information with regard to the benefits and limitations of the various test methods available to them and, thus, how best to assess surface cleanliness. When taken collectively, this data should enable food businesses to ensure their cleaning and disinfection procedures are effective, that cross-contamination is minimised and ultimately that the food produced is safe and of the highest quality.

8.2. Recommendations for future research

- Trial the proposed cleaning assessment strategy within different food processing environments and assess the effect of its implementation upon surface cleanliness

- Determine the factors that influence the recovery of microbial contaminants from a dry stainless steel surface using the traditional swabbing technique
    - use atomic force microscopy to investigate the effect of drying upon cell surface structure
    - use epifluorescence microscopy and appropriate staining techniques to determine microbial viability on a swabbed surface and/or within swab fibres
    - use impedance microbiology to determine microbial viability within swab fibres
    - investigate alternative swab materials and their effect upon bacterial recovery

- Investigate alternative surface sampling techniques but in addition work to increase the speed and sensitivity of traditional swab-based microbiological methods

- Refine the performance characteristics of the rapid enzymatic coliform detection method developed as part of this thesis
    - assess its ability to detect the presence of coliforms within a range of different processing environments
    - investigate its ability to detect viable but non-culturable bacteria
    - determine the influence of both non-target bacteria and extraneous substances upon the chemiluminometric reaction

- Increase the range of residue detection methods available to the food industry

- Enhance the performance characteristics of the fat residue detection method developed as part of this thesis
    - investigate alternative swab/surface wetting solutions
    - assess the effect of a mixed sudan solution upon overall assay performance

- Construct a model food soil, comprising fat, carbohydrate, protein and microorganisms. Inoculate a test surface and 'clean' using sanitation procedures of increasing hygienic rigorousness. Assess the ability of different test methods to detect the presence of contaminating debris.

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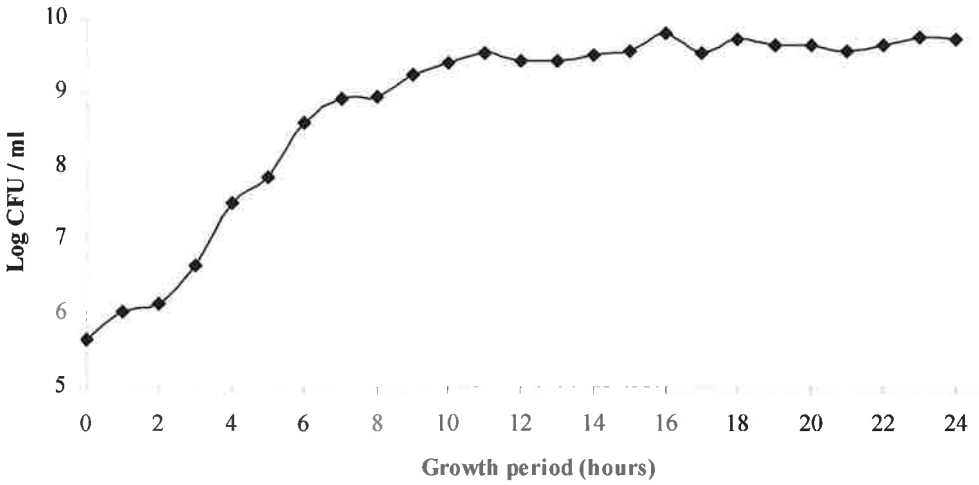
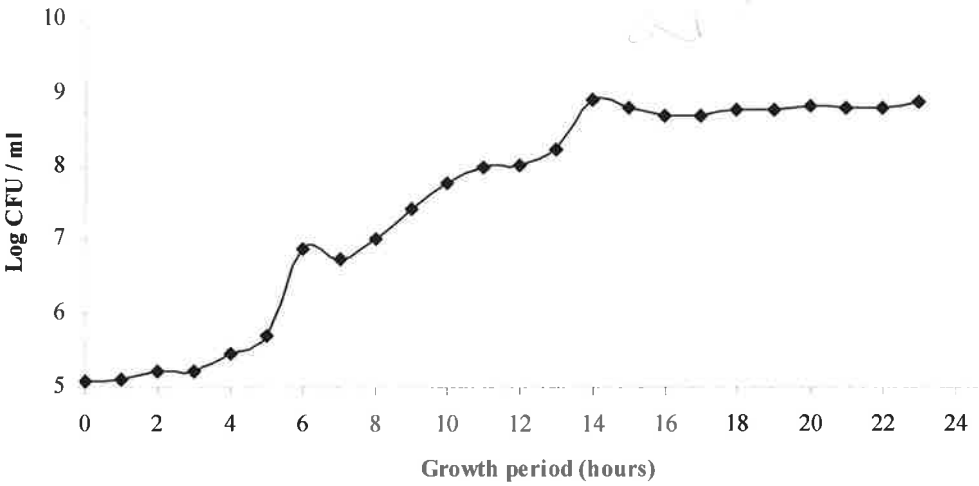
Appendix I. Bacterial Growth Curves (see Section 2.2.2)

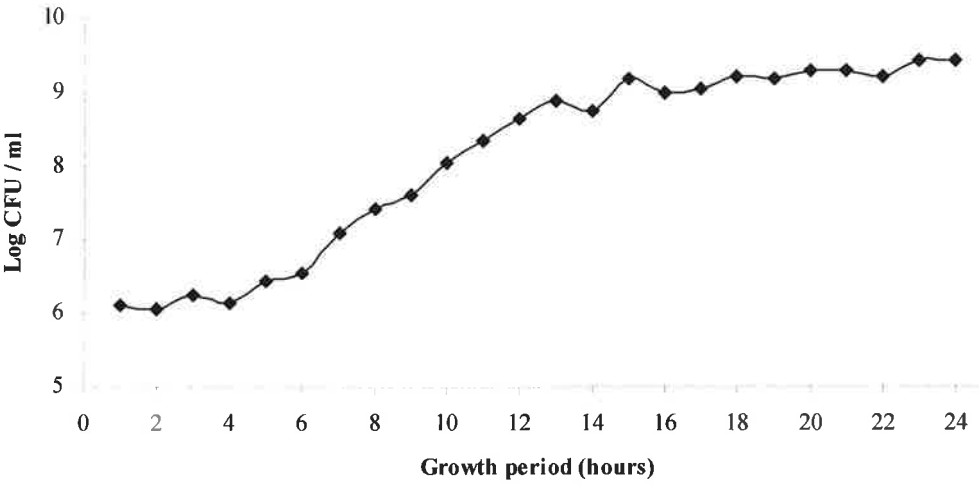
***Salmonella* sp.**



***Listeria* sp.**



Pseudomonas aeruginosa



Appendix II. Detection of variety of different food residues from a wet stainless steel surface using a range of different test methods (see Chapter 3)

Food Type	Minimum detection limit (dilution factor)									
	ATP bioluminescence									
	Swab plate	Pour plate	Dipslide	CleanTrace / UniLite	PocketSwab / Firefly	PHMK	Protect	Check Pro	Check It	VERIcleen
Raw chicken	1:1000	1:1000	1:1000	1:100	1:100	1:10	1:100	1:50	1:100	1:10
Ready-to-eat ham	ND	ND	ND	ND	ND	ND	ND	ND	1:10	
Carrot	1:1000	1:50	1:1000	1:1000	1:1000	ND	ND	ND	ND	1:1000
Raw minced beef	1:1000	1:1000	1:1000	1:1000	1:1000	1:100	1:100	1:100	1:100	1:10
Meat drip	1:1000	1:100	1:1000	Neat	1:10	Neat	Neat	1:10	1:100	
UHT milk	ND	ND	ND	Neat	1:10	1:10	1:10	1:10	1:100	
Milk	ND	ND	Neat	1:100	1:100	1:10	1:50	1:50	1:50	1:10
Rice	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Tomato	ND	ND	ND	1:10000	1:10000	ND	ND	ND	ND	1:1000
Salami										1:100
Egg										1:300
Ice cream										1:3000
Orange juice										1:3000
Coke										1:3000
Marmalade										1:3000
Cooked chicken										1:30

ND: non detectable

Appendix II. Detection of variety of different food residues from a dry stainless steel surface using a range of different test methods (see Chapter 3)

Food Type	Minimum detection limit (dilution factor)									
	ATP bioluminescence									
	Swab plate	Pour plate	Dipslide	CleanTrace / UniLite	PocketSwab / Firefly	PHMK	Protect	Check Pro	Check It	VERIcleen
Raw chicken	1:50	1:10	1:100	1:100	1:100	1:10	1:50	1:500	1:100	1:50
Ready-to-eat ham	ND	ND	ND	ND	ND	ND	ND	1:50	1:10	
Carrot	ND	ND	1:100	1:1000	1:1000	ND	ND	ND	ND	1:1000
Raw minced beef	1:100	1:100	1:1000	1:1000	1:1000	1:10	1:10	1:1000	1:100	1:30
Meat drip	1:100	1:10	1:100	1:100	1:100	Neat	Neat	1:10	1:10	
UHT milk	ND	ND	ND	ND	Neat	Neat	1:10	1:100	1:100	
Milk	ND	ND	Neat	1:10	1:10	1:10	1:10	1:100	1:50	1:50
Rice	ND	ND	ND	ND	ND	ND	ND	1:10	ND	ND
Tomato	ND	ND	ND	1:1000	1:1000	ND	1:10	1:50	ND	1:1000
Salami										1:300
Egg										1:300
Ice cream										1:3000
Orange juice										1:3000
Coke										1:3000
Marmalade										1:3000
Cooked chicken										1:100

ND: non detectable

Appendix III. Raw data obtained from factory trials (see Chapter 4)

Product	Time	Site	Surface dryness	Visual assessment	ATP count	ATP result	Protein level	Protein result	ACC count	ACC result
Cheese	BC	Bowl mixer 1	dry	dirty	9054	Fail	4	Fail	hg	Fail
Cheese	BC	Waxing table (under)	dry	clean	1055	Fail	1	Pass	15	Pass
Cheese	BC	Churn of reiser	dry	dirty	5469	Fail	4	Fail	hg	Fail
Cheese	BC	Bowl of reiser	dry	dirty	1885	Fail	4	Fail	mg	Fail
Cheese	BC	Table top	dry	dirty	5873	Fail	4	Fail	54	Pass
Cheese	BC	Floor	wet	dirty	3937	Fail	4	Fail	mg	Fail
Cheese	BC	Wall of drain	dry	clean	2381	Fail	4	Fail	9	Pass
Cheese	BC	Wall	dry	clean	2230	Fail	1	Pass	6	Pass
Cheese	BC	Screws	dry	dirty	1364	Fail	4	Fail	hg	Fail
Cheese	BC	Mixer (outer surface)	dry	dirty	255	Pass	4	Fail	sg	Fail
Cheese	AC	Bowl mixer 1	wet	clean	124	Pass		FALSE	0	Pass
Cheese	AC	Waxing table (under)	dry	clean	721	Fail	2/3	Fail	96	Fail
Cheese	AC	Churn of reiser	wet	clean	36	Pass	1	Pass	mg	Fail
Cheese	AC	Bowl of reiser	wet	clean	10	Pass	1	Pass	mg	Fail
Cheese	AC	Table top	dry	clean	20	Pass	1/2	Caution	88	Fail
Cheese	AC	Floor	wet	clean	264	Pass	2	Caution	sg	Fail
Cheese	AC	Wall of drain	wet	dirty	2977	Fail	4	Fail	sg	Fail
Cheese	AC	Wall	dry	clean	29	Pass	1	Pass	1	Pass
Cheese	AC	Screws	dry	clean	707	Fail	4	Fail	61	Pass
Cheese	AC	Mixer (outer surface)	dry	clean	358	Pass	2/3	Fail	28	Pass
Frozen Ready-meals	BC	Line B	dry	clean	56853	Fail	1	Pass	1	Pass
Frozen Ready-meals	BC	Metal meat tray	dry	dirty	278934	Fail	4	Fail	14	Pass
Frozen Ready-meals	BC	Table (Line C)	dry	dirty	14642	Fail	2/3	Fail	62	Pass
Frozen Ready-meals	BC	Boiler (inner surface)	dry	clean	91	Pass	1	Pass	1	Pass
Frozen Ready-meals	BC	Boiler (lid)	dry	dirty	21549	Fail	1	Pass	5	Pass
Frozen Ready-meals	BC	Plastic hopper (bowl)	dry	dirty	overload	Fail	1	Pass	0	Pass
Frozen Ready-meals	BC	End table (Line A)	dry	clean	873	Fail	4	Fail	18	Pass
Frozen Ready-meals	BC	Food bin / trolley	wet	dirty	5961	Fail	4	Fail	4	Pass
Frozen Ready-meals	BC	Scales (Line B)	dry	clean	1553	Fail	1	Pass	11	Pass
Frozen Ready-meals	BC	Line A (belt)	wet	dirty	55	Pass	4	Fail	105	Fail
Frozen Ready-meals	AC	Line B	dry	clean	1744	Fail	1	Pass	116	Fail
Frozen Ready-meals	AC	Metal meat tray	wet	clean	69486	Fail	1	Pass	21	Pass
Frozen Ready-meals	AC	Table (Line C)	wet	clean	3933	Fail	1	Pass	0	Pass
Frozen Ready-meals	AC	Boiler (inner surface)	wet	clean	14453	Fail	1	Pass	7	Pass
Frozen Ready-meals	AC	Boiler (lid)	wet	clean	4920	Fail	1	Pass	10	Pass
Frozen Ready-meals	AC	Plastic hopper (bowl)	wet	clean	414	Pass	1	Pass	67	Fail
Frozen Ready-meals	AC	End table (Line A)	wet	clean	688	Fail	1	Pass	8	Pass
Frozen Ready-meals	AC	Food bin / trolley	dry	clean	14666	Fail	1	Pass	41	Pass
Frozen Ready-meals	AC	Scales (Line B)	dry	clean	123	Pass	1	Pass	1	Pass
Frozen Ready-meals	AC	Line A (belt)	wet	clean	2926	Fail	1	Pass	27	Pass

Product	Time	Site	Surface dryness	Visual assessment	ATP count	ATP result	Protein level	Protein result	ACC count	ACC result
bakery	BC	Mixer blade (pastry)	dry	dirty	1192	Fail	3/4	Fail	13	Pass
bakery	BC	Mixer bowl (pastry)	dry	dirty	1231	Fail	2/3	Fail	sg	Fail
bakery	BC	Microwave turntable	dry	clean	713	Fail	3	Fail	34	Pass
bakery	BC	Pastry cutter line	dry	dirty	844	Fail	4	Fail	hg	Fail
bakery	BC	Mixing bowl (bakery)	dry	dirty	4386	Fail	3/4	Fail	33	Pass
bakery	BC	Scales	dry	dirty	3393	Fail	3	Fail	hg	Fail
bakery	BC	Bread roll tray	dry	clean	437	Pass	3	Fail	35	Pass
bakery	BC	Pallet knife	dry	clean	209	Pass	1/2	Caution	64	Fail
bakery	BC	Table (pastry)	dry	dirty	282	Pass	4	Fail	115	Fail
bakery	AC	Mixer blade (pastry)	wet	clean	516	Fail	3/4	Fail	15	Pass
bakery	AC	Mixer bowl (pastry)	dry	clean	131	Pass	3	Fail	3	Pass
bakery	AC	Microwave turntable	dry	clean	66	Pass	1	Pass	47	Pass
bakery	AC	Pastry cutter line	dry	clean	79	Pass	2/3	Fail	28	Pass
bakery	AC	Mixing bowl (bakery)	dry	clean	11403	Fail	4	Fail	hg	Fail
bakery	AC	Scales	dry	clean	47	Pass	2/3	Fail	34	Pass
bakery	AC	Bread roll tray	dry	clean	3652	Fail	2/3	Fail	103	Fail
bakery	AC	Pallet knife	wet	clean	38	Pass	1	Pass	6	Pass
bakery	AC	Table (pastry)	wet	clean	150	Pass	1/2	Caution	39	Pass
meat	BC	Rotating table (top)	dry	dirty	6258	Fail	3/4	Fail	sg	Fail
meat	BC	Rotating table (under)	dry	clean	193	Pass	1	Pass	2	Pass
meat	BC	Stripping table (top)	wet	dirty	5168	Fail	4	Fail	vhg	Fail
meat	BC	Stripping table (grill)	wet	dirty	2769	Fail	4	Fail	hg	Fail
meat	BC	Stripping table (under)	dry	clean	1185	Fail	1/2	Caution	0	Pass
meat	BC	Bagging table (top)	wet	dirty	11291	Fail	1	Pass	0	Pass
meat	BC	Bagging table (under)	dry	clean	3376	Fail	4	Fail	hg	Fail
meat	BC	Knife	wet	dirty	12041	Fail	4	Fail	vhg	Fail
meat	BC	Sink	dry	clean	36	Pass	1	Pass	0	Pass
meat	BC	Door handle (to chiller)	dry	clean	22673	Fail	1	Pass	2	Pass
meat	BC	Rotating table (top)	dry	dirty	21693	Fail	4	Fail	mg	Fail
meat	BC	Rotating table (under)	dry	clean	49	Pass	1	Pass	1	Pass
meat	BC	Stripping table (top)	wet	dirty	22704	Fail	4	Fail	45	Pass
meat	BC	Stripping table (grill)	wet	dirty	157088	Fail	4	Fail	mg	Fail
meat	BC	Bagging table (top)	wet	dirty	75785	Fail	4	Fail	2	Pass
meat	BC	Bagging table (under)	wet	clean	375	Pass	1	Pass	6	Pass
meat	AC	Rotating table (top)	wet	clean	885	Fail		FALSE	2	Pass
meat	AC	Rotating table (under)	wet	clean	162	Pass		FALSE	3	Pass
meat	AC	Stripping table (top)	dry	clean	14	Pass	1	Pass	1	Pass
meat	AC	Stripping table (grill)	dry	clean	25	Pass	1	Pass	0	Pass
meat	AC	Stripping table (under)	wet	clean	152	Pass	1	Pass	4	Pass
meat	AC	Bagging table (top)	wet	clean	80	Pass		FALSE	0	Pass
meat	AC	Bagging table (under)	wet	clean	49	Pass	1	Pass	0	Pass
meat	AC	Knife	dry	clean	574	Fail	1	Pass	41	Pass
meat	AC	Sink	dry	clean	48	Pass	1	Pass	0	Pass
meat	AC	Door handle (to chiller)	dry	clean	2906	Fail	1	Pass	0	Pass
meat	AC	Rotating table (top)	wet	clean	327	Pass	1	Pass	2	Pass
meat	AC	Rotating table (under)	wet	clean	52	Pass	1	Pass	4	Pass
meat	AC	Stripping table (top)	wet	clean	1881	Fail	1	Pass	mg	Fail
meat	AC	Stripping table (grill)	wet	clean	49	Pass	1	Pass	9	Pass
meat	AC	Bagging table (top)	wet	clean	63	Pass	1	Pass	0	Pass
meat	AC	Bagging table (under)	wet	clean	108	Pass	1	Pass	0	Pass

Appendix IV (see Chapter 6)

Assay Stability trials: Methods

Growth medium (LNM/neutralising solution mix)

Pipette 100 µl of growth medium (stored (< 5°C) and fresh) into two separate cuvettes
Add 20 µl of appropriate microbial dilution
Incubate for 4 h at 37°C
Add 100 µl of polymyxin B to cuvettes
Incubate for 1 min at room temperature
Add 200 µl of reaction buffer
Incubate for 1 h at room temperature
Attach Hold-Tite and measure light output using Biotrace UniLite
Compare RLU values

Buffer diluent

Pipette 100 µl of microbial dilution (pre-incubated for 4 h) into a cuvette
Add 100 µl of polymyxin B to cuvette
Incubate for 1 min at room temperature
Pipette 300 µl of reaction buffer (made using either stored or fresh buffer diluent) into second cuvette
Transfer 20 µl of microbial/extractant mix to reaction buffer
Incubate for 1 h at room temperature
Attach Hold-Tite and measure light output using Biotrace UniLite
Compare RLU values

Galacton-Star®/polymyxin B mix

Pipette 20 µl of microbial dilution (pre-incubated for 4 h) into a cuvette
Add 100 µl of Galacton-*Star*®/polymyxin B mix (stored and fresh) to separate cuvettes
Add 200 µl buffer diluent
Incubate for 1 h at room temperature
Attach Hold-Tite and measure light output using Biotrace UniLite
Compare RLU values