An Evaluation of Some Microbiological and ATP Bioluminescence Methods for the Recovery and Detection of Bacterial Contamination from Food Contact and Environmental Surfaces

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May 2001

A thesis submitted to the University of Wales in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the School of Applied Sciences at the University of Wales Institute, Cardiff.

Contents

Declaration

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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STATEMENT 1

This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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Abstract

Plant hygiene and food contact surface cleanliness are key prerequisites to the management of food quality and safety, and may form a critical control point within Hazard Analysis Critical Control Point food safety management systems. Several methods exist with which to monitor food contact surface cleanliness, with a recent survey of the UK food industry indicating that ATP bioluminescence, cotton hygiene swabbing and agar contact methods are the most commonly adopted. Despite their widespread use, little is known about the relative efficiency with which these methods recover contaminating surface bioburden. The purpose of the work reported was to critically evaluate these hygiene methods for assessing food contact and environmental surface cleanliness within the food industry.

On surfaces sampled while dry, cotton swabbing was found to be the least efficient of the methods, with bacterial recovery rates ranging from < 0.1% on surfaces sampled while dry, and from 0.25% to 16% on surfaces sampled while wet. Minimum detection limits (MDLs) ranged from 10^2 to 10^8 cfu/100 cm^2 depending upon surface moisture level, organism type and the nature of the organism release method used. Absolute recovery rates were influenced by organism type and by a number of sampling variables, with surface moisture level having the greatest effect on recovery. Organism recovery rates were not found to vary greatly over swab storage times typical of those found in industry during swab transportation, but the method was found to have poor reproducibility with coefficients of variation of up to 164% being recorded for sampling marginally unclean stainless steel surfaces.

Agar contact dip slides were found to be more reproducible than cotton swabbing, with minimum detection limits on inoculated surfaces sampled while wet being consistent at 10^2 cfu/100 cm^2, and from 10^2 cfu/100 cm^2 to $>10^7$ cfu/100 cm^2 on inoculated surfaces sampled while dry.

Different ATP detection systems were found to have different minimum detection limits when individual components of total ATP detection limit were evaluated. These ranged from 10^4 to 10^6cfu/100 cm^2 when used to sample inoculated stainless steel surfaces while dry. On identical inoculated surfaces sampled when either wet or dry, the minimum detection limit was found to be consistent at 10^4cfu/100 cm^2. A technique for determining microbial ATP levels was developed. Microbial ATP values from a range of food contact and environmental surfaces within different food processing environments correlated well with microbial colony count data, with R^2 values ranging from 0.65 to 0.93 before cleaning and from 0.50 to 0.94 after cleaning.

Results are discussed within the context of surface cleanliness assessment in the food industry and should help industry develop appropriate strategies for surface hygiene monitoring.

List of Publications and Presentations

Publications

Davidson, C.A., Griffith, C.J., Peters, A.C., & Fielding, L.M., (1999). Evaluation of Two Methods for Assessing Surface Cleanliness – ATP Bioluminescence and Traditional Hygiene Swabbing. *Luminescence,* 14:33-38.

Griffith, C.J., Davidson, C.A., Peters, A.C., & Fielding, L.M. (1997). Towards a Strategic Cleaning Assessment Programme: Hygiene Monitoring and ATP Luminometry, An Options Appraisal. *Food Science and Technology Today,* 2: 15-24.

Griffith, C.J., Davidson, C.A., & Fielding, L.M. (1997). Casting Light on Reproducibility. *International Food Hygiene,* 8:35-36.

Presentations

Davidson, C.A., Griffith, C.J., Peters, A.C., & Fielding, L.M., (2000). A Comparison of ATP Bioluminescence and Microbiological Methods for Assessing Food Contact Surface Cleanliness. IAFP 87th Annual Meeting, Atlanta, USA.

Peters, A.C., Griffith, C.J., Fielding, L.M., & Davidson, C.A. (1998). Validating ATP bioluminescence in Hygiene Monitoring. Microbiological Methods Innovation Forum, Chipping Campden, Glocs.

Davidson, C.A., Griffith, C.J., Peters, A.C., & Fielding, L.M. (1997). Assessing Surface Cleanliness – An Integrated Approach using ATP Bioluminescence and Microbiological Analysis. IAMFES 84th Annual Meeting, Orlando, USA.

Davidson, C.A., Griffith, C.J., Peters, A.C., & Fielding, L.M. (1997). Sensitivity and Reproducibility of ATP Bioluminescence in Determining Microbial Contamination. American Society for Microbiology, 97th General Meeting, Miami, USA.

Davidson, C.A., Peters, A.C., Griffith, C.J., & Fielding, L.M. (1997). Monitoring Surface Contamination within HACCP Plans. 1st International Conference on HACCP. The Netherlands.

Davidson, C.A., Griffith, C.J., & Fielding, L.M. (1997). ATP Bioluminescence and the Validation and Monitoring of Cleaning Programmes. *Journal of Bioluminescence and Chemiluminescence,* 12:96.

Davidson, C.A., Griffith, C.J., Peters, A.C., & Fielding, L.M. (1996). A Comparison of ATP Bioluminescence and Microbiological Analysis in the Assessment of Food Contact Surface Cleanliness. MAFF/BBSRC Link Programme – Hygienic Food Processing for Food Engineers and Microbiologists. London.

Acknowledgements

I remain deeply indebted to so many people for their help and encouragement throughout the research for, and writing of this thesis. In particular, most sincere thanks are extended to the members of my supervisory team, Dr. Chris Griffith, Dr. Adrian Peters, and Dr. Louise Fielding for their friendship, academic support and never ending patience throughout the duration of this research project. For showing me just how exciting (and frustrating!) research can be, and for not having allowed me to give up during the tough times, I thank them all. Without their never-ending help and infinite patience this project would never have been completed.

The work reported on ATP Bioluminescence would not have been possible without the very generous support from the following companies: Biotrace Ltd., Celsis Lumac, Charm Sciences, Hughes Whitlock, Idexx, Merck Ltd., and Rhone Poulenc. Special thanks to Dr. Catherine Ramsay, Technical Director of Biotrace Ltd., for many invaluable discussions and suggestions.

I am particularly grateful to Mr. David Lloyd of the Food Industry Centre in UWIC for kindly arranging the factory visits that enabled the research for Chapter Five to be executed. Sincere thanks are also due to all the companies within the Welsh Food Industry who so willingly agreed to participate in the study, and without whose help the work would not have been possible.

For technical assistance I am extremely grateful to Mrs. Jean Fleri, Mr. Mark Huntley and Mrs. Carole Tucker, and for having done an excellent job of turning hand-scribbled pieces of paper into legible tables and figures, I thank Mrs. Linda Morris, Miss. Sarah Probert and Mrs. Sue Wild. For having given so generously of their time to help format this thesis, I am extremely grateful to my very good friends Lydia Davies and Chris Longford. For providing that never-ending supply of fresh ground coffee, very special thanks Lydia. I haven't forgotten about those Welsh cakes!

Special thanks must go to my research colleagues, Elizabeth Redmond, Dr. Wendy Harrison, Matthew Mortlock, Ginny Moore, Carys Davies, Dr. Karen Elvers and Dr. Gareth Rees for their

help and friendship over the past five years. Special thanks are also extended to all my other friends and colleagues in the School of Applied Sciences at UWIC for their support and encouragement, especially while writing-up.

Heartfelt thanks are also extended to my many very good friends out with UWIC for their continued friendship and support throughout the period of this research project that I (and them too, I guess!!) often thought would never end. Very special thanks to you all: Dr. Collin Royed, Dr. Nick Dudley Ward, Frederic Gaudin, Thomas Heidenfeld, Mark Christie, Allen Doherty, Dr. Ajay Shah, John Rodger, Linda Canning, Morag Boyd, Dr.Donald Stewart, Dr. Charles Juwah, Allison Tennant, Gwyn Hicks, Emil Carvalho, Dr. Nizam Badri, Rosemary Hawkin, Dr. Hilary Dodson, Terry Sheerin, Pankaj Desai, Andrea Coe, Dr. Anne Doherty, Dr. Denise Mortimore, Professor Rajat Roy, and Dr. Kofi Aidoo for all your support, patience and understanding through the many ups and downs during my time as a Ph.D. researcher – a once in a lifetime experience which I have no desire to repeat ever again!

Last, but by no means least, it is with love and gratitude that I thank my parents for all that they themselves have sacrificed to give to me, not only throughout my Ph.D. research, but throughout the many years I have spent in pursuit of knowledge. Without their very generous financial support, and faith in my ability to succeed, I would never have even started, far less reach the finishing line. It is to them that I owe the most, and to whom I dedicate this thesis.

The secret of getting ahead is getting started. The secret of getting started is breaking your complex overwhelming tasks into small manageable tasks, and then starting on the first one.

Mark Twain.

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Chapter 1

Introduction and Review of Literature

1.0 Introduction

Never before has the quality and safety of food been in the public domain to quite the same extent as it has been over the past few years (Wolf and Lechowich, 1989; Frewer *et al.,* 1996; Barendsz, 1998). Constant media and press coverage have heightened consumer awareness of both the quality, and perhaps more importantly, the microbiological safety of food (Ollinger-Snyder and Matthews, 1994). Whereas a decade ago the names of bacteria were the domain of microbiologists only, today the names of organisms such as *E. coli* and *Salmonella* have become much more familiar household terms.

This increase in public awareness of the importance of, and need to ensure, food quality and safety has, in part at least, been due to the increase in the number of reported cases of food contamination, of whatever sort, that has been seen nearly every year (Ollinger-Snyder and Matthews, 1994; Baird-Parker, 1994; Sharp and Reilly, 1994; Gould *et al,* 1995). Undesirable microbiological contamination of food, whether in terms of food spoilage or food poisoning, may be attributed to a number of factors, including improved reporting mechanisms for food poisoning; changes in agricultural practices; changes in food marketing and eating habits; identification of new pathogens, and the development of improved microbiological methods resulting in more reliable detection of such organisms.

One of the most important means by which industry is able to help prevent food contamination, and reduce the potential risks from food related illness, is through the implementation of effective cleaning programmes that are part of good hygiene practice. In addition, food safety management systems such as HACCP, which to be successful, are dependent upon the

generation of real-time results, often identify the cleanliness of food contact surfaces as being critical to ensuring food quality and safety (Griffith *et al.,* 1997), to the extent that cleaning is a pre-requisite to effective HACCP implementation, and may be designated as a critical control point.

Several techniques are available with which to monitor the effectiveness of surface cleaning protocols within industry, and these include microbiological and non-microbiological monitoring methods (Griffith *et al.,* 1997). The overarching aim of this thesis is, therefore, to critically evaluate these methods for use within the food industry for assessing surface cleanliness.

1.1 Microorganisms and Food

Human survival is dependent on the consumption of food, and it is expected that the food people eat is both safe and of an acceptable quality (Waites and Arbuthnott, 1990). Only food that is known to be free from chemical, physical and undesirable microbiological contamination will be of the nature and quality demanded by the consumer (Wijtzes *et al.,* 1998). In spite of this a significant volume of the total food sold each year in the UK and elsewhere is contaminated resulting in human illness or unnecessary deterioration (Baird-Parker, 1994). This in turn has led to a lack of consumer confidence and increased concern over the microbiological quality and microbiological safety of the food we eat, with Bruhn (1997) reporting that microbiological safety is consumers' most frequently volunteered concern, which Flores (1991) indicates is a critical issue for consumers' and producers alike.

This concern might, in part at least, be due to the fact that throughout the world the manufacture, distribution and retailing of food has become a highly complex business (Zink 1997; McMeekin *et al.,* 1997). Raw materials are sourced on a global scale, wide ranges of processing technologies are used, and the number of food products available for purchase by the consumer continues to increase annually (Jouve *et al.,* 1999).

The complexity of the food chain as we enter the new millennium therefore necessitates the development of comprehensive control procedures and monitoring systems in order to ensure that the consumer can have confidence in both the quality and microbiological safety of food consumed. It is therefore necessary that at all stages in the food chain, from raw material through to product sale, consideration be given to the quality and safety issues associated with food production.

1.1.1 Food Spoilage, Quality and Shelf Life

All food was once living tissue, of organic origin, and it is because of its organic nature that food is susceptible to deterioration or spoilage by microorganisms. Microbial spoilage leads to undesirable sensory changes in food and may also compromise the food in terms of its safety and quality for consumption.

While the dictionary definition of quality includes some mention of "degree of excellence" possessed by a particular product, food quality is the result of numerous factors including physical, biochemical and microbiological characteristics (Wijtzes *et al.,* 1998). The official International Standards Organisation (ISO) definition of quality is the totality of features and characteristics of a product or service that bear on its ability to satisfy stated or implied needs (ISO 8402-1994). Quality might therefore be described simply as "fitness for use" (Oakland, 2000). In the microbiological context, quality comprises three aspects that include safety, acceptability/shelf-life and consistency. It is important that a food does not contain levels of a pathogen or its toxin likely to cause illness when the food is consumed. Nor should the food contain levels of microorganisms sufficient to render it organoleptically spoiled (Buchanan, 1997). It is also essential that food be of a consistent quality with respect to both its safety and shelf life. Rejections of a food on the basis of quality is often the result of decay in the widest sense of the word, with the presence or growth of microorganisms being major problems relating to food quality and safety.

In order to ensure that the safety and quality of foods are not affected through microbiological contamination from equipment surfaces during manufacture, and during storage and transportation, it is important that the hygienic condition of such surfaces are controlled through routine cleaning and disinfection (Dunsmore, 1981), where the purpose of these processes is to remove all undesirable material including food residues, microorganisms and foreign bodies that may pose a risk to the quality or safety of food products (Holah, 1995). It is important that microbial numbers are reduced to an acceptable level, and that numbers of pathogens are reduced to safe levels. However, despite the importance of cleaning and disinfection in the food industry, there is still a lack of information pertaining to the efficiency with which various hygiene methods are able to assess the effectiveness of cleaning processes. In addition, if the hygienic condition of food contact and equipment surfaces is to be improved, the performance of the cleaning processes must also be improved, and this must involve some form of monitoring of the effectiveness of the processes used (Dunsmore, 1981).

The two groups most actively engaged in determining and controlling the microbiological quality of foods are the food industry and certain regulatory bodies (Jouve, 1999), with the extent of their intervention being determined by the food legislation of the country in which they operate. Procedures currently in use that assess and control possible deviations from quality and safety standards include Good Manufacturing Practice (GMP) and the Hazard Analysis Critical Control Point (HACCP) system, both of which may identify the cleaning of food contact surfaces as being critical to ensuring food quality and safety which, if compromised, may have a significant impact on the credibility of food manufacturers (McMeekin *et al.,* 1993). Additionally, cleaning is a legal requirement identified in both the Food Safety Act (1990) and the 1995 Food Safety (General Food Hygiene) Regulations. One of the consequences of food contamination due to poor implementation of cleaning programmes is the potential for foodborne illness.

1.1.2 Foodborne Illness

While spoilage of foods due to the action of microorganisms is important in terms of quality, of greater significance in terms of human health is foodborne transmission of pathogenic and toxigenic microorganisms, which has been a recognised hazard for decades (Foster, 1997). Although most microorganisms are not harmful to humans, with some being beneficial, many microorganisms are capable of causing disease (Bower and Daeschel, 1999). Over the past decade Britain, along with many other countries, has seen a dramatic increase in the incidence of reported foodborne illness (Pritchard and Walker, 1998), which continues to present itself as a major problem of both health and economic significance (Griffith *et al.,* 1998). Despite improvements in both medicine and food science and technology, foodborne illness has been described as one of the most widespread problems of the contemporary world (Notermans *et al.,* 1994), with notified incidence having increased worldwide (Todd, 1989; Maurice, 1994), with the range of organisms capable of causing disease now being more extensive (Notermans *et al.,* 1994). Of particular concern has been the increase of enterohaemorrhagic *Escherichia coli* in addition to Rotavirus and Small Round Structured Virus (SRSV) infections (Sockett *et al.,* 1993).

In 1990, an average of 120 cases of foodborne illness per 100,000 of population were reported from 11 European countries, with more recent estimates indicating that in some European countries there are at least 30,000 cases of acute gastroenteritis per 100,000 population annually (Notermans and van der Giessen, 1993) much of which was thought to be foodborne. It is also clear that the extent of foodborne illness, and the data from national surveillance of infectious intestinal disease represent only a small proportion of the total number of people affected (Richmond *et al.,* 1990; Wall *et al.,* 1996; Handysides, 1999). A similar situation is reported elsewhere with the World Health Organization estimating that in most European countries only about 10% of incidents are reported (WHO, 1992). In a UK study looking at infectious intestinal diseases in the community it was found that one in five members of the population of England (9.4 million cases) develops infectious intestinal disease in a year (Wheeler et *al.,* 1999), with 17-45% of these cases thought to be attributable to some form of food related illness.

The economic costs of food poisoning, therefore, represent a significant burden to the economy (Griffith *et al.,* 1994). There is obviously a health cost to be paid by the individual suffering from the illness, but increasingly the wider economic costs are being recognised (Pritchard and Walker, 1998). Estimates on the economic consequences of foodborne illness (Sockett, 1991) suggest that in England and Wales in 1991, for example, some 23,000 cases of salmonellosis were estimated to have resulted in an overall cost of between £40 and £50 million. An estimated loss of approximately eight million working days has been proposed (Aston and Tiffney, 1993), while the annual national cost has been estimated at between £500 million and £1 billion (Sockett, 1993). The cost to the health service over a three-year period has been calculated at £83,139,685, for inpatient treatments of infectious intestinal diseases (Djuretic *et al,* 1996). Most recently, the Food Standards Agency estimate that the average cost of a case of infectious intestinal disease in England is £79, with the total cost amounting to at least £743 million p.a. (Food Standards Agency, 2000). The management and control of food quality and safety is, therefore, an important issue for both Government and the food industry

1.2 The Management and Control of Food Quality and Safety

1.2.1 The Role of Government

Government plays a key role in ensuring the quality and safety of the food supply through a number of pieces of legislation. Such legislation is the product of a long evolution, and is aimed at supporting and encouraging the supply of safe, wholesome food (Craddock, 1999). Important is the need for food legislation to be based upon scientific evidence, and which must be acceptable to all relevant stages of the food chain (Craddock, 1999). Current food legislation, which is discussed more specifically in Section 1.2.2, acknowledges the need for routine cleaning. It is important, especially where the cleanliness of surfaces is designated as a CCP within HACCP, that the methods of assessing surface cleanliness are reliable. Determining the reliability of these methods can only be done through the generation of scientific evidence on the performance of the methods. The work of this thesis aims to contribute to this aim.

Long before the recent increased interest in the quality and safety of the food supply, national governments had already taken responsibility for the protection of public health, and the EU is now considering what changes might be necessary to current legislation, be it a general directive on food law, consolidation and simplification or reformulation of existing legislation, or recommendations of a non-legislative nature. The underpinning aims for all future food legislation include a high level of protection of consumer health and safety. That is legislation that is primarily based on scientific evidence and risk assessment (Jouve, 1998). The research findings reported in this thesis attempt to provide some scientific evidence on the efficiency with which selected hygiene monitoring methods are able to assess food contact and environmental surface cleanliness.

1.2.2 Food Safety Legislation

While much food legislation has been developed in a co-ordinated manner, other legislation has been introduced in response to specific issues at any given time. This has lead to a marked lack of consistency in terms of the level of detail given, with European Food Hygiene Legislation being a good example (Craddock, 1999). Future developments in food safety legislation need, therefore, to reconcile science, the interests of consumers, the legitimate interests of food operators and the exigence of free trade (Jouve, 1998).

The EC Commission's Food Hygiene Directive (93/43/EEC) requires all food businesses that carry out production, processing, manufacturing, packaging, storing, transportation, distribution, handling and sale of foodstuffs to comply with certain hygiene rules (Jacob, 1992). Regard must also be taken of other aspects including critical points in process control, hygiene of premises, structure of buildings, and training of food handlers in food hygiene. Given the wide range of businesses to which the Directive applies, a series of Codes of Practice in good hygienic practice supplement the Directive and have been, or are being developed for each particular sector of the food industry.

The Codes of Practice are based on the recommended international code of practice of the Codex Alimentarius Commission of FAO/WHO. While not part of legislation, or subject to enforcement, the Good Hygiene Practice Codes in the UK are voluntary.

Council Directive 89/337/EEC on the Official Control of Foodstuffs lays down criteria for inspection of food premises unless more detailed rules apply to specific sectors. There are obligations arising from the Directive for member states to report to the EC Commission on the levels of inspection activity in the various sectors of the food industry. Provisions are also made within the Directive for a Commission inspectorate.

Codes of practice can be used to refine necessary food safety controls. Quality and safety standards are applied by the industry, and increasing emphasis on industry self-regulation using techniques such as HACCP will certainly be beneficial.

The new legislative framework outlined began to be introduced to the UK in 1990, and September 1995 saw the advent of the new Food Safety (General Food Hygiene) Regulations based upon the EC Hygiene Directive. The purpose of these regulations is to implement Council Directive 93/43/EEC on the Hygiene of Foodstuffs. These require all food business owners to identify relevant hazards and points critical to food safety, and to introduce effective controls at those points. This implicitly requires some form of risk assessment (Griffith *et al.,* 1998). These regulations also stipulate that premises must be kept clean and maintained in good repair and condition, be designed to allow proper cleaning and protect against the accumulation of dirt (Dillon and Griffith, 1999). To enable relevant hazards to be identified, the food industry is now adopting the Hazard Analysis Critical Control Point (HACCP) Food Safety Management System, in addition to good manufacturing practice and good hygiene practice, in order to ensure that the food they produce is of acceptable quality and safety.

1.2.3 The Food Industry

It is clear that food quality and safety are major concerns facing the food industry, with much publicity being associated with undesirable microbiological and chemical contamination (Jouve *et al.,* 1999). Modern trading conditions and legislation require food businesses to demonstrate their commitment to food quality and safety through the adoption of an appropriate management programme. It is important that such a programme takes account of the role of businesses in the food chain, whether they are primary producers, manufacturers, retailers or caterers. Ultimate success will be dependent upon the correct use of appropriate methods and tools identified earlier, and including Good Hygiene Practice (GHP), Good Manufacturing Practice (GMP) and the HACCP system.

1.3 Quality Management and HACCP within the Food Industry

With the demand for safer foods, new approaches to the management of food quality and safety such as HACCP, ISO, and Total Quality Management (TQM) have received widespread support (Barendsz, 1998).

Quality systems encompass the organisational structure, responsibilities, procedures, processes and the resources required to implement comprehensive quality management within a firm. Such systems are relevant to, and should interact with, all stages of a food product's cycle. It is not sufficient merely to implement Good Manufacturing Processes (GMP) in order to ensure product quality and safety (Barendsz, 1998) since GMP only represents a set of non-standardised guidelines that contribute to food production that lead to food of acceptable quality and safety. However, in conjunction with HACCP, GMP can be very effective when control measures are considered. In view of the TQM concept, it is important that the HACCP system is compatible with the ISO 9000 series as this is the global quality standard (Barendsz, 1998). For this reason, the strong points of HACCP, i.e. the systematic approach adopted, are combined with appropriate ISO 9000 standards. The standards in the ISO 9000 series are predominantly directed at the standardisation of the industrial organisation, but with little attention being given to the professional quality dimension (Barendsz, 1998). Therefore, when a HACCP food safety

management system is implemented, along with efforts with respect to TQM, it is advisable that all efforts directed towards HACCP are focussed towards realising a HACCP process control plan that satisfies the format requirements of an ISO quality plan.

Quality systems are therefore designed to ensure that all factors potentially having an effect on the quality of a food product are under control, with the control being oriented towards the reduction, elimination and prevention of deficiencies relating to product quality. The two basic functions that a quality management system must perform are quality control, which deals with the operational techniques, and an activity that eliminate causes of unsatisfactory standards, and also covers the monitoring processes, and quality assurance. Quality assurance provides confidence, both internally and externally that the food business and/or its operational processes will fulfil the requirements for quality. Because quality in its entirety is covered by this means, it is important that food safety considerations are included in a food company's quality system.

Total quality management and quality systems therefore provide the philosophy, culture and discipline necessary to commit every food business operator to the achievement of all of the company's quality objectives. Within this framework, the inclusion of HACCP as the key specific assurance plan provides the essential confidence that the food product will conform to safety requirements, and consumer expectations, in order that no microbiologically unacceptable or unsuitable product will leave the production unit. Included in this is the use of pre-requisite programmes (PRPs).

Prerequisite programmes are not part of the formal HACCP system, and may include objectives other than those specifically relating to ensuring food quality. Since it may not always be easy to associate performance of a prerequisite programme element specifically to food production, it is generally agreed that it is more effective to manage them within the overall quality framework. This is considered more appropriate than including their performance and control as part of the HACCP plan (Sperber *et al.,* 1998). Such pre-requisite programmes may, for example, include cleaning and disinfection where it would be expected that the cleaning and

disinfection activities within a specific company were being performed to a suitably high standard before implementing HACCP. It is also recognized that many of these prerequisite programmes, including cleaning and disinfection, are based upon Good Manufacturing Practices, and may include other systems such as ingredient specifications, supplier approval auditing and customer complaint management procedures.

1.3.1 HACCP Food Safety Management Systems

The adoption of the Codex Alimentarius text "Guidelines for the Application of the Hazard Analysis Critical Control Point (HACCP) System" (Codex, 1993) and its subsequent revision entitled "Hazard Analysis and Critical Control Point System and Guidelines for its Application (Codex, 1997), have provided international endorsement of the use of the HACCP system as a cost effective procedure for assuring the quality and safety of food (Mayes, 1998).

The principles of the approach have been extensively reviewed, most recently by Notermans *et al.,* 1994; Griffith and Worsfold, 1994; Notermans *et al.,* 1995; Notermans and Mead, 1996; Khandke and Mayes, 1998 and Sperber, 1998.

The HACCP system was developed in the early 1960s in the USA in order to produce safe food for the space programme (Bauman, 1990). In its original form the system consisted of three principles. Later, in the late 1980s the system was extended by the International Committee on the Microbiological Safety of Food (ICMSF) in 1988 and by the National Advisory Committee on the Microbiological Control of Food (NACMCF) in 1989 to the seven principles that have now been incorporated into Codex Alimentarius (FAO/WHO Codex Alimentarius Commission, 1997).

The HACCP system of Codex Alimentarius is a systematic approach to the identification, assessment and control of hazards within food production, and has been defined by ICMSF (1988) as:

" A systematic approach to the identification and assessment of microbiological hazards and risks associated with the manufacture, distribution, and use of a particular foodstuff and the definition of means for their control".

The process involves the evaluation of all procedures in the production, distribution and use of raw materials and food products. The seven principles are structured into three elements, namely hazard analysis (principle 1), measures for hazard control (principles 2-5), and verification and documentation of the system (principles 6-7) (Untermann, 1999).

Since its initial use some thirty years ago, practitioners now realize that the HACCP philosophy cannot be applied in a vacuum, and that it must be supported by a strong foundation of prerequisite programmes (Sperber *et al.,* 1998) that should include cleaning and disinfection.

In addition, promoted most actively is the use of risk analysis procedures, and in some cases the use of specific elements of quantitative risk analysis within HACCP systems. A number of authors have recently proposed the use of risk-based approaches within HACCP (Baird-Parker, 1994, 1995; Buchanan, 1995; Notermans and Jouve, 1995; Elliott, 1996; Notermans and Mead, 1996).

Within the context of surface cleanliness, which may be a designated CCP within HACCP, another consideration is the need to be able to validate the methods used for assessing surface cleanliness levels, and to verify that the results from monitoring using the methods are reliable. The processes of validation and verification within HACCP are therefore important.

1.3.2 Validation and Verification within HACCP

In order to ensure that the procedures put in place for controlling CCPs are effective, it is important that such procedures are monitored, validated, and verified to ensure that confidence in their ability to control the CCP(s) exists. The adoption of the Codex Alimentarius text (Alinorm 97/13A) as the authoritative text on Principles and Guidelines for HACCP has

contributed to a more harmonized approach and agreed terminology, but there is still lack of clarity over the specific activities of validation and verification in relation to monitoring CCPs (Mayes, 1999). It is important, therefore, that the relevant terminology is clarified.

Although definitions differ depending upon their source, increasingly the use of the Codex Alimentarius Alinorm definitions are becoming accepted as standard. Validation is defined as being the process of obtaining evidence that the elements of the HACCP plan are effective, with no additional explanation of the use of validation being given in the Codex text (Codex Alimentarius, 1997). Verification is defined as the application of methods, procedures, tests and other evaluations, in addition to monitoring to determine compliance with the HACCP plan (Codex Alimentarius, 1997). Codex provides additional explanatory text:

"Verification and auditing methods, procedures and tests, including random sampling and analysis, can be used to determine if the HACCP system is working correctly. The frequency of verification should be sufficient to confirm that the HACCP system is working effectively……..where possible, validation activities should include actions to confirm the efficacy of all elements of the HACCP plan".

The relationship between the activities of validation and verification still cause confusion (Mayes, 1999). Separate definitions for the two terms are provided in the Codex text, but include validation under Principle 6 – Verification, thereby promoting the concept that validation is a sub-set of verification. Confusion is maintained by Codex providing no clarification of the activities comprising validation, yet explicitly describes verification activities.

In the context of HACCP, it is not intended to apply validation or verification to the hazard analysis principle alone, nor to any other principle in isolation because all seven Principles contribute to the overall HACCP plan.

Within the specific context of Hazard Analysis, therefore, validation should be performed as part of the design of the HACCP plan prior to implementation, while verification of hazard analysis should consist primarily of an examination of the records of the validation activities to determine if there is evidence that validation has been executed correctly. An important consideration within HACCP is obviously the methods used to assess surface cleanliness, and the need for these to be properly validated. The work reported in this thesis aims to help those responsible in industry for the assessment of cleaning to better understand the way in which some of these methods recover and detect microbial contamination from food contact and environmental surfaces.

1.4 Design, Construction and Cleaning of Food Premises

1.4.1 The Design and Construction of Food Premises

Fundamental to the control of microbial contamination and its transmission within food processing environments is good hygienic design, which will help to facilitate cleaning to an appropriate standard (Holah, 1995). Such cleaning must ensure that levels of contamination present on surfaces are reduced to acceptable levels, where the risks associated with food spoilage and the potential for cross-contamination are minimised. An important aspect of such design includes the selection of appropriate materials for the construction of food contact surfaces that may be involved in direct or indirect contamination of food (Taylor and Holah, 1996).

The selection of appropriate materials for the construction of food contact surfaces is, therefore, important due to the potential risks associated with food spoilage and cross-contamination, which may in turn result in unacceptable quality changes in foods or in food poisoning. Another important issue is the potential for biofilm formation on food contact surfaces, and this has been extensively reviewed by a number of authors (Carpentier and Cerf, 1993; Zottola and Sasahara, 1994; Hood and Zottola, 1995; Ganesh Kumar and Anand, 1998). The food industry provides diverse environments suitable for biofilm formation (Peters *et al.,* 1999) and this should be a fundamental consideration in the design process.

Within good manufacturing practice, an effective control measure in attempting to limit the risks associated with contaminated food contact surfaces will include the implementation of an effective cleaning and disinfection programme. Because of the contribution made by cleaning to ensuring food quality and safety it is a legal requirement that "all articles, fittings and equipment with which food comes into contact shall be kept clean". The main purpose of ensuring high standards of cleanliness in food premises is to protect public health. The Food Safety Act 1990 makes it an offence to contaminate food, while the Food Safety (General Food Hygiene) Regulations 1995 require the proprietor of a food business to identify any step in activities of the business which are critical to ensure food quality and to introduce effective controls at those points. There is, therefore, a clear obligation in law on the business proprietor to keep food premises clean wherever there is risk to the food (Dillon and Griffith, 1999). Cleaning will, therefore, help to ensure that the transmission of microbial contamination via food contact surfaces is prevented. To be successful, it will, in part at least, be dependant upon the nature and composition of the materials used in the initial design of the working environment, with particular importance being placed upon the nature of the food contact surfaces. In addition, within HACCP, the cleanliness of food contact surfaces may be designated as a CCP.

1.4.2 The Process of Cleaning and Disinfection

Cleaning, within the context of food hygiene, is defined as any process of physical removal of "soil", i.e. any matter present that should not be part of an item, defined by Jennings (1965) as "matter out of place" and can contain microbes that are responsible for food poisoning or spoilage. Disinfection, on the other hand, is defined as the elimination of microorganisms or their reduction to acceptable levels, this being achieved either through the application of heat or chemicals. In this thesis, however, the term *cleaning* is used to describe both of these processes unless otherwise indicated.

The success of any cleaning programme will be dependant upon the integration and co-ordination of the cleaning programme with the manufacturing operations. In order to achieve effective cleaning within an operation, it is important to have an understanding of the type and nature of the soil present, information on the design and construction of the operation and the equipment present, some idea of what the cleaning process is meant to be accomplishing, and knowledge of the types of cleaning agents available and what their primary functions are. Should the cleaning programme employed not be effective, microorganisms and food product residues will remain on the surface at concentrations that may in turn affect the quality and safety of the food product.

The cleaning process involves a number of stages which includes wetting of the surface and soil by the cleaning agent, action of the cleaning agent to facilitate removal of the soil from the surface, prevention of re-deposition onto the surface, and disinfection of residual microbes (Jennings, 1965; Koopal, 1985; Holah, 1992). An important stage of the cleaning process is surface drying, which may have an effect on organism survival, in addition to the ability of some methods to recover and detect any residual organisms remaining on surfaces after cleaning has been completed. While surface drying may result in loss of viability, it may also lead to surface attachment of the organisms, and this will have implications for surface monitoring. Depending upon the effect of drying on the organisms, monitoring methods may or may not be able to recover them, and this is an important consideration in determining when monitoring should be performed.

Four main factors are involved in combination with each other that help to achieve the stages of cleaning outlined above. These are chemical energy in the form of the detergent/cleaning agent, mechanical or kinetic energy, temperature or thermal energy through the use of hot water in which the detergent is dissolved, and time. Chemical energy is important in both the cleaning and disinfection processes. In the cleaning stage, the chemicals act by breaking down surface soils and aid the reduction of their attachment strength in order to facilitate their removal from

the surface. In the disinfection phase, the viability of residual microorganisms is reduced as a result of chemical action. Mechanical or kinetic energy is employed to remove soils from surfaces physically, and may include a number of activities such as wiping, brushing, and pressure jet washing. Temperature affects cleaning and disinfection in a number of ways, with increases in temperature generally enhancing the activity of disinfectants (Gelinas *et al.,* 1984). An increase of temperature was found to greatly enhance the activity of glutaraldehyde and chlorhexidine acetate, whereas contact time mainly enhanced the efficiency of sodium hypochlorite, a quaternary ammonium compound and an amphoteric surfactant when evaluated by Gelinas *et al.*(1984). In a similar manner, increasing time through using foams or gels to increase contact time can lead to improved cleaning effectiveness through longer association of the cleaning chemical with the surface soil (Gibson *et al.,* 1999), which is in agreement with the findings of Gelinas *et al.* (1984). It is thought that it is the cleaning stage which is the most important in effecting reduction in microbial numbers on food contact surfaces (Carpentier and Cerf, 1993; Dunsmore, 1981).

A comparison of the bactericidal efficiency of eighteen disinfectants used in the food industry for destroying *Escherichia coli* O157:H7 and *Pseudomonas aeruginosa* was investigated by Taylor, *et al.,*(1999). The authors found that the disinfectants exhibiting reduced efficacy at a temperature of 10°C were amphoterics and quaternary ammonium compounds. However, Gelinas *et al.,* (1984) reported that increasing contact time was more important than temperature in the use of these compounds. Of the eighteen disinfectants tested, only eleven were found to perform effectively at both 10°C and 20°C. This work demonstrated the importance of ensuring that such products are used according to manufacturers instructions that will include an indication of what temperature to use in order to achieve optimum results. In addition, it is important that the disinfection stage of any cleaning programme is verified for its efficacy, and the methods available to establish this will be discussed in Section 1.5 – Methods for Assessing Cleaning Effectiveness. Other work reported by Ronner and Wong (1993) evaluating biofilm formation and sanitiser inactivation of *Listeria monocytogenes* and *Salmonella typhimurium* on

stainless steel and Buna-n rubber found that the type of surface, nutrient level and organism type influenced biofilm development. Buna-n rubber was found to have a strong bacteriostatic effect on *Listeria monocytogenes*, with biofilm formation being reduced under low nutrient conditions. Buna-n was, however, less bacteriostatic towards *Salmonella typhimurium,* with inhibition of growth of several other pathogens being achieved to varying degrees. Biofilms were challenged with four types of detergent and non-detergent sanitizers, and it was found that resistance to these substances was strongly influenced by the type of surface. Bacterial biofilm populations on stainless steel were reduced between three and five log cycles by all the sanitizers, but those on Buna-n were resistant to these sanitizers and were reduced by less than one to two log cycles. It was interesting to note that planktonic cells were reduced by between seven and eight logs by the same sanitizers, and that scanning electron microscopy revealed that biofilm cells and extracellular matrices remained on sanitized surfaces from which no viable cells were recovered. In another study, Frank and Koffi (1990) found that surface-adherent growth of *Listeria monocytogenes* was associated with increased resistance to surfactant sanitizers and heat. Adherent microcolony cells on glass slides decreased by two to three log cycles immediately after exposure to benzalkonium chloride and anionic acid sanitizers. The remaining cell population survived for twenty minutes demonstrating resistance to both sanitisers at all concentrations. Adherent single cells exhibited an initial three to five log cycle decline in numbers and reached undetectable levels after twelve to sixteen minutes of exposure to the sanitizers, with planktonic cells being reduced to undetectable levels after thirty seconds exposure to the lowest concentration of each sanitiser. Clearly, these findings emphasize the need to ensure that in cleaning food contact surfaces, the nature of the substances selected, and the concentration and temperature at which they are used is important in ensuring that they work effectively. The cleaning process is important since it ought to disrupt surface biofilm prior to disinfection, which is why the cleaning process is more significant than the disinfection stage.

1.4.3 The Cleanability of Stainless Steel

It has already been established that an important consideration in the selection of any material for use as a food contact surface within the food and catering industries is its ability to be cleaned and sanitised effectively. Current research reported by Steiner *et al.,* (2000) suggests that the ease of cleaning stainless steel is one of the foremost concerns relating to its use in the food and dairy industries. This in turn will help to reduce the risks of pathogen transmission through the food chain. A substantial body of work exists on the cleanability of stainless steel as a food contact surface. While it was recognised in the 1950s that stainless steel may show a low microbiological count and still retain significant quantities of soil, much of the early work on the cleanability of stainless steel involved only microbiological methods of cleanliness assessment (Holland *et. al.*, 1953; Jennings, 1959; Kaufman *et al.,* 1960; Abele 1965), and such methods are designed to detect microorganisms and not other components of total surface contamination such as organic food debris.

The work of Kaufmann (1960) investigating various microbiological methods of cleanliness assessment on dairy equipment with different surface finishes concluded that there was no significant difference in the cleanability of several different stainless steel surfaces tested, yet Stone and Zottola, (1985a) found that suboptimum cleaning protocols when used in two different clean-in-place systems did have an effect. They found that under normal clean-in-place procedures those cells that remained in the system did not exhibit attachment fibrils. Under conditions of lowered detergent water temperature and detergent and sanitizer concentrations, those cells that were present after clean-in-place showed attachment fibrils. Organisms remaining after normal clean in place were not viable, whereas those present after suboptimum cleaning were.

In contrast to Kaufman *et al.,* (1960), Masurovsky and Jordan, (1958) found that certain surface finishes did influence efficiency of cleanability, with electro polished and 240 grit finishes exhibiting superior cleanability than other finishes. Timperley and Lawson (1979) found a statistically significant relationship between surface roughness and cleanability using Clean-in-

Place (CIP) techniques for stainless steel pipe of five different finishes using microbiological methods of assessment. Ease of cleaning was reported to be related to surface roughness, with surfaces of low roughness (Ra) values being more easily cleaned, while Stone and Zottola, (1985) have illustrated the importance of using optimum cleaning conditions. Pflug, *et al.,* (1961) in a study of both cleaning and soil build up found that surface finish had a small but measurable effect on soil deposition, but no significant effect on the rate of soil removal. Holah and Thorpe (1990) in a study on the cleanability of various unused sink surfaces found no difference in cleanability, but reported that stainless steel, abraded to produce a finish not dissimilar to that observed on naturally worn domestic stainless steel sinks, was approximately ten times more cleanable than the other sink materials subjected to the same treatment. Extending the cleaning time did not enhance bacterial removal from other surfaces compared with stainless steel. These differences were thought to be caused by the characteristic surface changes due to the abrasion, with stainless steel being most resistant to surface change. Reported in a study of clean in place of stainless steel lines Holland *et al.*, (1953) found that there was no correlation between film deposition and bacterial counts and it was recommended that bacterial counts should not be used as the sole measure of cleanliness in clean-in-place lines. In assessing the relative cleanability of artificially abraded stainless steel, enamelled steel, mineral resin and polycarbonate domestic sinks, Stevens and Holah (1993) found that after a combined wiping combined with a spray wash procedure, stainless steel retained 0.5 to 1.0 log order fewer bacteria than enamel sinks which were reported to be 0.5 log order cleaner than the mineral resin and polycarbonate sinks. After spray washing only, stainless steel retained 0.5 log order fewer bacteria than enamel which in turn was 0.5 log order cleaner than the polycarbonate and mineral resin sinks.

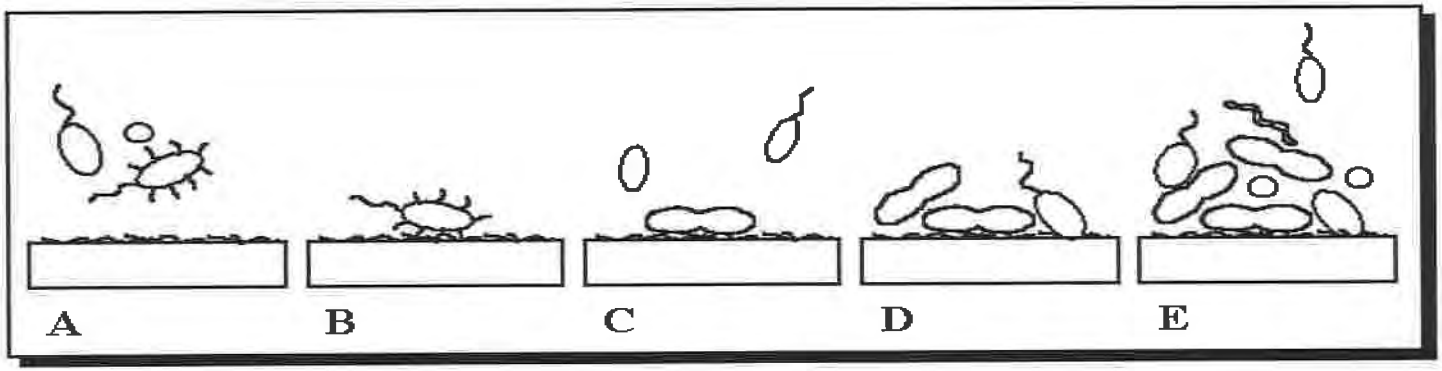
Most recently, Frank and Chmelewski (1997) investigated the relative ability of various materials used for domestic and food service sinks and worktops to be sanitised. Surfaces tested included stainless steel, polycarbonate, and mineral resin. Results indicated that stainless steel and smooth polycarbonate were more readily sanitised by quaternary ammonium compounds than the mineral resin. Chlorine was found to be most effective on mechanically polished

stainless steel, and was less effective on mineral resin surfaces. It was found that sanitation with quaternary ammonium compounds and chlorine reduced *Staphylococcus aureus* populations by a three log order on all surfaces except unabraded mineral resin.

The importance of implementing effective cleaning programmes within the food industry to help ensure that the quality and safety of the food produced is not compromised has been discussed in detail. Should cleaning programmes be inadequate, a potential problem facing the industry will be microbial attachment and biofilm formation, which will, in turn, have a marked effect on product quality and safety.

1.4.4 Microbial Attachment and Biofilm Formation

Microbial attachment and biofilm formation in food processing environments are potential sources of contamination of food that may lead to food spoilage and/or the transmission of food-borne pathogens (Hood and Zottola, 1997 a, b), and is of major health significance (Mettler and Carpentier, 1998). Biofilm formation is described as the ability of microorganisms to attach and grow on food and food contact surfaces under favourable conditions, and is particularly important since the food industry provides a number of diverse environments suitable for their development (Peters *et al.,* 1999). Figure 1.1 provides a diagrammatic representation of biofilm formation and illustrates the stages in its formation. These include the deposition of a conditioning film followed by the adhesion of planktonic cells, cell division and further attachment of new cells, the formation of micro-colonies and over a period of days, the development of a mature biofilm. The term *biofilm* refers to the biologically active matrix of cells and extracellular substances in association with a solid surface (Bakke *et al.,* 1984). Costerton *et al.,* (1985), however, describes a biofilm as being a consortium of microorganisms attached to a surface and is embedded in the extracellular polymeric substances (EPS) produced by the microorganisms. Biofilm formation specifically relating to food contact surfaces has been extensively reviewed by a number of authors, and including Notermans, *et al.,* 1991; Holah and Kearney, 1992; Mattila-Sandholm and Wirtanen, 1992; Carpentier and Cerf, 1993; Zottola and



Stage	Time scale
A: deposition of a conditioning film	seconds
B: adhesion of planktonic cells	minutes
C: cell division and further attachment	hours
D: formation of micro-colonies	hours
E: development of mature biofilm	days

Figure 1.1 Diagramatic Representation of Biofilm Formation

(Peters, A.C. & Elvers, K.T., personal communication)

Sasahara 1994; Sjoberg, *et al.*, 1995; Hood and Zottola, 1997a; Ganesh Kumar and Anand, 1998; and Peters *et al.*, 1999.

Biofilm formation is dependent upon attachment, with the mechanisms of bacterial attachment being complex, with numerous different interactions between the bacterial cell surface and the surface to which it is attached (Husmark, 1988). Of importance is the role of pili, which are thin hair-like proteinaceous appendages that are several micrometers long. These structures are generally associated with the adhesion of bacteria to surfaces, and can often be seen projecting from the surface of bacterial cells. The most generally accepted mechanisms for the attachment of bacteria to solid surfaces are a two-stage process proposed by Marshall, *et al.*, (1971) and a three-stage process suggested by Busscher and Weerkamp (1987). In the two stage process, the first step involves the bacteria being transported close enough to the surface in order that they may be adsorbed onto it, which is a reversible step. The second stage is known to be time dependent and involves the production of extracellular substances that secure the attaching bacteria to the surface. The three-stage process outlined by Busscher and Weerkamp, (1987) view the process in terms of the distance of the bacteria from the surface. At distances of more than 50nm only long range forces are in operation and the attachment is reversible. As the separation distance decreases to around 20nm, both long range and electrostatic interactions exist. It is thought that this leads to weak attachment, but that over time the attachment becomes stronger. The third stage in the attachment process, which occurs at distances of less than 15nm, involves the production of adhesive polymers that lead to strong attachment

In both these models, the final stage depends upon the ability of the microorganism to metabolise and produce adhesive material In addition to the two models of microbial attachment outlined above, attachment can also be divided into another two major types, these being specific and non-specific (Marshall, 1984). The attachment of bacterial cells to inanimate surfaces such as stainless steel and other food contact surfaces is known to follow chemical and physical laws (Husmark, 1988). In non-specific attachment the attraction and binding of the cell

to the surface are caused by hydrophobic interactions and covalent, ionic H-linkage or electrostatic interactions. Specific attachment is governed by the same physical parameters as non-specific attachment with the addition of a requirement of stereochemical interaction between the bacteria and the surface to which attachment is taking place. Distinction can also be made between passive and active mechanisms of surface attachment. When bacteria produce surface molecules, generally the accumulation of polysaccharides known as the glycocalyx, in order to influence the attachment process this is referred to as active attachment.

The attachment and colonisation of bacteria on food contact surfaces has, not surprisingly, been of considerable interest in the context of food hygiene (Ganesh Kumar and Anand, 1998). Those microorganisms found upon food contact surfaces have been reported to exist in a complex environment where surfaces, soil residues, detergent residues, moisture, temperature, the population density of the microorganisms and various other factors influence each other (Chaturvedi and Maxcy, 1969). When several biotic and abiotic elements interact in a limited space, the sum total off their interactions may be called an *ecosystem.* In applying this term to food contact surfaces, the term *hard surface ecosystem* may be used, and refers to a system capable of maintaining an equilibrium that controls microbial survival, and perhaps proliferation, which is of importance in the food industry (Chaturvedi and Maxcy, 1969).

In situations where the formation of a biofilm is considered detrimental, the term *microbial fouling* or *biofouling* is generally used (Ganesh Kumar and Anand, 1998). The term biofouling refers to the undesirable formation of a layer of living microorganisms and their decomposition products as deposits on the surfaces in contact with liquid media. Serious problems are caused in the food and dairy industry due to biofouling, and several studies have investigated microbial attachment specifically on stainless steel and other types of food contact surfaces, Stanley, 1983; Stone and Zottola, 1985 (a,b); Herald and Zottola, 1988; Mafu *et al.,* 1990; Helke *et al.,* 1993; Hood and Zottola, 1997(a,b) and Smoot and Pierson, 1998.

Studies investigating biofilm formation on food contact surfaces due to specific pathogens have been reported by Kim and Frank, 1995; Dewanti and Wong, 1995; Oh and Marshall, 1995; and

Blackman and Frank, 1996. In investigating the effect of nutrients on biofilm formation by *Listeria monocytogenes*, Kim and Frank (1995) found that the degree to which biofilm initially developed was associated with amino acid concentrations within the range of 0.12 to 6 grams per litre. After twelve days, biofilm formation was not affected by amino acid concentration. Of five carbohydrates tested, mannose and trehalose were found to enhance biofilm formation. These findings alone would suggest that time is a critical factor in allowing biofilm to establish on food contact surfaces, and that such surfaces should not be left uncleaned for extended periods of time. This was confirmed by Oh and Marshall (1995) who found that planktonic cells of *Listeria monocytogenes* were more sensitive to heat and monolaurin than were cells attached to stainless steel. Young cells were found to be more sensitive to each treatment than old (7-day) cells. Cells that were in a rich nutrient environment were more resistant to treatment than cells in a depleted nutrient environment. These results clearly illustrate the benefits of cleaning food contact surfaces before residual microorganisms have the opportunity to manifest themselves as biofilm. Despite Oh and Marshall (1995) reporting increased resistance of cells to treatment when present in a high nutrient environment, Dewanti and Wong (1995) found that biofilm develops faster when organisms are grown in low nutrient conditions. Growth of *Listeria monocytogenes* as a biofilm on various food-processing surfaces was investigated by Blackman and Frank (1996). Biofilm formation was found to be greatest on polyester floor sealant and least on nylon, with the use of a chemically defined minimal medium resulting in a reduction in biofilm formation on polyester floor sealant, and reduced biofilm levels on stainless steel. It was concluded that biofilm growth of *Listeria monocytogenes* was sufficient to provide a substantial risk of the pathogen contaminating food-processing plants if wet surfaces are not maintained in a hygienic condition.

The relative cleanability of stainless steel food contact surfaces is, therefore, an important consideration in relation to preventing biofilm formation (Steiner *et al.,* 2000). Additionally, product shelf life and quality will be enhanced if adequate cleaning procedures are in place. Steiner, *et al.,* (2000) suggest that industry concerns regarding stainless steel cleanability relate to finding a surface finish that will better release adhering particles.

1.5 Methods for Assessing Surface Cleanliness

Several methods exist with which to monitor food contact surface cleanliness, and these have been reviewed by a number of authors (Favero *et al.*, 1968; Patterson, 1971; Baldock, 1974; Sveum *et al.,* 1992). The principles underpinning the use of these methods have been explained in sections 1.5.1 and 1.5.2. The importance of surface cleaning assessment has already been outlined, but is essential in ensuring that consumers' are provided with safe and wholesome food products that are free from undesirable microbial contamination. The assessment of surface cleanliness levels can be achieved by a number of means including microbiological and non-microbiological methods. However, there is no agreed method adopted by industry for assessing cleanliness, and there is a lack of data on the ability of certain methods to assess surface cleanliness levels. Figure 1.2 outlines the range of methods currently available, which have been conveniently grouped into microbiological and non-microbiological based methods. These methods of surface cleanliness assessment form an important part of the food quality control process since, by sampling food contact and environmental surfaces, it is possible to help prevent the dispatching of low-quality food from the food processing environment. However, the quantification of microorganisms from surfaces is difficult, not least because of strong microbial adherence and because of the formation of biofilms outlined earlier.

A number of factors will influence choice of monitoring methods (Griffith *et al.,* 1997) and these have been summarised in Table 1.1 – Factors Influencing the Choice of Cleanliness Assessment Methods. Several key issues will determine the choice of methods used to monitor the effectiveness of surface cleanliness, not least of which will be the nature of the information required by industry. Information on total surface cleanliness, comprising residual organisms and product residues, can only be obtained using ATP bioluminescence, yet details of residual microorganisms on a cleaned surface, which many microbiologists consider to be more important, can be obtained by a number of microbiological based methods which differ in terms of cost, ease of use, minimum detection limits and reproducibility. In addition, the threshold for detecting microorganisms on surfaces will vary depending upon the method used. Some

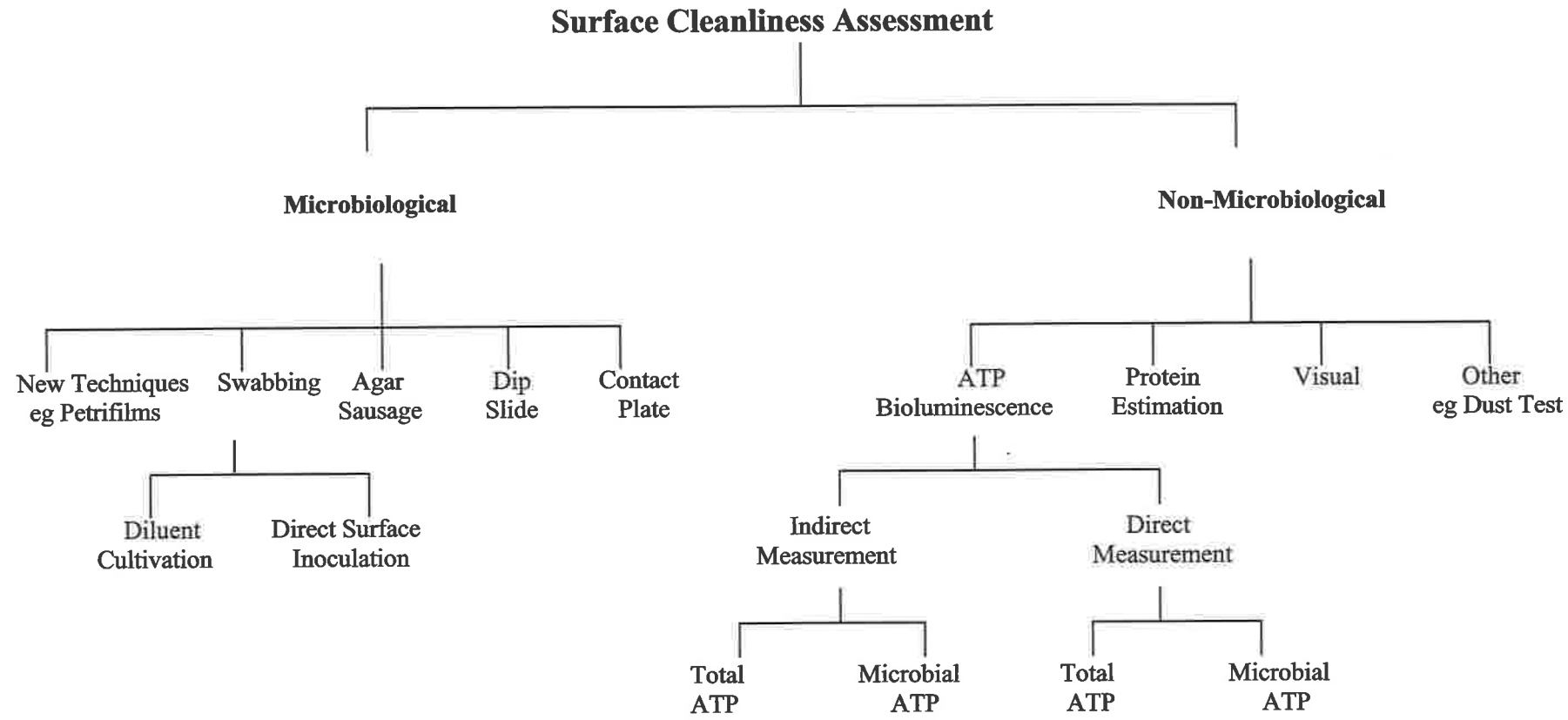


Figure 1.2 Methods of Surface Cleanliness Assessment

Table 1.1 Factors Influencing the Choice of Cleanliness Assessment Methods

Factor	Comment
Cost	The cost of cleaning and the assessment of cleaning efficiency must be optimised especially for designated critical control points. This may include capital as well as running costs.
Time	The speed with which results are required. For a designated critical control point this should be in time for corrective action to be taken. Speed of results may be especially important in a 24-hour production environment.
Information Required	Is information on residual surface microorganisms needed or is the level of surface cleanliness (including product debris/biofilm and micro-organisms) more important
The Type of QA System	The type of quality management systems in use and who implements it. This includes equipment and laboratory availability, staff responsibilities, use of outside laboratories etc.
Staff	Level of technical training.
Ease of Use	Considered in relation to availability of technical staff.
Type of Product and Predicted Level of Contamination	Levels of cleanliness required and amount of monitoring needed can be product related. Considered in relation to sensitivity required and possible levels of contamination. Method used should offer appropriate sensitivity.
Position in Plant	Do surfaces need to be checked as part of GMP or are they CCPs. Relation to other CCPs and processing methods?
Reliability and Reproducibility	Does the test give reliable and reproducible results? Are target values and critical limits required, if so, can they be achieved?
Management Needs	Requirements for due diligence defence, second or third party audits
Nature of Food Contact Surface	The composition and shape of the surfaces being monitored may influence the choice of hygiene assessment method

(from Griffith *et al.* 1997)

methods may underestimate the numbers of organisms present (Boulange-Petermann, 1996), while others may have poor repeatability and reproducibility (Holah, *et al.*, 1990). In addition, it is important that, in selecting a particular method, the user understands the fundamental differences in what microbiological and non-microbiological methods are measuring. While ATP bioluminescence is capable of providing information on total surface cleanliness through detecting and measuring ATP from both microorganisms present on a surface, and from any residual food debris, microbiological methods of assessment have been designed to provide information only on residual microorganisms remaining on a surface after cleaning. In selecting a specific microbiological method for use, several issues relating to the nature of the information required need to be considered. These include whether a precise count of organisms is necessary, or whether an approximation on the numbers of organisms present will be sufficient. More quantitative data on the numbers of organisms present on a surface will be more easily obtained using surface swabbing, while approximate numbers of organisms can be more easily determined using agar contact dip slides, which are semi-quantitative in nature.

The time available before results are required and/or available will be another important determining factor since microbiological methods are dependent upon incubation periods of up to forty eight hours in comparison to ATP bioluminescence that has the ability to produce results within minutes. The time taken to obtain results is especially important in HACCP since corrective action at critical control points within manufacturing processes can only be taken once results are gained. The time of sampling may also be important, especially since allowing surfaces to dry thoroughly after cleaning may have an effect on the ability to recover any residual organisms still remaining on the surface.

Other considerations may include the ease of use of the method, especially where non-technically trained staff are involved in the monitoring of surface cleaning. In addition, microbiological methods generally require laboratory facilities that are not always necessary for ATP bioluminescence, especially given the introduction of single-shot assays. It is important also that the hygiene monitoring methods chosen offer an appropriate level of sensitivity, and that there is confidence in both the reliability and reproducibility of the method chosen. The nature of the surfaces being sampled may also influence the method chosen. For example, agar contact methods are only suitable for flat, smooth surfaces, yet hygiene swabs have the ability to access surfaces that are less easily sampled using agar contact methods, and may be better at removing surface biofilms. In addition to these considerations, probably the most important factor determining choice of methods will be their cost. The cost of cleaning and the assessment of cleaning efficiency must be optimised, and this may include both capital as well as running costs.

Table 1.2 outlines some of the main issues surrounding the decision to perform microbiological assessment "In House" or to contract out to an external laboratory. While the decision to contract out such work will be dependent upon the facilities available "In House", the skills of staff, and the time and costs involved will also be important considerations. In terms of cost, both capital and running costs need to be considered, as do the costs of any staff that need to be employed to perform the analyses. The time taken to obtain results is another factor, as is perhaps the need for results to be accepted by a third party. This will be more likely if the results have been obtained from an accredited external laboratory. Also of importance may be the safety issues surrounding the cultivation of potentially harmful organisms near food production areas, in which case contracting out sample analysis would be better.

Table 1.2 Comparison Of 'In House' And External Microbiological Assessment

Attribute	In House	External
Cost	High capital outlay (£2000+). Lower running costs: 60-90p The greater the number of tests the more cost effective	No capital outlay. Running costs £5 - £17 per swab External laboratories must make a profit. Costs of transportation. Can be more cost effective for small operations. May be discount for large numbers
Safety	Organisms and possible pathogens may be cultivated near food production environment	Organism cultivated in site remote from production
Speed	Quick availability of results	May be a delay in results. Time for transportation
Methodology	May or may not be standardised. Laboratory may or may not be accredited May not be cost effective to introduce sophisticated new techniques	Increasingly, laboratories are externally accredited e.g. NAMAS. Standard methods likely to be used. Can use the latest sophisticated technique of analysis – sensitivity, economy of scale
Acceptance	Results may or may not be accepted by third party	Results more likely to be accepted by third party
Transport of Swab	Minimal time delay between sampling and analysis. Immediate response to problems	Variable delay due to transport may result in loss of viability of organisms
Staffing	Technically trained staff needed by company, maybe more highly motivated than externals In house staff more knowledgeable of product, expected flora etc. Can make suggestions on alternative or additional sampling. Technical expertise can be an asset to company e.g. in 2nd, 3rd party audit and HACCP In house staff less likely to misunderstand requirements. Will engage in dialogue	Technically trained staff provided by external laboratory External staff no knowledge of product sample sites etc. Mistakes in communications can occur. Dialogue with an 'expert' may cost additional money

(from Griffith *et al.*, 1997)

1.5.1 Microbiological Methods for Assessing Surface Cleanliness

While microbiological based methods of hygiene monitoring are of limited value for monitoring surface cleanliness, their importance must not be underestimated. Within HACCP, they can be useful tools for validation and verification. Their ability to provide information on the presence of specific pathogens is of considerable value, as is their ability to provide quantitative information on the numbers of organisms present.

The main microbiological methods available for use in assessing surface cleanliness include surface swabbing, which includes cotton and alginate swab variations, and agar-contact methods which comprise agar contact plates, dip-slides and the traditional agar sausage. Other more recent developments include the use of petrifilms that are a modification on the method of organism cultivation, and involve the inoculation of a dehydrated media film with recovery diluent.

1.5.1.1 Surface Swabbing

This particular method has many forms and is probably one of the most widely used methods in the food industry today. A sterile swab, pre-moistened in an appropriate wetting agent, is rubbed over the surface of interest and then either streaked directly onto the surface of an appropriate culture medium, or released into a known quantity of sterile recovery diluent that is then used to prepare agar plates for incubation. Patterson (1971) has indicated that there is often poor organism recovery using this method, either through characteristics of the surface being sampled or as a result of the amount of pressure applied to the swab, or due to the time and speed of application of the swab to the surface. Additionally, different people use swabs in different ways, so the results may not be comparable between samples, or between laboratories. Specifically with regard to cotton swabs, organism retention within the cotton fibres may also lead to reduced counts and, in turn, inaccurate results. Various modifications of the method have been introduced to reduce these errors, and these are discussed in detail in Chapter 2.

1.5.1.2 Agar Contact Methods

Subject to many modifications, the agar contact method involves pressing a sterile agar surface onto the surface to be sampled. The agar is then incubated for an appropriate length of time and the adhering microorganisms enumerated. By the very nature of the method, it is best suited to smooth, flat surfaces and since dilution is not possible, it might be used best on surfaces where low levels of contamination are expected. The agar contact method was compared with agar contact dip slides and the swab method for the enumeration of *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* from artificially contaminated stainless steel and formica surfaces before and after sanitizing with chlorine (Restaino, *et al.*, 1994). The correlation coefficients found between the methods were 0.81 for swab versus agar contact plate, 0.84 for swab versus dip slide, and 0.89 for dip slide versus agar contact plate. These results suggest that agar contact methods can be used as an alternative method to assess surface microbial contamination levels.

Pictorial methods of interpreting results are useful, such as in the interpretation key used to interpret dip slide results (Ref: Appendix 2.). This may be especially helpful when non-technically trained staff are responsible for surface hygiene monitoring. In addition, dip slides are quick and easy to use, and provide a semi-quantitative measure of the numbers of organisms present per square centimetre of the surface sampled. Furthermore, the cost per test is much cheaper than that of surface swabbing, especially when the additional costs of agar plate preparation is taken into account. For these reasons of ease and speed of use, and cost, agar contact methods are becoming more frequently used and have therefore been selected as one of the methods evaluated in this thesis.

1.5.1.3 Direct Surface Agar Plating

While not always practical, contaminants of microbial origin can be detected *in situ* by the direct surface agar plating method. This involves pouring sterile agar on to the surface to be sampled and left to solidify under a sterile cover. After incubation, the colonies at the interface are counted. Small items can be placed in a Petri dish and covered with agar. Due to practical problems, this method is always used in the laboratory since food plant surfaces are generally

large, fixed, and cannot be incubated at a desired temperature., in addition to the potential detrimental effect on the product coming into contact with surfaces sampled in this way. The method has been evaluated for detecting bacterial contamination on non-porous surfaces by Angelotti and Foter (1958). Based on averages of triplicate determinations, the direct surface agar plate technique detected from 87.6% to 100.9% of the number of *B. globigii* spores inoculated onto china surfaces. The authors report that the technique is capable of detecting bacterial contamination on surfaces with a high degree of precision. While limited to use on flat surfaces, nonporous surfaces, and not practical for use in the food industry, it has the advantage over other methods because *in situ* determinations are made, thus eliminating errors associated with intermediate manipulations.

1.5.2 Non-Microbiological Methods for Assessing Surface Cleanliness

Non-microbiological methods consist of the visual inspection, protein estimation and ATP bioluminescence. Of these, visual inspection is generally the first means of assessing the hygiene status of food contact surfaces since, if gross deficiencies are evident from this initial inspection, other forms of monitoring cleanliness are relatively pointless on unclean surfaces, (Dillon and Griffith, 1999).

1.5.2.1 Visual Inspection

The visual inspection is often the first stage in an integrated cleaning assessment strategy (Dillon and Griffith, 1999). The method relies solely on the subjective opinion of the person performing the test, and is often regarded as being unreliable since several authors have reported that there appears to be no correlation between the results of visual inspections and other test methods (Tebbutt, 1991b; Powell and Atwell, 1995). Some attempts to make the method more quantitative in its output have resulted in numerical scoring scales being used such as that reported by Powell and Atwell (1995) to indicate the extent of either cleanliness or residual contamination remaining on the surface after cleaning.

Under normal circumstances visual inspections of food processing environments will only reveal gross visible food soil residues. However, in a small number of occasions the presence of microbial growth may be observed, e.g. mould growth in bakeries (Dillon and Griffith, 1999), but this would be dependent upon the specific circumstances under which the inspection was being performed. Other tests that may be performed during a visual inspection include wetting a dry surface to observe water dispersion that will indicate possible grease residues remaining on a surface. Wiping a dry paper towel over a surface can also indicate residual grease or fat, and while not strictly visual in nature, smelling a surface can indicate the presence of residual and/or deteriorating product, e.g. beer and dairy products.

Visual inspection has the ability to detect gross deficiencies due to the presence of visible food soil but without magnification cannot be used as an assessment of disinfection effectiveness. In a comparative study of food retail premises by means of visual inspection and microbiological quality of food, Powell and Atwell (1995) found no correlation between potential risks of foodborne infection, as assessed by visual inspection, and bacteriological counts in food. Similar work reported by Tebbutt (1991b), in which the relationship between visual inspections performed by environmental health officers and microbiological examinations in eighty-nine restaurants, resulted in similar results and it was reported that there were no highly significant associations between microbiological examination and visual assessments.

1.5.2.2 Protein Estimation

Introduced as a means of determining surface cleanliness only within the past two or three years, this method, as its name suggests, detects protein residues on surfaces. The method produces results within a few minutes, but is not capable of detecting low levels of residual food soil on surfaces, especially those with low protein contents. Current developments in this area are now leading to test procedures that are quicker and easier to perform in addition to having lower minimum detection limits. Most recently, Tebbutt (1999) in comparing traditional and rapid methods for assessing bacterial cross-contamination from cutting boards found that in a total of 212 tests, there was a relatively good correlation between the numbers of bacteria present on boards and the amount of protein detected ($r=0.67$). Further work comparing protein, ATP

bioluminescence and agar based microbiological methods (Moore, G., *et al.*, 2001) found that the most sensitive protein detection assays were superior or comparable to ATP bioluminescence when used to detect bioburden high in protein. In the presence of bioburden with a low protein content but with a high microbiological count, none of the protein detection assays indicated that surfaces were unsuitable for food production, despite agar based microbiological methods indicating the presence of large numbers of bacteria.

1.5.2.3 ATP Bioluminescence

ATP bioluminescence is beginning to be used widely in the food industry for monitoring surface cleanliness. Despite its relatively recent rise in popularity it was in the 1960s that the method was first described by NASA scientists who were primarily interested in clinical applications of the technique (Griffiths, 1996). Even though the potential of the technique was realised forty years ago, it was not until the early 1990s that food microbiologists realised the full potential of the method in the context of surface hygiene monitoring.

1.5.2.3.1 Biochemistry of ATP Bioluminescence

The biochemistry underpinning the reaction has been extensively reviewed by a number of authors (Stannard and Gibbs, 1986; Meighen, 1991; Baker *et al.,* 1992). All living cells contain adenosine triphosphate (ATP) that has often been described as being the universal energy donor for metabolic processes (Griffiths, 1996). The method detects microbial cells and food residues that may persist on surfaces following inadequate cleaning, and which provide a source of nutrients for microbial growth (Corbitt *et al.,* 2000). An enzyme-substrate complex, luciferin-luciferase, present in the tails of the firefly *Photinus pyralis* converts the chemical energy associated with the ATP into light through a stoichiometric reaction where one photon of light is produced by the hydrolysis of one molecule of ATP.

The level of ATP within cells can vary depending upon the environment and the phase of cell growth, but the ATP pool in living cells is normally kept constant by the cells regulatory mechanisms (Stanley, 1989) and, as a result, the amount of light produced is directly proportional

to the number of metabolically active cells present in the initial sample. Figure 1.3 illustrates the reaction.

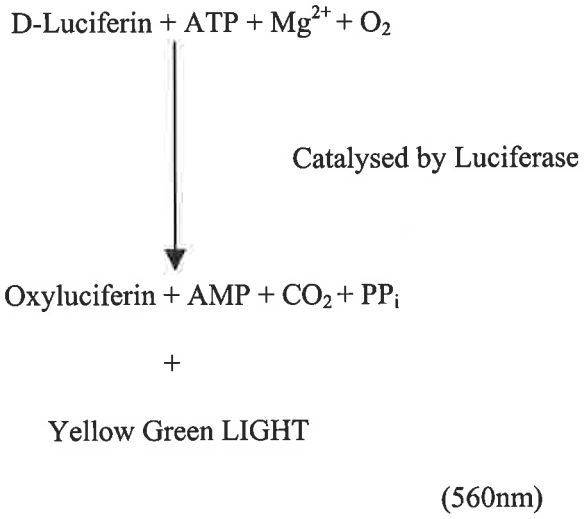


Figure 1.3 Schematic Representation of the ATP Bioluminescence Reaction.

1.5.2.3.2 ATP Bioluminescence within the Food Industry

The increasing use of the technique for monitoring plant hygiene might be attributed to the adoption of food safety and quality management systems discussed earlier. These are considered to be more proactive and preventative in nature than those used in the past (Griffiths, 1996). Earlier systems tended to rely almost entirely on the use of end product testing and visual inspection (Griffith *et al.,* 1997).

Increased adoption of the Hazard Analysis Critical Control Point System (HACCP), and the need for real-time monitoring of CCPs means microbiological based methods are not suitable for monitoring CCPs within HACCP. Real-time data is not possible where incubation of samples is necessary for extended periods of time before results become available. Many microbiologists now therefore argue that both the time lapse in between sampling and results becoming available, and the nature of the sampling strategy needed in order to generate meaningful results make

microbiological methods of limited value within the context of HACCP (Griffiths, 1996). However, they are of importance with respect to verification and validation in HACCP.

While microbiological methods are often considered to be time consuming and laborious (Stannard and Gibbs, 1996) their role cannot be underestimated in verifying the safety of individual processes which must rely on some microbiological data. Griffiths (1996) argues that while microbiological data is essential, it is not necessary for such data to be generated "real-time". He suggests that ATP bioluminescence is the only technique currently available that comes anywhere near to offering the potential of "real-time" data. It is the rapidity with which results become available which makes the technique highly desirable to the food industry as it enables corrective action to be taken immediately, and therefore has the potential to be used in monitoring plant hygiene (Stannard and Gibbs, 1996).

1.6 Aims of Research Work

A number of issues relating to the use of microbiological and ATP bioluminescence based methods for assessing surface cleanliness within the food industry have been highlighted and discussed in this chapter. A large number of variables have been identified that may potentially affect the recovery of organisms from surfaces using microbiological methods. In addition, it has been acknowledged that ATP bioluminescence may be of value within HACCP as a rapid means of determining surface cleanliness levels, with the ability of being able to detect microbial ATP being particularly useful. In addition, the surface moisture level at the time assessment of cleaning effectiveness is performed, i.e. the sampling of wet or dry surfaces, may have a marked effect on the performance of both microbiological and ATP based methods. The aims of the work reported in this thesis are, therefore, to:

1. Critically evaluate commonly used microbiological and non-microbiological methods of hygiene monitoring for use within the food industry for assessing surface cleanliness;

2. Investigate selected sampling variables potentially influencing bacterial recovery rates from stainless steel surfaces using cotton hygiene swabbing;

3. Evaluate the technical performance of a range of commercial ATP bioluminescence detection systems for use within hygiene monitoring;

4. Determine the minimum bacterial detection limits of microbiological and ATP bioluminescence monitoring methods for the recovery and detection of bacterial contamination on stainless steel contact surfaces;

5. Develop and evaluate the use of a microbial ATP bioluminescence protocol for use within food processing environments;

6. Recommend a strategy for the use of cleanliness assessment methods in the food industry.

Chapter 2

An Evaluation of Selected Variables potentially affecting the Recovery of Bacteria from Inoculated Stainless Steel Food Contact Surfaces using Cotton Surface Swabbing

2.1 Introduction

The existence of microorganisms on food contact surfaces within food processing environments presents a very real problem (Ganesh Kumar and Anand, 1998). This lies not only in the fact that such organisms have the potential to cause both food spoilage and human illness, but also in the ability of current microbiological methods to detect them in a way that reflects the numbers present on a food contact surface at any given time.

Despite the apparent limitations of microbiological methods in monitoring surface cleanliness, cotton surface swabbing is still widely used by large numbers of food manufacturers for routine surface cleanliness assessment (Griffith *et al.,* 1997). While unable to provide "real-time" results on surface cleanliness for use within HACCP, quantification of the relative numbers, and when necessary the types of microorganisms present on a surface, can only be achieved using microbiological methods (Chapter One). The importance of cotton surface swabbing as one of the main methods of assessing the microbiological status of food contact surfaces in the food industry should, therefore, not be underestimated.

Even though the method is extensively used in the food industry and elsewhere, there is still a lack of scientific knowledge and understanding of the effects of many of the sampling variables potentially affecting microorganism recovery rates when using the method as part of a routine hygiene assessment programme. In addition, no one accepted sampling protocol has been adopted by industry when using the method (Collins and Lyne, 1989; PHLS, 1995; Harrigan, 1998). Table 2.1 provides details of selected published surface hygiene swabbing protocols, while Table 2.2 identifies the main sampling variables potentially affecting organism recovery rates, and provides details of their possible effects.

Table 2.1 Selected Published Surface Hygiene Swabbing Protocols

Sampling Variable	Collins and Lyne (1989)	PHLS (1995)	Harrigan and McCance (1998)
Type of Swab	Cotton Wool or Alginate	Cotton Wool	Cotton Wool or Alginate
Area of Surface sampled	5cm x 5 cm	5cm x 5cm	Not Specified
Swabbing Protocol	Swab within 25cm^2 card or cellophane template area using one swab	Using aluminium template, swab entire area using 2 swabs, one moist and one dry, rotating swabs. Return both swabs to one diluent tube	Swab predetermined area by rubbing firmly over the surface in parallel strokes, with slow rotation of the swab
Type of Diluent	Nutrient broth	M.R.D. or ¼ St. Ringers	¼ St. Ringers
Release Method	Soak and squeeze swab	Shake until cotton wool broken down into fibres	Agitate/shake swab in tube up and down 10 times
Type of Culture Media	Not Specified	Plate Count Agar	Nutrient Agar or Trypticase Soya Agar
Cultivation/ Plating Method	Not Specified	Spread/Pour Plates	1ml pour plates for cotton swabs, 0.1ml and 1ml plates for alginate swabs
Time and Temperature of Sample Storage before Analysis	Not Specified	Not Specified	Not Specified
Time and Temperature of incubation	Not Specified	30°C, 48-72 hours	Not Specified
Expression of Results	Count/25cm^2	Count/cm^2	Count/cm^2

MRD – Maximum Recovery Diluent (Oxoid) – Microbiological diluent

¼ Strength Ringers (Oxoid) – Microbiological diluent

Table 2.2 Sampling Variables Potentially Affecting the Recovery of Microorganisms From Stainless Steel Food Contact Surfaces using Cotton Surface Swabbing

Sampling Variable	Potential Effect on Recovery of Microorganisms from Food Contact Surface
Swab Size	Increased/decreased pick-up of microorganisms from surface Possible retention of microorganisms in or release of microorganisms from swab bud.
Composition of Swab	Possible retention of microorganisms in, or poor release of microorganisms from, swab bud
Composition of Swab Wetting Agent	Increased/decreased pick-up of microorganisms from sampled surface
Composition of Recovery Diluent	Promotion or inhibition of microbial survival over time
Composition of Culture Media	Promotion or inhibition of microbial growth during incubation period
Method of Cultivation/Plating	Increased/decreased recovery of recovered microorganisms
Nature of Organism Release Method	Increased/decreased release of microorganisms from swab bud
Time and Temperature of Sample Storage prior to Analysis	Possible increase or decrease in microbial numbers
Level of Surface Contamination	Increased/decreased pick-up of microorganisms from surface
Food Contact Surface Moisture Level	Increased/decreased pick-up of microorganisms from wet or dry surfaces
Surface Swabbing Protocol	Increased/decreased pick-up of microorganisms from surface

A number of studies have evaluated the use of surface hygiene swabbing for determining *in situ* microbial levels on food contact surfaces. One of the earliest studies was that by Saelhof and Heinekamp (1920) who investigated the recovery of *Streptococcus haemolyticus* from restaurant tableware. No information on the surface swabbing protocol used was given, but a total of sixty-three food contact surfaces were examined for the presence of microbial contamination. Only four surfaces (6.35%) were found to be positive for *Streptococcus haemolyticus*, while just over fifty percent of the total surfaces sampled were found to be positive for *Staphylococcus albus*. The results were discussed within the context of cleaning efficiency, and the personal habits of food handlers. Similar work by Krogg and Dougherty (1936) investigated methods of dish and utensil washing in public eating and drinking establishments. Bacterial counts from a range of eating and drinking utensils were presented along with bacterial counts from wash and rinse waters. Given the excessively high bacterial counts gained from all utensils and water samples, the authors concluded that the methods of washing employed in the different establishments visited merely acted through partial removal of food particles, but did not have any significant bactericidal properties. While the high bacterial counts gained suggested that the surface swabbing protocol employed was effective at removing contaminating bioburden, no details of the surface swabbing protocol used was given. Another study investigating the sanitary quality of glassware was reported by Fellers *et al.,* (1936). Before deciding upon surface swabbing as the method of choice, eighteen different methods of sampling glasses and dishes were compared for utility, accuracy, speed and simplicity. In laboratory controlled studies the authors found that the recovery of known levels of bacteria using surface swabbing was always over forty percent, and often as much as seventy five percent for some test organisms, although no indication as to how these values were calculated was provided. The use of mixed cultures always resulted in lower recovery rates than when pure cultures were used. Details were also provided on some aspects of the sampling protocol used in which swabs were released into recovery diluent tubes by hand shaking twenty five times, yet no information was provided on the method of surface swabbing used to sample the glasses. Bacterial counts were provided for a range of glasses in three different types of establishments, and were compared to the nature of the cleaning, rinsing and drying regimes in place within the individual establishments.

The first report of the use of the surface swabbing method where some attempt was made to investigate its use under controlled experimental conditions is that by Speck and Black (1937) who investigated the effectiveness of the method for examining the bacteriological status of paper ice cream containers. The protocol employed involved using a double swabbing procedure in which one moist and one dry swab were used. The moist swab was first released into one recovery diluent tube, and then placed in a second tube where it was released for a second time. The dry swab was released into a separate third recovery diluent tube. The authors report that by merely pressing out the first moist swab, an average of fifty eight percent of the total bioburden present on the swab was recovered. By shaking out (releasing) the same swab, additional organism recovery averaging twenty one percent was gained. Swabbing with an additional dry swab also recovered an additional twenty one percent of the bioburden present on the swab. The authors stress, however, that it cannot be assumed that the surface swabbing protocol employed recovered all the bioburden present from the container surfaces. This particular study has clearly illustrated that a single swabbing protocol may not be as efficient at removing contaminating surface bioburden from food contact surfaces. However, the protocol used by Speck and Black (1937) may not be particularly practical for use within food processing environments, especially where several organism release stages are involved.

A similar study by Buchbinder *et al.,* (1947) in which different variations of a surface swabbing protocol were investigated for examining the cleanliness of eating and drinking utensils found that variations in the method used did give rise to different organism recovery rates. It was found that with high-count utensils, the mean count with five strokes was about sixty percent greater and with ten strokes, eighty percent greater than the mean count obtained with three strokes. However, the exact definition of a "stroke" is not provided making it difficult to ascertain exactly what is meant by the use of the term. On utensils with low counts, the mean count with five strokes was approximately twenty percent higher and with ten strokes thirty percent higher than that obtained with three strokes. The authors also note that with high-count utensils (40,000 to 100,000 organisms) that reversing the direction of strokes decreased the count obtained on all occasions, while with low-count utensils (800 to 1200 organisms) reversing the direction of the

strokes increased the counts obtained from five to fifteen percent. In swabbing an area of four square inches the authors recovered more than three times as many organisms using ten strokes, reversing direction between strokes, as was obtained with ten single strokes in a single direction. While many aspects of the protocol used are not explicit, the authors suggest that further work on standardising the surface swabbing method is needed.

Further development of the method as a means of monitoring surface cleanliness continued with Hansen (1962) reporting on an adaptation of the method in which samples taken with cotton swabs were transferred directly to agar slopes by direct streaking. Agar slopes were then grouped according to the numbers of colonies formed during incubation, similar to the process involved in using and interpreting the results gained from agar-contact dip slides. The method was used to evaluate the efficiency of hydraulic pressure cleaners in a pig slaughterhouse, and it was found that as a result of the daily use of pressure cleaners, the bacterial surface contamination on the machinery and equipment was no higher than 100 cfu/cm^2 after cleaning. Counts of this order from cleaned surfaces were, however, considered to be unacceptably high by the authors.

In comparing the use of agar contact (rodac) plates with cotton hygiene swabs for assessing the sanitary quality of food preparation surfaces, Silverman, *et al.*, (1981) found that the cotton swab method appeared to recover a higher percentage of the contaminating bioburden, but that microbial counts using the agar contact method were higher than the swab method. This was thought to be due to a failure of the cotton fibres of the swab buds to release entrapped organisms. In the study, the cleanliness of twenty seven tables routinely used for food preparation was measured by monitoring ten locations per table using the two methods. The total bioburden at each location was determined by taking five successive contact plates or three successive swabs. To obtain the percentage recovery by the initial rodac plate or swab count, the total microbial population was estimated on each of ten selected locations by adding the colony forming units (cfu) of either the five successive rodac plates or three successive swab counts. A large number of locations were found in which 90% or more of all the recoverable bioburden was removed from the surface after the fourth rodac plate or second swab. It was found that the first contact plate or swab recovered on average about fifty percent of the microflora at bioburden levels ranging from

lass than 25 cfu/25 cm^2 to over 200 cfu/25 cm^2. The swab technique generally gave higher average percentage recovery than the contact plate although the range for both was extremely broad (17% - 100%). The authors report that microbiological counts gained from the first contact plate or first swab were found to serve as a good indication of the total bioburden present.

Although not directly concerned with assessing food contact surface cleanliness, Anderson, *et al.,* (1987), in evaluating the swab and tissue excision methods for recovering microbial contamination from washed and sanitised beef carcasses, found that excised tissues produced much higher counts than the cotton swabbing method, especially when washed and sanitised carcasses were sampled. It was found that percentage recovery rates from surface swabbing were influenced by the characteristics of the area of the carcass sampled, with the numbers of *Enterobacteriaceae* and *Escherichia coli* recovered by swabbing washed and sanitised carcasses usually being to low to count.

More recently, Gill and Jones (1998, 2000) have compared a number of methods, including surface swabbing, for sampling and enumerating carcasses for *Escherichia coli.* In the earlier study, three variations of the swab method were compared for the detection of *Escherichia coli* on pig carcasses. The methods used included swab sampling with a single, cellulose acetate sponge, sampling with a gauze swab over an area approximating 100 cm^2, and sampling with gauze swabs as indicated but in conjunction with the enumeration of *Escherichia coli* using a hydrophobic grid membrane filtration technique. All three methods were found to recover the organism with similar frequency, and with the recovery of similar numbers of colony forming units. In the later study, samples were collected from six groups of pig or beef carcasses by excision or swabbing with sponge, gauze or cotton wool. It was found that the numbers of bacteria recovered by excision or swabbing with sponge or gauze swabs were similar, while the numbers recovered by cotton wool swabbing were at the lower end or below the range of the numbers recovered by the other methods. It was, however, interesting to note that the reported areas of surface swabbed by the different methods varied considerably. For gauze swabs, areas of 10cm x 10cm were swabbed, while for cotton swabbing an area of 5cm x 5cm was used. Direct comparison of the

results in terms of organism recovery rates is, therefore, inappropriate due to the vastly different areas of surface sampled.

It is clearly evident from the reported findings of the above studies that a number of factors influence the recovery of microbial contamination from food-contact and other types of surfaces using surface hygiene swabbing, and that these published findings remain inconclusive as to the efficiency with which surface swabbing is able to detect levels of microbial bioburden on such surfaces. In addition, it would appear that there is no uniform approach to the use of the method in industry, and that the development of a standard protocol for its use would be beneficial.

Figure 2.1 illustrates the sampling variables potentially affecting microbial recovery rates using cotton swabbing, and includes those evaluated in this study. The variables selected were chosen on the basis that they represented some of the main variables listed in the published swabbing protocols outlined in Table 2.1. The test organisms used in the study, *E. coli* and *S. aureus*, were selected on the basis that *E. coli* is a common indicator organism, while *S. aureus* is a skin organism commonly found on the skin of about 50% of the population.

2.2 Aims

The aims of the work reported in this chapter were, therefore, to:

1. Determine the effects of selected sampling variables on the recovery rates of *Staphylococcus aureus, Escherichia coli* and an environmental isolate of the genus *Staphylococcus* from stainless steel food contact surfaces using cotton hygiene swabbing.

2. Propose a standard surface swabbing protocol that optimises organism recovery from stainless steel food contact surfaces.

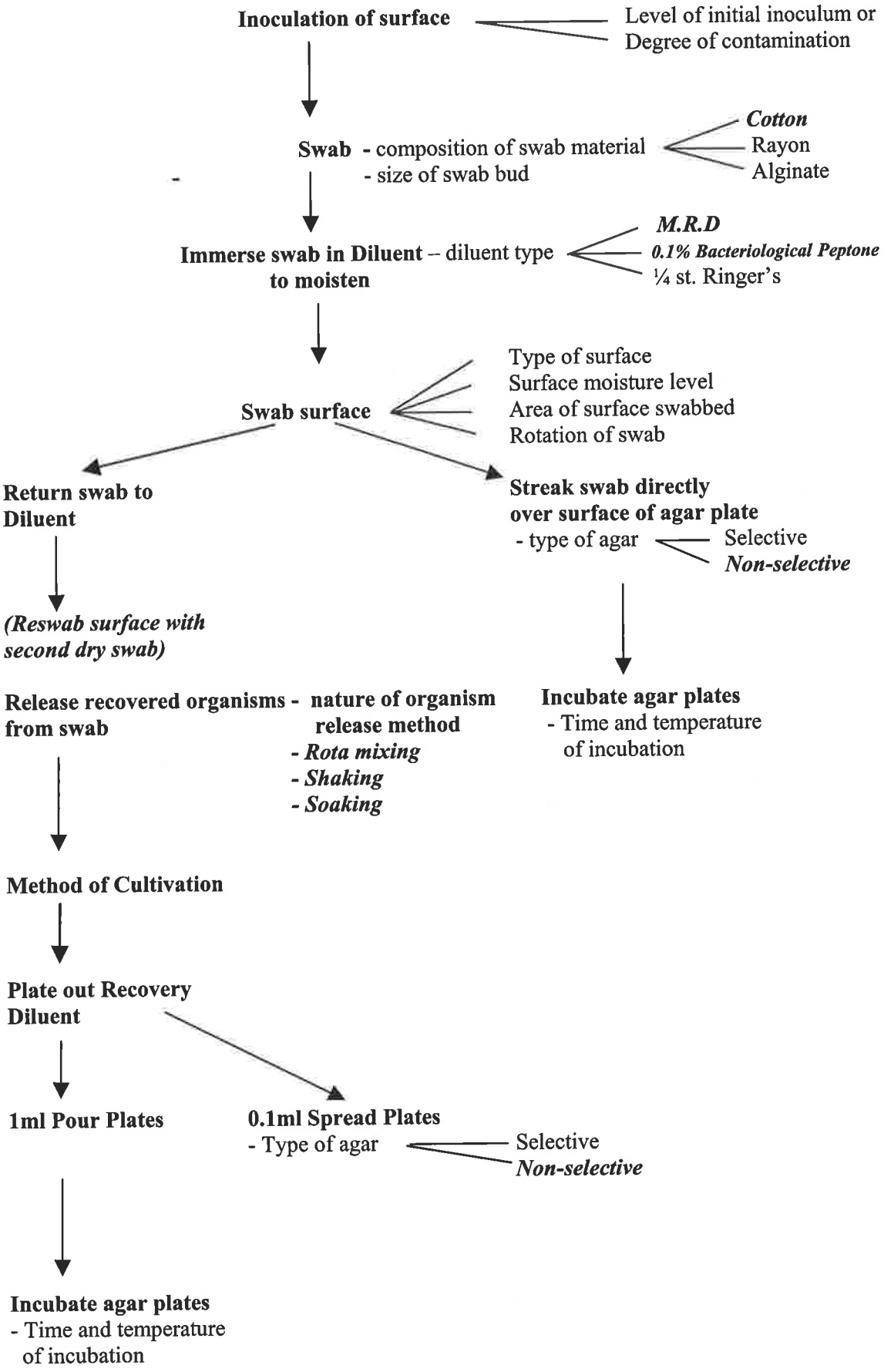


Figure 2.1 **Surface Hygiene Swabbing Protocols and Variables potentially affecting Bacterial Recovery Rates.**
(Variables given in bold italics are those evaluated in this study).

2.3 Materials and Methods

2.3.1 Bacteria and Culture Conditions

Cultures of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (NCTC 6571), and an environmental isolate of the Genus *Staphylococcus* were grown in sterile Nutrient Broth No.2 (CM 67, Oxoid, Basingstoke, UK) in unshaken batch culture volumes of 10 ml under aerobic conditions at 37°C for 18 hours. Serial dilutions of each culture, prepared in either sterile Maximum Recovery Diluent (MRD, CM733, Oxoid) or sterile 0.1% Bacteriological Peptone (BP, L37, Oxoid) were used to inoculate a food grade stainless steel surface or cotton hygiene swabs.

2.3. 2 Hygiene Swabs

Sterile cotton hygiene swabs with plastic applicator sticks (PBI, Cherwell Laboratories, Bicester, UK) pre-moistened in sterile MRD or 0.1% BP immediately before use, were used for all surface swabbing experiments. Swabs used in experiments involving direct swab inoculations were not pre-moistened prior to use to help prevent potential loss of sample, except in those experiments in which swab inoculations were used to assess the effect of different plating methods on bacterial recovery rates where direct streak plates were used. In these experiments swab buds were pre-moistened in order to ensure that that inoculated bacterial cells did not become completely absorbed into the fibres of the swab bud that might inhibit their release onto the surface of the culture media.

2.3.3 Standard Surface Swabbing Protocol

In all surface sampling experiments, a standard surface swabbing protocol was used in which individual sampling variables were altered in turn. The protocol involved ensuring that the swab bud, pre-moistened in MRD, came into contact with the entire 100 cm^2 surface area, that the swab was rotated constantly during sampling, and that the surface was swabbed from top to bottom, swabbing from side to side until the entire surface had been swabbed. Each swab was placed into a 10 ml tube of recovery diluent and released through vortexing for fifteen seconds before pour plates were produced in duplicate, either from the recovery diluent itself or from serial dilutions thereof. In an attempt to standardise the amount of pressure applied to swabs during swabbing, swabs were held by their handles, and not by their applicator sticks.

2.3.4 Surface Preparation

Prior to inoculation, the stainless steel surface, marked with 100 cm^2 areas, was pre-sanitised for thirty minutes using a 1:80 dilution of Bioscan containing < 5% cationic surfactants, 5-15% non-ionic surfactants and < 5% phosphoric acid. (Henkel Ecolab Ltd, Swindon, UK), which acts through disrupting cell membrane integrity. The surface was then cleaned using an "in house" validated cleaning protocol. This involved washing with hot water and detergent (<5% amphoteric, 5-15% non-ionic and 15-30% anionic surfactants), applying kinetic energy for two minutes using a new rayon cloth, thoroughly rinsing with hot water to remove all traces of detergent, and a final rinse with boiling water before being left to air dry at room temperature. This was demonstrated to give negative microbiological results and ATP bioluminescence readings of less than 100 RLU using the Biotrace Xcel detection system and Clean Trace Rapid Cleanliness Test.

2.3.5 Surface Inoculation, Sampling and Organism Cultivation

In experimental protocols involving surface inoculations, ten 100 cm^2 areas of stainless steel were each inoculated with 0.1ml of culture that was spread over the entire area to within 2 mm of the edges using a sterile plastic spreader (Technical Service Consultants, Heywood, UK). Surfaces were then sampled when the inoculum applied to the surface had dried completely, or for wet surfaces, immediately after inoculation.

Dry inoculated surfaces were achieved through allowing the inoculum applied to the stainless steel surface to remain resident on the surface for sixty minutes at an ambient temperature of approximately 22°C prior to sampling. This resulted in no visible liquid culture remaining on the surface at the time of swabbing.

Organisms recovered from inoculated surfaces using hygiene swabs were released into 10 ml tubes of MRD or 0.1% BP by mechanical agitation for fifteen seconds and duplicate 1 ml nutrient agar (CM3, Oxoid) pour plates prepared. All pour plates were incubated at 37 °C for twenty four hours.

2.3.6 Assessment of the Effects of Sampling Variables on Bacterial Recovery

The effect of different sampling variables on the recovery of bacteria from stainless steel food contact surfaces using cotton hygiene swabbing were assessed either through swabbing inoculated stainless steel surfaces when wet or dry, or through direct swab inoculations. Each experimental procedure was repeated on at least three occasions using ten replicates (n=30), except where otherwise stated. The variables that were evaluated included:

2.3.6.1 Organism Release Methods

Two experimental protocols were used to assess the efficiency of organism release from cotton swabs. In the initial protocol, organisms on swab buds, recovered from swabbing inoculated stainless steel surfaces sampled when dry, were released using either mechanical agitation (vortexing) or hand shaking. The mechanical agitation (vortexing) method of releasing recovered organisms was evaluated using a five second release time, while for hand shaking both five and fifteen-second release times were compared. The results from these surface inoculation experiments indicated that organism recovery rates were very low, and that an alternative experimental protocol using direct swab inoculations would be necessary in order that the effects of different release times on organism recovery from cotton swabs could be determined more easily.

In the experiments using direct swab inoculations, organism recovery rates from vortexed samples using release times of 0, 10, 20, 30, 40, 50 and 60 seconds were compared. Each of five swabs for each release time was inoculated with 0.1ml of a known level of inoculum of either *Escherichia coli* or *Staphylococcus aureus.* Each inoculated swab was placed into a 10 ml MRD recovery diluent tube and vortexed for its specified release time. Duplicate 1ml pour plates were then prepared for each swab sample using nutrient agar, which were incubated at 37°C for twenty four hours.

2.3.6.2 *Diluent Type*

0.1% Bacteriological Peptone (B.P.) and Maximum Recovery Diluent (M.R.D.) were compared as recovery diluents in which to both release organisms, and as storage diluents for swab samples prior to plating out. Ten 100 cm^2 stainless steel surfaces were inoculated with a known level of inoculum of *Escherichia coli, Staphylococcus aureus* or the environmental isolate and allowed to dry at ambient temperature for up to sixty minutes. Each of the ten inoculated dry surfaces was then swabbed using one pre-moistened swab that had been moistened in the test diluent. Swabs were returned to their diluent tubes and released using mechanical agitation (vortexing) for thirty seconds. Duplicate 1ml pour plates using nutrient agar were prepared for each sample, which were incubated at 37°C for twenty four hours.

2.3.6.3 *Culture Media*

The growth of recovered bacteria from inoculated stainless steel surfaces when sampled dry was compared using nutrient agar (NA, CM3, Oxoid) and plate count agar (PCA, CM463, Oxoid). Each of ten 100 cm^2 stainless steel surfaces was inoculated with a known level of either *Escherichia coli* or *Staphylococcus aureus.* Inoculated surfaces were allowed to dry for sixty minutes at ambient temperature before sampling. Each surface was swabbed using one pre-moistened swab, which was then released in its diluent tube using mechanical agitation (vortexing) before duplicate 1ml pour plates were prepared using each of the two culture media. All plates were incubated at 37°C for twenty four hours.

2.3.6.4 *Time and Temperature of Sample Storage before Plating*

Organisms recovered from inoculated stainless steel surfaces when sampled dry were stored in MRD recovery diluent tubes at ambient temperature for up to four hours before plating was carried out. This attempted to simulate the effect of storage of swab samples between sampling and analysis, in order to establish the effect of time and temperature of sample storage on resulting organism recovery rates.

2.3.6.5 Single versus Double Swabbing Procedure

A single swabbing procedure in which one moist cotton swab was used to sample each 100 cm^2 inoculated surface area was compared to a double swabbing procedure in which two swabs, one moist and one dry, were used for each 100 cm^2 area to recover organisms from inoculated stainless steel surfaces sampled while dry. In the double swabbing procedure, each swab was used in different directions at 90° to each other, with both swabs being returned to the same 10 ml recovery diluent tube, and released in the same way as the swabs used in the single swabbing procedure. Duplicate 1ml pour plates were prepared from each recovery diluent using nutrient agar, and were incubated at 37°C for twenty four hours.

2.3.6.6 Method of Organism Cultivation

Three different methods of organism cultivation were compared to establish the effect of any differences that existed in organism recovery rates using different plating methods. Ten swabs were each inoculated with 0.1ml of a known level of either *E.coli* or *S. aureus*. Of the ten inoculated swabs, five were streaked directly onto the surface of nutrient agar plates, while the other five swabs were released into each of five 10ml MRD recovery diluent tubes, released for thirty seconds using mechanical agitation (vortexing), serial decimal dilutions prepared, and then these were plated out on nutrient agar using both duplicate 0.1ml spread plates and 1ml pour plates. All plates were incubated at 37°C for twenty four hours.

2.3.6.7 Re-moistening Inoculated Dry Surfaces

Surface swabbing experiments in which an attempt was made to reproduce the way in which the swabbing technique may be used in industry, through swabbing dry surfaces, resulted in very low bacterial recovery rates, often of less than one percent of the initial inoculum applied to the surface. As a result of the low organism recovery rates gained from swabbing dry surfaces, an experimental protocol was designed in an attempt to ascertain the effect on bacterial recovery of re-moistening an inoculated dry surface before sampling. This involved inoculating each of fifteen 100 cm^2 areas of stainless steel with 0.1ml of culture that was spread over the entire surface area to within 2mm of the edges. Of the fifteen inoculated surfaces, five were sampled

immediately after inoculation while still wet using pre-moistened cotton swabs. The remaining ten inoculated surfaces were allowed to dry for sixty minutes after which five surfaces were sampled while dry using pre-moistened swabs, with the remaining five dry inoculated surfaces being re-moistened through the addition of 0.1ml of MRD which was spread over the entire surface to within 2mm of the edges using a sterile plastic spreader. Each of the surfaces was then sampled using one pre-moistened swab as outlined earlier. All swabs were placed in individual 10ml MRD diluent recovery tubes, released for thirty seconds by mechanical agitation (vortexing) and duplicate 1ml pour plates prepared using nutrient agar. All plates were incubated at 37°C for twenty four hours.

2.3.7 Statistical Analysis

Data from all experimental protocols were analysed using two tailed Student's *t* tests (Excel) to establish the significance of any differences ($p<0.05$) that existed in bacterial recovery rates, in order that the effect of individual sampling variables on organism recovery could be determined.

2.4 Results

Table 2.3 classifies the sampling variables into those that were not found to influence bacterial recovery rates for all organisms, and those that did have an effect on recovery rates. It has been shown that the variables not influencing recovery rates from stainless steel food contact surfaces using cotton swabbing were the cultivation media used, the organism release method and the use of a single versus a double swabbing procedure. Results for these variables were not found to give statistically significant results when analysed using two tailed Student's *t* tests ($p>0.05$). The sampling variables which were found to influence recovery rates were the status of the surface at time of swabbing, i.e. wet or dry, the nature of the organism being recovered, the release time for swab samples, the type of recovery diluent used, and the method of organism cultivation. Results for these variables were found to be statistically significant when analysed using two tailed Student's *t* tests ($p<0.05$).

Table 2.3 Cotton Swabbing Sampling Variables Classified into Two Groups depending upon their Effect on Bacterial Recovery Rates from Inoculated Stainless Steel Food Contact Surfaces.

Sampling variables not influencing bacterial recovery rates from surfaces	Sampling variables found to influence bacterial recovery rates from surfaces
Cultivation media	Surface moisture level
Organism release method	Nature of organism
Single versus double swabbing procedure	Organism release time
	Method of organism cultivation
	Diluent type

Based upon statistically significant results being found through two tailed Student's *t* tests (Excel)

The effect of different release methods and release times on the recovery of *Staphylococcus aureus* and *Escherichia coli* from inoculated stainless steel surfaces when sampled while dry is presented in Table 2.4. Recovery rates for both organisms were less than 0.1%, ranging from 0.001% to 0.008% for *Staphylococcus aureus*, and from 0.0008% up to 0.001% for *Escherichia coli*. When direct swab inoculations were used to ascertain the effect of increasing release time, it was found that increasing the release time using mechanical agitation (vortexing) increased the mean percentage recovery of *Escherichia coli* from 2% after no mechanical agitation to over 140% after sixty seconds, that is maximum recovery of the organism being achieved. Increasing the release time did not, however, result in as much of an increase in the recovery of *Staphylococcus aureus.* Mean percentage recovery rates for this organism ranged from 0.7% after no mechanical agitation up to 35.2% recovery after sixty seconds mechanical agitation.

Statistically significant differences in mean percentage recovery rates were observed when two different diluent types were compared for the recovery of *Staphylococcus aureus* and the environmental isolate. Table 2.5 shows that for *Staphylococcus aureus*, MRD gave a mean percentage recovery rate of 6.25% compared to a mean percentage

Table 2.4 The Effect of Organism Release Method and Release Time on Bacterial Recovery Rates from Inoculated Stainless Steel Food Contact Surfaces Sampled while Dry

Organism	Estimate Level of Surface Inoculum/100cm^2	Release Method	Release Time	Mean Percentage Recovery/100 cm^2 based upon initial inoculum applied +/- Standard Deviation
Staphylococcus aureus (n = 90)	4.22 x 10^6	Mechanical Agitation (vortexing)	5 seconds	<0.1 +/- 0.008
		Handshaking	5 seconds	<0.1 +/- 0.007
		Handshaking	15 seconds	<0.1 +/- 0.002
Escherichia coli (n = 90)	2.0 x 10^6	Mechanical Agitation (vortexing)	5 seconds	<0.1 +/- 0.003
		Handshaking	5 seconds	<0.1 +/- 0.002
		Handshaking	15 seconds	<0.1 +/- 0.0004

Table 2.5 The Effect of Diluent Type on Bacterial Recovery Rates From Inoculated Stainless Steel Food Contact Surfaces Sampled while Dry

Organism	Estimate Level of Surface Inoculum/ 100 cm^2	Diluent Type	Mean Percentage Recovery/100 cm^2 based upon initial inoculum applied +/- Standard Deviation
Staphylococcus aureus (n = 60)	3.85×10^6	0.1% B.P.	0.96 +/- 0.010
		M.R.D.	6.25 +/- 2.26
Environmental Isolate (Staphylococcus sp) (n = 60)	3.82×10^6	0.1% B.P.	35.53 +/- 9.17
		M.R.D.	23.51 +/- 5.18

recovery of 0.96% for 0.1% BP. In comparison, the use of 0.1% BP resulted in better recovery of the environmental isolate than MRD. Mean percentage recovery rates for this organism were found to be 35.53% using 0.1% BP and 23.51% using MRD.

The effect of different culture media on the recovery of the organisms is presented in Table 2.6. No statistically significant differences in recovery were found in cultivating *Staphylococcus aureus,* recovered from inoculated stainless steel surfaces when sampled while dry in either nutrient agar or plate count agar. Mean percentage recovery rates were found to be 0.39% for nutrient agar and 0.43% for plate count agar. In comparison, the mean percentage recovery rates for *Escherichia coli* using nutrient and plate count agars were found to be 0.11% and 1.70% respectively. When direct swab inoculations were used to determine the effect of different methods of cultivation on organism recovery, highest recovery rates were achieved using 1ml pour plates with mean percentage recovery rates of 80% and 157% being gained for *Staphylococcus aureus* and *Escherichia coli* respectively. Lowest recovery rates were achieved using direct streaking, with mean percentage recovery rates of 16% for *Staphylococcus aureus* and 15% for *Escherichia coli* being gained. Spread plates gave recovery rates of 70% for *Staphylococcus aureus* and 56% for *Escherichia coli.*

Table 2.7 illustrates the effect of retaining swab samples in their recovery diluents for up to four hours at ambient temperature on mean percentage recovery rates after releasing followed by cultivation and incubation. It was found that slight variations in recovery did occur after a four-hour storage period. These variations in recovery ranged from a 0.4% reduction in recovery of *Staphylococcus aureus* when stored in 0.1% BP to a 3.7% reduction in the recovery of the same organism when stored in MRD. In comparison, the mean percentage recovery of the environmental isolate was reduced by 5.3% after a four hour storage period in 0.1% BP, with no reduction in percentage recovery being observed when stored in MRD for four hours, with a 1.1% increase in recovery being noted.

Table 2.6 The Effect of using Different Culture Media on Mean Percentage Recovery Rates of *S. aureus* and *E.coli* Recovered from Inoculated Stainless Steel Food Contact Surfaces Sampled while Dry

Organism	Estimate Level of Surface Inoculum/ 100 cm^2	Mean Percentage Recovery/100 cm^2 Nutrient Agar +/- Standard Deviation	Mean Percentage Recovery/100cm^2 Plate Count Agar +/- Standard Deviation
Staphylococcus aureus (n = 30)	2.0×10^6	0.39 +/- 0.06	0.43 +/- 0.04
Escherichia coli (n = 30)	2.0×10^6	0.11 +/- 0.23	1.70 +/- 1.52

Recovery rates are based upon level of initial inoculum applied to surface.

Media composition (g/L): PCA: Yeast Extract 2.5g; Pancreatic digest of Casein 5.0g; Glucose 1.0g; Agar 15.0g.

NA: Lab Lemco Powder 1.0g; Yeast Extract 2.0g; Peptone 5.0g; NaCl 5.0g; Agar 15.0g.

Table 2.7 The Effect of Soaking Released Bacteria in Recovery Diluent Tubes at Ambient Temperature for up to Four Hours on Mean Percentage Recovery Rates

Organism	Estimate Level of Surface Inoculum/100cm^2	Diluent Type	% Recovery immediately after Swabbing	% Recovery after 1 Hour Soak	% Recovery After 2 Hour Soak	% Recovery after 3 Hour Soak	% Recovery after 4 Hour Soak
Staphylococcus aureus (n = 150)	8.4 x 10^6	0.1% B.P.	1.4 +/- 1.04	1.2 +/- 1.05	1.3 +/- 1.13	1.3 +/- 1.01	1.0 +/- 0.90
		M.R.D.	4.9 +/- 5.38	2.1 +/- 3.50	1.6 +/- 1.96	1.3 +/- 1.56	1.2 +/- 1.53
Environmental Isolate *(Staphylococcus Sp)* (n = 150)	6.4 x 10^6	0.1% B.P.	32.5 +/-11.15	30.7 +/- 10.38	28.2 +/- 10.28	28.7 +/-11.08	27.2 +/- 10.04
		M.R.D.	23.3 +/- 3.81	23.8 +/- 3.81	22.9 +/- 3.86	23.1 +/-3.90	24.4 +/- 4.55

Recovery rates are based upon level of initial inoculum applied to surface.
0.1% B.P. – Bacteriological Peptone
M.R.D. – Maximum Recovery Diluent
All percentage recovery results are presented with standard deviation values

Table 2.8 provides mean percentage recovery rates gained from using both a one-directional single and a two-directional double swabbing procedure for the recovery of *Staphylococcus aureus* and the environmental isolate from inoculated stainless steel surfaces when sampled while dry. No statistically significant differences in organism recovery rates were found between both swabbing procedures. Recovery rates for *Staphylococcus aureus* were 1.30% for the single swabbing procedure and 1.44% for the double swabbing procedure. For the environmental isolate mean percentage recovery rates were 20.3% and 20.5% for the single and double swabbing procedures respectively.

Table 2.9 summarises mean percentage recovery rates gained for *Staphylococcus aureus* and *Escherichia coli* from stainless steel surfaces when sampled while wet or dry, and after re-moistening surfaces that had been allowed to dry completely before sampling. Highest organism recovery rates were gained from sampling wet surfaces, with mean percentage recoveries of 34.3% and 56.5% being achieved for *Staphylococcus aureus* and *Escherichia coli* respectively. Mean percentage recovery rates gained from dry surfaces were similar to those gained in earlier experiments, these being 0.20% for *Staphylococcus aureus* and 0.01% for *Escherichia coli.* Re-moistening inoculated surfaces that had been allowed to dry completely beforehand was not found to improve recovery rates to any great extent. Mean percentage recovery rates were found to be 0.70% for *Staphylococcus aureus* and 0.50% for *Escherichia coli* after re-moistening the surface.

Table 2.8 The Effect of Single Versus Double Swabbing on Mean Percentage Recovery Rates of *Staphylococcus sp* from Inoculated Stainless Steel Food Contact Surfaces Sampled while Dry

Organism	Estimate Level of Surface Inoculum/100cm^2	Method of Swabbing	Mean Percentage Recovery/100cm^2 based upon initial level of inoculum +/- Standard Deviation
Staphylococcus aureus (n = 60)	8.5 x 10^6	Single	1.3 +/- 0.86
		Double (Two-Directional)	1.4 +/- 0.56
Environmental Isolate *(Staphylococcus Sp)* (n = 60)	1.6 x 10^5	Single	20.3 +/- 4.97
		Double (Two-Directional)	20.5 +/- 5.66

Table 2.9 A Comparison of the Effect of Surface Moisture Level on the Recovery of Bacteria from Stainless Steel Food Contact Surfaces using Cotton Surface Swabbing

Organism	Estimate Level of Surface Inoculum/100cm^2	Mean % Recovery from Surfaces Sampled while Wet +/- Standard Deviation	Mean % Recovery from Surfaces Sampled while Dry +/- Standard Deviation	Mean % Recovery from Re-moistened Surfaces after Drying +/- Standard Deviation
Staphylococcus aureus (n = 45)	6.73 x 10^6	34.3 +/- 6.98	0.20 +/- 0.18	0.70 +/- 0.26
Escherichia coli (n = 45)	5.56 x 10^7	56.5 +/- 4.36	0.01 +/- 0.008	0.50 +/- 0.17

Recovery rates based upon level of initial inoculum applied to surface.

2.5 Discussion of Results

The work reported in this chapter evaluated a number of sampling variables potentially affecting the recovery of bacteria from stainless steel food contact surfaces using cotton hygiene swabbing under conditions of use typically found in the food industry, and has shown that a number of these sampling variables do have an effect of the efficiency with which bacteria are recovered and detected using the swabbing method.

It is clear that bacterial recovery rates achieved using cotton hygiene swabbing are influenced by a number of the sampling variables evaluated, and that the most important of these is surface moisture level at the time of sampling.

One of the most important findings has been the very low bacterial recovery rates obtained from surfaces when sampled while dry, and that the initial experimental protocols, which were designed to represent as closely as possible the way in which the swabbing method is used in industry, were not as efficient at enabling the assessment of the effects of these sampling variables as was initially hoped. The inability to recover over 99% of contaminating surface bioburden from dry surfaces when using laboratory type cultures raises the question of why such large amounts of bioburden are not being recovered after relatively short residence times on the stainless steel food contact surface. Whether this loss in recovery may be due to cell death as a result of desiccation, or attachment of the organisms to the surface to the extent that the swab method is unable to remove the attached cells remains uncertain. While both of these explanations may be possible, another important consideration is the possibility of the method recovering non-culturable cells, or perhaps a partial inability of the swab to release the recovered bioburden for cultivation and enumeration. It was interesting, however, to note that the recovery rates for the environmental isolate used were considerably greater, and that percentage recovery levels of up to 36% were achieved using the surface inoculation protocol. This suggests that over time organisms present on food contact surfaces may adapt to their environment, attaching to the surface and becoming more resistant to drying, giving rise to

improved recovery rates. In addition, the effects of surface conditioning over time must not be ignored, and this may account for the variations in recovery rates seen in Table 2.4 and those in Tables 2.5 and 2.6. Slight variations in bacterial recovery rates from dry surfaces may be attributable to this effect. Subsequent work in which inoculated surfaces were sampled while wet clearly illustrate that improved bacterial recovery rates are achieved under these conditions, and this is investigated further in Chapter Four.

In experimental protocols not involving surface inoculations, the effects of some sampling variables have been illustrated very clearly. The length of time used in which to release recovered bacteria from swab buds was found to be a key variable influencing organism recovery, and it has been shown that over time periods from zero to sixty seconds, recovery rates for the test bacteria used ranged from under 1% up to a maximum of 100%. However, these findings also suggest that the low recovery rates gained from sampling inoculated surfaces while dry may be attributable, in part at least, to the inability of swab buds to release the recovered organisms. This is an important issue, especially when recommending a sampling protocol for the use of surface swabbing to industry. It would not be practical, for example, to recommend that individual swabs are released for sixty seconds, especially when large numbers of swabs require analysis. Release times of this order would significantly increase the total time required to analyse large volumes of swab samples. It was, therefore, decided that a thirty second release time for all swabs would be used. This facilitated the release of recovered organisms, but yet did not require large amounts of time, which would be a critical consideration in industry.

The use of different cultivation media was not found to influence recovery rates from inoculated surfaces when sampled while dry to any great extent, yet comparisons of this sort may not be as valid since the bacterial recovery rates gained from the inoculated surfaces sampled while dry were very low through reasons explained above. No increases in mean recovery rates were found when a double swabbing procedure was compared to a single swab procedure. Clear distinction needs to be made between what is meant by these terms. In this study, a single swab

procedure is described as one in which one swab is used to swab each surface area in one direction from the top left to the bottom right of the surface. The double swabbing procedure uses two swabs, one moist and one dry, but each is used to swab the surface in different directions at 90° to each other. No differences in bacterial recovery rates were found, and as a result subsequent swabbing experiments in this thesis used a single swab procedure where swabbing was performed in two directions at 90° to each other. Despite the fact that different cultivation media were not found to influence organism recovery rates, it was found, however, that different methods of organism cultivation did result in different recovery rates, with pour plates resulting in the best levels of recovery of up to 100%.

No statistically significant differences in mean percentage recovery rates were found when swab samples were stored for up to four hours at ambient temperature before plating. This indicates that short time intervals between swabbing and subsequent laboratory analysis will not have any marked effect on resulting bacterial recovery rates, although in some instances a small reduction in recovery rates were observed.

Within industry, it is possible that the variables potentially affecting bacterial recovery rates using cotton swabbing can be classified into those which can be controlled through the sampling process and those which cannot. Clearly, the nature of the surface sampled and the contaminating bioburden present on it are uncontrolled variables, while the swabbing protocol used and when swabbing is performed can be controlled. The findings of this study demonstrate that many of the controlled variables would appear to have little or no effect on the ability of the swabbing method to recover organisms when used to sample food contact surfaces. It has been suggested by Whyte *et al.,* (1989) that to establish the number of bacteria on a surface it is necessary to know the efficiency of any proposed method of sampling, and that for many sampling methods this efficiency is unknown, and where it is supposedly known, it has been obtained by some artificial method. The authors state that it is common to calculate the efficiency of a sampling method by drying a bacterial suspension of known concentration onto a surface, and that the efficiency of a method calculated in this way may be misleading, especially

when factors such as the type of bacteria, penetration of bacteria within the surface, and the adhesion of bacteria to the surface are variable. The rate of recovery of organisms from a surface is largely dependent upon the consistency of the surface contamination (Favero *et al.*, 1968) and will also be influenced by the nature of organism attachment, as described in Chapter One.

Very little published work exists in which the cotton swabbing method has been evaluated under controlled laboratory conditions, yet several studies have used the method for assessing levels of *in situ* microbial contamination on a number of different types of surfaces, and these have been outlined in the Introduction to this Chapter. It is difficult to compare the present data with that from other studies since the sampling protocols used and the types of surfaces sampled are not identical. One of the most important considerations, however, is the issue of what exactly is being sampled. Organism recovery rates reported in the present study have been expressed as a percentage of the initial inoculum level applied to the surface, where the level of organism viability on the surface at the time of sampling was unknown. In published studies in which *in situ* contamination levels on surfaces have been assessed, the level of surface contamination is obviously unknown, and therefore the recovery rates gained represent only what the sampling protocol used was able to recover. For this reason, it is difficult to make any conclusions on the efficiency with which *in situ* methods recover contaminating surface bioburden.

Gill and Jones, (2000) found that in sampling beef carcasses for levels of *in situ* contamination by a number of methods, the numbers of organisms recovered using cotton swabbing were lower or below the range of numbers of organisms recovered using sponges or gauze swabs, where recovery rates using cotton swabbing were generally about one log unit less than the log total numbers recovered by other methods. Similar work reported by Anderson *et al.*, (1987) found that when cotton swabs were used to sample dressed beef carcasses, recovery rates were much lower and appeared to be influenced by the nature of the area sampled. It was concluded that the swab method was less efficient.

Work involving the examination of paper ice cream containers using the cotton swab method (Speck and Black, 1937) in which several swabs were used to sample each ice cream container has shown that the majority of the microbial contamination recovered from the container surfaces was removed by the first moist and second dry swab, with up to 83% of the total contaminating bioburden recovered being removed by one moist and one dry swab. While no significant differences in organism recovery rates were found in the present study when a two swab protocol, where one moist and one dry swab was used, was compared to a single swab procedure, the results of the study by Speck and Black (1937) would suggest that most of the recoverable microbial bioburden from a surface is in actual fact recovered by the initial swabs used. This is in agreement with the work of Silverman *et al.*, (1981) who, in using the swab method to assess the cleanliness levels of food preparation surfaces, indicates that over 50% of the microbial bioburden on surfaces is removed by the first swab when a three swab protocol was used, but acknowledges that the range of recovery rates obtained was variable. Other work by Buchbinder (1947), that examined the cleanliness of eating and drinking utensils, suggests that swabbing a surface in two directions, and increasing the number of swab strokes, increases the rate of organism recovery. These findings are supported by the work of Yamayoshi *et al.,* (1984) who investigated surface sampling using a single swab method. Vinyl, stainless steel, glass and enamelled tiles were used as test surfaces, and were inoculated with known levels of *Escherichia coli.* Each surface area (20cm x 20cm) was swabbed on up to seven occasions, but exact details of the swabbing protocol employed are not provided. It was found that, regardless of surface type, by increasing the number of occasions on which a surface was swabbed, an increase in the percentage recovery of *Escherichia coli* was found. After swabbing a surface once, percentage recoveries ranged from 50 to 60%, while after swabbing a surface twice, percentage recoveries increased to between 75 and 85%. After swabbing surfaces four times, percentage recoveries were greater than 95% of the inoculum level applied prior to sampling. However, no information is provided on whether surfaces were sampled while wet or after a period of drying. These findings support the recommendation to industry to always swab surfaces in two directions, and to ensure that in sampling surfaces that the swab bud comes into complete contact with the entire surface area being sampled.

Other work examining the removal of known levels of bacteria from wet glass surfaces using swabs made from a range of materials by Barnes (1952) found that cotton swabs were no more reliable than alginate ones, and that recovery rates using cotton swabs were very low at 9.1%. While it is not possible to directly compare the results gained in that study with those in the present one because of the use of different types of surface, the low recovery rates gained from sampling glass surfaces are not too dissimilar to some of the results reported in the present study. Similar work examining *in situ* microbial levels on both glass and china surfaces by Fellers and Levine (1936) found the cotton swab method to be the most satisfactory in terms of organism recovery rates, which ranged from 40 to 80%. Present research findings along with those from other published work clearly suggest that the cotton swabbing method is capable of recovering microbial bioburden from food contact surfaces, but that the success with which microbial bioburden is recovered is dependent upon a range of sampling variables. These variables include surface moisture level at the time of sampling and the nature of the swabbing process. Sampling variables found to influence organism recovery rates from food contact surfaces, determined through the experimental protocols in this present study, in addition include the nature of the organism being recovered, organism release time, diluent type and method of organism cultivation.

The work reported in this chapter has illustrated that a number of sampling variables influence the efficiency with which cotton hygiene swabbing is able to recover bacteria from stainless steel food contact surfaces. In comparison, the work reported in Chapter Three investigates the use of ATP bioluminescence as a method for determining levels of surface contamination.

2.6 Conclusions

A number of factors have been shown to influence the recovery of bacteria from stainless steel food contact surfaces using cotton hygiene swabbing, and these include surface moisture level at the time of sampling, the nature of the organisms being recovered, the organism release time, the diluent type and the method of organism cultivation used.

The most significant variable influencing organism recovery from stainless steel food contact surfaces using cotton swabbing is surface moisture level, with highest recovery rates being achieved from sampling surfaces while wet.

Current research findings indicate that in order to achieve optimum recovery of microbial contamination from stainless steel food contact surfaces using cotton hygiene swabs, sampling should be performed on surfaces while still wet. In addition, it is important to ensure that the swab is in constant contact with the surface, and that the swab bud is rotated constantly during swabbing. It is recommended that surfaces be swabbed in two directions at 90° to each other. In addition, swab samples should be stored for no longer than one hour at room temperature before analysis is performed. Recovered organisms from swab buds should be released using mechanical agitation (vortexing) for at least 30 seconds, except where direct surface inoculation of agar plates is used, with organism cultivation being carried out using either nutrient or plate count agar pour plates. It is suggested that organism recovery should be expressed as count (cfu)/100 cm^2.

Chapter 3

A Laboratory Evaluation of Selected Commercial ATP Bioluminescence Detection Systems for Surface Cleanliness Assessment

3.1 Introduction

Background information on the history and biochemistry of ATP bioluminescence has been included in Chapter One. Within the context of the food industry, ATP bioluminescence technology, specifically for use as a means of hygiene monitoring is evolving quickly with new developments continually appearing on the market. Most recently, developments in swab technology, including the introduction of single-shot assays, and assay systems that have improved minimum detection limits, have contributed to the continued increase in the use of ATP bioluminescence within the food industry for monitoring surface cleanliness, (Flickinger, 1996). Other important advances within the field have included the development of trend analysis software that enable those responsible for the hygiene function within an organisation to review data generated over a number of weeks or months. This ability to down-load data from the bioluminescence monitor to PC software is important within the context of HACCP since it allows data generated over long periods of time to be evaluated for trends (Griffith *et al.,* 1997). This, in turn, should provide valuable information to management on the effectiveness of cleaning regimes within a factory. These trends may, for example, highlight specific sites sampled which are continually giving rise to high ATP readings, indicating inadequate cleaning, or perhaps cleaning staff who continually fail to clean specific areas within the factory to an acceptable standard. Such information would not be available using traditional microbiological analysis without considerable time and effort devoted to maintaining records of the microbiological data generated. In addition, the ability to download data from the bioluminescence monitor to PC is especially useful given the introduction of Laboratory Information Management Systems (LIMS) for data management.

To be successful, HACCP is dependent upon generating real-time data. Not only have these ATP assay systems made this a practical reality given their ability to provide results within minutes, the ease of use of single-shot tests in particular has enabled the responsibility for surface hygiene monitoring to be devolved to factory operatives. Where cleaning has been identified as a control measure, for example, rapid cleanliness assessment using ATP bioluminescence may be helpful in establishing that surfaces are clean and that any residual organisms are not being provided with the opportunity to proliferate due to presence of organic residues. However, use under these circumstances would require validation of ATP bioluminescence by microbiological methods beforehand. In addition, ATP monitoring is now being used to help estimate the risks from cross-contamination within food processing environments, and to allow them to be identified and minimised more rapidly.

Despite being recognised as a valuable tool for monitoring the efficacy of cleaning and disinfection within food plants, and as a means of quickly verifying that effective cleaning and disinfection have been performed, debate still exists as to the capabilities and limitations of ATP bioluminescence hygiene monitoring systems (Flickinger, 1996). This may, in part at least, be due to the extensive range of ATP bioluminescence detection systems and assay types now available on the market. In addition, a number of other issues are important in deciding upon whether to adopt the technology within a food plant. These include, for example, the quenching effect of sanitizer residues present on surfaces that will affect the ATP signal through degradation of the firefly luciferin-luciferase substrate-enzyme system (Velazquez and Feirtag, 1997). Light signal quenching of between 6-47% resulted when five cleaning solutions were used at standard working concentrations, while ethanol at 1% inhibited bioluminescence by 15%, yet concentrations above 4% were found to enhance light output. The light signal was quenched by 20-25% at pH values below pH 4 and above pH 10 (Calvert *et al.,* 2000). However, ensuring that cleaned surfaces are adequately rinsed after being sanitised should help to eliminate these potential problems. Also important is the inherent ATP content of certain foods, or absence of ATP, that may give rise to either higher or lower than expected ATP readings, or false positive/negative results, when used in some food processing environments,

which again can be avoided through effective cleaning and rinsing of surfaces before sampling. However, despite what may be considered to be minor limitations of ATP bioluminescence, the ability of the technique to produce a measure of total surface cleanliness almost instantaneously make it one of the most valuable contributions to the science of hygiene monitoring over the past decade.

Regardless of the obvious potential that the technique has within the food industry, very few studies evaluating and comparing different ATP bioluminescence detection systems and assay procedures, with experimental protocols representing typical in-use conditions, have been executed within the context of surface hygiene monitoring. This has been partly due to ATP technology only becoming more readily available and more widely accepted by the food industry since the early 1990s. Some studies (Griffith, *et al.,* 1994; Flickinger, 1996; Colquhoun, *et al.,* 1998) have focussed on specific parameters potentially affecting assay minimum detection limit or reproducibility, but the experimental protocols used in these studies aimed at evaluating technical performance have varied. This in itself may suggest the need for the development of a series of standard experimental protocols with which to evaluate these systems under conditions representing typical use in industry. Some studies (Storgards and Haikara, 1996; Powell and Attwell, 1997; Illsley *et al.,* 2000) have focussed on evaluating detection systems under such conditions, but the work reported has been limited.

Within the context of rapid hygiene monitoring, the most important issue is whether or not an ATP assay and detection system is capable of accurately determining the efficiency of cleaning and providing a reliable indication of the outcome from cleaning processes. That is, can the ATP assay and detection system reliably determine whether a surface is free from contaminating organic soil and microorganisms?

Given the range of protocols used in previously published comparison studies, an important consideration in the present study was the need to ensure that the "technical performance" of the ATP assays and detection systems was evaluated under comparable defined sets of conditions

and that these conditions, as far as possible, represented use of the systems under typical conditions. Technical performance encompasses a number of aspects including the ability of the surface swabbing stage to remove total and microbial ATP, for the extractant to extract the ATP, for the ATP to be converted into light, and for the detection system photomultiplier tube to detect and measure the light produced from the ATP bioluminescence reaction. The present study, therefore, developed experimental protocols that assessed these individual components of total ATP minimum detection limit under typical conditions of use, as shown in Figure 3.1 – Components of Total ATP Minimum Detection Limits and Assay Procedures used in this Study. The figure illustrates the components of ATP assay and detection systems that contribute to the overall minimum detection limit achieved. These components include the ability of the swab device to remove both residual food and microbial contamination from a surface, from which total and microbial ATP levels are derived. In addition, the ATP detection limit achieved will be dependent upon the ability of the assay procedure used to extract the ATP and convert it into light, which must in turn be detected by the photomultiplier tube within the ATP detection system. In order that these components could be assessed, a series of experimental protocols were developed. These included raw milk surface inoculations that enabled all components contributing to total ATP minimum detection limits to be assessed, and *Escherichia coli* surface inoculations that assessed the removal of microbial ATP and its subsequent extraction and detection. The ability of individual detection systems to extract ATP and then convert that ATP into light for detection by the detection system was assessed through *Escherichia coli* swab inoculations, while the ability of detection systems to convert ATP into light and detect that light was assessed through swab inoculations with pure ATP solutions. Figure 3.2 illustrates the different assay procedures used by the ATP bioluminescence detection systems available for assessment, along with information on the main variables potentially affecting assay performance. These variables include the efficiency of the swab wetting agent at removing surface bioburden, the nature of the ATP extractant, the solubilisation of the assay reagents, the effect of quenching on ATP signal due to the presence of, for example, sanitizer residues on a surface at the time of sampling, and the time lapse between sampling and measuring ATP.

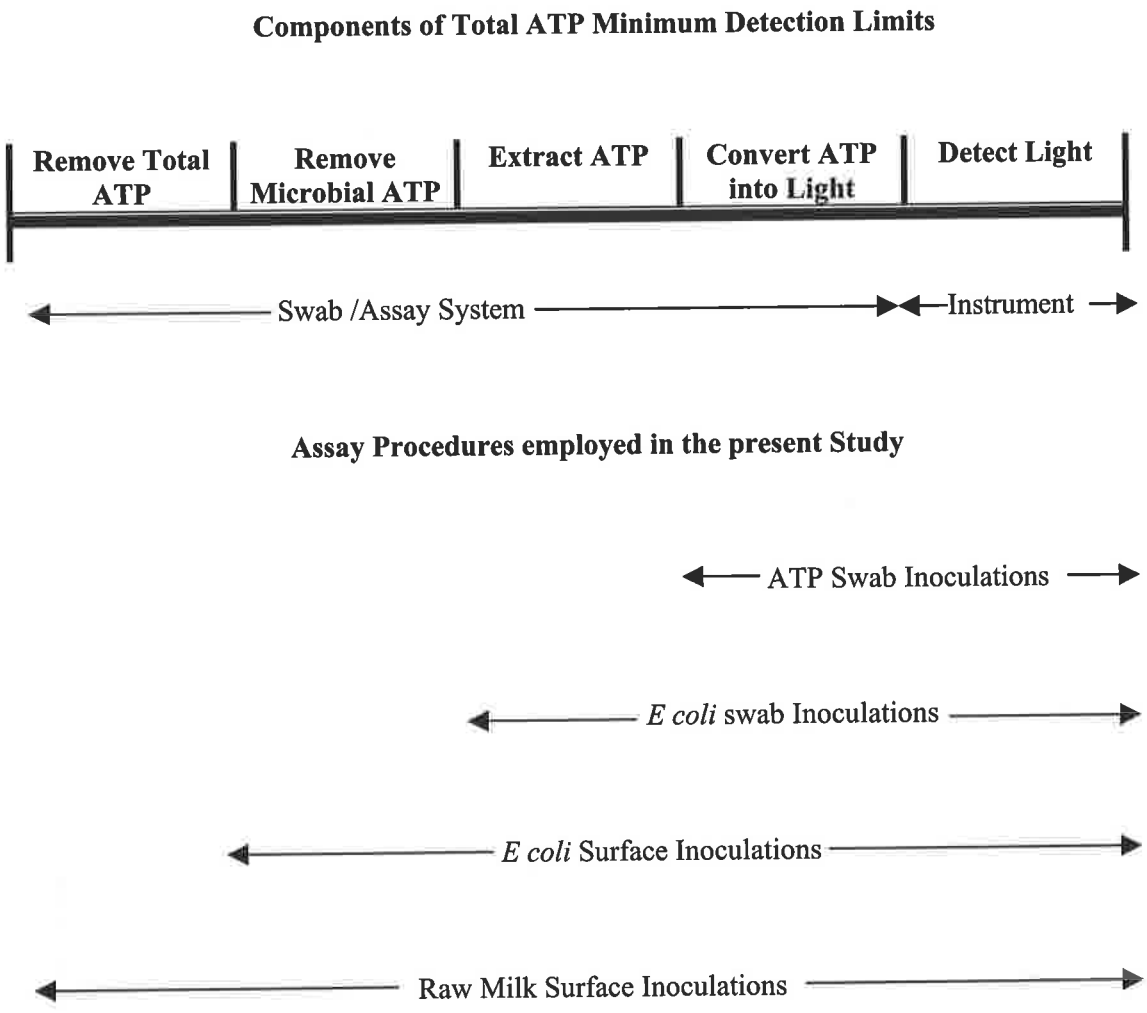


Figure 3.1 Components of Total ATP Minimum Detection Limits and Assay Procedures used in this Study.

It is clear that a number of variables are important in determining the efficiency of ATP bioluminescence testing, in addition to several factors that may influence the decision of whether or not to use ATP bioluminescence for assessing surface cleanliness within the food industry. Some of these factors include the achievable minimum detection limits of the ATP assay, the repeatability and reproducibility of the results gained, and the ease and suitability of use of the assays and detection systems. The work reported in this chapter evaluated some of these variables using *E. coli* as the test organism, which was selected on the basis that it is a typical indicator organism.

3.2 Aims

The aims of the work reported in this chapter were, therefore, to:

1. Determine the minimum detection limits of nine ATP bioluminescence assay and detection systems when used to detect bacterial contamination on stainless steel food contact surfaces;

2. Determine the minimum detection limits achieved by nine ATP bioluminescence assay and detection systems when used to detect solutions of pure ATP and levels of *Escherichia coli* inoculated directly onto hygiene swab buds;

3. Determine the repeatability and reproducibility of results gained from nine ATP bioluminescence assay and detection systems when used by two operatives to monitor clean and marginally unclean stainless steel surfaces;

4. To evaluate the ease of use and suitability for use of nine ATP bioluminescence assays and detection systems when used by both technically trained and untrained operatives.

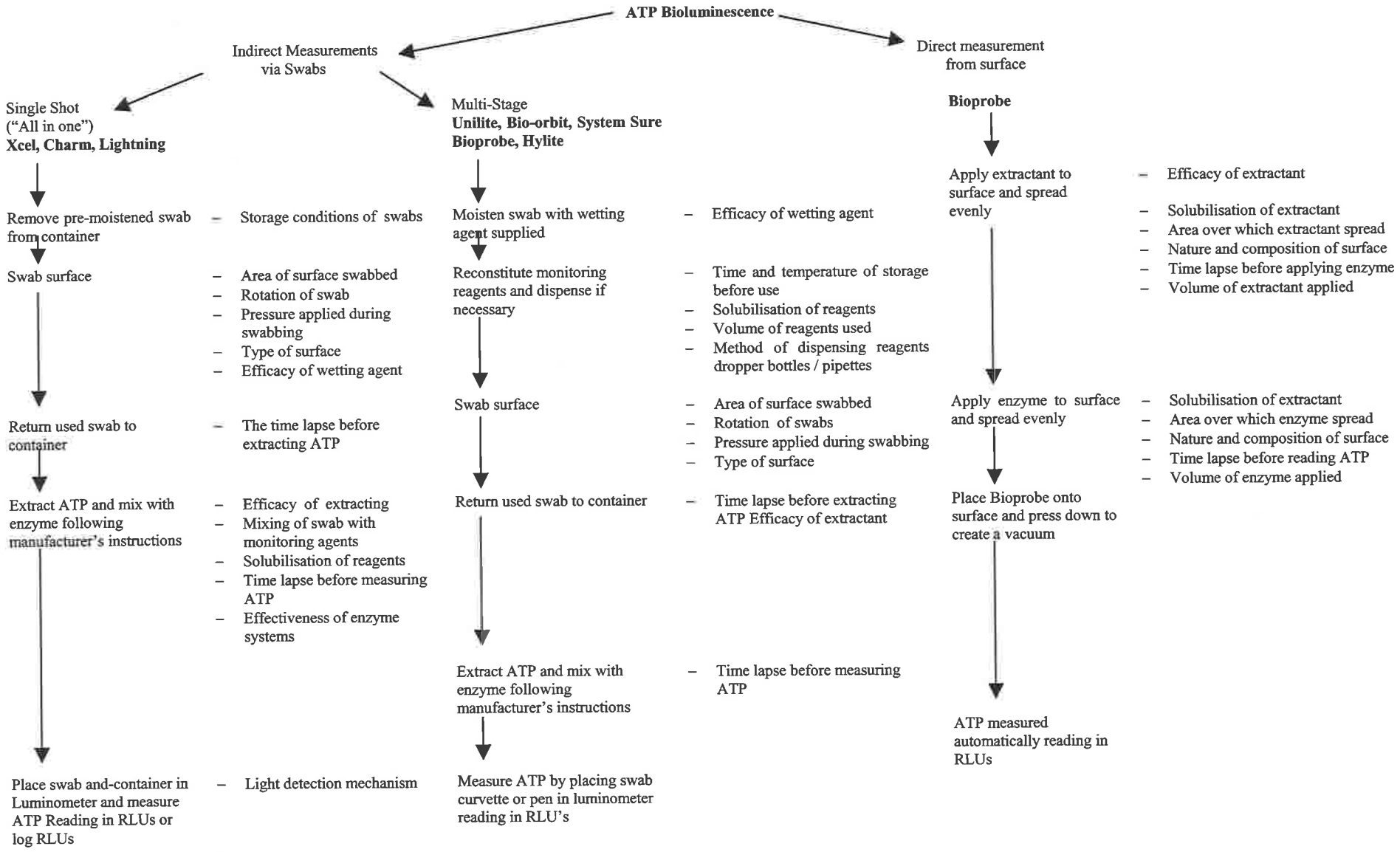


Figure 3.2 ATP Bioluminescence Monitoring Protocols and Variables potentially affecting ATP Assay Performance

3.3 Materials and Methods

3.3.1 Bacterial culture

Escherichia coli (ATCC 25922) was grown in sterile Nutrient Broth No. 2 (CM67, Oxoid, Basingstoke, UK) in unshaken batch culture volumes of 10ml under aerobic conditions at 37°C for eighteen hours. Serial decimal dilutions of the eighteen hour culture, prepared in sterile Maximum Recovery Diluent (MRD, CM733, Oxoid) were used to inoculate a food-grade stainless steel table and hygiene swabs.

3.3.2 ATP Bioluminescence Detection Systems

Nine commercially available ATP bioluminescence detection systems and assay reagents were included in the comparison study, which have been outlined in Table 3.1. These included four "single-shot" systems and one system that were capable of performing direct ATP measurements from surfaces, in addition to making indirect ATP measurements via swabs. The remaining four systems involved pipetting procedures as part of the assay protocol.

3.3.3 Preparation of ATP Standard Solutions

An 80nM ATP standard supplied by Celsis-Lumac (Cambridge, UK) was used to prepare a series of pure ATP standard solutions ranging from 1nM (10 femtomoles) down to 0.025nM (0.25 femtomoles). Detailed instructions for the preparation of these ATP standard solutions are provided in Appendix One.

3.3.4 Surface Preparation

Prior to inoculation, the stainless steel table, marked with 100 cm^2 areas, was pre sanitized for 30 minutes using a 1:80 dilution of Bioscan containing <5% cationic surfactants, 5-15% non-ionic surfactants and <5% phosphoric acid (Henkel Ecolab Ltd, Swindon, UK), which acts through disrupting cell membrane integrity. The surface was then cleaned using an "in-house" validated cleaning protocol. This involved washing with hot water and detergent (<5% amphoteric, 5-15% non-ionic, and 15-30% anionic surfactants), applying kinetic energy for two

minutes using a new rayon cloth, thoroughly rinsing with hot water to remove all traces of detergent, and a final rinse with boiling water before being left to air dry at room temperature. This was demonstrated to give microbiological counts less 2.5 cfu/cm^2 using agar contact dip slides and ATP bioluminescence readings consistently below 100 relative light units (RLU) using the Biotrace Xcel detection system and Clean-Trace Rapid Cleanliness Test.

3.3.5 Surface Inoculation and Sampling

Aliquots of 0.1ml of an eighteen hour culture of *Escherichia coli* containing 10^7 cfu in 0.1ml and dilutions giving inoculation levels of 10^6 - 10^3 cfu/0.1ml were inoculated onto each of ten 100 cm^2 areas of stainless steel for each dilution and spread over the entire surface to within 2mm of the edges using a sterile plastic spreader (Technical Service Consultants, Henlow, UK). Surfaces were sampled after a sixty minute residence time of the inoculum on the surface by which time the inoculum had dried completely so that there was no visible moisture present on the surface. All surfaces were sampled using a standard swabbing protocol (See 3.3.7). Each experimental protocol was repeated on at least three occasions, with ten replicates in each.

3.3.6 Direct Swab Inoculations

Aliquots of 0.1ml of an eighteen hour culture of *Escherichia coli,* prepared through serially diluting the culture in sterile MRD to give inoculation levels of between 10^6 and 10^1, and 0.1 ml of the prepared standard ATP solutions, were inoculated onto each of ten pre-moistened swab buds in separate experiments. Inoculated swabs were then assayed using the manufacturers recommended procedures. For each of the ATP detection systems under test a series of pre-moistened uninoculated swabs were treated as blanks against which all other data could be compared during statistical analysis. Each experimental protocol was repeated on at least three occasions, with ten replicates in each.

3.3.7 Surface Swabbing Protocol

In all experiments, including those evaluating operator repeatability and reproducibility, a standard surface swabbing protocol was used. The protocol involved ensuring that the pre-

moistened swab bud came into contact with the entire 100 cm^2 surface, that the swab was rotated constantly during swabbing, and that each surface was swabbed in two directions at 90° to each other. Swabs were held by their handles and not by their applicator sticks in an attempt to standardize the amount of pressure applied to the swab during sampling.

3.3.8 Determination of Assay Minimum Detection Limit

For the purpose of this study, *assay minimum detection limit* was defined as the minimum level of inoculum necessary to produce a positive test result above background ATP levels, and which was confirmed as being statistically significant using a one-tailed Student's *t* test.

Assay minimum detection limits were determined through a series of three experimental protocols that focussed on *Escherichia coli* surface and direct swab inoculations, and through swab inoculations using standard ATP solutions. In each experimental procedure each of ten 100 cm^2 areas of stainless steel or ten hygiene swabs were inoculated with 0.1ml of a known concentration of either bacterial culture or of standard ATP solutions in order to detect ATP directly. All surfaces were sampled after a residence period of sixty minutes, by which time the bacterial culture had completely dried on the surface. ATP determinations from direct swab bud inoculations were performed immediately after inoculation. All experimental procedures were repeated in duplicate, with the achieved minimum levels of detection being confirmed through at least one further repeat of each experimental procedure.

3.3.9 Determination of Operator Repeatability and Reproducibility

For the purpose of this study, the terms *assay repeatability* and *assay reproducibility* were defined as follows:

Assay repeatability: the ability of any operator to achieve the same test result on the same sample on each occasion the sample is tested.

Assay reproducibility: the ability of the swab, assay reagents and ATP bioluminescence detection system, when used by any operative under the same sampling conditions, to produce the same end result given a consistent level of initial surface bioburden.

Studies designed to assess operator repeatability and reproducibility involved two technically trained operatives each swabbing ten 100 cm^2 stainless steel areas. The stainless steel surfaces were sampled under three different sets of conditions which included inoculation with a known concentration of *Escherichia coli*, inoculation with fresh milk to represent organic food debris, each after a sixty minute residence time, or sampling after the surfaces had been cleaned and allowed to air dry. Mean percentage coefficients of variation for sampling under each set of conditions were recorded for each operative.

3.3.10 Ease of Use and Suitability of Use

Each of the ATP detection systems and assay procedures were demonstrated by a competent user of the equipment to a group of twenty-one volunteers comprising both technically trained and non-technically trained individuals. The demonstration procedure consisted of explaining and demonstrating the operation of each ATP bioluminescence monitor, and its corresponding assay procedure. Demonstration volunteers were then asked to score on a scale from one to five each of the ATP detection systems and assay procedures. A score of one represented greatest difficulty in use/ least appealing/ least suitable for use by non-technically trained staff, and a score of five the least difficulty in use/most appealing/most suitable for use by non-technically trained staff. A mean score for each attribute for each of the detection systems was then calculated.

3.3.11 Statistical Analysis

All data, excluding data on ease and suitability of use, were compared with appropriate control data using a one-tailed Student's *t* test (Excel) to estimate the significance of any difference

($p < 0.05$). Minimum detection limits were recorded as those levels at which statistically significant results were obtained. Levels of operator repeatability and reproducibility were determined through calculating coefficients of variation expressed as percentages.

3.4 Results

Table 3.1 provides details of the general characteristics of the different assay reagents and procedures used in this study. This includes the typical cost per assay, details on the stability and shelf life of the assay reagents, details on reconstitution requirements for reagents, and the maximum time interval recommended between swabbing and analysis. Table 3.2 provides summary data gained from a comparison of nine ATP detection systems when used to detect different components of total ATP detection limits. It can be seen that when used to detect *Escherichia coli* on stainless steel inoculated surfaces when sampled after a sixty minute residence time, the Celsis System Sure and the Hughes Whitlock Bioprobe when used in its direct mode had the lowest minimum detection limit, both at 10^4 cfu/100 cm^2. Three detection systems, the Biotrace Unilite, Biotrace Xcel, and the Merck Hylite had a minimum detection limit of 10^5 cfu/100 cm^2 when used to detect *Escherichia coli* inoculated onto the stainless steel surface. For the same experimental protocol it was found that four detection systems had a minimum detection limit of 10^6 cfu/100 cm^2. These were the Charm Luminator, the Rhone Poulenc Bio-Orbit, Idexx Lightning and the Hughes Whitlock Bioprobe in its indirect mode.

Different minimum detection limits were achieved when the detection systems were used to detect *Escherichia coli* inoculated directly onto swab buds. The lowest minimum detection limit achieved was 10^2 cfu/swab, this being achieved by the Rhone Poulenc Bio-Orbit system. The Biotrace Xcel and Celsis System Sure both detected down to 10^3 cfu/swab, while the Biotrace Unilite, Idexx Lightning and the Hughes Whitlock Bioprobe in its indirect mode detected 10^4 cfu/swab. The Charm Luminator and Merck Hylite both detected 10^5 cfu/swab. When pure ATP solutions were inoculated onto swab buds, the Hughes Whitlock Bioprobe had the lowest minimum detection limit of 0.25 femtomoles. Two detection systems, the Celsis System Sure and the Merck Hylite detected 2.5 femtomoles.

Table 3.1 General Characteristics of Eight Different ATP Bioluminescence Detection Systems and Assay Reagents

	Assay Reagents				Assay Procedure			
Detection System	**Typical cost per test (in 1997)**	**Reconstitution of reagents required**	**Stability of reagents open and reconstituted**	**Typical shelf-life of reagents (unused)**	**Single-shot or multi-stage**	**Swab moistening required**	**Pipetting required**	**Maximum time between swabbing and testing**
Biotrace Unilite	£1.25-£1.85	Yes	24 hours – 5 days	1 year below 8°C	Multi-stage	Yes	Yes	3 hours
Biotrace Xcel	£1.25-£1.85	No	N/A	1 year below 8°C	Single-shot	No	No	3 hours
Hughes Whitlock Bioprobe	£1.10-£1.20	Yes	1 day at 30°C 5 days at 4°C	1 year at 4°C	Multi-stage	Yes	No	3 hours
Rhone Poulenc Bio-Orbit	£1.20	Yes	48 hours at 25°C 1 week at 4°C	Minimum 5 Months at 4°C or at -18°C	Multi-stage	Yes	Yes	8 hours
Celsis System Sure	£0.95-£1.10	Yes	5 days at 4°C 1 day at 25°C	1 year below 5°C	Multi-stage	Yes	Yes	2 hours
Charm Luminator	£1.70	No	N/A	1 year below 5°C, 1 week at room temperature	Multi-stage	Yes	No	6 hours
Merck Hylite	£1.50-£1.80	No	N/A	5 months below 5°C	Multi-stage	Yes	No	48 hours
Idexx Lightning	£1.45-£1.60	No	N/A	Approx. 6-7 months	Single-shot	No	No	3 hours

Table 3.2 Comparison of Nine ATP Detection Systems and Different Components of Total ATP Detection Limits

Detection system/ Assay	Biotrace Unilite	Biotrace Xcel	Charm Luminator	Celsis System Sure	Rhone Poulenc Bio Orbit	Idexx Lightning	Hughes Whitlock Bioprobe (Direct)	Hughes Whitlock Bioprobe (Indirect)	Merck Hylite
Viable *E. coli* cells inoculated onto surface (sampled dry)	10^5	10^5	10^6	10^4*	10^6	10^6	10^4*	10^6	10^5
***Viable E. coli* cells inoculated onto swab**	10^4	10^3	10^5	10^3	10^2*	10^4	NA	10^4	10^5
ATP Swab Inoculations (Femtomoles)	10	10	10	2.5	>10	10	NA	0.25*	2.5

All values represent levels at which statistically significant results were achieved ($p<0.05$) when compared to control data using a one tailed Student's *t* test.

* Indicates the ATP detection system with the lowest minimum detection limit.

n = 30 for each assay using each ATP detection system.

The Biotrace Unilite, Biotrace Xcel, Charm Luminator and Idexx Lightning all detected ten femtomoles per swab, while the Rhone Poulenc Bio-Orbit had a detection limit greater than ten femtomoles per swab.

Table 3.3 shows data for levels of statistical significance obtained for each of the nine assay procedures from sampling *Escherichia coli* inoculated stainless steel surfaces following a sixty minute residence time. Levels of statistical significance are provided for each of the assay procedures at surface inoculation levels ranging from 10^7 cells/100 cm^2 down to 10^1 cells/100 cm^2. Data show that the detection system with the best level of detection under the experimental test conditions reported was the Celsis System Sure, being significant at the 10% level when used to detect 10^3 cells/100 cm^2. All statistically significant differences reported were determined through comparing ten ATP readings in RLUs for inoculated surfaces with ten ATP readings in RLUs for clean, uninoculated control surfaces using one tailed Student's *t* tests. Given that the differences existing between individual detection systems may be only marginal for the experimental protocols used, it was deemed worthwhile to report levels of statistical significance at the 1%, 5% and 10% levels.

Two assay procedures were able to detect 10^4 cells/100 cm^2 with results being statistically significant. These were the Celsis System Sure and the Hughes Whitlock Bioprobe in its direct mode, with results being significant at the 1% and 5% levels respectively. In its indirect mode, the Hughes Whitlock Bioprobe's minimum detection limit was found to be two log factors greater at 10^6 cells/100 cm^2.

Of the other assay procedures tested, five were found to have statistically significant minimum detection limits at 10^5 cells/100 cm^2. The least sensitive assay procedure, that is the one with the highest minimum detection limit, was found to be the Rhone Poulenc Bio-orbit, with a statistically significant minimum detection limit of 10^7 cells/100 cm^2.

Table 3.3 Levels of Statistical Significance Achieved by Nine ATP Detection Systems for Detecting Different Inoculum Levels of *Escherichia coli* on Stainless Steel Food Contact Surfaces

	ATP Detection System								
Level of Surface Inoculum/100 cm^2	**Biotrace Unilite**	**Biotrace Xcel**	**Charm Luminator**	**Celsis System Sure**	**Rhone Poulenc Bio-Orbit**	**Idexx Lightning**	**Bioprobe Direct**	**Bioprobe Indirect**	**Merck Hylite**
***Escherichia coli* 10^7 cells**	NT	5%	NT	NT	1%	NT	NT	5%	5%
***Escherichia coli* 10^6 cells**	5%	10%	5%	1%	NS	1%	ND	5%	5%
***Escherichia coli* 10^5 cells**	5%	1%	NS	10%	NS	10%	1%	NS	5%
***Escherichia coli* 10^4 cells**	NS	NS	NS	1%	ND	NS	5%	NS	NS
***Escherichia coli* 10^3 cells**	NS	ND	ND	10%	ND	ND	NS	NS	NS
***Escherichia coli* 10^2 cells**	NS	ND	ND	ND	ND	ND	NS	NS	NS
***Escherichia coli* 10^1 cells**	NS	ND	ND	ND	ND	ND	NS	ND	ND

Statistical significance is that estimated by comparison of test data with control results using a one tailed Student's *t* test.
NT – Not Tested; ND – Not Detected; NS – Not Significant.
n = 30 for each level of inoculum using each ATP detection system.

Table 3.4 summarises the results gained from experiments involving direct swab inoculations with various inoculation levels of *Escherichia coli.* It was found that the detection system with the lowest minimum detection limit under these experimental conditions was the Rhone Poulenc Bio-orbit, with a minimum detection limit of 10^2 cells/swab, with results being significant at the 5% level. The Celsis System Sure and Biotrace Xcel detection systems were found to have a minimum detection limit of 10^3 cells/swab, with results for each being significant at the 1% level. Three further detection systems, the Biotrace Unilite, Idexx Lightning and Hughes Whitlock Bioprobe (indirect mode) were found to have minimum detection limits of 10^4 cells/swab, with results for all being significant at the 5% level. Two systems, namely Charm Luminator and Merck Hylite were found to have a minimum detection limit of 10^5 cells/swab, with results being significant at the 1% and 5% levels respectively.

Results for ATP swab inoculations are provided in Table 3.5. As was the case with other experimental protocols, different detection systems were found to have different minimum detection limits. The detection system with the lowest minimum detection limit was found to be the Hughes Whitlock Bioprobe (in its indirect mode) being able to detect 0.25 femtomoles with results being statistically significant at the 5% level. The Merck Hylite and Celsis System Sure detection systems detected 2.5 femtomoles, with results for both detection systems being significant at the 5% level. Four of the remaining five detection systems were found to have a minimum detection limit of 10 femtomoles, with results being statistically significant at the 5% level for one, and at the 10% level for the other three detection systems. One detection system, the Rhone Poulenc Bio-Orbit, was unable to detect any of the ATP standard solutions. Given the ability of this detection system to produce results for other experimental protocols, its inability to detect the pure ATP standard solutions was not thought to be due to system failure.

Table 3.4 Levels of Statistical Significance Achieved by Eight ATP Detection Systems for Detecting Different Inoculum Levels of *Escherichia coli* Inoculated Directly onto Swab Buds

	ATP Detection System							
Swab Inoculum Level	**Biotrace Unilite**	**Biotrace Xcel**	**Charm Luminator**	**Celsis System Sure**	**Rhone Poulenc Bio Orbit**	**Idexx Lightning**	**Bioprobe Indirect**	**Merck Hylite**
Escherichia coli **10^6 cells**	1%	1%	1%	1%	ND	ND	1%	1%
Escherichia coli **10^5 cells**	1%	5%	1%	1%	1%	1%	1%	5%
Escherichia coli **10^4 cells**	5%	1%	NS	1%	1%	5%	5%	NS
Escherichia coli **10^3 cells**	NS	1%	ND	1%	10%	NS	ND	ND
Escherichia coli **10^2cells**	ND	ND	ND	ND	5%	ND	ND	ND
Escherichia coli **10^1 cells**	ND	ND	ND	ND	ND	ND	ND	ND

Statistical significance is that estimated by comparison of test data with control results using a one tailed Student's *t* test.
ND – Not Detected; NS – Not Significant.
n =30 for each level of inoculum and ATP detection system.

Table 3.5 Levels of Statistical Significance Achieved by Eight ATP Detection Systems for Detecting Different Levels of Pure ATP Inoculated Directly onto Swab Buds

	ATP Detection System							
ATP Applied to Swab	**Biotrace Unilite**	**Biotrace Xcel**	**Charm Luminator**	**Celsis System Sure**	**Rhone Poulenc Bio Orbit**	**Idexx Lightning**	**Bioprobe Indirect**	**Merck Hylite**
10 Femtomoles	5%	10%	10%	1%	NS	10%	1%	5%
5 Femtomoles	NS	NS	NS	5%	NS	NS	5%	10%
2.5 Femtomoles	NS	NS	ND	5%	NS	NS	5%	5%
0.5 Femtomoles	ND	NS	ND	NS	ND	ND	5%	ND*
0.25 Femtomoles	ND	ND	ND	ND	ND	ND	5%	ND

Statistical significance is that estimated by comparison of test data with control results using a one tailed Student's *t* test
ND – Not Detected; NS – Not Significant.
n = 30 for each ATP solution tested using each detection system.

Table 3.6 shows results for assay repeatability and reproducibility gained from two separate operatives under a number of different experimental procedures for each of the detection systems and assay procedures evaluated. For individual experimental protocols, data are expressed as mean percentage coefficients of variation (CV) that resulted from each operative sampling ten 100 cm^2 surfaces under the experimental conditions described. Detection systems deemed to be the most repeatable and reproducible were those with the lowest mean CVs for the individual experimental protocols evaluated.

Five detection systems, Biotrace Unilite, Celsis System Sure, Idexx Lightning, and the Hughes Whitlock Bioprobe in both direct and indirect modes, were found to have an overall mean CV of less than 40%, while another two, the Biotrace Xcel and Charm Luminator had an overall mean CV of less than 60%. Two further detection systems, the Bio-Orbit and the Merck Hylite, were found to have a mean CV of greater than 60%. Mean coefficient of variation values for individual experimental protocols for each of the two operatives are shown in Table 3.6. Within the realms of microbiological experimental error it can be seen that individual values for each experimental protocol for each operative are very similar.

Table 3.7 provides the mean scores for ease of use along with mean percentage values for suitability of use by technical and non-technical staff, for each of the ATP detection systems. The highest mean score for ease of use was 5.0 gained by the Biotrace Xcel detection system indicating that it was perceived to be the easiest to use. The lowest score of 1.86 was gained for the Hughes Whitlock Bioprobe when used in its direct mode.

Table 3.6 Reproducibility of Results Expressed as % CV for Different Experimental Protocols Achieved by Two Operatives using Nine ATP Detection Systems

	ATP Detection System								
Test materials / **Operator**	**Biotrace Unilite** A B	**Biotrace Xcel** A B	**Charm Luminator** A B	**Celsis System Sure** A B	**R. Poulenc Bio - Orbit** A B	**Idexx Lightning** A B	**Bioprobe Direct** A B	**Bioprobe Indirect** A B	**Merck Hylite** A B
Raw Milk	43 17	32 35	32 27	17 32	47 68	22 33	14 9	19 16	68 79
***Escherichia coli* Suspension**	26 31	53 60	46 70	34 28	57 212	39 44	36 15	27 53	171 26
Cleaned Surface	21 38	78 71	63 110	48 31	71 60	34 27	36 55	58 56	87 36
± Standard Deviation	10	19	31	10	62	8	18	20	52

Values expressed as % Coefficient of Variation (CV) obtained by two operatives.
For each detection system the data presented in the left hand column relate to operative A, while the data presented in the right hand column relate to operative B.
n = 30 for each test material tested using each detection system

Table 3.7 Opinions on Ease of Use and Suitability of Use of Nine ATP Detection Systems

ATP Detection System	Mean Score for Ease of Use (Max 5)	Suitable for Use by Technical Staff Only	Suitable for Use by Non Technical Staff
Bioprobe (Direct Mode)	1.8	76%	24%
Bioprobe (Indirect Mode)	2.1	71%	29%
Biotrace Uni Lite	3.1	24%	76%
Biotrace Xcel	5.0	0%	100%
Celsis System Sure	2.6	57%	43%
Charm Luminator	4.7	0%	100%
Idexx Lightning	4.0	5%	95%
Merck Hylite	2.9	24%	76%
Rhone Poulenc Bio Orbit	2.1	76%	24%

Two detection systems were considered suitable for use by non-technical staff by all (100%) of demonstration participants. These were the Biotrace Xcel and the Charm Luminator, both of which utilise "single-shot" assay procedures. Another system based upon the "single-shot" principle, the Idexx Lightning, was also scored as very suitable for use by non-technical staff by 95% of the demonstration participants. Detection systems that were considered least suitable for non-technical staff use were the Bio-orbit and the Bioprobe (in its indirect mode), both of which were only considered to be suitable for use by such people by 24% of demonstration participants. Both these systems involve several pipetting stages in the assay procedure, while the Hughes Whitlock Bioprobe in both its direct and indirect modes, and the Rhone Poulenc Bio-orbit were considered to be suitable for use by technically trained staff only.

3.5 Discussion of Results

The work reported in this Chapter evaluated nine ATP assay and detection systems through a series of experimental protocols representing typical conditions under which the detection systems may be used. These focussed both on swab and surface inoculations using *Escherichia coli* and pure ATP solutions to evaluate different components of total ATP minimum detection limit. In addition, assay repeatability and reproducibility was determined for two operatives through surface inoculations using milk. Ease and suitability for use by technically trained and untrained operatives was also determined.

Experimental results clearly show that differences did exist in the minimum detection limits (MDL) of the different ATP detection systems evaluated. In addition to MDL differences between detection systems, differences in MDLs within detection systems were also found when individual components of total ATP detection limit were being assessed, as shown in Figure 3.1. Several factors might contribute to these differences, and these have been listed in Figure 3.2 - ATP Bioluminescence Monitoring Protocols and Variables Potentially Affecting ATP Assay Performance. These factors include the efficiency of the swab wetting agent, the

solubilization of the assay reagents, the volume of reagents used, the efficiency with which ATP is extracted, and the effectiveness of the enzyme systems. In order that reliable test results are gained, it is important that the swab wetting agent used is capable of removing surface bioburden, and that the extractant is able to lyse cells open to release cellular ATP. These two stages in the assay procedure are critical to ensuring that an accurate end result is achieved. It is also necessary that the assay reagents solubilize fully, and that the volumes used are sufficient in order that the assay performance is optimal.

Differences were also found in the repeatability and reproducibility of the results gained for different experimental procedures using the different detection systems. While variations in minimum detection limits may be the result of the enzyme and reagents used, differences in repeatability and reproducibility may be the result of a number of factors, not least of which will be the system operators and the surface swabbing protocol they employ. Being able to determine the achievable minimum detection limits of the individual detection systems included in the present study was of particular importance for a number of reasons. Given that the minimum detection limit achieved by any of the systems represents the lowest level at which ATP detection was possible, the detection limit values gained represent threshold values below which ATP detection would not be possible. This may be a critical consideration where the difference between what would be deemed clean and unclean is negligible, especially within HACCP where pass and fail critical limits have been determined. However, ATP detection systems with very low minimum detection limits may be considered unsuitable for use, especially where the number of surfaces failing cleanliness assessment is high due to the low minimum detection limit of the system used. It is important, therefore, that in using detection systems with low minimum detection limits that realistic pass and fail limits for surface cleanliness levels are set. Assuming that appropriate limits are determined, through understanding what is achievable through effective cleaning, and through reference to trend analysis data, ATP bioluminescence can be a valuable tool for assessing surface cleanliness within HACCP.

It is clear, however, that a number of key variables contribute to the achievable minimum detection limits and the repeatability and reproducibility of the end results gained from ATP bioluminescence monitoring, some of which are evident from the results in this chapter. These include the nature of the bioburden being detected; the swabbing protocol employed for removal of the bioburden; the nature of the wetting agent used to moisten swab buds, and to aid in bioburden removal; the efficiency of the extractant and enzyme used; the nature of sample measurements, either directly from swab buds or from a recovery diluent, and with measurements directly from swabs, the distance of the swab device from the photomultiplier tube in the detection system.

Other comparative studies on detection system technical performance (Griffith *et al.*, 1994; Flickinger, 1996; Flowers *et al.,* 1997; Colquhoun *et al.,* 1998) have confirmed that differences do exist in assay minimum detection limits. However, direct comparison of different detection systems is difficult for a number of reasons. Each system uses a unique measurement scale. Some systems display results as log transformations, while others use linear scales and report relative light units (RLU). This in itself is a problem as the RLU is not a standard unit of measurement, and different systems may employ different scales. Additionally, each of the systems will have a different background light level that the end user may be unaware of (Flickinger, 1996).

Results gained for direct inoculation of swabs with standard ATP solutions, given in Table 3.5 were found to be in general agreement with those of Flowers *et al.,* (1997), while Colquhoun *et al.*, (1998) found that both the Idexx Lightning and Biotrace Xcel detection systems had better minimum detection limits, being able to detect 0.04 - 0.4nMol of ATP, than the Charm Luminator detection system which he found only able to detect 0.4 - 4.0 nMol of ATP. Absolute comparison of the results is not possible given the use of initial ATP standards of different sources, and because of the reporting of theoretical detection limits (TDL) in ranges of femtomoles by Flowers *et al.,* (1997), where the theoretical detection limit is defined as the mean of the swab background plus two standard deviations, and was calculated as the limit of

detection (d) where $d = 10^{(\log (x + 2s) b)/m}$ with "x" being the mean swab background results, "s" is the standard deviation of the swab background, "b" is the intercept of the regression and "m" the slope of the regression. The authors report that analysis of the ATP solutions indicated that the calculated theoretical detection limits for the detection systems ranged from 0.48 femtomoles to 14 femtomoles, with the most and least sensitive systems differing by 29-fold. Reported theoretical detection limit levels of thirteen femtomoles for the Merck Hylite and fourteen femtomoles for the Charm Luminator are higher than the results achieved in the present study. A minimum detection limit of 10 femtomoles was found for Charm Luminator, with a lower minimum detection limit of 2.5 femtomoles being achieved for the Merck Hylite detection system. A theoretical detection limit of 0.48 femtomoles for the Celsis System Sure was not dissimilar to the results of the present study. Results for detection of 0.5 femtomoles were not statistically significant but with a detection level of 2.5 femtomoles statistically significant results were achieved at the 5% level. These results suggest that the actual minimum detection limit was somewhere in the range of 0.5-2.5 femtomoles. Only the results gained for the Idexx Lightning were in complete disagreement. This present study reported a minimum detection limit of 10 femtomoles (P=0.1), while Flowers *et al.,* (1997) reported a theoretical detection limit of 2.2 femtomoles.

The results from Flowers *et al.,* (1997) for the detection of pure cultures are difficult to interpret. Results are presented as "dilutions" where a series of five different dilutions were used. Detection limits for bacteria were dilutions of 30 to 3000 starting with a concentration of 4.0×10^6, which the authors suggest represents a detection limit of 1.3×10^3 to 1.3×10^5. From the data given, it would appear that the systems with very low minimum detection limits were the Celsis System Sure, Idexx Lightning, and Merck Hylite, with the Charm Luminator being considerably poorer in its detection ability. All data from Flowers *et al.*, (1997) pertain to direct swab inoculations. Data gained from direct swab inoculations with *Escherichia coli* in the present study is not in complete agreement with the published data of Flowers *et al.*, (1997). In comparing the systems mentioned, Celsis System Sure was found to be capable of detecting the

lowest inoculum level, this being 10^3 cells/100 cm^2. Minimum detection limits of 10^4 and 10^5 cells/100 cm^2 were found for the Idexx Lightning and Merck Hylite machines respectively.

It was particularly interesting to note from the data generated in the present study that the results for the Rhone Poulenc Bio-Orbit detection system were markedly different for each of the experimental protocols executed. For *Escherichia coli* surface inoculations the Bio-Orbit system was found to have the poorest minimum detection limit of all detection systems, with a limit of 10^7 cells/100 cm^2 being achieved, while for direct swab inoculations this system was found to have the lowest minimum detection limit of 10^2 cells/100 cm^2. One explanation for such different results may rest in the presence or absence of an appropriate wetting agent in the swab moistening solution. While the success of swabbing based hygiene monitoring methods can be significantly improved by the use of wetting agents such as some cationic detergents, (Salo and Wirtanen, 1999), not all assay systems use such substances. While such detergents break up biofilm and reveal surface bacteria without affecting their survival rate (Salo and Wirtanen, 1999), quaternary ammonium compounds are known to affect organism survival (Frank and Chmielewski, 1997). ATP detection systems in which only water is used as the swab wetting agent may be less efficient at removing contaminating surface bioburden for this reason.

Once again, in directly comparing results gained for system repeatability and reproducibility, it is only possible to make some general comments, both because of differences in the experimental procedures used, and in the way data is presented. Colquhoun *et al.,* (1998) investigated assay reproducibility through direct swab inoculations with solutions of food products. They found the Idexx Lightning detection system to be the most reproducible with mean % CV values of 9-10% for swab inoculations with pure ATP solutions. The Unilite Xcel was found to give % CVs of 17 and 21% for such solutions, depending on the concentration of ATP used. Reproducibility data for the Charm Luminator detection system was found to be least reproducible with % CV data of 54% and 74% being achieved for orange juice and milk solutions respectively. Data on repeatability and reproducibility from the study reported in this thesis is of the same general order as that of Colquhoun *et al.*, (1998) with a mean % CV for

each of the three detection systems evaluated by Colquhoun *et al.,* (1998) being: Idexx Lightning (33%), Charm Luminator (58%) and Biotrace Xcel (55%). With these comments in mind, it is important to acknowledge the fact that the study by Colquhoun *et al.,* (1998) attracted considerable controversy, with comments subsequently being published in the Journal of Food Protection, (Vol. 61 (7) 781-783). The controversy surrounded the nature of the protocol design and the statistical analyses performed on the data that were deemed to be inappropriate. It was noted in the published correspondence that the protocols and statistical analyses used in this present study (Griffith *et al.,* 1997) were appropriate for the nature of the work being executed. The study by Flowers *et al.,* (1997) was also subject to debate by at least one other leading manufacturer for a number of reasons. These included the fact that it was felt inappropriate to compare single-shot swabs to assay procedures involving a number of separate stages. This is primarily due to differences in the design of the assay procedures. For example, the potential for operator error using a multi-stage assay is significantly greater than when using a single-shot device. Sample volumes in the study by Flowers *et.al.,* (1997) were also felt to be low, again increasing the potential for experimental error. Some of the tests performed involved testing liquids with the swabs. This was considered inappropriate given the availability of test kits specifically designed to analyse liquid samples. It was also acknowledged by Biotrace (Personal communication), that the experimental protocols used had not been designed to represent in-use conditions. This was one of the main criteria used in developing the experimental protocols used in the present study.

It is noted that in the study by Griffith *et al.,* (1994), in which some detection systems evaluated were the same as those in the present study, the mean % CV for repeatability and reproducibility for three detection systems which were evaluated in both studies were different. In the 1994 study the Merck Hylite detection system was reported as having a mean % CV of 43%, while in the present study the same system had a mean CV of 78%. Similarly the mean % CV for the Biotrace Unilite detection system in the present study was found to be 29% in comparison to 42% in the 1994 study. Markedly different results were found for the Rhone Poulenc Bio-Orbit that was found to have a mean % CV of 86% in this study, but only 23% in the earlier one.

Presenting experimental results using %CV data in this way does allow for evaluating detection system performance over a number of experimental protocols all at once. Expressing the results of one operator's sample repetitions from one discreet experiment in this way will, however, not give rise to any experimental error such as that outlined above.

Despite the difficulties encountered in trying to present repeatability and reproducibility data in a format that allows for easy interpretation, and which facilitates straight forward evaluation of the detection systems, data of this nature is important in terms of the overall evaluation of the detection systems. Presenting data in this way results in each detection system being allocated one numerical value against which it can be compared to all others. Operator confidence in ATP detection systems that give rise to considerable variation in repeatability and reproducibility data will be reduced, since wide variations in data gained may suggest that a large number of test readings may be the consequence of both false positive and false negative test results.

Many of the differences reported both for minimum detection limit and repeatability and reproducibility may be the result of the use of different experimental protocols. Additionally, in comparing current data with that reported in previous studies, differences may be the result of developments both in detection system performance and/or assay formulation.

Another ATP bioluminescence hygiene study published by Flickinger (1996) involved participants from eight food companies evaluating seven ATP detection systems within their own work environments. The study was described as "open-ended", and was not highly controlled. Participants were provided with no more than 150 swabs over a ten-day period, and were instructed to maintain some level of consistency in the applications and procedures used for all systems. What each participant did during each test period depended upon both individual priorities and interests. Participants evaluated the systems in a series of performance categories using a scale from one to ten, where one was the poorest and ten being excellent. While a number of issues from the study were raised, it was interesting to note that the most prominent distinguishing characteristic among systems was self-contained swab devices versus

the need to manually prepare reagents or tests. This comment is in general agreement with the scores awarded for ease of use of the ATP detection systems evaluated in the present study. Participants in the study by Flowers (1996) also noted that there was no practical correlation between RLU readings and microbiological testing. Participants also indicated that being able to correlate RLU readings to total plate counts, and a system's ability to distinguish between microbial and non-microbial ATP, were also key considerations. Across all detection systems the ability to achieve consistent, reliable results was generally good, but establishing cut-off values or pass-fail limits was felt to be the critical first step in using ATP technology, and these values obviously vary widely between different types of food processing environments and surface types.

These findings are of particular importance in evaluating the potential use of commercial ATP bioluminescence surface hygiene monitors (detection systems) within HACCP, and this is discussed in more detail in Chapter Six. Results have shown that different ATP detection systems do possess different detection limits, and that some systems are very much easier to use than others, especially by non-technically trained staff. In deciding which particular system to use it is likely that compromises will need to be made. The system that is easiest to use may not necessarily be the one with the lowest minimum detection limit, for example. It has also been clearly illustrated that, in detecting the different components contributing to overall total ATP minimum detection limits, different systems again possess different detection limits. The most important detection limit result achieved by each system is that gained for the recovery and detection of surface bioburden, since this represents typical use of the system in industry.

The successful implementation of ATP monitoring within a food plant as part of a HACCP based food safety management system to assess surface cleanliness is also going to depend upon appropriate validation and verification of ATP bioluminescence as a monitoring method. The validation process will be dependent upon the collection of a substantial bank of data generated from the use of the system in the food plant in order that appropriate pass and fail benchmark

values are established. Verification of the method can then be done in conjunction with established microbiological methods.

The work reported in this chapter has evaluated the use of ATP Bioluminescence as a means of assessing surface cleanliness levels, while Chapter Two evaluated the efficiency with which surface hygiene swabbing could detect and recover bacteria on stainless steel food contact surfaces. The work of Chapter Four investigates the use of both microbiological and ATP Bioluminescence methods, and determines minimum detection limits for these methods when used to sample food contact surfaces when wet and dry.

3.6 Conclusions

Differences were found in both assay minimum detection limits and in repeatability/reproducibility for all ATP assay and detection systems evaluated. Such differences might be the result of a number of factors that have been identified and discussed. These may include the nature of the swab wetting agent, and its ability to remove surface bioburden, the efficiency of the extractant used, and the position of the swab in the detection system, which may result in light shielding. These factors may influence the RLU readings gained from swab samples.

A comparison of the current data with that in the literature is problematic given the use of different experimental protocols in individual studies. However, some agreement between the data generated in the present study and that reported in others was found.

Experimental protocols employed for evaluating ATP detection systems for surface hygiene monitoring must reflect "in-use" conditions in order that meaningful data, which is analysed using appropriate statistical tests, is generated. The protocols used in this study assessed different components of total ATP minimum detection limits and clearly demonstrate that differences in assay detection limits do exist depending upon which components are being evaluated.

The experimental protocols developed in this study may be considered as benchmarks upon which all future ATP test system developments might be evaluated.

ATP bioluminescence has been shown to have many advantages as a rapid means of assessing surface cleanliness, but implementation of the technique in industry will only be worthwhile if its limitations are clearly understood and appreciated.

Chapter 4

A Comparison of Minimum Bacterial Detection Limits of Microbiological and Non- Microbiological Methods of Surface Cleanliness Assessment on Inoculated Stainless Steel Surfaces Sampled Wet and Dry

4.1 Introduction

Chapter 1 outlined the range of hygiene monitoring methods available and discussed the most important considerations in relation to their choice. In selecting the most appropriate method with which to monitor the cleanliness of food contact surfaces, microbiologists are now, in entering the 21st century, presented with a more extensive range of methods than that which was available in the early 1900s. At that time, choice of hygiene monitoring methods was limited to cotton or alginate swabbing and simple agar contact methods such as agar sausages. However, the wide range of methods now available has meant that the selection of the most appropriate technique for a specific task is no longer as straightforward as it once was. Additionally, a wide range of factors may influence the choice of methods as outlined Table 1.1 in Chapter One.

After the use of initial visual inspection, one of the most important decisions surrounds whether to use microbiological or non-microbiological based methods for verification of surface cleanliness, especially in terms of microbiological safety. In comparing the results of visual inspection with microbiological quality of foods, Powell and Atwell (1995) found no correlation between the results gained. In a later study by the same authors, Powell and Atwell (1997) found no relationship between surface contamination, reflected in high ATP readings, and visual inspection ratings when used to assess cleanliness levels in food retail premises. The experimental design adopted in both of these studies is, however, questionable given the fact that it has long been recognised that the results of a visual inspection, which are very subjective, are unlikely to reflect the results of more quantitative monitoring methods.

Ultimately, choice between both types of method will be determined by the nature of the information required. Information on total surface cleanliness, as opposed to information only

on residual microorganisms, for example, can only be achieved using non-microbiological methods. The most important features of both microbiological and ATP bioluminescence methods are compared in Table 4.1.

The traditional understanding of surface cleanliness, and the means of monitoring it, has until recently, however, been based largely upon microbial enumeration (Griffith *et al.,* 1997). Methods based on microbial enumeration are relatively simple to use and can provide qualitative, semi-quantitative or quantitative information, but require incubation periods of up to forty eight hours before results become available (Griffith *et al.,* 1997). "Modernists", however, argue that for initial hygiene monitoring, it is more appropriate to consider total organic soil, which includes microorganisms and food debris, rather than to sample for bioburden of microbial origin only. This can be easily achieved using ATP bioluminescence. This philosophy of assessing total surface cleanliness rather than the individual components of which it is composed is particularly important within the context of HACCP where surface cleanliness may be designated as a critical control point. In such circumstances ATP levels above specified target values would be deemed unacceptable regardless of the source of the ATP detected. The use of microbiological methods would be of no value under these circumstances. These methods are best suited to situations where information on specific pathogens is required, perhaps where problems with high ATP readings have been occurring over long periods of time, or to confirm that a surface disinfection stage during the cleaning process has been effective.

Several studies have focussed on comparisons of traditional microbiological and rapid ATP based methods for assessing *in situ* surface cleanliness. In two of these studies (Tebbutt and Midwood, 1990; Tebbutt, 1999) the methods were used to assess surface cleanliness within hospital kitchens, and for assessing the cleanliness of kitchen chopping boards. In the earlier study, a good correlation was found to exist, on some occasions, between agar contact plate results and ATP readings, but in some cases marked differences between the two methods were observed.

Table 4.1 Comparison of Microbiological and ATP Bioluminescence Surface Cleanliness Assessment Methods

Attribute	Microbiological (Cultivation)	ATP Bioluminescence
Acceptance	Widely accepted by food industry world wide	Widely accepted in UK and some countries. Less well used and accepted elsewhere. Acceptance increasing rapidly.
Method/Principle Tested	Microorganisms derived from surface grow and multiply. Laboratory facilities required.	ATP derived from microorganisms and food debris analysed using a luminometer. ATP can be assessed directly on surface or indirectly via a swab. No laboratory facilities needed.
Time for Results	18 - 48 hours.	2 minutes.
Sensitivity to a standard raw milk suspension	Inferior to ATP Bioluminescence	Superior and improving.
Limits of sensitivity using standardised *E. coli* test on inoculated surfaces sampled while dry	10^6 cells	10^3 cells
Reproducibility for raw milk contaminated surface	CV 84 - 300%	CV 9 - 79%
Approximate consumable cost	60 - 100p (In House)	95 – 135p
Capital Costs	Variable but, Incubator, £130 - £2000 Autoclave, £600 - £10,000	Around £2000 Discounts and trade ins possible
Staff Requirements	Some level of microbiological training preferable	Relatively little training

(from Griffith *et al.* 1997)

High bacterial counts with low ATP readings were reported as being more common than samples that cultivated fewer bacteria and yet contained significant amounts of ATP. The authors report also that the final ATP readings were not easily related to a specific level of contamination. The occurrence of different levels of contamination on adjacent areas, whilst not ruled out by the authors, was thought to be an unlikely explanation for the presence of relatively high numbers of bacteria on some surfaces that had zero or very low ATP readings. Data provided to the authors by the manufacturer of the ATP detection system indicated that at least 10^3 bacteria are needed before a reading is registered by the detection system, and this is in agreement with the data presented in Chapter Three. From the discussion presented by the authors (Tebbutt and Midwood, 1990) it is apparent that they were unaware of the minimum detection levels of the methods chosen before using them, and that these would be influenced by the moisture levels of the surfaces at the time of sampling. This is an important issue in determining choice of hygiene methods as outlined earlier in Chapter Three.

In the later study (Tebbutt, 1999) traditional swabbing was compared with rapid methods to detect protein and ATP on kitchen chopping boards both before and after cleaning. Positive correlations were found to exist between the microbiological count, protein and ATP detection methods, but the overall ranges for plate counts at various levels of protein and ATP showed a large amount of variability to the extent that the rapid methods could not be relied upon to accurately predict the level of bacterial contamination. The pass level reported for ATP readings of 4.0×10^2 is below the minimum detection limits of all the ATP detection systems evaluated in Chapter Three, and also below the minimum detection limit reported for the ATP monitor used by the same author in the study of hospital kitchens reported above (Tebbutt and Midwood, 1990). Given the fact that microbiological methods often display considerable variation in results, as reported in Chapter Two, it is interesting to note that the authors appear to trust the microbiological data from their study to a much greater extent than the data gained from the non-microbiological methods. Perhaps a more detailed understanding of the concept of assay minimum detection limits may have led the authors to a clearer interpretation of the results gained.

Other studies have found a poor correlation between rapid and traditional methods (Poulis *et al.*. 1993). When used to sample food factories, poor correlation existed between ATP bioluminescence and agar contact plates. This disparity in findings is not surprising given the fact that *in situ* details of the nature of surface contamination were unknown. High ATP readings, for example, may be the result of varying combinations of ATP derived from food and microbial origin (Griffith *et al.,* 1997). It is, however, important that those involved in monitoring surface cleanliness using ATP bioluminescence can detect as low numbers of microorganisms as possible in the absence of ATP derived from food residues.

A wide number of other studies in which different microbiological methods have been compared for assessing surface cleanliness have also been reported. Cousin (1982) reported that an absorbent pad test strip gave results one log factor greater than both swabbing and contact plates when used to assess cleaned surfaces. All three methods were, however, reported to give good correlation on surfaces that had been cleaned and disinfected. A similar adhesive tape method for monitoring meat samples reported by Fung *et al.,* (1980) indicated that results were equally as good as a conventional rinse technique. The use of a sponge technique (Dorsa *et al.,* 1997) for recovering low levels of foodborne pathogens from beef carcasses was found to recover fewer organisms than excision. A comparison of sponge based methods with traditional swabbing for the recovery of *Escherichia coli* on pig carcasses showed that both methods gave similar results (Gill and Jones, 1998). Such findings would suggest that, while choice of sampling method may be important, the nature of the surface being sampled is also a key consideration. Niskanen and Pohja (1977) found that while an agar contact method gave better recovery on flat surfaces, a swabbing method was better for flexible and uneven surfaces. Yet, when used to recover bacteria on pork carcasses, Corday and Huffman (1985) also found that significant differences in recovery did exist, with the swab method giving better recovery.

In assessing cleaning and sampling methods for food contact surfaces in premises selling high risk foods, Tebbutt (1991) found that agar contact methods were at least as sensitive as alginate swabbing, which was in agreement with Restaino *et al.,* (1994). In evaluating an agar contact

dip slide with rodac plates in comparison to the swab method for the recovery of organisms from artificially contaminated stainless steel and Formica surfaces, Restaino *et al.*, (1994) found that there was a strong linear relationship between both agar contact methods, and that the agar contact dip slide method was as effective as the swab method. Other studies (Angelotti *et al.*, 1964; Corday and Huffman, 1985; Niskanen and Pohja, 1977) have also found good correlations.

Despite these reported findings, another important consideration in hygiene monitoring relates to when surfaces should be sampled after cleaning. For the assessment of cleaning to be worthwhile, it must follow surface cleaning that has adhered to a well-designed cleaning schedule. This should clearly state when and how cleaning, and its assessment, should be performed (Griffith *et al.*, 1997). Food contact surfaces that are sampled while wet may give different results to those that are allowed to dry thoroughly before sampling. Such differences in recovery rates of organisms from cleaned surfaces using different techniques may be the result of a number of factors, including attachment of the organisms to the surfaces being sampled, organism death and the recovery of viable but non-culturable bacteria (Bovill and Mackey, 1997; Besnard *et al.*, 2000).

Findings of these published studies clearly show that differences do exist in the ability of different methods to recover and detect microorganisms on a range of different surface types. Many of these studies have evaluated individual methods, but *in situ* as opposed to under controlled laboratory conditions. The purpose of the work reported in this Chapter was, therefore, to evaluate individual methods using standard laboratory protocols, with specific reference to the effect of sampling inoculated surfaces when both wet and dry.

4.2 Aims

The aims of the work reported in this Chapter were, therefore, to:

1. Determine the minimum bacterial detection limits achieved using ATP bioluminescence, cotton swabbing and agar contact dip slides when used to sample inoculated stainless steel surfaces when wet and dry.

2. Compare the repeatability and reproducibility of results gained from ATP bioluminescence, cotton hygiene swabbing and agar contact dip slides when used by six technically trained operatives to sample inoculated stainless steel surfaces when wet and dry.

4.3 Materials and Methods

4.3.1 Bacterial Cultures

Cultures of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (NCTC 6571), *Pseudomonas aeruginosa* (ATCC 27853), and *Listeria monocytogenes* (ATCC 7644) were grown in Nutrient Broth No.2 (CM67, Oxoid, Basingstoke, UK), or Tryptone Soya Broth (CM129, Oxoid, Basingstoke, UK) for *Listeria monocytogenes,* in unshaken batch culture volumes of 10 ml under aerobic conditions at either 30 or 37°C for eighteen hours. Serial dilutions of each culture, prepared in Maximum Recovery Diluent (CM733, Oxoid, Basingstoke, UK) were used to inoculate a food grade stainless steel surface.

4.3.2 ATP Bioluminescence Test Method

A range of ATP Bioluminescence equipment and assay reagents was available, but previous work had shown that the Biotrace Clean-Trace Rapid Cleanliness Test was the most widespread commercially available, and typical of those available in terms of its minimum detection limit and reproducibility, and it was found to be the easiest to use (Chapter Three).

The test consists of a swab device containing a Dacron bud, pre-moistened with swab diluent XT, containing a cationic agent and non-ionic agent to release ATP from any intact food or microbial cells, and to aid soil removal from surfaces. Firefly reagent released on activation of the device reacts with ATP collected on the swab bud and produces light that is measured by the photomultiplier tube within the Biotrace Xcel detection system.

4.3.3 Hygiene Swabs and Swabbing Protocol

Details of the swabbing protocol used are provided in the Materials and Methods section in Chapter 3, Section 3.3.7. Swabs, which consisted of cotton buds, were supplied by *PBI*, Italy.

4.3.4 Agar Contact Dip Slides

One plate count agar (PCA) or tryptone soya agar (TSA) dip slide (Dimanco, Henlow, UK) was applied to the centre of each 100 cm^2 area of stainless steel, ensuring that the agar surface came into complete contact with the surface, for a period of three seconds. All dip slides were incubated at 30°C for *Listeria monocytogenes* and at 37°C for all other test organisms for twenty four hours.

4.3.5 Surface Preparation

Details of surface preparation procedures are provided in the Materials and Methods section in Chapter 3, Section 3.3.4.

4.3.6 Surface Inoculation, Sampling and Organism Cultivation

0.1ml of each culture (10^7 cfu) and serial decimal dilutions, using MRD, giving inoculation levels of 10^6 - 10^2 cfu were inoculated onto each of sixty 100 cm^2 areas of stainless steel and spread over the entire surface area to within 2mm of the edges using a sterile plastic spreader. Of the sixty inoculated surfaces, thirty were sampled immediately after inoculation while wet, and thirty after a sixty minute residence time on the surface by which time the inoculum had completely dried. A dry surface was defined as one on which no visible moisture was present. Using the same sampling protocol for both wet and dry surfaces, ten surfaces were sampled

using the Biotrace Clean-Trace Rapid Cleanliness Test, ten using hygiene swabbing, and the remaining ten with agar contact dip slides. This experimental procedure was repeated on at least two occasions, with the minimum detection limits achieved for each method being confirmed through repeating the experimental protocol using these inoculum levels on two additional occasions.

Dry surfaces were achieved through allowing the inoculum applied to the surface to remain resident on the stainless steel for sixty minutes at an ambient temperature of approximately 22°C prior to sampling.

Organisms recovered from inoculated surfaces using hygiene swabs were either released into 10 ml tubes of MRD by rota mixing (30s) and duplicate 0.1 ml plate count agar spread plates prepared or swabs were streaked directly over the surface of plate count agar plates in two directions in order to maximise release of the recovered organisms from the swabs. All nutrient agar plates were incubated at 37°C for twenty four hours. For *Listeria monocytogenes*, tryptone soya agar (CM131, Oxoid, Basingstoke, UK) was used, with plates being incubated at 30°C for twenty four hours.

4.3.7 Determination of Minimum Bacterial Detection Limits

For ATP bioluminescence test results, data from inoculated surfaces were compared with control data from a cleaned surface using a two tailed Student's t test to estimate the significance of any difference. The minimum detection limit was recorded as the inoculum level that resulted in a significant ($p < 0.05$) difference.

For plate count data from hygiene swabs the minimum bacterial detection limit was noted as the level of inoculum that resulted in consistent counts of between 30 and 300 colonies (ISO standard). In addition, minimum bacterial detection limits are indicated in brackets where counts of less than 30 cfu/plate were recorded consistently for all ten replicates. Percentage recovery rates were calculated for all organisms from inoculated surfaces sampled when wet and dry.

These related to percentage recovery in relation to level of initial inoculum applied to the surface, and not to viable cell numbers present after surface drying. For agar contact dip slides, the minimum bacterial detection limit was the level of inoculum applied to the surface that resulted in consistent recovery equating to approximately 2.5 organisms per cm^2 using the results interpretation key (ref. Appendix 2). In addition, where consistent numbers of colonies were recorded on all dip slides, but which was less than the level required which represented 2.5 cfu/cm^2, an additional detection limit is provided in brackets.

4.3.8 Determination of Extracellular ATP levels in Culture Growth Medium

Extracellular ATP levels were determined to ensure that ATP present in the spent growth medium did not significantly affect the ATP signal being detected. Eighteen-hour cultures of each organism were divided into two 5 ml portions and centrifuged at 2000 rpm for fifteen minutes. In order to ensure that both portions of each culture received identical processing, the supernatant from each was removed. The supernatant from one portion was replaced and the microbial cells resuspended. The supernatant from the second portion was filtered through a 0.45 um filter (Whatman, Maidstone, UK) to remove residual cells and retained for ATP analysis. To resuspend the microbial cells of the second portion of each bacterial culture, 5 ml of quarter-strength Ringer's solution were added to each.

For the culture suspended in nutrient broth, the culture suspended in quarter-strength Ringer's solution, and the filtered supernatant for each organism, ten Clean Trace Rapid Cleanliness Test swabs were inoculated with 0.1 ml of a 10^{-2} dilution of the suspended microbial cells, or the filtered supernatant. All swabs were assayed as described previously. The mean ATP readings for the filtered supernatant was calculated as a percentage of the mean ATP reading for the culture suspended in Ringer's solution.

4.3.9 Determination of Operator Reproducibility

Studies designed to assess operator reproducibility involved six technically qualified operatives swabbing each of ten 100 cm^2 areas of stainless steel which were either clean or marginally

unclean through inoculation with low but detectable numbers (10^5 cfu/100 cm^2) of *Escherichia coli.* All operatives received training in the use of both ATP bioluminescence and hygiene swabbing prior to surface sampling. Experiments were repeated with all operatives using both the Biotrace Clean Trace Rapid Cleanliness Test and conventional cotton hygiene swabbing. Cotton swabs were streaked directly onto plate count agar plates and incubated for twenty four hours at 37°C.

4.3.10 Statistical Analysis

All data for ATP bioluminescence and surface swabbing were compared with appropriate control data using a two-tailed Student's *t* test (Excel) to estimate the significance of any difference. Minimum detection limits were recorded as those levels at which statistically significant results were obtained $p<0.05$), while for agar contact dip slides, the minimum detection limit was recorded as the level of detection that represented approximately 2.5 cfu/cm^2 using the results interpretation key provided by the manufacturer, illustrated in Appendix Two.

4.4 Results

Table 4.2 illustrates the minimum bacterial detection limits achieved by the four test methods when used to sample wet and dry stainless steel surfaces inoculated with four test organisms. For the ATP bioluminescence method, the minimum bacterial detection limit was found to be 10^4 cfu/100 cm^2 that was consistent for all organisms. This minimum bacterial detection limit was not influenced by sampling surfaces when wet or dry. Minimum detection limits for the other three methods varied, and were influenced by whether surfaces were sampled when wet or dry. For hygiene swabbing using diluent spread plates as the method of organism cultivation, detection limits varied from 10^3 up to 10^8 per 100 cm^2. For the Gram-positive organisms, detection limits were found to be 10^3 and 10^6 cfu/100 cm^2 for *Staphylococcus aureus* on wet and dry surfaces respectively, whilst for *Listeria monocytogenes* the detection limits were found to be 10^2 cfu/100 cm^2 on inoculated surfaces sampled while wet, and 10^8 cfu/100 cm^2 on inoculated surfaces sampled while dry. For Gram negative organisms the minimum detection limits were 10^3 and $>10^7$/100 cm^2 for *Escherichia coli* on wet and dry surfaces respectively,

Table 4.2 **Minimum Bacterial Detection Limits of Four Organisms using Four Surface Cleanliness Assessment Methods on Inoculated Stainless Steel Food Contact Surfaces Sampled Wet and Dry**

Bacterium	Surface Status	ATP Bioluminescence Detection Limit	Hygiene Swabbing/Diluent Spread Plates Detection Limit	Hygiene Swabbing/Direct Streaking Detection Limit	Agar Contact Dip Slides Detection Limit
Staphylococcus aureus	WET	10^4	$10^4(10^3)$	10^2	$10^2(10^1)$
	DRY	10^4	10^6	10^5	10^2
Listeria monocytogenes	WET	10^4	$10^3(10^2)$	$10^3(10^2)$	10^2
	DRY	10^4	10^8	10^7	10^6
Escherichia coli	WET	10^4	$10^4(10^3)$	10^2	$10^2(10^1)$
	DRY	10^4	$>10^7$	10^7	10^7
Pseudomonas aeruginosa	WET	10^4	10^5	$10^3(10^2)$	10^2
	DRY	10^4	$>10^7$	10^5	$>10^7$

Detection limits given in brackets indicate consistent recovery of less than 30cfu/plate or less than 2.5cfu/cm^2 on dip slides.
All detection limits relate to cfu/100 cm^2.
n = 30 for each organism recovered from wet or dry surfaces using each test method

while for *Pseudomonas aeruginosa* the values were 10^5 and $>10^7$ /100 cm^2 on inoculated surfaces sampled wet and dry respectively.

For direct streaking of swabs onto the surface of agar plates detection limits were found to range from 10^2 up to 10^7 per 100 cm^2. For both Gram-positive organisms the minimum bacterial detection limit on wet surfaces was 10^2/100 cm^2, while on dry surfaces it was found to range from 10^5 to 10^7/100 cm^2. For Gram-negative organisms the minimum bacterial detection limit on wet surfaces was found to be 10^2/100 cm^2 for *Escherichia coli* and 10^3/100 cm^2 for *Pseudomonas aeruginosa.* On inoculated surfaces sampled while dry the detection limit for both organisms ranged from 10^5 to 10^7/100 cm^2.

Minimum detection limits achieved using agar contact dip slides ranged from 10^2 cfu/100 cm^2 to $>10^7$cfu/100 cm^2 on inoculated surfaces when sampled dry, and were consistent at 10^2 cfu/100 cm^2 on inoculated surfaces when sampled while wet. On inoculated surfaces sampled while dry, *Staphylococcus aureus* was found to have a minimum detection limit of 10^2 cfu/100 cm^2 which was several log factors lower than the other test organisms, and this is thought to be due to the greater ability of *Staphylococcus aureus* to tolerate dry conditions.

Hygiene swabbing percentage recovery rates for all organisms, based upon the actual inoculum level applied to the surface, and not on viable numbers present immediately before sampling, are provided in Table 4.3. Recovery rates varied depending upon which organism was used, and whether surfaces were sampled when wet or dry. Percentage recovery rates ranged from less than 1% when surfaces were sampled dry, up to 16% for *Pseudomonas aeruginosa* when sampled while wet.

Table 4.3 **Mean Percentage Recovery Rates for Four Organisms from Inoculated Stainless Steel Food Contact Surfaces Sampled Wet and Dry using Cotton Surface Swabbing**

Bacterium	Surface Status	% Recovery From Diluent Spread Plates	% Recovery From Direct Streaking
Staphylococcus aureus	WET	0.3	9.0
	DRY	<0.1	<0.1
Listeria monocytogenes	WET	0.1	4.0
	DRY	0	<0.1
Escherichia coli	WET	0.3	8.0
	DRY	0	<0.1
Pseudomonas aeruginosa	WET	2.5	16.0
	DRY	0	<0.1

n = 20 for each organism recovered from wet or dry surfaces using each method of cultivation

Table 4.4 provides coefficients of variation (CV) and 95% confidence limits for ATP bioluminescence and hygiene swabbing when used to sample clean and marginally unclean stainless steel surfaces by six technically trained operatives. Regardless of surface moisture levels, mean coefficients of variation ranged from 24% to 32% for the ATP bioluminescence method. For marginally unclean surfaces sampled using hygiene swabbing the mean coefficient of variation was found to be 130%. Surface hygiene swabbing of clean sanitised surfaces gave no detectable results.

Extracellular ATP levels in a 10^{-2} dilution of the culture growth medium, that was used for surface inoculations were found to account for between 0.47% and 1.7% of the ATP signal detected when using *Escherichia coli* or *Staphylococcus aureus* as the surface inoculants. A value of 14.8% gained for one replicate of *Escherichia coli,* which was considerably higher than the other values gained, was thought to be the result of chance use of dirty glassware.

4.5 Discussion of Results

The work reported evaluates four methods commonly employed by the food industry for assessing food contact surface cleanliness. Minimum bacterial detection limits for both the Biotrace Clean-Trace Rapid Cleanliness Test and three microbiological based methods were determined when used for sampling wet and dry surfaces inoculated with known levels of pure bacterial cultures.

The minimum bacterial detection limit of the ATP bioluminescence test method was found to be the same for all test organisms on inoculated surfaces sampled when either wet or dry at 10^4 cfu/100 cm^2, with extracellular ATP levels present in the culture growth medium having little effect on the ATP signal detected. Agar contact dip slides used to sample inoculated surfaces while wet resulted in the lowest minimum detection limit of 10^2 cfu/100 cm^2. When the same was used to sample dry surfaces, the

Table 4.4 **Mean Coefficients of Variation and 95% Confidence Limits gained from Six Operatives when using ATP Bioluminescence and Cotton Surface Swabbing to Sample Clean and Marginally Unclean Stainless Steel Food Contact Surfaces.**

Surface Status and Sampling Method	Mean C.V. for 6 Operatives + 95% Confidence Limits (2 x Standard Error of Mean)
Clean sanitised sampled using Biotrace Clean Trace™ Rapid Cleanliness Test.	24% +/- 4.38
Marginally unclean (inoculated with *E.coli*) sampled using Biotrace Clean-Trace™ Rapid Cleanliness Test.	32% +/- 7.89
Marginally unclean (inoculated with *E.coli*) sampled using hygiene swabs followed by Cultivation.	130% +/- 34.16

Microbiological sampling of clean surfaces using surface hygiene swabbing gave no detectable results.

minimum detection limit for the four test organisms varied, with detection limits ranging from 10^2 cfu/100 cm^2 for *Staphylococcus aureus,* and up to $>10^7$ cfu/100 cm^2 for *Pseudomonas aeruginosa.* Hygiene swabbing of wet surfaces followed by direct streaking also gave good results in terms of organism detection, and was superior to the ATP bioluminescence assay in the detection of bacterial contamination. Hygiene swabbing of inoculated surfaces sampled while dry, especially if using a diluent in the recovery stage, gave poor results, which were several log factors inferior to those of the ATP assay, and varied between test organisms. A key consideration in evaluating the results gained from these experiments is the nature of the bacterial cultures on the surface. Both the loss of viability of organisms through drying on the surface, and the potential for the presence of non-culturable bacteria will have an effect on both minimum detection limits and recovery rates. In addition, it is acknowledged that fresh environmental isolates may behave differently. The data presented in Chapter Two has shown that an environmental isolate of the genus *Staphylococcus* gave superior recovery rates to a laboratory type culture of the same genus.

The minimum detection limit of the ATP assay was the same for inoculated surfaces sampled either wet or dry, and on dry surfaces the ATP assay's minimum detection limit was superior to that achieved on the same surfaces sampled using hygiene swabbing. However this might, in part at least, be due to the presence of an agent present in the ATP swab wetting agent that acts through lysing surface attached bacteria resulting in the pick up of ATP and not the intact cells which hygiene swabbing is dependent upon removing.

The results obtained for inoculated surfaces sampled while wet could, theoretically, explain why in some circumstances it might be possible to recover organisms from a surface that an ATP test indicates is clean. This would require no food debris to be present, coupled with an optimum swabbing recovery technique from a wet surface. This is, however, unlikely to occur often *in situ.* Microorganisms are rarely likely to be present as pure culture, and organic food debris in the form of product residue is likely to be the major component of unclean surfaces. Given

increasing concerns over cross-contamination and the low minimum infective dose of pathogens such as *E. coli* 0157, detecting low levels of contamination is now much more important. While future developments in swab wetting agents might improve the recovery of dry microbial bioburden from food contact surfaces, enhancing the detection limits of ATP bioluminescence detection systems is currently the research focus of at least two companies. While future developments in ATP technology may help to ensure the wider adoption of the method within industry, it is important that ATP systems with very low detection limits do not result in large numbers of sampled sites failing through being able to detect very low levels of residual surface bioburden. This in turn may lead to unnecessary re-cleaning of surfaces that invariably would necessitate increased "down time" periods. However, it is important that surface contamination due to organisms with low minimum infective doses such as *E. coli* 0157:H7 can be detected.

An important consideration is when and how cleaning performance should be assessed. It is clear that, with microbiological methods at least, that sampling surfaces while still wet would appear to give more accurate results. However, the most important issue to bear in mind is the effect that surface drying is having on any residual microorganisms still present on the surface. Should the result of surface drying be that of loss in organism viability, then the risks attached to residual microorganisms remaining are reduced to a negligible level. If, however, surface drying merely results in the attachment of the organisms to the surface, without any loss in viability, then this would suggest that surfaces should be sampled while still wet when low levels of microbial contamination are still able to be detected, especially when using surface hygiene swabbing for the detection of specific pathogens such as *Campylobacter* and *Listeria.* It is also important to determine exactly the nature of the information required through using any particular type of hygiene assay. This may be information solely on contaminating microorganisms, or on levels of organic food debris present.

Studies on *Listeria* spp. (Cox *et al.*, 1989) indicate that drying quickly leads to loss in viability. However, studies on other pathogens (Scott and Bloomfield, 1990; Humphrey *et al.,* 1995) indicate that these may survive for extended periods in dry product residues on food contact

surfaces. While survival is one issue, it is important that the methods chosen to monitor surface cleanliness have the ability to detect these residual organisms, and results achieved in this present study, for hygiene swabbing in particular, would suggest that this is not always the case. It is important, therefore, that the monitoring methods chosen to check surface cleanliness are capable of detecting this residue. The difference in recovery rates of Gram positive and Gram-negative organisms from dry surfaces may be due to the ability of the former to tolerate dry conditions (Scott and Bloomfield, 1990).

Growth of recovered microorganisms will be influenced by the composition of the growth media used and the time and temperature of incubation. Ginn *et al.,* (1984) and Beuchat *et al.,* (1998) found good correlation between standard plating and petrifilm methods, while Linton *et al.,* (1997) found variable results depending upon the nature of the initial samples being examined. Specific to the ATP bioluminescence detection method used is the requirement that the enzyme system is active throughout the reported shelf life of the test kit, and that the photomultiplier tube can detect and measure the light produced from the buffer into which the recovered ATP is released.

In order to accurately establish the number of bacteria present on a surface it is first necessary to know the efficiency of the method of sampling chosen (Whyte *et al.,* 1989). Several factors have been identified which may contribute to the levels of efficiency achieved. However, for many sampling methods this level of efficiency is unknown, and where it is supposedly known, it has often been obtained by some artificial method (Whyte *et al.,* 1989). It is common to calculate the efficiency of sampling methods through drying a bacterial suspension of known concentration onto a surface. Bacteria are then sampled and the efficiency of the method calculated. The type of bacteria and their potential attachment to the surface are two factors previously identified which may affect the efficiency of bacterial recovery rates. Calculating the efficiency of surface sampling methods in this way is, therefore, misleading, yet is the means by which industry have evaluated the performance of these methods. It is, therefore, important and worthwhile to assess the efficiency of the methods used in this way, in order that the limitations

of the methods are better understood, and in order that industry may be advised on how best to improve their surface sampling protocols to maximise organism recovery.

The work reported in this study acknowledges these potential problems and has shown that the type of organism and the effect of drying the bacterial inoculum onto the surface influence recovery rates. Results gained for inoculated surfaces sampled while wet, which were very different in their minimum bacterial detection limits, clearly support this.

Corday and Huffman (1985) in using several microbiological based methods for estimating bacterial contamination levels on pork carcasses found that differences in bacterial recovery did exist depending upon whether agar contact or surface swabbing methods were used, and this is in full agreement with the findings of this present study. Similarly, in comparing traditional and rapid methods for assessing the risk of bacterial cross-contamination from cutting boards, Tebbutt (1999) found that rapid methods, which included ATP bioluminescence and protein estimation, could not be relied upon to accurately predict levels of bacterial contamination. While a correlation between bacterial numbers and amount of protein detected, and between bacterial numbers and amount of ATP detected did exist, further analysis of the data indicated that overall and interquartile ranges for plate counts at various levels of protein or ATP showed a large amount of variability such that neither ATP nor protein estimation could be relied upon to give an accurate prediction of bacterial count. Several factors that may contribute to these findings have been discussed earlier, and include, for example, the unsuitability of the protein estimation method for detecting organic food debris that may have low protein content.

An earlier study by Tebbutt (1991) in which an agar contact method was compared to alginate swabbing for assessing the cleanliness of food contact surfaces in premises preparing and selling high-risk foods, found that in addition to being more convenient to use, the agar contact method was at least as sensitive as the alginate swabbing method.

Niskanen and Pohja, (1977) in comparing the recovery of bacteria from a range of food contact surface types found that the agar contact method was best suited for flat surfaces in terms of both recovery rate and repeatability of results, but was influenced by the type of organism used. Data from the work reported in the present study support these findings in that some of the lowest minimum bacterial detection limits were achieved using the agar contact dip slides, and that some variation in detection limits between organisms using the same methods were found. It is, however, acknowledged that agar contact dip slides provide only an estimate of the numbers of contaminating organisms present on a surface, and that determination of the numbers present is dependent upon accurately matching the results gained with those illustrated on the interpretation key provided by the manufacturer. The presence of low numbers of colony forming units on dip slides used in the experiments reported in this study may, in addition to being a reliable result, be due either to chance contamination of the surface, or to the uneven distribution of the inoculum on the surface prior to sampling. It is important that those who use dip slides acknowledge these potential limitations given the semi-quantitative nature of the method.

Surface cleanliness testing is no longer the preserve of dedicated quality control departments, and the methods used should be as simple and reproducible as possible. The data reported in this study shows that the ATP bioluminescence method had better reproducibility than traditional hygiene swabbing, and this confirms previous findings (Griffith *et al.,* 1997). Recovery rates from hygiene swabbing were typical of those found in previous studies (Humphrey *et al.,* 1995). It is recognised, however, that problems could be encountered in determining the level of microbial contamination on surfaces using hygiene swabbing due to the presence of viable but non-culturable bacteria (Bovill and Mackey, 1997; Besnard *et al.,* 2000).

If cleaning is prescribed as a control measure within a HACCP plan, then the cleaning protocol must be validated with target values and critical limits designated for monitoring. If performed by more than one operative, reproducibility of results becomes an important issue. Poor reproducibility (lack of precision) can be problematic and result in an unnecessary high failure

rate (i.e. critical limits exceeded) or in the initial setting of an overgenerous target value. This in turn may lead to marginally unclean surfaces being accepted.

The present data on minimum bacterial detection limits and reproducibility for different organisms, coupled with real-time results, indicate that ATP testing should be the initial method of choice in hygiene monitoring, especially within HACCP plans. However, this should be integrated with microbiological testing using optimum recovery techniques as part of a coherent surface cleanliness monitoring programme.

The work of this chapter has evaluated the use of microbiological and ATP bioluminescence methods for assessing surface cleanliness, and has illustrated that differences in minimum detection limits do exist between methods. The most important factor influencing differences in minimum detection limit has been shown to be the moisture level of the surface at time of sampling, i.e. wet or dry. Chapter Five evaluates the use of these methods in different types food processing environment, and reports on the development and evaluation of a rapid microbial ATP assay for assessing microbial contamination levels on food contact and environmental surfaces within the food industry.

4.6 Conclusions

Differences in minimum detection limits for each of the methods assessed were found, and these ranged from 10^1 up to > 10^7 cfu/100 cm^2 depending upon method used and surface moisture level.

The minimum detection limit of the ATP bioluminescence test method remained consistent at 10^4 cfu/100 cm^2 regardless of whether used to sample inoculated surfaces when wet or dry.

Agar contact dip slides were found to have some of the lowest minimum detection limits of all techniques, being able to detect 10^2 cfu/100 cm^2 on inoculated surfaces sampled while wet for

all test organisms. On dry inoculated surfaces, minimum detection limits increased, and varied between test organisms.

Minimum detection limits achieved using hygiene swabbing varied between test organisms and with surface moisture level. Superior detection limits were obtained when the technique was used to sample inoculated surfaces while wet, and when the method of cultivation used was direct streaking onto plate count agar.

Levels of organism recovery were poorest from inoculated surfaces sampled while dry, and when the method of cultivation used was diluent spread plates following organism release into MRD recovery diluent tubes.

Results gained suggest that the nature of the test organism may, in part, influence minimum detection limits achieved using different techniques. Differences observed between test organisms in this study might be the result of Gram-positive organisms being more resistant to dry conditions than Gram-negative organisms

Chapter 5

Development and Evaluation of a Microbial ATP Assay within Food Processing Environments

5.1 Introduction

In food processing environments, product and detergent residues, and various ions from rinse water, in addition to pathogenic microorganisms, comprise the soil on food contact surfaces (Chaturvedi and Maxcy, 1969) and have often been implicated in food poisoning due to cross-contamination. Additionally, the potential for biofilm formation on such surfaces by pathogens is another food safety concern (Hood and Zottola, 1995; Ganesh Kumar and Anand, 1998; Peters *et al.,* 1999). Hygiene standards within the food industry are, therefore, important and are primarily concerned with the prevention of food contamination by direct or indirect modes (Taylor and Holah, 1996). To achieve this adequate cleaning of food contact surfaces is, therefore, a key function of all food operations, and is necessary in order to remove the contaminating soil which will be primarily a combination of microorganisms and organic food debris.

While the process of cleaning itself requires validation, the products used to perform cleaning are also important. Of equal importance, especially within HACCP, is the requirement that the methods used to monitor cleaning effectiveness are also validated, with appropriate benchmark values, including pass and fail limits, being set. Only then can the monitoring methods chosen be reliably used to verify that adequate cleaning has been performed. Confirmation that adequate cleaning has been carried out is especially important within food operations, as cleaning practices are critical to ensuring that opportunities for post-processing contamination do not exist.

Despite technological advances in the areas of rapid food microbiology and surface hygiene monitoring, microbiological methods of cleanliness assessment have remained relatively unchanged since the days of Louis Pasteur (Ogden, 1993). These traditional methods of surface cleanliness assessment have the ability to detect microbial contamination on surfaces only after a period of incubation. Given the inherent hazards associated with foodborne pathogens, and the potential of other microorganisms to cause food spoilage, the ability to detect contamination of microbial origin is, for some microbiologists, the most important concern in terms of food safety. It is because of this need to establish whether or not residual microbial contamination exists on surfaces that ATP bioluminescence has not yet been adopted industry wide, even though ATP monitoring provides information on total surface cleanliness which microbiological methods are unable to do (Griffith *et al.,* 1997).

While some microbiologists may be more willing to adopt ATP technology, others are reluctant, still viewing the microbial component of surface contamination as more important. The inability of current ATP hygiene assays to distinguish between ATP from microbial and non-microbial origin is therefore an important issue facing the bioluminescence industry over the next decade, which has been highlighted by Flickinger (1996).

While both rapid and microbiological methods of cleanliness assessment have advantages in terms of monitoring cleaning effectiveness, limitations do also exist. Those pertaining to microbiological methods have been discussed in earlier chapters. Specifically in relation to ATP monitoring is the effect of residual traces of both detergent and/or sanitizer on a surface that may cause quenching or amplification of the ATP signal detection, and this has been acknowledged by at least two research groups (Calvert *et al.,* 2000; Velazquez and Feirtag, 1997). However, while Lappalainen *et al.,* (2000) have also acknowledged that ATP signal may be affected by cleaning agents and disinfectants, they have shown that the use of ATP monitoring in combination with a microbiological residue testing method can help to prevent false positive and false negative results. ATP bioluminescence, therefore, continues to become increasingly popular as a means of assessing surface cleanliness within the food industry. In

addition to its role in assessing surface cleanliness, studies are reported in the literature in which modified ATP based methods have been used to detect microbial contamination on beef, pork and chicken carcasses (Siragusa *et al.,* 1995; Bautista *et al.,* 1995; Siragusa *et al.,* 1996). These studies report that good correlations exist between ATP and the microbiological methods used.

In determining contamination levels on beef and pork carcasses, Siragusa *et al.,* (1995) found that a rapid microbial ATP (R-mATP) assay was as accurate as a standard plate count method for estimating bacteria in bovine or porcine faecal samples. Correlations between the microbial ATP assay and the standard aerobic plate count for beef and pork carcasses sampled in commercial processing areas were found to be 0.91 and 0.93 respectively. In a later study by the same authors (Siragusa *et al.*, 1996) in which microbial contamination on poultry carcasses was examined, the correlation coefficient between aerobic colony counts and microbial ATP was found to be 0.82. Given both the rapidity and accuracy of the microbial ATP assay, the authors suggest that it could be used for monitoring critical control points within food processing environments. In another study of the microbiological quality of poultry carcasses by Bautista *et al.,* (1995) a modified ATP bioluminescence assay gave about 90% agreement (R^2=0.85) with plate counts for carcass rinses, and again it is suggested as being suitable for monitoring critical control points within HACCP in industry.

The dairy industry, in particular, has been proactive in adopting ATP methods, not only for monitoring cleanliness (Griffiths, 1993; Bell, *et al.,* 1994; Murphy, *et al.,* 1998), but also for rapid determination of milk quality (Bautista *et al.,* 1993). Other sectors of the food industry are now beginning to adopt the technology, with the use of the method in monitoring hygiene control of draught beer dispensing systems being reported by Storgards and Haikara, (1996) who found no correlation between ATP and plate count results when used to monitor cleanliness levels. A good correlation was, however, reported by Illsley *et al.*, (2000) when both methods were used to assess surface cleanliness levels within a bakery environment.

While Griffiths (1993) has comprehensively reviewed the potential use of ATP bioluminescence within the dairy industry, Bell *et al.*, (1994) report that the method was found to be a practically useful and rapid technique for assessing the hygienic status of milk tankers. Results from two different ATP detection systems demonstrated a good correlation when classified for indicating a clean or dirty condition of the surfaces tested. Reported levels of agreement between the two ATP detection systems ranged from 66% to 91%. Similar findings are reported by Murphy *et al.,* (1998) who reports that an ATP bioluminescence detection system proved to be just as effective as standard microbiological methods for monitoring the cleanliness of food contact surfaces in four fluid milk plants which were experiencing product shelf-life problems.

Novel work on the use of an ultrasonic methodology in conjunction with ATP bioluminescence for the non-invasive detection and removal of biofilm from stainless steel and polypropylene food processing equipment surfaces in the dairy industry has recently been reported by Oulahal-Lagsir *et al.*, (2000). The ultrasonic methodology was found not to be detrimental to the use of ATP bioluminescence for quantifying biofilm removal. The ultrasonic apparatus was found to remove twice the amount of industrial milk biofilm compared with the swabbing method when used to monitor polypropylene surfaces.

Werlein and Fricke (1997) found that microbiological swabbing was less efficient when used on its own to determine the microbiological quality of poultry carcasses. Lower microbial counts were found when using this method on its own. The use of a destructive ATP based method was reported to provide improved results when used to determine levels of microbiological quality of poultry carcasses.

Despite increasing use of ATP bioluminescence within the food industry, a commercially available microbial ATP assay for assessing surface cleanliness does not yet exist, and its development may lead to the wider adoption of ATP bioluminescence within the food industry, especially by those who believe determination of microbial contamination is most important. The development of such an assay, and its potential value in the food industry, has been

acknowledged by a number of food processors involved in an ATP hygiene monitoring evaluation study (Flickinger, 1996). In addition, little if anything is known about the relative proportions of microbial and product residue ATP present within different types of food processing environments. This may be of value in helping to determine which cleaning agents might be best suited to different environments, and on specific food contact surfaces, as different types of cleaning agents may be more or less efficient at removing microbial and organic food debris from surfaces. It is these two issues that form the basis for the work reported in this Chapter.

5.2 Aims

The aims of the work reported in this chapter were, therefore, to:

1. Develop a surface assay protocol to distinguish between total, free and microbial ATP for use within the food industry;

2. Evaluate the microbial ATP assay protocol in a range of different food processing environments;

3. Determine the relative levels of microbial and product residue ATP present in different types of food processing environments both before and after appropriate cleaning;

4. Determine the correlation between microbial ATP and aerobic colony count data.

5.3 Materials and Methods

5.3.1 Food Processing Environments

An opportunistic range of ten food-processing environments was recruited via the Food Industry Centre at UWIC to participate in this study. These covered a range of food industry sectors including general chilled food, dairy and meat processing environments. Each establishment that was of SME size was visited on two occasions, once during normal working hours, and again after routine cleaning had taken place. In each food processing environment a range of both food contact and environmental surfaces were chosen for sampling. These surfaces were selected on the basis of a small pilot study in which certain surfaces were identified as being contaminated with high numbers of microorganisms and/or food product residues.

5.3.2 Surface Sampling Methods

All surfaces were sampled using a modified ATP bioluminescence test method (Figure 5.1) that enabled microbial ATP determinations to be made. In addition, this method allowed estimations on the relative proportions of surface contamination attributable to both microorganisms and product residues to be determined. Agar contact dip slides, which are semi-quantitative in nature, consisting of plate count agar (PCA) (Dimanco, Henlow, UK) were used in addition to the microbial ATP assay as a comparison. All surfaces were sampled in triplicate by both monitoring methods.

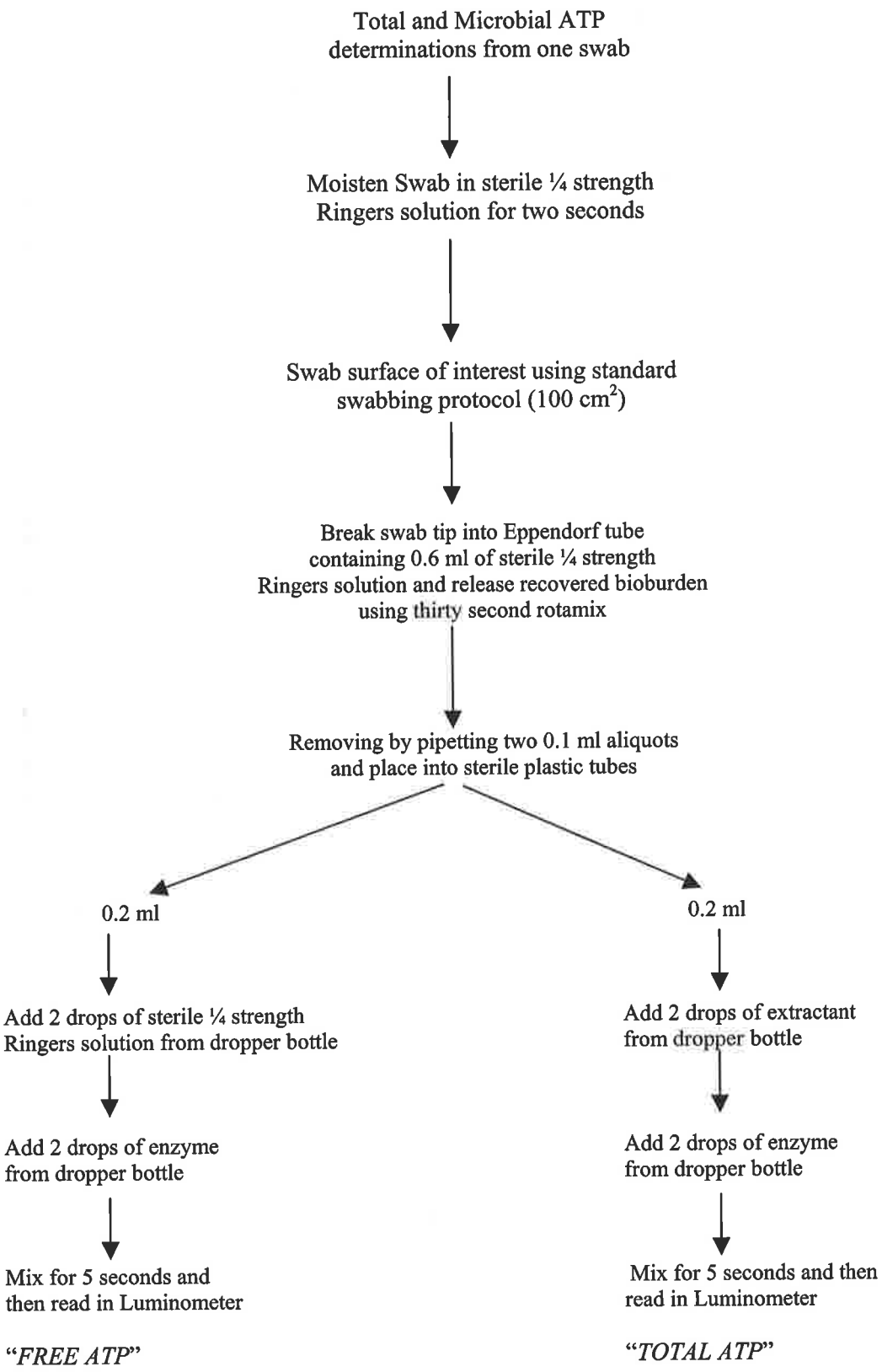


Figure 5.1 Microbial ATP Assay Procedure.

5.3.3 Microbial ATP Assay Procedure

A standard commercially available ATP hygiene assay kit (Biotrace) was adapted according to the procedures outlined in Figure 5.1. The first stage of this development process involved establishing the optimum quantity of diluent in which to release the recovered bioburden present on swab buds. An important consideration was the need to ensure as complete as possible release of recovered bioburden into a volume of recovery diluent that would allow for two separate ATP readings to be made – one for total ATP and one for free ATP. It was necessary, however, to ensure that the volume of recovery diluent used for releasing the recovered bioburden was not excessive to the extent that only a small proportion of the total volume was used for ATP determinations. In conjunction with this was the requirement to determine the optimum quantities of ATP extractant and enzyme to add to the recovery diluent containing the recovered bioburden. This was essential in order that an optimum ATP signal was achieved. It was found that highest RLU readings were achieved when 0.2ml aliquots of recovery diluent were used in conjunction with equal volumes of extractant and enzyme. The final assay procedure involved eluting each swab into 0.6ml of quarter strength Ringer's solution (BR52, Oxoid, Basingstoke, UK) through vortexing for thirty seconds. Two 0.2ml aliquots of the recovered bioburden sample were dispensed into small plastic tubes, with two drops of ATP extractant being added to one tube, and two drops of MRD being added to the other, each being dispensed using dropper bottles supplied by the manufacturer. Two drops of enzyme complex was then added to each tube, with tubes then being placed in the ATP bioluminescence detection system in turn for reading.

ATP readings were therefore gained for each site that enabled both total and free ATP levels to be determined. Total ATP determinations were made from the aliquot of each swab recovery diluent that was extracted, while the non-extracted aliquot provided an indication of free ATP within the initial sample. Microbial ATP determinations were established through subtracting free ATP readings (non-extracted) from total ATP readings (extracted).

5.3.4 Agar-Contact Dip Slides

Dip slides (Dimanco, Henlow, UK) with plate count agar (PCA) surfaces were used to sample each site in triplicate within every food processing environment, both before and after cleaning. Both sides of each dip slide were applied to the food contact or environmental surface being sampled for five seconds, before being returned to their holding containers. All dip slides were incubated at 37°C for twenty-four hours, after which estimate colony counts were determined using the interpretation of results key provided (ref. Appendix 2.).

5.3.5 Correlation of Microbial ATP with Aerobic Colony Counts

One food-processing environment from each of the three categories (general chilled foods, meat processing and dairy) was sampled using ATP bioluminescence and surface hygiene swabbing, instead of agar contact dip slides. Within each of the three processing environments selected, each surface was sampled in triplicate using both methods, with the surface swabbing protocol employed being the optimum one achieved in earlier work (Chapter 2). Hygiene swabs were released into 10ml tubes of MRD (CM733, Oxoid, Basingstoke) for thirty seconds and then serially diluted before diluent pour plates were prepared in duplicate for each swab. All plates were incubated at 37°C for twenty four hours. Aerobic colony counts were then used in conjunction with microbial ATP data to determine whether any correlation between microbial ATP and colony counts existed.

5.3.6 Statistical Analysis

All ATP (RLU) and colony count data gained for each surface from the pre-cleaning sampling session were compared to data gained for the same site after appropriate cleaning procedures had been performed. ATP data and aerobic colony counts from diluent pour plates were analysed using a two tailed Student's *t* test (Excel) to estimate the significance of any difference ($p<0.05$). Aerobic colony count data gained from agar contact dip slides were analysed using a Mann-Whitney U test (SPSS).

5.4 Results

Table 5.1 provides summary information on the food processing environments used in this study, and includes information on the types of food products produced, the mean air temperature of the processing environments at the time of sampling, mean relative humidity data, the nature of the cleaning processes employed and the products used, the sites cleaned using these protocols, and the frequency of cleaning.

Table 5.2 illustrates the total number of surfaces sampled in each food processing environment, and the number of surfaces from each that had statistically significant total ATP, microbial ATP and colony count results after the post-cleaning sampling session when post-cleaning results were compared to those gained for the same surfaces before cleaning. It can be seen that of a total of seventy six surfaces sampled from all food processing environments, only one third of those surfaces resulted in statistically significant differences in the levels of total and microbial ATP present following cleaning. Results for aerobic colony counts following cleaning, where agar contact dip slides were used, were not found to be statistically significant when analysed using the Mann Whitney U test, even though differences in the counts gained were observed. This was thought to be partly due to both the power of the statistical test employed, and the number of data values used in the analysis. In the three processing environments where aerobic colony counts were obtained using surface swabbing and cultivation, it was found that of twenty two surfaces sampled, thirteen gave statistically significant different results following cleaning. It was interesting to note that the results from different surface monitoring methods resulted in different numbers of surfaces being confirmed as clean following statistical analysis of the data gained.

Table 5.3 outlines the number of food contact and environmental surfaces sampled within each food processing environment after cleaning that achieved benchmark RLU and colony count values indicating that adequate cleaning had been performed. These values were less than 500 RLU/100 cm^2 for total ATP, and less than 2.5 cfu/cm^2, equivalent to 2.5 x 10^2/100 cm^2 for aerobic colony counts. Of sixty nine cleaned surfaces sampled, it was found that forty two had

Table 5.1 General Factory Information

Food Processing Environment	Food Products Produced	Number of Employees	Air temperature at time of sampling °C	Humidity recorded at time of sampling	Cleaning Schedule	Cleaning Products Used	Supplier of Cleaning Agents	Surfaces Cleaned	Frequency of Cleaning Surfaces
General Chilled Foods 1	Filled sandwiches and rolls	35	10	94	Clean as you go, some schedules	CP204, Divosan, Handex	Diversey	All sites	Daily
General Chilled foods 2	Sauces, spare ribs	125	16 -11.5	80.5 -100	Various schedules	Detergent, sanitizer (QAC)	Clennol Group Ltd	All sites	Daily
General Chilled Foods 3	Ready-to-eat	600	19 -20	68 -52	Various schedules	Terminol (QAC)	Holchem	All sites	Daily at night
General Chilled Foods 4	Ready-to-eat curry, catering packs, cook-chill-freeze	9	20.7 -17.8	58.3 -58.5	No specific schedules, clean as you go	Dettox, bactericidal cleaner, bleach	Bluecol Brands Ltd, Reckitt & Coleman	All sites	Clean at break times, at the end of shift, major clean Friday pm
Meat Processing 1	Cooked meats	28	10.9-9	77.3-79.5	Hot water rinse, detergent, rinse terminal sanitizer	Sanarinse (Biguanide, QAC)	Techtron Ltd	All sites except chiller	After morning break, lunchtime, and end of day
Meat Processing 2	Cooked frozen chicken halves and pieces	50	13.8	Not determined	Hot water rinse, detergent, rinse terminal sanitizer	Chlorfoam Holquat	Holchem Lab. Ltd	Hot strip Conveyor ceilings air conditioning chiller other sites	Every break with hot water and QAC once a month every three months twice a week daily over night clean
Meat Processing 3	Hot dogs, black puddings, saveloys	400	17	57	Various schedules	Wash room foam, terminal disinfectant, quat blocks	Not known	All sites	Daily/weekly
Dairy 1	Pasteurised milk	119	17.8	57	Clean-in-Place	Sodium Hydroxide, Hypochlorite	Not known	Floors other sites	Every day (hypochlorite and bleach)
Dairy 2	Cheese	37	19 -20	79 -86	Clean-in-Place, fogging	Eversan	Diversey	Cheese area tanks and pipe work draining table butter packing	Fogged daily, daily wash , major clean once a week
Dairy 3	Cheese	60 - 70	15 14	63.5 -45.7	No specific schedules	Quad X, Hypochlorite, dairy detergent	Selden Research	All sites	Daily

Table 5.2 Numbers of Sampled Surfaces within each Food Processing Environment giving Statistically Significant differences in Total ATP, Microbial ATP and Aerobic Colony Count Data after Cleaning

Food Sector	Factory	Total Number of Sites Sampled	No. Sites with Statistically Significant Levels of Total ATP after Cleaning	No Sites with Statistically Significant Levels of Microbial ATP after cleaning	No. Sites with Statistically Significant Colony Counts after cleaning
General Chilled Foods	1	7	0	0	0
	2	6	2	1	0
	3	8	2	2	4
	4	6	1	1	0
Meat Processing	1	7	2	2	3
	2	11	4	3	0
	3	7	4	4	0
Dairy	1	6	3	3	0
	2	7	4	4	6
	3	11	3	4	0

Food processing environments in which statistically significant different results were gained after cleaning for aerobic colony counts were those in which pour plates were used.

Table 5.3 **Numbers of Sampled Surfaces within each Food Processing Environment Passing and Failing Surface Cleanliness Assessments when compared to Benchmark Values For Microbiological and ATP Bioluminescence Methods.**

Food Processing Environment	Surface Type	Total Number of Surfaces Sampled After Cleaning	ATP Bioluminescence		Aerobic Colony Count	
			No. Surfaces Passing	No. Surfaces Failing	No. Surfaces Passing	No. Surfaces Failing
Gen. Chilled Foods 1	Food Contact	2	2	0	1	1
	Environmental	5	0	5	2	3
Gen. Chilled Foods 2	Food Contact	3	1	2	2	1
	Environmental	3	3	0	1	2
Gen. Chilled Foods 3	Food Contact	3	2	1	0	3
	Environmental	0	-	-	-	-
Gen. Chilled Foods 4	Food Contact	3	3	0	3	0
	Environmental	3	2	1	2	1
Dairy 1	Food Contact	4	4	0	4	0
	Environmental	3	3	0	3	0
Dairy 2	Food Contact	3	2	1	1	2
	Environmental	2	0	2	0	2
Dairy 3	Food Contact	6	3	3	4	2
	Environmental	5	2	3	2	3
Meat Processing 1	Food Contact	2	2	0	2	0
	Environmental	3	3	0	3	0
Meat Processing 2	Food Contact	5	4	1	4	1
	Environmental	7	4	3	3	2*
Meat Processing 3	Food Contact	2	1	1	1	1
	Environmental	5	1	4	1	4
	Total No. Food Contact Surfaces Passing/Failing		**24**	**9**	**22**	**11**
	Total No. Environmental Surfaces Passing/Failing		**18**	**18**	**17**	**17**

Benchmark values used were <500RLU/100 cm^2 for ATP bioluminescence and < 2.5 x 10^2 /100 cm^2 for Aerobic colony Counts.

* Two surfaces not sampled using dip slides.

ATP RLU values of less than 500 RLU, and that 39 surfaces had colony counts of less than 2.5 x 10^2/100 cm^2. Identical numbers of sites passing and failing cleanliness assessment using both sampling methods were gained for both food contact and environmental surfaces in four food processing environments, these being general chilled food environment four, dairy one, and meat processing environments one and three. Of the remaining six food processing environments, identical numbers of sites passing and failing cleanliness assessment were obtained using both sampling methods for environmental surfaces in dairies two and three, and for food contact surfaces in meat processing environment two. Overall, a total of twenty four food contact surfaces passed and nine surfaces failed cleanliness assessment using the ATP method. Using the aerobic colony count method twenty two food contact surfaces passed, while eleven surfaces failed cleanliness assessment when the results gained were compared to the benchmark values. With regard to environmental surfaces, when using ATP bioluminescence, eighteen surfaces passed and eighteen surfaces failed when results were compared to the benchmark value of less than 500 RLU/100 cm^2. The aerobic colony count method resulted in seventeen environmental surfaces passing and seventeen surfaces failing when the results gained were compared to the benchmark value of less than 2.5 x 10^2/ 100 cm^2.

Figures 5.2a and b illustrate the correlation between log microbial ATP and log aerobic colony count data for food contact and environmental surfaces sampled in general chilled food environment number three before and after cleaning. An r value of 0.94 was achieved before cleaning, while after cleaning an r value of 0.51 was obtained. Figures 5.3a and b illustrate the extent of the correlation that exists for log microbial ATP and log aerobic colony count data gained for surfaces sampled before and after cleaning in dairy environment number two. Before cleaning, an r value of 0.91 was achieved, while after cleaning an r value of 0.91 was obtained.

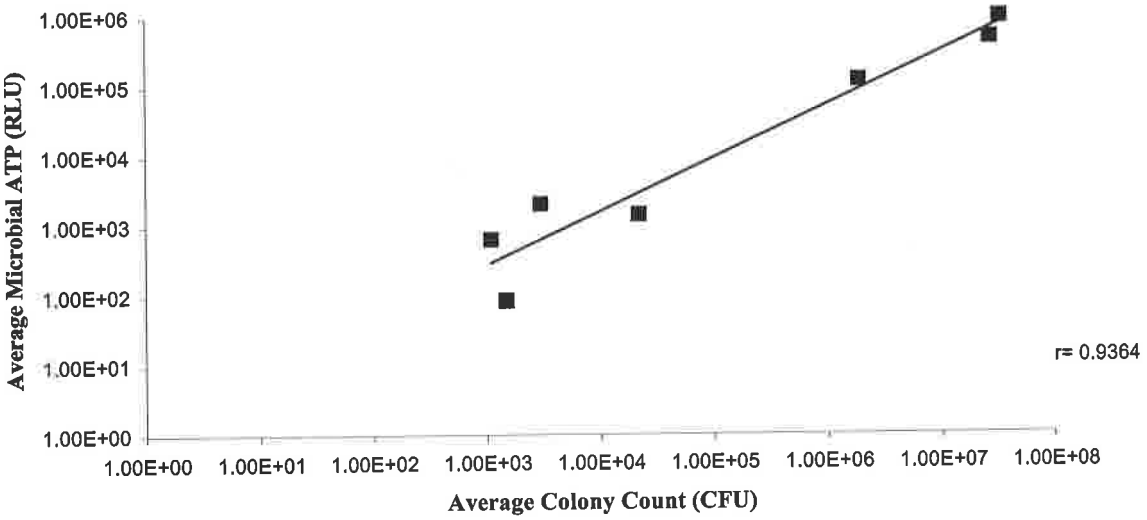


Figure 5.2 (a): Correlation of Microbial ATP with Aerobic Colony Count Before Cleaning in General Chilled Food Environment 3.

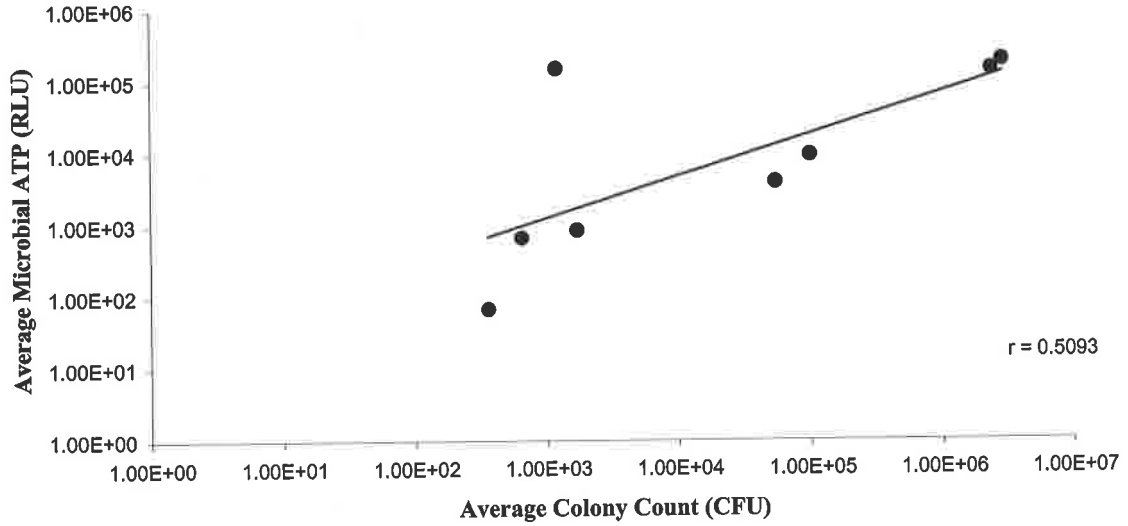


Figure 5.2 (b): Correlation of Microbial ATP with Aerobic Colony Count After Cleaning in General Chilled Food Environment 3.

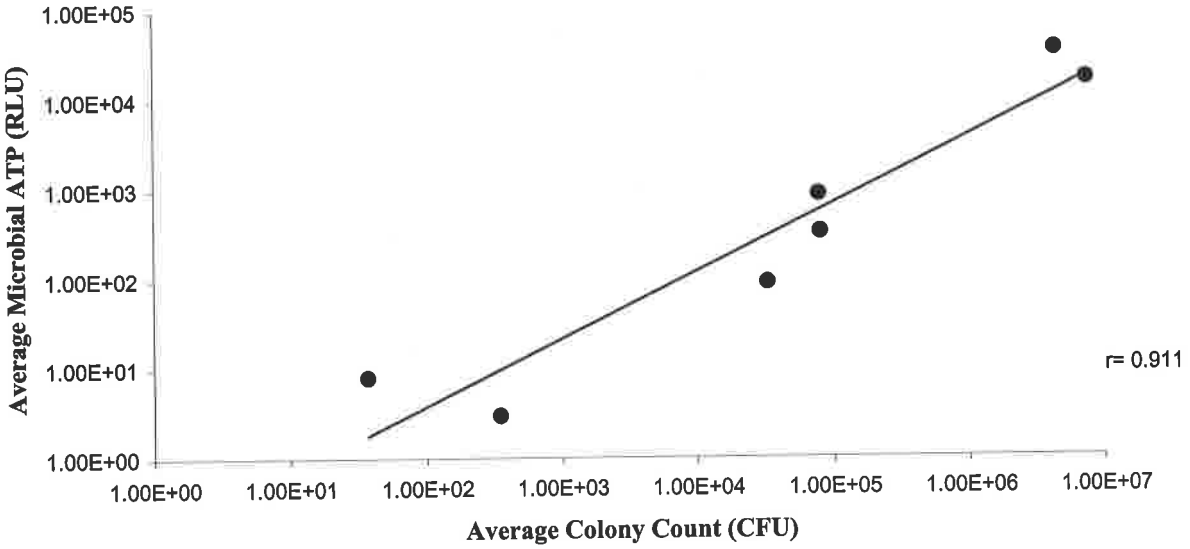


Figure 5.3 (a): Correlation of Microbial ATP with Aerobic Colony Count Before Cleaning in Dairy Environment 2.

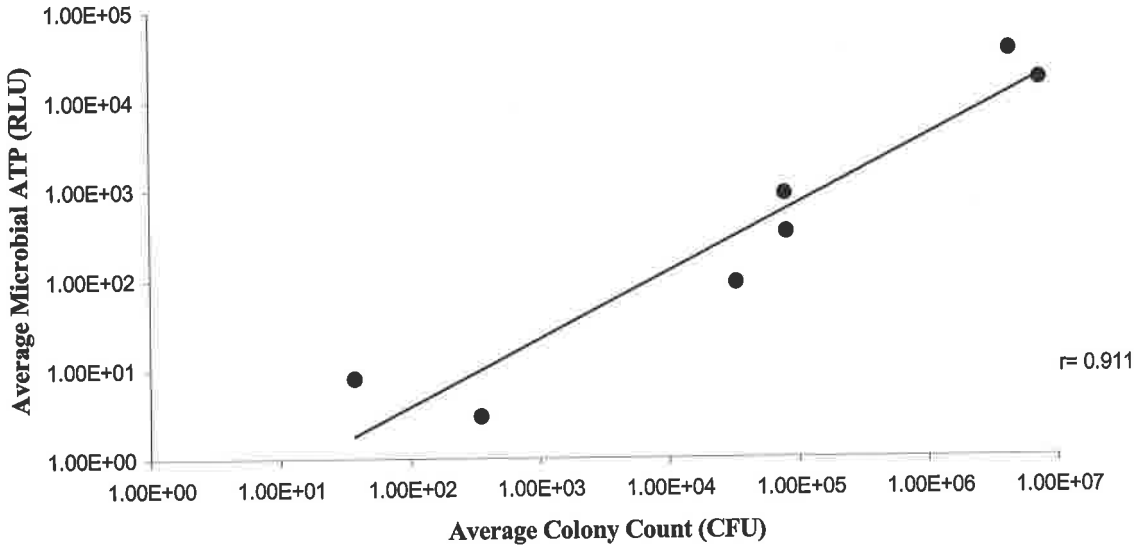


Figure 5.3 (b): Correlation of Microbial ATP with Aerobic Colony Count After Cleaning in Dairy Environment 2.

Figures 5.4a and b illustrate the correlation obtained for log microbial ATP and log aerobic colony count data for meat processing environment number three before and after cleaning. An r value of 0.65 was obtained before cleaning, while after cleaning the r value achieved was 0.94.

Table 5.4 provides overall mean summary data on ATP and aerobic colony count data for pre and post-cleaning sampling sessions in the three types of food processing environment, for both food contact and environmental surfaces. In all three types of food processing environments it was found that in sampling before cleaning had been performed, the mean percentage product residue contamination on food contact surfaces was higher than the mean percentage levels of microbial contamination on the same surfaces. On environmental surfaces, however, it was found that in dairy and meat processing environments, that a higher proportion of the surface contamination was attributable to contamination of microbial origin.

The mean relative levels of the two types of contamination on environmental surfaces in the general chilled foods environments were found to be almost identical. Post-cleaning, however, it was found that on food-contact surfaces the greatest proportion of the contamination present was product residue contamination. This was also found to be true for environmental surfaces, except in the case of dairy environments where microbial contamination predominated.

Aerobic colony counts, determined using agar-contact dip slides, ranged from no detection of growth up to 10^5 cfu per 100 cm^2 of surface sampled for food contact surfaces before cleaning. It is important, however, to emphasise that a negative microbiological result, i.e. no growth, may indicate that any microbial contamination on the surface was present at a level below the minimum detection limit of the cleanliness assessment method used. Detailed information on minimum detection limits of different methods are provided in Chapter Four. In addition, in using agar contact dip slides, it is important to remember that any results gained will be from a sampled area of approximately 2.5cm x 5cm, and this is considerably less than the 10cm x 10cm areas sampled using the other methods.

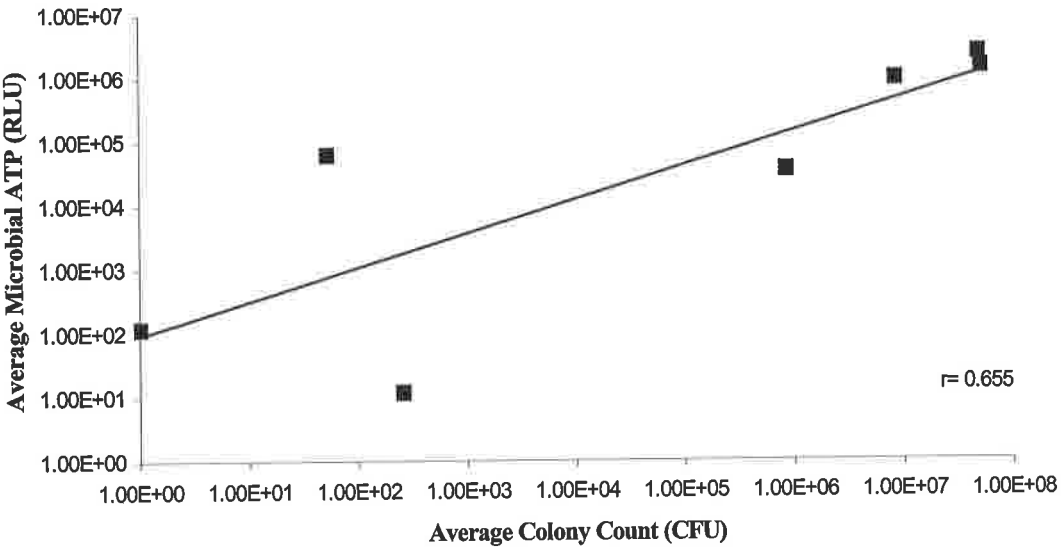


Figure 5.4 (a): Correlation of Microbial ATP with Aerobic Colony Count After Cleaning in Meat Processing Environment 3.

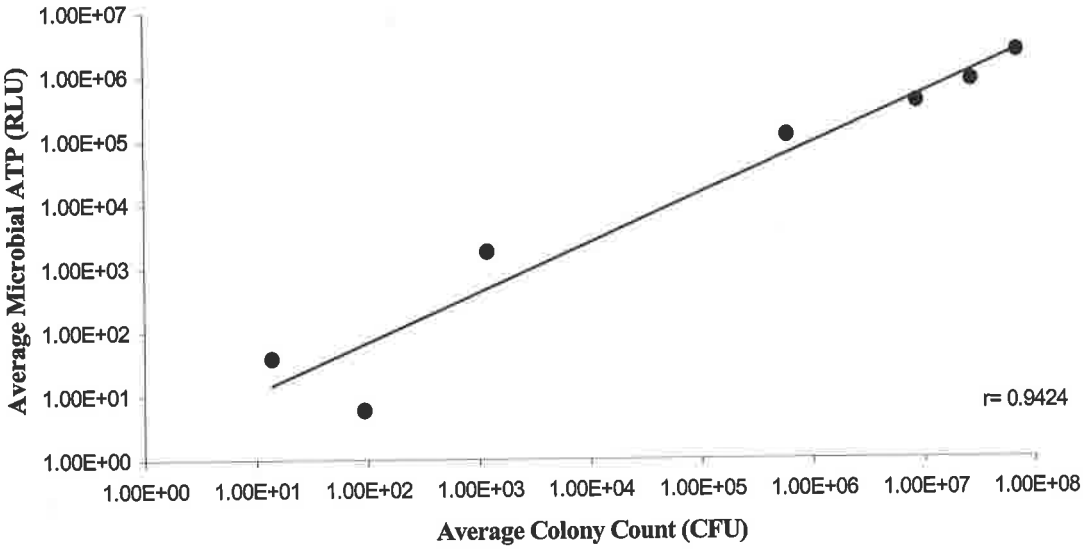


Figure 5.4 (b): Correlation of Microbial ATP with Aerobic Colony Count After Cleaning in Meat Processing Environment 3.

Table 5.4 **Summary Mean ATP and Aerobic Colony Count Data for Pre and Post-Clean Sampling Sessions in Three Types of Food Processing Environments**

		Pre-Clean Sampling					Post-Clean Sampling				
Factory Type	**Surface Type**	**Mean Total ATP/100cm² (RLUs)**	**Mean Micro. ATP/100cm² (RLUs)**	**Mean Micro. ATP/100cm² as % of Total ATP**	**Mean Product Residue ATP/100cm² as % of Total ATP**	**Range of Colony Counts/100cm²**	**Mean Total ATP/100cm² (RLUs)**	**Mean Micro. ATP/100cm² (RLUs)**	**Mean Micro. ATP/100cm² as % of Total ATP**	**Mean Product Residue ATP/100cm² as % of Total ATP**	**Range of Colony Counts/100cm²**
General Chilled Foods	**Food Contact**	7319	3401	35	65	$<10^2 - 10^3$	544	230	31	71	$10^2 - 10^4$
	Environ-mental	19500	15421	49	51	$<10^2 - 10^7$	13935	23266	47	53	$<10^2 - 10^4$
Dairy	**Food Contact**	8150	7295	43	57	$<10^2 - 10^5$	5812	4827	25	41	$10^1 - 10^6$
	Environ-mental	15721	6183	56	44	$<10^2 - 10^5$	1725	1282	59	42	$<10^2 - 10^4$
Meat Process.	**Food Contact**	43743	13037	27	73	$<10^2 - 10^4$	285	93	10	57	$<10^2 - 10^4$
	Environ-mental	59317	54137	54	46	$<10^2 - 10^7$	43871	18554	43	57	$<10^2 - 10^7$

Colony count data gained using either agar contact dip slides or surface swabbing followed by cultivation using pour plates.
For ease of clarity and presentation, standard deviation values for all surfaces within each factory are provided in Appendix 3 (page 208 onwards).

Slight variations in the upper limits of growth detected were found in the three types of processing environment. Similar results were found on environmental surfaces before cleaning, with levels of growth ranging from no detection up to 10^7 cfu per 100 cm^2 of surface sampled. Similar aerobic colony counts were found after cleaning, with values on food contact surfaces ranging from no detection of growth up to 10^4 cfu/100 cm^2 of surface sampled. On environmental surfaces it was found that the upper limit of growth, post-cleaning, determined using dip slides was found to be 10^7 cfu/100 cm^2.

Table 5.5 provides summary ATP and aerobic colony count data for pre and post-cleaning sampling sessions in general chilled food environments. In all but one occasion it can be seen that the levels of microbial contamination on food contact surfaces before cleaning were lower than levels of product residue contamination. Mean values for microbial contamination on food contact surfaces before cleaning ranged from 12 to 63%. Only in chilled food environment two did the level of microbial contamination on food contact surfaces exceed the level of product residue contamination, the values being 63% and 37% respectively. Excluding this value of 63%, mean percentage microbial contamination levels ranged from 12% to 35%, with an overall mean level of 35% being obtained for all four general chilled food environments. On environmental surfaces before cleaning, it was found that levels of microbial contamination were lower in two chilled food environments, and higher in two others. Values ranged from 31% to 72%, with an overall mean value of 49%.

Sampling food contact surfaces after cleaning had been performed resulted in lower levels of microbial contamination than product residue contamination on food contact surfaces in all but one chilled food environment. The proportion of ATP of microbial origin ranged from 17% to 52%, with an overall mean level for all food contact surfaces of 31% being recorded. Mean percentage levels for product residue contamination on food contact surfaces after cleaning ranged from 48% to 83%, with an overall mean of 71% being recorded.

Table 5.5 Summary Mean ATP and Aerobic Colony Count Data for Pre and Post-Clean Sampling Sessions in General Chilled Food Environments

		Pre-Clean Sampling					Post-Clean Sampling				
	Surface Type	Mean Total ATP/100cm (RLUs)	Mean Micro. ATP/100cm^2 (RLUs)	Mean Micro. ATP/100cm^2 as % of Total ATP	Mean Product Residue ATP/100cm^2 as % of Total ATP	Range of Colony Counts /100cm^2	Mean Total ATP/100cm (RLUs)	Mean Micro. ATP/100cm^2 (RLUs)	Mean Micro. ATP/100cm^2 as % of Total ATP	Mean Product Residue ATP/100cm^2 as % of Total ATP	Range of Colony Counts/ 100cm^2
Chilled Foods 1	**Food-Contact**	395	116	29	71	$<10^2$ - 10^3	397	118	30	70	$<10^2$ - 10^4
	Environ-mental	9675	7259	53	47	$<10^2$ - 10^4	38588	65363	69	31	$<10^2$ - 10^4
Chilled Foods 2	**Food-Contact**	27259	13044	63	37	$<10^2$ - 10^3	1341	455	52	48	$<10^2$ - 10^3
	Environ-mental	10506	3111	31	69	$\leq 10^2$	159	56	26	74	$<10^2$ - 10^3
Chilled Foods 3	**Food-Contact**	796	150	12	88	10^3	341	85	17	83	10^2 - 10^3
	Environ-mental	56210	50637	72	28	$<10^2$ - 10^7	Surface not clean	–	–	–	–
Chilled Foods 4	**Food-Contact**	825	293	35	65	$<10^2$ - 10^3	96	33	23	77	$<10^2$
	Environ-mental	1609	678	40	60	$<10^2$ - 10^3	3058	4380	47	53	$<10^2$ - 10^3
Overall Mean	**Food-Contact**	7319	3401	35	65	$<10^2$ - 10^3	544	230	31	71	10^2 - 10^4
	Environ-mental	19500	15421	49	51	$<10^2$ - 10^7	13935	23266	47	53	$<10^2$ – 10^4

Colony count data for General Chilled Foods 3 gained using pour plates. All other colony count data gained using dip slides.
For ease of clarity and presentation, standard deviation values for all surfaces within each factory are provided in Appendix 3 (page 208 onwards).

Results for the proportion of microbial contamination on environmental surfaces after cleaning were more variable, with the mean proportion for each chilled food processing environment ranging from 26% to 69%. An overall mean proportion of microbial contamination on environmental surfaces of 47% was recorded, with no mean proportion being recorded for chilled food environment three due to a combination of both clean surfaces giving no detection, and some surfaces not being cleaned prior to the second sampling session.

Proportions of product residue contamination (i.e. free ATP) were, in general, found to be higher than proportions of microbial ATP on food contact surfaces prior to cleaning. Mean percentage proportions ranged from 37% to 88%, with an overall mean proportion of 65% being recorded for food contact surfaces before cleaning. Greater variation was found on environmental surfaces before cleaning, with values for proportions of product residue ATP ranging from 28% to 69%, with an overall mean proportion for all general chilled food environments of 51% being recorded. Values for the proportions of product residue ATP on food contact surfaces after cleaning had taken place were generally higher than those of microbial ATP. Mean percentage levels ranged from 48% to 83%, with an overall mean proportion for all environments of 71% being recorded. Results for proportions of product residue ATP on environmental surfaces after cleaning were more variable, but with an overall mean proportion for all environments of 53% being recorded.

Table 5.6 provides summary mean ATP and aerobic colony count data for pre and post-clean sampling sessions in dairy environments. It can be seen that in two of the three dairy environments, the proportions of microbial ATP on food contact surfaces prior to cleaning were found to be lower than the proportions of product residue ATP. Mean percentage values for microbial ATP ranged from 23% to 69%, with an overall mean percentage level of 43% being recorded on food contact surfaces prior to cleaning. Proportions of product residue ATP on the same surfaces prior to cleaning ranged from 31% to 77%, with a mean of 57% being recorded.

Table 5.6 Summary Mean ATP and Aerobic Colony Count Data for Pre and Post-Clean Sampling Sessions in Dairy Environments

		Pre-Clean Sampling					Post-Clean Sampling				
	Surface Type	Mean Total ATP/100cm^2 (RLUs)	Mean Micro. ATP/100cm^2 (RLUs)	Mean Micro. ATP/ 100cm^2 as % of Total ATP	Mean Product Residue ATP/100cm^2 as % of Total ATP	Range of Colony Counts/ 100cm^2	Mean Total ATP/100cm^2 (RLUs)	Mean Micro. ATP/100cm^2 (RLUs)	Mean Micro. ATP/100cm^2 as % of Total ATP	Mean Product Res. ATP/ 100cm^2 as % of Total ATP	Range of Colony Counts/ 100cm^2
Dairy 1	**Food-Contact**	1499	739	23	77	Not Sampled	Not Detected	Not Detected	Not Detected	Not Detected	$<10^2$
	Environ-mental	37538	9463	46	54	Not Sampled	Surfaces not clean	–	–	–	–
Dairy 2	**Food-Contact**	7549	5939	69	31	10^4 - 10^5	13905	11583	28	72	10^1 - 10^6
	Environ-mental	6717	6276	49	51	10^3 - 10^5	1003	606	59	41	10^4
Dairy 3	**Food-Contact**	15401	15207	36	64	$<10^2$ - 10^4	3530	2897	48	52	$<10^2$ - 10^4
	Environ-mental	2908	2810	72	28	$<10^2$ - 10^4	2447	1957	58	42	$<10^2$ - 10^4
Overall Mean	**Food-Contact**	8150	7295	43	57	$<10^2$ - 10^5	5812	4827	25	41	10^1 - 10^6
	Environ-mental	15721	6183	56	44	$<10^2$ - 10^5	1725	1282	59	42	$<10^2$ - 10^4

Colony count data for Dairy 2 gained using pour plates. All other colony count data gained using dip slides. Surfaces that were not clean during the post clean sampling session (Dairy 1, Environmental Surfaces) were, as a result, not sampled, therefore no results are provided, indicated by: -

For ease of clarity and presentation, standard deviation values for all surfaces within each factory are provided in Appendix 3 (page 208 onwards).

On environmental surfaces, the proportions of microbial ATP in two dairy environments prior to cleaning were found to be lower than those of product residue ATP, with values of 46% and 49% being recorded. In one food-processing environment, however, the proportion was found to be considerably higher at 72%. An overall mean proportion of microbial ATP of 56% on such surfaces was recorded. Lower proportions of microbial ATP on these surfaces resulted in slightly higher proportions of product residue ATP, with values ranging from 28% to 51%. The food-processing environment with a mean product residue value of 28% did, however, have a much higher proportion of microbial ATP present on surfaces, with a mean proportion of 72% being recorded.

After cleaning had been carried out, ATP was not detected above background levels on both food contact and environmental surfaces in one dairy environment. These results were confirmed through no aerobic colony counts being obtained using agar-contact dip slides. Proportions of microbial ATP on food contact surfaces in the other two dairy environments were found to range from 28% to 48%, with these proportions being lower than those of product residue ATP being recorded on the same surfaces. Mean proportions of product residue ATP ranged from 52% to 72%. Overall mean percentage values for microbial and product residue ATP on food contact surfaces after cleaning were 25% and 41% respectively. On environmental surfaces after cleaning proportions of microbial ATP were found to be higher than proportions of product residue ATP. Values were consistent at 58% to 59%, as were values for product residue ATP that ranged from 41% to 42%.

Table 5.7 provides summary mean data on ATP and aerobic colony counts gained from meat processing environments both before and after cleaning. It can be seen that both before and after cleaning, the proportion of microbial ATP detected on food contact surfaces was lower than the proportions of product residue ATP detected, with mean percentage values of 27% and 10% being recorded. However, on environmental surfaces, it was found that the proportion of microbial ATP being detected prior to cleaning (54%) was higher than the proportion of product residue ATP being detected, with an overall mean percentage of 46% being recorded. After cleaning, the overall mean percentage proportion of microbial ATP was reduced to 43%, whilst the proportion of product residue ATP had increased from 46% to 57% on environmental surfaces. Results for levels of microbial growth, detected through agar contact dip slides, indicated that both prior to and after cleaning the range of levels of contamination recorded were essentially the same, with levels of between no growth to 10^7 per 100 cm^2 of surface being recorded.

In evaluating the results gained for each of the three meat processing environments sampled, it was noted that prior to cleaning, the proportions of microbial ATP detected on food-contact surfaces were always lower than the proportions of product residue ATP, with percentage values of 20% to 34% microbial ATP, and 66% to 80% product residue ATP being detected. On environmental surfaces, prior to cleaning, it was found that in two meat processing plants the percentage values of microbial ATP were higher than those of product residue ATP, with values of 57% and 77% being noted. A much lower proportion of 28% microbial ATP was recorded in the third plant on environmental surfaces.

Post-cleaning sampling results for each of the three processing environments show that in one processing facility no ATP was detected at all on food contact surfaces, but with 13% microbial and 87% product residue ATP being detected on environmental surfaces.

Table 5.7 Summary Mean ATP and Aerobic Colony Count Data for Pre and Post-Clean Sampling Sessions in Meat Processing Environments

		Pre-Clean Sampling					Post-Clean Sampling				
	Surface Type	Mean Total ATP/100cm^2 (RLUs)	Mean Micro. ATP/100cm^2 (RLUs)	Mean Micro ATP/ 100cm^2 as % of Total ATP	Mean Product Residue ATP/100cm^2 as % of Total ATP	Range of Colony Counts/100cm^2	Mean Total ATP/ 100cm^2 (RLUs)	Mean Micro. ATP/ 100cm^2 (RLUs)	Mean Micro ATP/100cm^2 as % of Total ATP	Mean %Product Residue ATP/100cm^2 as % of Total ATP	Range of Colony Counts/100cm^2
Meat Processing 1	Food-Contact	10068	2537	27	73	$<10^2$ - 10^4	Not Detected	Not Detected	Not Detected	Not Detected	$<10^2$
	Environ-mental	7549	4002	57	43	$<10^2$ - 10^4	82	32	13	87	$<10^2$
Meat Processing 2	Food-Contact	106585	33379	34	66	$<10^2$ - 10^4	215	130	17	83	$<10^2$-10^4
	Environ-mental	14997	4241	28	72	$<10^2$ – 10^3	10217	4544	37	63	$<10^2$-10^4
Meat Processing 3	Food-Contact	14576	3195	20	80	10^1	639	150	13	87	10^1-10^3
	Environ-mental	155404	154169	77	23	10^2 - 10^7	121315	51086	78	22	10^1-10^7
Overall Mean	Food-Contact	43743	13037	27	73	$<10^2$ - 10^4	285	93	10	57	$<10^2$-10^4
	Environ-mental	59317	54137	54	46	$<10^2$ - 10^7	43871	18554	43	57	$<10^2$-10^7

Colony count data for Meat Processing 3 gained using pour plates. All other colony count data gained using dip slides.
For ease of clarity and presentation, standard deviation values for all surfaces within each factory are provided in Appendix 3 (page 208 onwards).

Microbiological analysis using agar contact dip slides detected no growth on either food-contact or environmental surfaces. However, the minimum detection limit of this test when used to sample dry surfaces was found to be of the order of 10^6 to 10^7 cfu/100 cm^2. For the two other meat processing environments after cleaning, it was found that food contact surfaces had lower proportions of microbial ATP than product residue ATP, with values of 13% and 17% being detected. On environmental surfaces it was found that in one processing environment a lower proportion of microbial ATP to product residue ATP was detected with values of 37% and 63% being recorded. A much higher proportion of microbial ATP at 78% was recorded in the third processing environment, with product residue ATP accounting for only 22% of the total contamination detected on environmental surfaces.

Table 5.8 illustrates mean microbial ATP and aerobic colony count data gained for selected food contact and environmental surfaces, common to all three types of food processing environment, before cleaning had been carried out. Additionally, percentage values for the proportions of microbial contamination detected on the surfaces sampled are given. These percentage values relate to the contribution that microbial contamination had to the total contamination present on the surfaces sampled, i.e. total surface contamination comprising microbial and product residue contamination. Data for the same surfaces after cleaning had been performed are provided in Table 5.9. It can be seen in Table 5.8 that prior to cleaning, the percentage microbial contamination on stainless steel food preparation surfaces ranged from 26% for surfaces in dairy environments up to 34% in general chilled food environments. Aerobic colony count data for these surfaces ranged from no growth up to 10^5 cfu/100 cm^2. After cleaning, the mean percentage microbial contamination on the same surfaces in dairy environments had increased to 54%, while for the same surfaces in general chilled food environments, the proportion of microbial contamination detected decreased from 34% to 21%. Aerobic colony count data after cleaning ranged from no growth being detected up to 10^6 cfu/100 cm^2. For plastic contact surfaces, percentage values for proportions of microbial contamination before cleaning had been performed ranged from 26% in general chilled food environments up to 99% in dairy environments.

Table 5.8 Comparison of Mean Microbial ATP and Aerobic Colony Count Data for Selected Surfaces within Three Types of Food Processing Environments Before Cleaning

	General Chilled Foods		Dairy		Meat Processing	
Food Contact Surfaces	**Mean Microbial ATP/100 cm^2 +% of Total ATP**	**Range of Colony Counts/100 cm^2**	**Mean Microbial ATP/100 cm^2 +% of Total ATP**	**Range of Colony Counts/100 cm^2**	**Mean Microbial ATP/100 cm^2+ % of Total ATP**	**Range of Colony Counts/100 cm^2**
Stainless Steel Preparation Surfaces	2923 (34)	$<10^2$ - 10^3	770 (26)	$<10^2$ - 10^5	53101 (32)	$<10^2$
Plastic Contact Surfaces	237 (26)	10^2 - 10^3	86403 (99)	10^4	4797 (35)	$<10^2$
Environmental Surfaces	**Mean Microbial ATP/100 cm^2+ % of Total ATP**	**Range of Colony Counts/100 cm^2**	**Mean Microbial ATP/100 cm^2+ % of Total ATP**	**Range of Colony Counts/100 cm^2**	**Mean Microbial ATP/100 cm^2+ % of Total ATP**	**Range of Colony Counts/100 cm^2**
Chilled Area Floors	83100 (90)	10^4 - 10^7	6456 (67)	10^2 - 10^5	103584 (51)	10^2 - 10^7
Ambient Area Floors	98312 (70)	$<10^2$ - 10^6	NS	NS	97327 (85)	$<10^2$ – 10^7
Stainless Steel Surfaces	9840 (42)	$<10^2$ – 10^7	75 (34)	$<10^2$	649 (51)	$<10^2$ – 10^3

NS – Indicates surface type not sampled within this category of food processing environment.
For ease of clarity and presentation, standard deviation values for all surfaces within each factory are provided in Appendix 3 (page 208 onwards).

Table 5.9 **Comparisons of Mean Microbial ATP and Aerobic Colony Count Data for Selected Surfaces within Three Types of Food Processing Environments After Cleaning**

	General Chilled Foods		Dairy		Meat Processing	
Food Contact Surfaces	**Mean Microbial ATP/100 cm^2 + % of Total ATP**	**Range of Colony Counts/100 cm^2**	**Mean Microbial ATP/100 cm^2 +% of Total ATP**	**Range of Colony Counts/100 cm^2**	**Mean Microbial ATP/100 cm^2+ % of Total ATP**	**Range of Colony Counts/100 cm^2**
Stainless Steel Preparation Surfaces	63 (21)	Up to 10^2	4992 (54)	$<10^2$ - 10^6	Not Detected	10^2
Plastic Contact Surfaces	115 (26)	$<10^2$ - 10^4	514 (53)	Up to 10^2	325 (44)	$<10^2$ - 10^4
Environmental Surfaces	**Mean Microbial ATP/100 cm^2+ %**	**Range of Colony Counts/100 cm^2**	**Mean Microbial ATP/100 cm^2+ % of Total ATP**	**Range of Colony Counts/100 cm^2**	**Mean Microbial ATP/100 cm^2+ % of Total ATP**	**Range of Colony Counts/100 cm^2**
Chilled Area Floors	136 (19)	10^4	413 (47)	10^2 - 10^4	15747 (58)	10^3 - 10^5
Ambient Area Floors	8659 (97)	10^3	NS	NS	591105 (82)	$<10^2$ - 10^7
Stainless Steel Surfaces	418 (28)	10^2	88 (21)	$<10^2$	7 (4)	10^2

NS - Indicates surface type not sampled within this category of food processing environment.
For ease of clarity and presentation, standard deviation values for all surfaces within each factory are provided in Appendix 3 (page 208 onwards).

A mean level of 35% microbial contamination was found for meat processing environments. Colony count data for these surfaces ranged from no growth being detected up to 10^4 cfu/100cm^2. After cleaning, mean proportions of microbial contamination on plastic surfaces ranged from 26% up to 53%, with colony count data ranging from no growth up to 10^4cfu/100 cm^2.

Environmental surfaces common to all processing environments that were sampled included stainless steel surfaces and floors in both ambient and chilled areas. Mean proportions of microbial contamination ranged from 51% to 90% for floors in chilled areas prior to cleaning, while for floors in ambient areas proportions ranged from 70% to 85%. Aerobic colony count data for floors in chilled and ambient areas was found to be similar and ranged from no growth being detected up to 10^7 cfu/100 cm^2. After cleaning had taken place, mean proportions of microbial contamination ranged from 19% to 58% for floors in chilled areas and from 82% to 97% for floors in ambient areas. Aerobic colony count data for both ranged from no growth being detected up to 10^7 cfu/100 cm^2.

For environmental stainless steel surfaces before cleaning, proportions of microbial contamination ranged from 34% in dairy environments up to 51% in meat processing environments. After cleaning, proportions ranged from 4% in meat processing environments up to 28% in general chilled food environments. Prior to cleaning, aerobic colony count data on these surfaces ranged from no growth being detected up to 10^7 cfu/100 cm^2, while after cleaning ranged from no growth being detected up to 10^2 cfu/100 cm^2.

5.5 Discussion

A total of seventy six surfaces from ten different food processing environments were sampled in triplicate using ATP and microbiological methods before cleaning and sixty nine of these surfaces were sampled after normal cleaning procedures had been performed. Results clearly indicate that differences in the relative proportions of microbial ATP and product residue ATP

do exist on food contact and environmental surfaces, and that apparent differences might be attributable to a number of factors. In addition, the microbial ATP assay developed has been shown to correlate well with colony count data obtained from surface swabbing. In two of the three food processing environments in which microbial ATP data were compared to colony counts, r values in excess of 0.9 were obtained indicating that the correlation between both sampling methods was high. Similarly, after cleaning, two of the three environments sampled resulted in values of the same order. While good correlations were found between the methods both before and after cleaning, the most important of these are the values obtained after cleaning had been performed. Following appropriate cleaning, surfaces should be relatively free from food product residues leaving only residual microbial contamination to be detected by both methods.

Comparison of the ATP and colony count data, gained for all food processing environments after cleaning had been performed, with benchmark values of 500RLU/100 cm^2 for ATP and $<2.5 \times 10^2$ cfu/100 cm^2 for colony counts, representing acceptable levels of cleaning, showed that the total number of surfaces passing and failing when post-cleaning results gained from both methods were compared to the benchmark values were almost identical. The numbers of food contact surfaces passing and failing, when ATP and colony count data were compared to the benchmark values, were identical for five out of the ten food processing environments. Similarly, with regard to environmental surfaces, the numbers of surfaces passing and failing were identical for six out of the ten food processing environments in which sampling was performed. These findings suggest that both the ATP and microbiological methods of surface cleanliness assessment used in this study were reasonably reliable in determining the efficiency with which cleaning had been performed.

Some of the factors that may contribute to the variations in the proportions of microbial and product residue ATP detected on surfaces include the nature of the surface composition, which may influence the relative ease with which organisms and/or product residue attaches to surfaces. Related to this will be the relative ease with which surfaces can be cleaned. Certain

cleaning agents and procedures may be more or less efficient at removing these components of total surface contamination. The frequency of cleaning, and the opportunities that exist for contamination levels to increase over time, may also contribute to the relative levels of microbial and non-microbial contamination detected on surfaces within food processing environments.

The type of product produced, and the levels of contamination associated with such manufacturing may also have influenced the results gained. It is recognised, for example, that the existence of milk proteins on stainless steel surfaces may inhibit bacterial attachment (Helke *et al.*, 1993) and, therefore, in the dairy environments lower levels of microbial ATP, both before and perhaps after cleaning, may be attributable to this.

The efficiency of the cleaning processes, and the inclusion of a disinfection stage for reducing microbial numbers will influence the relative balance of microbial and product residue ATP detected after cleaning. While cleaning is undertaken primarily to remove all undesirable material (food residues, microorganisms, foreign bodies and cleaning chemicals) (Holah, 1995), a disinfection stage ought to significantly reduce microbial numbers to the extent that microbial ATP ought not to be detected at all, or at no more than very low levels. Certain cleaning agents will be more efficient at removing different types of surface contamination. This will result in significant changes to the ratios of microbial and product residue ATP present on surfaces after cleaning. A cleaning regime in which no disinfection stage exists, but which is effective at removing surface organic debris may, for example, give rise to higher microbial ATP levels and lower product residue ATP levels. In comparison, a cleaning regime in which a disinfection stage does exist should result in lower proportions of microbial ATP. The efficiency of the cleaning agents used will determine the extent to which product residue remains.

Of extreme importance in the interpretation of the results presented is not just the mean proportion of microbial contamination given, but also the mean total ATP value in RLUs recorded for each surface sampled. This is due to the fact that it is the mean total ATP value for

a surface that indicates the actual level of total contamination present. As an example, two surfaces each with mean microbial ATP values of 50% may have very different proportions of microbial contamination present if their respective mean total ATP values were 200 and 20,000 RLUs. On the former surface the level of microbial contamination would be very low, while on the latter it would be much higher.

Also of importance in evaluating results is the fact that each surface was sampled in triplicate using both detection methods. Under normal circumstances, each of the three swab or dip slide samples were taken from adjacent areas of the surface being sampled. The nature of construction of some pieces of equipment occasionally made this difficult resulting in triplicate samples being taken from identical pieces of equipment of the same type, but which were always located close together in the same area. It is, therefore, imperative when evaluating results not only to look just at mean values, but also at the individual results for each of the replicates taken. In some occasions, for example, the three replicate results for one particular surface, especially the ATP bioluminescence data, show significant variation ranging from no detection of ATP up to high levels being detected. Averaging these results to provide mean values, whilst essential, in order to provide a summary of the data, may lead to some misinterpretation of the results given if not viewed with these points in mind. In addition, some surfaces selected for sampling were not entirely flat making sampling difficult when using agar contact dip slides.

In general, on food contact surfaces, it was found that the proportions of microbial contamination detected before cleaning had been performed, were relatively low, with cheese-contact surfaces in dairies displaying particularly high microbial ATP levels. Post-cleaning, levels of microbial contamination generally decreased, often with no detection of ATP at all. Where mean proportions of microbial ATP were found to be greater after cleaning, this was probably due to an overall reduction in the proportion of product residue during cleaning, resulting in any residual microorganisms present on surfaces accounting for a greater proportion of the total residual contamination remaining.

Environmental surfaces, and floors in particular, generally displayed the highest levels of contamination, with high proportions of microbial ATP being present on floors. This is not surprising given the high volume of "traffic" passing over these surfaces almost continuously on a daily basis. Post-cleaning results for floor surfaces often showed little difference in contamination levels given this fact. The importance of sampling environmental surfaces cannot be underestimated. Post-processing contamination of food products may, for example, be the result of contamination from such surfaces. Recognition of this potential source of contamination within food processing environments is likely to lead to mandatory sampling of food contact and environmental surfaces in the USA for *Listeria* (Griffith, C.J., personal communication).

Matrix effects are another issue which require consideration, especially when sampling surfaces using ATP bioluminescence after cleaning has been performed. For example, the presence of detergent and/or sanitizer residues on surfaces may lead to a reduction in ATP signal through quenching, and this has been reported by Calvert *et al.,* (2000) and Velazquez *et al.,* (1997). This may especially be the case on surfaces that are sampled immediately after cleaning while still wet. It is therefore recommended that the effect of disinfectants and sanitizers on ATP signal is determined before they are routinely used during cleaning, and that all surfaces sampled using ATP methods are thoroughly rinsed beforehand. The potential for inhibition of colony growth due to the presence of detergent and/or sanitizer residues on agar contact dip slides has been overcome by the addition of neutralisers to the agar during manufacture. This is, however, an important consideration when selecting the type of dipslide to use.

Previous work on the use of ATP bioluminescence as a means of specifically detecting microbial contamination has very much focussed on the ability of the technique to detect contamination on poultry carcasses (Siragusa *et al.,* 1996; Werlein and Fricke, 1997) with no work to date being published on the development of a microbial ATP assay for determining levels of microbial contamination on inanimate food contact and environmental surfaces.

Siragusa *et al.,* (1996) found that a rapid microbial ATP bioluminescence test was adequate at determining microbial loads on poultry carcasses. Good correlations were found with aerobic colony counts. Similar work by the same authors (Siragusa *et al.,* 1995) on the use of an ATP bioluminescence assay to detect contamination on beef and pork carcasses found that a rapid microbial ATP assay was at least as accurate as standard plate counts for estimating levels of bacterial contamination. This correlation between microbial ATP and colony counts has also been demonstrated through the work reported in this Chapter on sampling food contact and environmental surfaces in industry. Werlein and Fricke (1997) found that microbiological swabbing was less efficient when used on its own to determine the microbiological quality of poultry carcasses. Lower microbial counts were found when using this method on its own. The use of a destructive ATP based method provided improved results on levels of microbiological quality of the poultry carcasses. Rapid assessment of the microbiological quality of poultry carcasses using ATP bioluminescence (Bautista *et al.,* 1995) also found that ATP tests used provided an acceptable correlation with plate count methods.

Work focussing on the use of ATP bioluminescence in the dairy industry (Bell *et al.,* 1994; Murphy *et al.,* 1998) has indicated that ATP bioluminescence was a practically useful means of monitoring surface cleanliness, and that it was as effective as traditional microbiological methods of hygiene assessment, especially for identifying possible sources of post pasteurisation contamination.

In determining levels of surface hygiene in a cheese plant, Kyriakides *et al.,* (1991) found 77% agreement in the results gained for 179 production sites sampled using ATP bioluminescence and plate counts derived from surface hygiene swabbing. While the ATP method used by the authors was not designed specifically to measure microbial ATP, the good correlation found between the methods is clearly in general agreement with the microbial ATP and colony count data reported in the present study. Similar findings have also been reported by Seeger and Griffiths (1994) who found good overall agreement in the results obtained from ATP bioluminescence and conventional swabbing using plate counts to assess the cleanliness of meat

slicing machines in health care institutions. However, it remains uncertain whether the correlations that are reported to exist between microbiological and ATP based methods exist due to the presence or absence of organic food debris in conjunction with contamination of microbial origin. This is especially important since commercially available ATP detection systems and assay procedures are designed to measure total ATP, while microbiological methods detect only microorganisms.

Other studies have, however, found a poor correlation between rapid and traditional methods (Poulis *et al.,* 1993). When used to sample food factories, poor correlations existed between ATP bioluminescence and agar contact plates. This disparity in findings is not surprising given the fact that *in situ*, details of the nature of surface contamination is unknown. High ATP readings may, for example, be the result of varying combinations of ATP derived from food and microbial origin (Griffith *et al.,* 1997). For this reason, the decision of whether or not to employ ATP bioluminescence for assessing surface cleanliness within industry may be influenced by the nature of the food production being carried out. Food processing environments that are involved in producing food products with low inherent ATP levels may not be suited to the use of ATP for surface cleanliness assessment. However, the ability to detect low numbers of microorganisms in the absence of ATP derived from food residues is still important.

5.6 Conclusions

Differences in the relative proportions of microbial ATP and product residue ATP do exist between different types of food processing environments.

Cleaning regimes do, in most cases, reduce levels of surface contamination within food processing environments, but some cleaning protocols appear more successful than others at removing microbial and product residue contamination as is evidenced in the data generated.

Ranges of estimate colony counts, determined mostly using agar-contact dip slides did not differ greatly before and after cleaning. This may be due to the poorer ability of this method at differentiating between different levels of microbial contamination when compared to other methods. In addition, this may also be due to colony count results being placed into a number of categories based upon the pictorial interpretation key provided by the manufacturer which enables estimate colony counts to be determined.

While the microbial ATP protocol developed has been successful in determining the relative levels of microbial contamination present on surfaces, expressing microbial contamination levels in percentage values must be done in conjunction with total ATP RLU data for reasons discussed earlier.

Data gained for the correlation of microbial ATP with aerobic colony count data in three food processing environments indicated that a good correlation exists between aerobic colony counts and the microbial ATP assay protocol developed.

Chapter 6

Overall Discussion, Conclusions and Recommendations for Future Research

6.1 Overall Discussion

The purpose of the work reported in this thesis was to evaluate the use of some microbiological and ATP bioluminescence methods of surface hygiene monitoring currently used by the food industry for assessing food contact and environmental surface cleanliness. The assessment of surface cleanliness is an extremely important function within any food-processing environment since it is one of the main ways that helps food producers ensure that the food they produce is safe and free from undesirable microbiological contamination (Gabis and Faust, 1988; Holah, 1995). The findings reported in this thesis confirm that a number of variables influence the recovery and detection of bacteria from such surfaces using these methods. In addition, the food industry should consider these variables in determining choice of appropriate methods for surface cleanliness assessment.

Much of the discussion focuses on the efficiency with which the methods evaluated are able to recover microbial bioburden from surfaces, and a definition of the term *recovery rate* is proposed. Within the context of laboratory evaluations, the term is used to describe the number of organisms recovered from a surface in relation to the level of organisms inoculated onto that surface. One of the main advantages of evaluating the surface cleanliness assessment methods included in this thesis under controlled laboratory conditions is the fact that information on the levels of inoculum used are known. This, in turn, enables accurate recovery rates and minimum detection limits for the methods to be determined, which would not be possible when using the methods to determine surface cleanliness levels on surfaces in industry where information on the precise levels of contamination present is not known.

It is acknowledged that in experiments involving the sampling of organisms that have been allowed to dry onto a surface, that some loss in organism viability may result (Cox *et al.,* 1989). This distinction between expressing recovery rate in relation to initial inoculum level, or as a percentage of the total recovered, as opposed to cell numbers present on the surface, is an important consideration in evaluating the data presented, and will be elaborated upon in more detail in this discussion.

The main factors found to influence the recovery and detection of bacteria on food contact and environmental surfaces using microbiological and ATP bioluminescence methods include:

- surface moisture level at time of sampling, i.e. wet or dry;
- the nature of the food contact or environmental surface;
- predicted levels of microbial/product reside contamination;
- the nature of the assessment (recovery) technique, including its minimum detection limit and repeatability/reproducibility;
- the method of organism cultivation;
- operator proficiency in using the assessment techniques.

The most important factor found to influence the rate of organism recovery from surfaces has been shown to be the moisture level of the surface at the time of sampling. Both the work reported in Chapter Two, and particularly that reported in Chapter Four, has shown that increased organism recovery rates from surfaces are achieved when sampling food contact surfaces which are sampled immediately after inoculation while still wet. Improved organism recovery rates gained from wet surfaces may be due to the fact that on surfaces which have been allowed to dry after cleaning, organisms may either die due to desiccation making them non-culturable once recovered, or they may attach to the surface making their recovery using swabbing or agar-contact methods more difficult. However, despite poor recovery rates gained from inoculated surfaces when sampled while dry, it has been reported that organisms may survive for prolonged periods of time in dry product residues (Scott and Bloomfield, 1990).

Another possible explanation accounting for poor recovery may be that the organisms recovered from dry surfaces remain viable but non-culturable using normal culture media and incubation conditions. However, it may be the case that in sampling inoculated surfaces while still wet, that the methods used to recover the organisms are not necessarily sampling the surface, but merely recovering the planktonic cells and not removing organisms attached to the surface. This, in turn, may be a contributing factor to the low recovery rates gained from inoculated surfaces sampled while dry. These findings are particularly important since when sampling surfaces while still wet results in apparently better organism recovery, the poor organism recovery rates gained from surfaces sampled while dry may suggest that residual organisms remaining on a surface after cleaning have undergone cell death. However, in order to be able to recommend to industry when surface sampling ought to be performed after cleaning, it is important first of all to establish whether the low organism recovery rates from dry surfaces are in actual fact due to cell death, or to strong attachment of the cells to the surface, making them more difficult to remove using the surface sampling methods used. In order to be able to recommend to the food industry the best approach to surface cleanliness assessment, it is important that further research focuses on these issues. Of all the findings reported in this thesis, the effect of sampling surfaces when wet or dry, and the vastly different organism recovery rates gained is, without doubt, one of the most significant findings. Further investigation into the effect of surface moisture level on organism recovery is suggested. It would, for example, be interesting to establish the effect of maintaining controlled surface moisture level over prolonged periods of time on resulting organism recovery rates. A series of experiments in which inoculated surfaces are kept moist would help ascertain whether the effect of surface drying on organisms was that of cell death, or whether drying simply results in the attachment of the cells to the surface to such an extent that they are not easily removed by the surface monitoring methods evaluated in this thesis. Should recovery rates of organisms from wet surfaces in these experiments be of a similar order to those gained from dry surfaces, this would indicate that organism attachment was the main factor contributing to the poor recovery rates achieved. However, an important consideration in designing such an experiment would be the potential for organism growth throughout the duration of the experiment, and this would need to be borne in mind in interpreting the results

gained. Another important issue that may contributing to the poor recovery of organisms from surfaces, especially inoculated dry surfaces, using cotton surface swabbing may be poor release of recovered organisms from swab buds. The ability of agar contact dip slides to detect lower levels of inoculum, for some test organisms, on surfaces that had been allowed to dry clearly supports this, as does the improved recovery rates achieved from swabs that were released for thirty seconds or more.

Another key variable that has been highlighted through the results in Chapter Four is that the minimum bacterial detection limits, in addition to organism recovery rates, of the microbiological assessment methods evaluated are also influenced by the wet or dry status of surfaces. Minimum detection limits were also found to be influenced by the nature of the organism being recovered, and by the monitoring method used to recover them. The minimum bacterial detection limits of the microbiological based methods were found to be superior on wet than on dry surfaces, ranging from 10^2 cfu/100cm^2 on wet inoculated surfaces up to 10^8 cfu/100 cm^2 on dry ones, yet for ATP bioluminescence the minimum detection limit was unaffected by whether surfaces were sampled while wet or dry, and was found to be consistent at 10^4 cfu/100 cm^2 for all test organisms used. Gram-positive organism had, on some occasions, lower minimum detection limits than Gram-negative organisms when microbiological methods were used. The superior detection limits achieved using ATP bioluminescence on dry surfaces, in comparison to hygiene swabbing may, for example, may be due to the presence of cationic detergents in the swab wetting agent of the ATP assay making recovery of contaminating surface bioburden easier (Salo and Wirtanen, 1999). Maximising organism recovery, especially from dry surfaces, using hygiene swabs may be significantly improved through including wetting agents in the diluent used to moisten swab buds, and this is worthy of further research. In addition, manufacturers of swab transport kits, that include swabs pre-moistened in transport media, ought to consider the inclusion of a wetting agent in the transport media to assist in bioburden removal from sampled surfaces.

Clearly, the improved recovery of organisms from wet surfaces supports the recommendation that industry ought to monitor the efficiency of surface cleaning immediately after it is completed, and before surfaces are allowed to dry. This will ensure that optimum recovery of residual surface organisms is achieved. Another important consideration is, however, the method of cultivation used. While optimum organism recovery from surfaces may be achieved through sampling surfaces while still wet, the method of organism cultivation selected needs to be efficient in detecting the recovered organisms. While organism pick-up from a surface may be optimal, unless appropriate culture media and incubation conditions are employed, the organism recovery rate achieved will not be truly representative of the contamination present on the surface (Griffith *et al.,* 1997). Another fundamental issue relating to organism recovery is that of negative microbiological results, and how such a result should be interpreted. The absence of microbial growth from a surface using microbiological methods is not an indication that the surface sampled was free from microbial contamination. Such results merely indicate that any microbial contamination present on the surface was present at a lower level than the minimum detection limit of the method used for assessing the surface, and this has been illustrated through the use of agar contact dip slides in the work reported in Chapter Five. The same is true for ATP bioluminescence. This issue alone is a key consideration for industry since the setting of critical limits, including pass and fail limits, for different types of surfaces using different methods of assessment will be influenced by the achievable minimum detection limits of the methods chosen. While these considerations are important, industry also needs to consider the nature of the information it requires from surface cleanliness assessment, which will influence the choice of methods, best able to provide it. In addition, the expected levels of bacterial contamination present on a surface may, to some extent, influence choice of method (Griffith *et al.*, 1997). Direct streaking of hygiene swabs, taken from surfaces with high levels of microbial contamination, onto agar surfaces, for example, will not provide quantitative results on levels of contamination. This particular method would be better suited to sampling surfaces on which low levels of microbial contamination are expected. Determination of microbial numbers on surfaces that are known to have high levels of contamination will be achieved more efficiently through releasing recovered organisms into a recovery diluent before

appropriate plating. It is necessary, in order for accurate results to be obtained, that recovered organisms are efficiently released from swab buds. However, swabbing as a method for enumerating bacteria remaining on surfaces after cleaning has been reported to be highly variable (Holah *et al.,* 1988). The high variability of the results gained from the method when used to sample surfaces would also suggest that the poor recovery rates gained from swabs may not be attributable entirely to problems with organism viability. This inherent variability of individual methods will obviously be one of the main considerations in deciding upon which variation of a particular method to use. These issues are obviously of critical importance in determining the reliability of different methods at being able to help estimate the potential risks from cross-contamination within food processing environments.

In addition to a lack of understanding of the efficiency with which individual monitoring methods recover and detect residual organisms on surfaces after cleaning, the predicted levels of contamination on surfaces, and the efficiency with which cleaning regimes remove this contamination is also perhaps not that well understood in industry. This is supported, in part at least, by some of the data presented in Chapter Five for post-cleaning sampling sessions in selected food processing environments where cleaning regimes appeared to have little effect on removing contaminating surface bioburden.

While the efficiency with which residual organisms on a cleaned surface can be recovered by individual methods is one issue, another important factor in determining choice of method is obviously its cost, both in terms of in-house analysis and contracting analysis out to an accredited laboratory. In deciding which is the most appropriate option, many of the discussion points raised above need to be considered. The potentially high cost of, for example, the analysis of a hygiene swab, analysed by an outside laboratory, may not be considered good value for money given the large number of variables potentially affecting the end results gained. Despite the higher initial cost of an ATP bioluminescence monitor, subsequent swab analyses are now relatively cheap, and this has been partly due to the large number of companies manufacturing ATP test kits. Therefore, ATP bioluminescence may be a better option, both in

terms of cost and in terms of the ability to provide information on total surface cleanliness rather than on residual microorganisms only, which would be the case with microbiological methods. Ultimately, there is no one ideal method suitable for all surface types in all types of food processing environments, and an integrated approach to surface cleanliness assessment is proposed in which a range of different types of methods are used.

Some of the work reported in Chapter Two illustrated that improved organism recovery rates were achieved using an environmental isolate as opposed to laboratory type cultures. This may suggest that, over time, organisms adapt to their environment and, in turn, their rate of survival improves through having become adapted to the various environmental conditions to which they have become subjected, and this is supported by the work of Scott and Bloomfield (1990). This in itself may suggest that the effect of drying on residual organisms present on a surface is only temporary in that such organisms, over time, adapt to their environment, giving rise to populations that are more difficult to control. Surviving organisms may, through time, multiply and establish a biofilm, especially on surfaces that remain inadequately cleaned over long periods. Previously published work that has been acknowledged in Chapter Five has indicated that ATP bioluminescence is more efficient than conventional hygiene swabbing at removing surface biofilm. Improved recovery rates will be partly the result of organisms surviving and multiplying, with any residual product residues potentially contributing to organism survival, but also due to the presence of any cationic detergents present in swab wetting agents that will aid organism removal from surfaces. In addition, organisms that come into contact with surfaces for only short periods of time may be less resistant to environmental factors resulting in lower survival and recovery rates through cell death (Cox *et al.,* 1989).

It is clearly evident, however, that despite both the nature of the food contact surfaces sampled, and the organisms present upon them, there are a number of factors specifically relating to the assessment techniques themselves that are important. Table 1.1 in Chapter One highlighted the variables considered most important by industry when selecting hygiene assessment methods. These variables have been more fully discussed in Chapter One, but those considered most

important by industry were the ability of the method chosen to provide results within minutes, the accuracy of the results gained, the reproducibility of the results, and the simplicity and ease of use of the method selected. The importance of many of these attributes has also been acknowledged by the ATP evaluation conducted by Flickinger (1996). The requirement of the method chosen to be able to produce results within minutes automatically excludes the use of microbiological methods as part of routine cleaning assessment programmes that are included within HACCP It is important, however, that the value of microbiological methods is not undermined, which raises the question of when should microbiological methods be employed? Figure 6.1 illustrates a suggested approach to cleaning assessment in which ATP and microbiological based methods are integrated into a system where microbiological methods are employed where ATP test results indicate failure, and where microbial ATP values have been consistently high over extended periods of time. This proposed approach to cleaning assessment is discussed in more detail later in this chapter, but suggests the use of microbiological methods of surface sampling when ATP analysis has indicated failure, and where reference to trend analysis data shows problems with previous failures.

The work on the repeatability and reproducibility of hygiene swabbing and ATP bioluminescence reported in Chapter Four illustrated that clear differences exist between the two types of methods in terms of their repeatability and reproducibility when used by a number of operatives to sample identical surfaces. Hygiene swabbing was found to be highly unreproducible with mean coefficients of variation for six operatives ranging from 88% to 164% when used to sample marginally unclean surfaces, and this is an important finding given the widespread use of the method in industry. The high variability in data gained from swabbing surfaces has also been reported by Holah *et al.,* (1989). ATP bioluminescence was found to be much more reproducible when used to sample the same surfaces, with coefficient of variation data for marginally unclean surfaces ranging from 21% to 42%. Again, these findings support earlier comments relating to the need to fully understand the limitations of the methods being used.

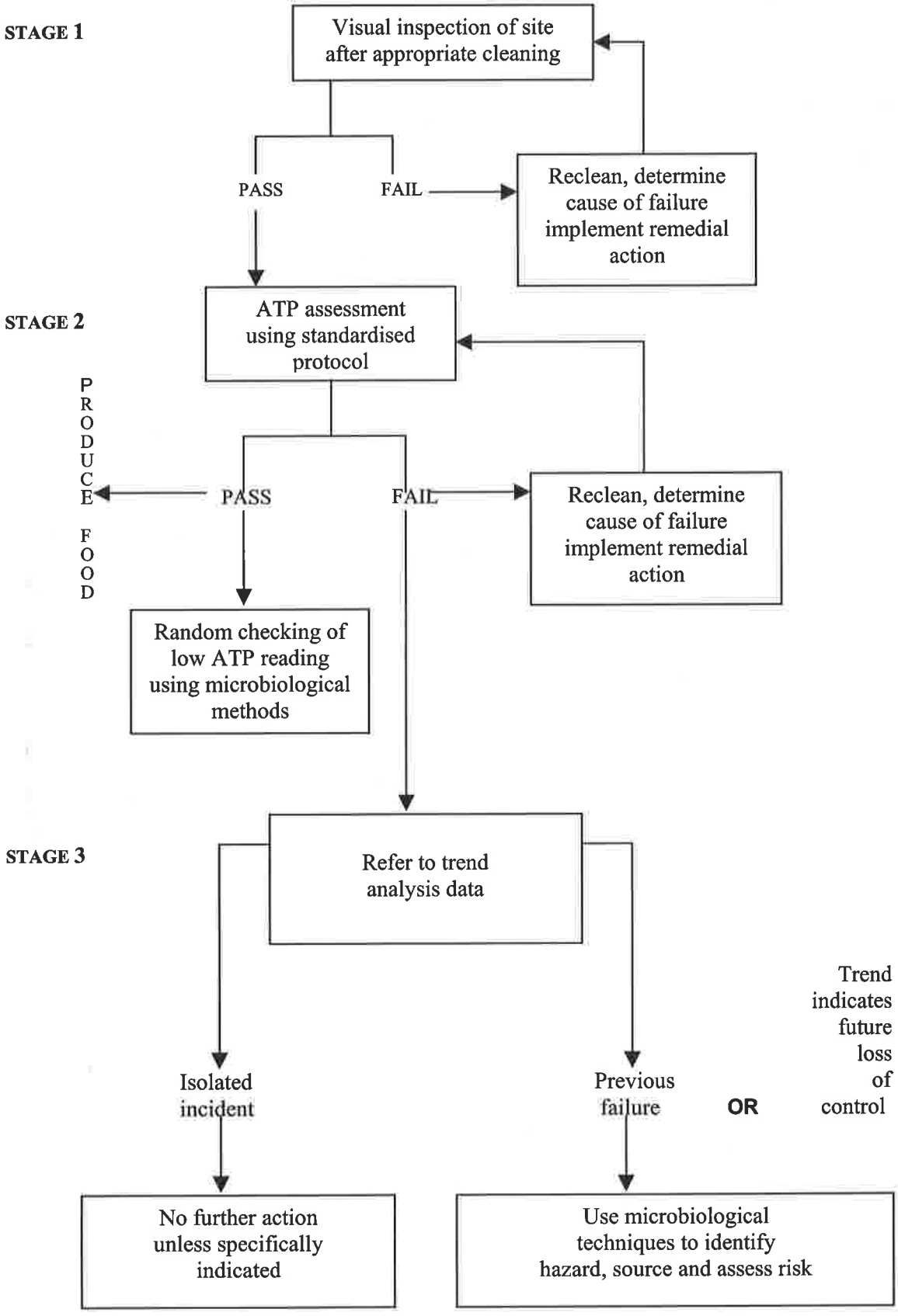


Figure 6.1 Proposed Strategy for the Use of Surface Cleanliness Assessment Methods in the Food Industry.

Despite limitations in terms of reproducibility, surface hygiene swabbing is still extensively used in industry for assessing levels of microbial contamination on surfaces. The results in Chapter Two clearly show that a number of factors contribute to the efficiency with which hygiene swabbing is able to recover and detect microbial bioburden. From the data presented it is clear that organism recovery rates from dry surfaces are considerably lower than those gained from wet surfaces, and this has been investigated more fully in Chapter Four. However, the improved recovery rates gained for the environmental isolate using hygiene swabbing suggests that the apparent problems in terms of achievable organism recovery rates may be due to loss of viability during drying, or perhaps be attributable to the fact that some organisms may attach to surfaces more strongly making their recovery more difficult. The apparent lack of efficiency of the technique in terms of organism recovery may be misleading in that the work reported in this thesis compares organism recovery rates with initial levels of inoculum applied to surfaces in laboratory controlled studies. Clearly, the experiments proposed earlier in this discussion in which inoculated surfaces are kept moist over prolonged periods of time is the first stage in beginning to understand the nature of the effect of surface drying on organism recovery.

Other important findings include the fact that, in using a diluent recovery stage, swabs required to be released for more than just a few seconds in order to optimise recovery. Current data presented in Chapter Two suggest that release times ought to be for at least thirty seconds, and this is clearly a recommendation to industry to ensure that optimum recovery of organisms from swab buds is achieved. However, while releasing organisms from swabs for longer time periods results in improved recovery, the release times adopted in industry need to be realistic.

ATP bioluminescence, on the other hand, has the ability to produce results within minutes, and has been shown to be more reproducible than traditional hygiene swabbing. A number of other issues are, however, also important in considering the potential use of ATP bioluminescence for monitoring surface cleanliness in industry. Figure 3.2 in Chapter Three illustrated the various components contributing to total ATP minimum detection limits, and it is important that individual assay and detection systems can detect these individual components. Chapter Three

data clearly illustrate that different assay and detection systems vary in terms of their ability to detect these individual components. While industry has highlighted the important features of ATP bioluminescence systems for use in industry, the data presented in Chapter Three would suggest that in order for optimum detection limits to be achieved, a number of variables contributing to assay performance are important. These include swab wetting agents that are critical to facilitating bioburden removal from surfaces, and future research ought to focus on this issue. In addition, the efficiency with which individual extractants release ATP from cells is also important to establishing overall assay performance. These two variables alone may be the most significant in terms of producing ATP detection systems with minimum detection limits of less than 10^3 cfu/100cm^2, which was the lowest detection limit found for ATP when used to assess inoculated surfaces as reported in Chapter Three. While these issues are not of immediate interest to industry when using ATP assays, future developments in ATP technology for surface cleanliness assessment need to bear these issues in mind.

Obviously, industry personnel need to fully understand the variables potentially affecting ATP monitoring results in order that data may be interpreted correctly. An ATP RLU reading of less than 100 RLU, for example, might suggest that the surface from which this reading was gained was very clean, and that no ATP above normal background levels was being detected. However, it is important to bear in mind that within industry, ATP derived from surfaces will be a combination of both microbial and product residue ATP. The minimum detection limits reported for different ATP detection systems in this thesis were achieved using pure cultures, and that in detecting combinations of microbial and product residue ATP within industry, minimum detection limits will be different. This suggests that ATP hygiene assays are good for determining total surface cleanliness, but not for determining information only on microbial contamination. This is, without doubt, one of the most important issues facing the bioluminescence industry over the next few years, especially if ATP technology is to be successfully adopted within HACCP food safety management systems. While the potential problems of using ATP systems with very low minimum detection limits have already been discussed, it is important, for example, that the risks from organisms such as *E. coli* 0157, which

have very low minimum infectious doses, can be detected. The development of a commercially available microbial ATP assay that is capable of detecting low levels of microbial ATP may prove useful in this regard.

It is important, however, that in employing such technology in industry, the nature of the contamination likely to be found on surfaces is understood, and that appropriate pass and fail limits for ATP are set. Such values ought not to be unrealistic, with pass values being achievable and fail values not being so high as to render them pointless.

Related to this is the need to be able to use individual methods to monitor surfaces that are marginally unclean. The choice of methods for monitoring such surfaces will be influenced by their achievable minimum detection limits, which must be low enough to detect low levels of residual microbial contamination. However, it is also important, especially with regard to ATP bioluminescence that the minimum detection limits of individual detection systems are not so low that companies find themselves constantly cleaning unnecessarily.

While ability to effectively detect contaminating surface bioburden is probably one of the main deciding factors in determining choice of method, devolving responsibility for surface cleanliness assessment to line operatives has increased the need for the methods used to be easy to use with relatively little training (Flickinger, 1996). The work in Chapter Three has shown that some ATP detection systems are suitable for this purpose, while others would require technically trained personnel to use them. Ultimately, industry must be prepared to compromise in terms of selecting an ATP detection system. While some systems have superior detection limits, others are easier to use. The recurrent costs of assay reagents for some systems will be cheaper than for others, which will, for some companies at least, be an important issue.

While ATP bioluminescence is now increasing in popularity within the food industry for assessing surface cleanliness, microbiological methods are still considered by some people to be more appropriate for surface cleanliness assessment given their ability to provide details on

contamination of microbial origin, which in terms of food safety, is often considered to be more important, especially where the risks of contamination from pathogens with low infective doses are thought to exist. Recognising this, and the fact that microbiological methods do have limitations, the work reported in Chapter Five has shown that a microbial ATP assay can provide an accurate indication of microbial contamination levels on surfaces, and at the speed required in order to be of value within HACCP systems. The developed protocol has been demonstrated to provide useful information, not just on microbial contamination, but also on product residue contamination which might often be a significant component of the total contamination present on a surface. Being able to distinguish between the levels of these individual components may lead to more effective cleaning programmes through the use of cleaning agents that have been designed to specifically deal with different types of contamination.

Ultimately, choice of cleanliness assessment method will be the result of answers to a number of questions including the nature of the information required, the speed with which that information is needed, who is responsible for obtaining it, and how much money is available to invest in order to obtain the information (Griffith *et al.*, 1997). Other pertinent considerations will obviously include the predicted levels of contamination present on a surface, the minimum detection limit of the proposed method, and the level of reproducibility achievable using the chosen method. Considered collectively, these issues should lead to the most appropriate choice of cleanliness assessment method for a specific task.

It is therefore proposed that both microbiological and ATP bioluminescence methods of surface hygiene assessment are of value to the food industry, but that their use should be combined within an integrated cleaning assessment programme as outlined in Figure 6.1. In this approach a visual inspection of the food processing environment and its food contact and environmental surfaces should constitute the first stage of verifying the adequacy of cleaning assessment. A satisfactory outcome from this would then permit ATP bioluminescence testing to confirm the results of the visual inspection. Appropriate pass and fail values for each area sampled would

need to be established, and these would generally be less than or equal to 100 RLU/ 100cm^2 for stainless steel surfaces. Assuming that ATP testing confirmed that surfaces were adequately clean, clearance could then be given for food production to begin. Any sampled areas failing ATP testing through having RLU readings in excess of the pass limits set would be subjected to ATP testing specifically for microbial ATP. Should low RLU values be obtained it is suggested that reference is made to trend analysis data to establish whether or not previous failures have occurred, or whether an isolated incident is being dealt with in which case no further action is needed. Previous failures would suggest that particular attention is given to the cleaning protocols used, and that they are reviewed in the light of the data gained. Only if high microbial ATP readings are gained should conventional microbiological methods be employed in an attempt to identify the hazard, its source, and to assess the risks posed.

To this end, a number of microbiological and non-microbiological based surface hygiene monitoring methods have been assessed in terms of their ability to determine food contact and environmental surface cleanliness. Several variables have been found to influence the reliability with which these methods determine surface cleanliness levels, and these have been discussed within the context of surface cleanliness assessment within the food industry. The findings of this thesis should assist the food industry in determining appropriate strategies for surface hygiene monitoring.

6.2 Conclusions

A number of factors have been shown to influence the recovery and detection of bacteria from food contact and environmental surfaces, and these include type of cleanliness assessment method, the nature of the organism, and the surface moisture level at time of sampling.

1. Cotton hygiene swabbing was found to give poor organism recovery rates, especially from inoculated surfaces that were sampled when dry. A number of sampling variables were not

found to significantly affect recovery rates including the use of a single versus a double swabbing procedure, the nature of the organism release method, and the type of cultivation media used. Variables that were found to influence organism recovery rates included the surface moisture level at time of sampling, the organism release time used, the diluent type and the method of organism cultivation used.

2. The minimum detection limits of microbiological based monitoring methods were found to vary widely, and were influenced by the type of method, type of organism being sampled, and the surface moisture level at the time of sampling.

3. Different ATP assay and detection systems were found to differ in their minimum detection limits depending upon which components of total ATP minimum detection limits were being evaluated.

4. The minimum detection limit of the ATP Bioluminescence assay, used to sample surfaces with different moisture levels, was found to remain constant regardless of whether inoculated surfaces were sampled when wet or dry.

5. ATP bioluminescence was found to be more reproducible than microbiological methods when used to sample marginally unclean stainless steel surfaces.

6. A good correlation was found to exist between microbial ATP and aerobic colony count data when used to sample food contact and environmental surfaces within different food processing environments before and after cleaning had been carried out.

6.3 Recommendations for Future Research

There remains considerable uncertainty surrounding the reasons why inoculated surfaces sampled after being allowed to dry, using cotton surface swabbing, should give rise to organism

recovery rates of less than 1%. It would, therefore, be valuable for further research to focus on this issue, and to attempt to determine whether the low recovery rates gained are due to surface attachment, death of the organisms, to the organisms entering a viable but non-culturable state, or indeed to problems with organism release from swab buds. However, it is acknowledged that the results presented in this thesis, from laboratory based studies, relate almost always to the use of laboratory type cultures, and that these do not exist in industry. Differences in recovery rates gained from laboratory studies, as opposed to those found in industry may be attributable, in part at least, to this. Experiments that may help to determine why these low recovery rates from laboratory type cultures are gained have been suggested, and include evaluating the inclusion of different swab wetting agents for promoting bioburden removal from surfaces. This work might also explain why differences exist in the minimum detection limits of the cleanliness assessment methods evaluated in this thesis.

ATP assays that include some form of detergent in the swab moistening solution may also improve organism recovery from dry surfaces. A series of experiments investigating the efficiency with which a range of different types of wetting agents removes dry microbial bioburden from surfaces would be useful. It would be important, however, especially when using ATP assays, that the swab wetting agents do not liberate ATP above normal background levels.

An investigation of the use of direct epifluorescent microscopy in conjunction with stains capable of distinguishing between viable and non-viable organisms would help to provide a better understanding of organism survival on surfaces over different time periods. The use of atomic force microscopy, capable of examining larger surface areas, may also assist in gaining a better understanding of organism survival on surfaces over prolonged time periods.

Many test developments have been introduced since the work in this thesis was completed. These include the introduction of protein estimation test kits, swab devices that detect specific pathogens, and ATP assays which are purported to have very much lower minimum detection

limits. These ought to be evaluated under controlled laboratory conditions using experimental protocols that are representative of typical "in-use" conditions.

This thesis has evaluated a number of methods available for assessing food contact and environmental surface cleanliness within the food industry, including microbiological and ATP bioluminescence based methods. In addition to evaluating the methods using typical "in use" conditions under laboratory controlled conditions, the work presented in this thesis has evaluated the performance of the methods within the food industry in a range of different types of food processing environments, both before and after normal cleaning had been performed. A rapid microbial ATP assay has been developed and used within the different food processing environments, and has been shown to correlate well with aerobic colony counts. Ultimately, the research findings presented in this thesis enhance current scientific knowledge and understanding of many of the variables potentially affecting the performance of microbiological and ATP bioluminescence based methods of surface cleanliness assessment within the food industry, and should help those responsible to develop appropriate strategies for surface cleanliness assessment within industry.

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Vulgo emin dicitur: lucundi acti labores

(For it is commonly said: completed labours are pleasant)

Cicero

APPENDIX 1.

Production of ATP Standard Solutions

Production of ATP Standard Solutions

A: Preparation of Stock Solutions

1. Reconstitute 80nM ATP standard following manufacturers instructions.

2. Pipette 9.5ml of pyrogen free water (PFW) in sterile ATP free centrifuge tube and add 0.5 ml of 80nM ATP solution. Pipette up and down a couple of times to ensure all ATP is transferred. This is a 4nM ATP stock.

3. Add 2.5ml of 4nM ATP stock to 7.5ml PFW (in ATP free centrifuge tube) - Gives 10ml of 1nM ATP stock.

4. Divide into 10 equal portions and freeze at - 18°C in Biotrace eppendorf tubes - (1ml volumes of 1nM ATP stock).

5. Divide 80nM and 4nM stocks into suitable volumes (just greater than 0.5ml and 2.5ml respectively?) and freeze at - 18°C. Avoid wasting any of the stocks.

B: Preparation of Standard Dilutions

1. Defrost 1ml of 1nM ATP solution - gives 1nM ATP solution.

2. Add 250µl of 1nM stock to 250µl PFW - gives 0.5nM ATP solution.

3. Add 100ml of 1nM stock to 300µl PFW - gives 0.25nM ATP solution.

4. Add 50ml of 1nM stock to 950µl PFW - gives 0.05nM ATP solution.

6. If any volumes show sensitivity with the 0.05nM ATP solution then prepare a 0.025 nM standard by adding 25µl 1nM ATP stock to 975µl PFW - gives 0.025 nM ATP solution.

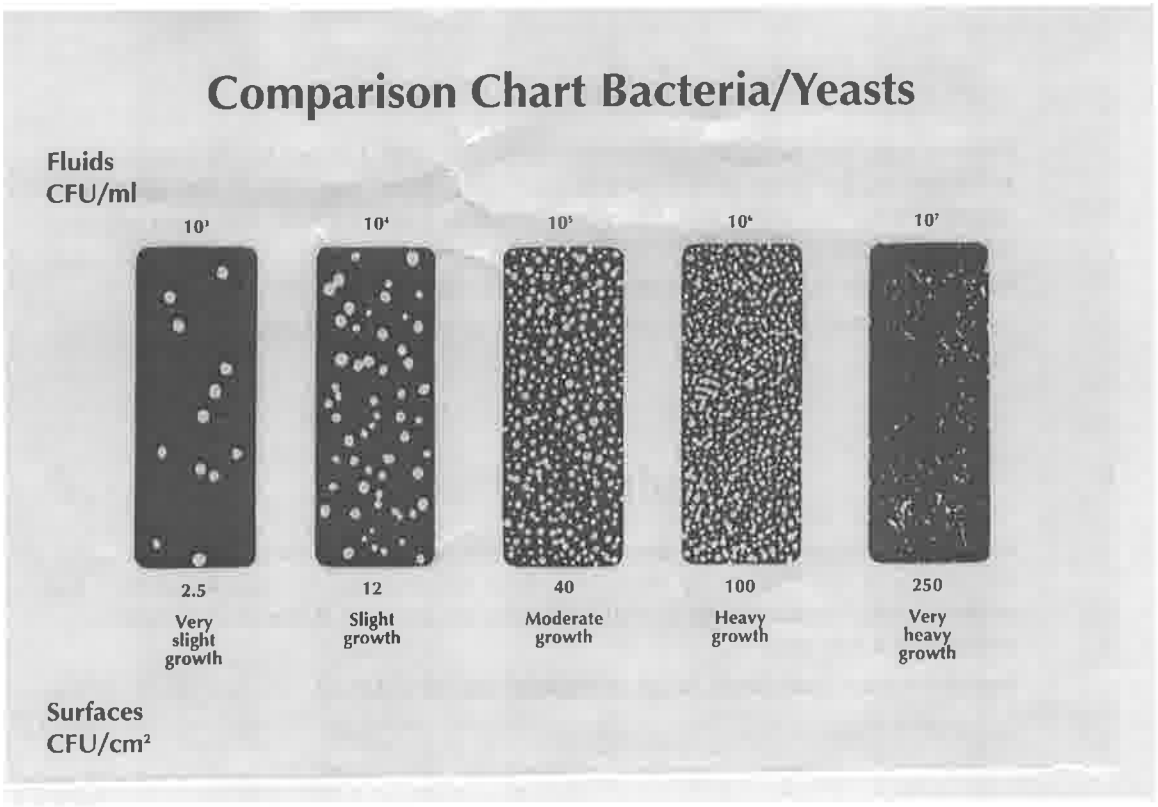
To test ATP detection system pipette10µl of standard dilutions onto pre-moistened swabs and read output.

Moles of ATP added to swab in 10µl of standard solutions

Molarity	*Moles in 10µl volume*
1nM	18×10^{-14} 10 femtomoles
0.5nM	5×10^{-15} 5 femtomoles
0.25nM	2.5×10^{-15} 2.5 femtomoles → approx. limit for System Sure
0.05nM	5×10^{-16} 0.5 femtomoles
(0.025nm	2.5×10^{-16} 0.25 femtomoles

APPENDIX 2.

Agar Contact Dip Slide
Results Interpretation Key


Comparison Chart Bacteria/Yeasts
Fluids
CFU/ml
10³
10⁴
10⁵
10⁶
10⁷
2.5
Very slight growth
12
Slight growth
40
Moderate growth
100
Heavy growth
250
Very heavy growth
Surfaces
CFU/cm²

APPENDIX 3.

Raw Microbiological and ATP Data
pertaining to Chapter 5.

KEY

n	=3
NG	No growth detected following incubation
ND	No detection of ATP above background levels;
APC	Aerobic plate count determined using agar contact dip slide;
*	Site clean on both sampling sessions

General Chilled Foods 1

Sampling Site	Pre-Clean Sampling				Post-Clean Sampling			
Food Contact Surfaces	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**
Plastic Chopping Board	429	170.27	130 (30)	10^2	397	175.58	118 (30)	10^4
SS Sandwich Preparation Surface	361	325.80	102 (28)	$<10^2$-10^3	ND	–	ND	$<10^2$
Mean of all surfaces	**395**	**248.03**	**116 (29)**	–	**397**	**175.58**	**118 (30)**	–
Environmental Surfaces	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count//100cm² Plate Count Agar**	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**
SS Handwash Sink	606	29.69	182 (30)	up to10^2	3394	5002.61	3284 (97)	up to10^2
Chill Floor	15039	10261.94	12398 (82)	10^4	723	139.61	136 (19)	10^4
SS Surface (Underside)	553	151.34	152 (27)	$<10^2$	627	99.80	205 (33)	$<10^2$
Floor near pot wash	22566	9922.65	19880 (88)	10^4	86918	11761.30	224219 (>99)	10^4
Floor of sandwich preparation area	9612	9190.53	3682 (38)	10^3 - 10^4	101278	77135.68	98970 (98)	10^3 -10^4
Mean of all Surfaces	**9675**	**5911.23**	**7259 (53)**	–	**38588**	**18827.8**	**65362.8 (69)**	–

General Chilled Foods 2

Sampling Site	Pre-Clean Sampling				Post-Clean Sampling			
Food Contact Surfaces	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count /100cm² Plate Count Agar**
SS Meat Preparation Surface	55550	39272.89	13717 (25)	$<10^2$ - 10^3	ND	--	ND	$<10^2$
Inside SS Vegetable Sink	25708	37082.02	25078 (98)	10^3 - 10^4	1056	797.93	697 (66)	10^2 - 10^3
SS Work Surface	520	175.52	338 (65)	10^2 - 10^3	570	117.09	213 (37)	up to10^2
Mean of all surfaces	**27259**	**25510.14**	**13044 (63)**	--	**1341**	**457.51**	**455 (52)**	--
Environmental Surfaces	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**
High Risk Sink SS outer surface	12200	6327.69	2727 (22)	10^2	118	31.19	14 (12)	$<10^2$
SS Cladding	5389	3063.64	2032 (38)	10^2	107	22.81	9 (8)	10^2 - 10^3
Inside Extraction Vent	13928	12502.94	4574 (33)	10^2	253	249.27	146 (58)	10^2 - 10^3
Mean of all surfaces	**10506**	**7298.09**	**3111 (31)**	--	**159**	**101.09**	**56 (26)**	--

General Chilled Foods 3

Sampling Site	Pre-Clean Sampling				Post-Clean Sampling			
Food Contact Surfaces	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Mean Colony Count/100cm² Plate Count Agar**	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Mean Colony count/100cm² Plate Count Agar**
Pasta Line	ND	--	ND	1.5×10^3	ND	--	ND	3.6×10^2
Cheese Jacket Chopping Board	1591	1059.54	343 (22)	3.0×10^3	531	71.28	112 (21)	6.5×10^2
Meat Spiral Feed	798	224.25	106 (13)	1.1×10^3	491	390.68	143 (29)	1.7×10^3
Mean of all surfaces	**796**	**427.93**	**150 (12)**	**1.9×10^3**	**341**	**153.98**	**85 (17)**	**9.0×10^2**
Environmental Surfaces	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Mean Colony Count/100cm² Plate Count Agar**	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Mean Colony Count/100cm² Plate Count Agar**
Floor in Fridge	158956	31307.51	153803 (97)	3.3×10^7	SNC	--	--	--
SS Conveyor Support	375	229.05	238 (63)	2.2×10^4	SNC	--	--	--
Leider Conveyor	14710	3629.38	2096 (14)	NG	SNC	--	--	--
Pasta Room Floor	20092	11058.23	19548 (97)	1.9×10^6	SNC	--	--	--
Blending Platform	86918	6330.02	77501 (89)	2.7×10^7	SNC	--	--	--
Mean of all surfaces	**56210**	**10510.83**	**50637 (72)**	**1.2×10^7**	--	--	--	--

General Chilled Foods 4

Sampling Site	Pre-Clean Sampling				Post-Clean Sampling			
Food Contact Surfaces	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**
Meat Prep. Surface	1201.4	1231.56	347 (29)	10^2	142	99.68	39 (27)	$<10^2$
Inside Vegetable Sink	825	978.26	421 (51)	10^2 - 10^3	ND	--	ND	$<10^2$
Potting Area SS Table	450	817.29	111 (25)	10^2 - 10^3	145	83.06	61 (42)	$<10^2$
Mean of all surfaces	**825**	**1009.03**	**293 (35)**	--	**96**	**60.91**	**33 (23)**	--
Environmental Surfaces	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**
Floor under high Risk Sink	2039	2567.43	876 (43)	$<10^2$- 10^3	8941	15336.72	8659 (97)	10^3
SS Cladding	2477	4807.96	1057 (43)	10^2 - 10^3	232	172.67	101 (44)	10^2
Inside Extraction Vent	312	154.61	102 (33)	$<10^2$	ND	--	ND	$<10^2$
Mean of all surfaces	**1609**	**2510**	**678 (40)**	--	**3058**	**5169.80**	**4380 (47)**	--

Dairy 1

Sampling Site	Pre-Clean Sampling				Post-Clean Sampling			
Food Contact Surfaces	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count100cm² Plate Count Agar**
SS Tank Wall	5087	2019.86	2517 (49)	Not Sampled	ND	--	ND	--
Fog Filler Bowl	909	143.92	386 (42)	Not Sampled	ND	--	ND	--
Inside Milk Tank Lid	ND	--	ND	Not Sampled	ND	--	ND	--
Inside Cream Tank Lid	ND	--	ND	Not Sampled	ND	--	ND	--
Mean of all surfaces	**1499**	**540.95**	**739 (23)**	--	**ND**	--	**ND**	--
Environmental Surfaces	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**
SS Blank End	4194	320.20	3202 (76)	Not Sampled	ND	--	ND	--
3 Way Valve	166848	288518.45	163 (<1)	Not Sampled	ND	--	ND	--
Overhead pipes	ND	--	ND	Not Sampled	ND	--	ND	--
Floor near tanks	10749	1676.79	10498 (98)	Not Sampled	SNC	--	--	--
Inside Hose Pipe	ND	--	ND	Not Sampled	SNC	--	--	--
Conveyor	43435	52024.83	42913 (99)	Not Sampled	SNC	--	--	--
Mean of all surfaces	**37538**	**57090.04**	**9463 (46)**	--	**ND/SNC**	--	--	--

Dairy 2

Sampling Site	Pre-Clean Sampling				Post-Clean Sampling			
Food Contact Surfaces	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Mean Colony Count/100cm² Plate Count Agar**	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Mean Colony Count/100cm² Plate Count Agar**
Outlet pipe of fermentation Vat	461	446.35	258 (56)	2.1×10^4	ND	--	ND	3.5×10^2
Whey storage tank lid	3562	1186.62	2455 (69)	4.1×10^5	41714	40985.30	34748 (83)	4.3×10^6
Cheese packing chute	18625	7139.88	15105 (81)	9.0×10^5	ND	--	ND	3.7×10^1
Mean of all surfaces	**7549**	**2924.28**	**5939 (69)**	**4.4×10^5**	**13905**	**13661.76**	**11583 (28)**	**1.4×10^6**
Environmental Surfaces	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Mean Colony Count/100cm² Plate Count Agar**	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Mean Colony Count/100cm² Plate Count Agar**
Pasteuriser Room Floor	25416	7831.01	24680 (97)	6.4×10^5	604	46.57	334 (55)	8.0×10^4
Cheese processing area floor	295	75.90	96 (38)	1.3×10^3	1402	561.36	877 (63)	7.9×10^4
Butter packing machine	1040	483.67	285 (25)	2.0×10^4	SNC	--	--	--
Floor in butter room	115	148.43	43 (37)	2.4×10^3	SNC	--	--	--
Mean of all surfaces	**6717**	**2134.75**	**6276 (49)**	**1.7×10^5**	**--**	**303.97**	**606 (59)**	**8.0×10^4**

Dairy 3

Sampling Site	Pre-Clean Sampling				Post-Clean Sampling			
Food Contact Surfaces	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**
Cheese Press	5268	2838.35	4808 (91)	10^3 - 10^4	15877	6411.27	15384 (97)	10^4
SS Screw Mixer *	ND	–	ND	$<10^2$ - 10^3	3627	966.14	1259 (35)	$<10^2$
Underside of mixer Lid	ND	–	ND	10^2	452	373.05	136 (30)	up to 10^2
Top of Batter Mixer	140	7.37	33 (24)	$<10^2$	114	85.80	57 (50)	10^2 - 10^4
Bottom of Batter Mixer *	ND	–	ND	$<10^2$	140	92.41	31 (22)	$<10^2$
Plastic Cheese Covers	86999	34579.52	86403 (99)	10^4	970	942.27	514 (53)	up to 10^2
Mean of all surfaces	**15401**	**6237.54**	**15207 (36)**	–	**3530**	**1478.49**	**2897 (48)**	–
Environmental Surfaces	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**
Underneath Conveyor	205	79.53	66 (32)	$<10^2$ - 10^3	3558	2969.74	2895 (81)	10^2 10^3
Dairy Food	1037	1358.11	1003 (97)	10^2 - 10^3	114	79.29	27 (24)	10^2
SS Underside of Conveyor *	219	82.36	75 (34)	$<10^2$	412	656.76	88 (21)	$<10^2$
Palate Surface	10467	4559.34	10378 (99)	10^2 - 10^3	3828	2406.36	2590 (68)	10^3
Bottom underside of mixer *	2612	4389.03	2527 (97)	10^2 - 10^4	4325	1476.80	4184 (97)	10^4
Mean of all surfaces	**2908**	**2903.67**	**2810 (72)**	–	**2447**	**1517.79**	**1957 (58)**	–

Meat Processing 1

Sampling Site	Pre-Clean Sampling				Post-Clean Sampling			
Food Contact Surfaces	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**
Bagging Area	21269	4575.98	5471 (26)	$<10^2$ - 10^3	ND	–	ND	$<10^2$
Steam Conveyor	688	349.64	227 (33)	10^3 - 10^4	ND	–	ND	$<10^2$
Mean of all surfaces	**10068**	**440064**	**2537 (27)**	–	**ND**	–	**ND**	–
Environmental Surfaces	**Mean Total AT/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**
Rack Area	8246	8276.31	1912 (23)	10^2 - 10^4	ND	–	ND	$<10^2$
Chill Floor	21811	17924.77	11603 (53)	10^4	SNC	–	–	–
Floor – Table Leg Area	642	288.48	335 (52)	$<10^2$	247	170.84	95 (38)	$<10^2$
Underside of Table	195	167.48	128 (66)	$<10^2$	ND	–	ND	$<10^2$
Mean of all surfaces	**7549**	**6126.91**	**4002 (57)**	–	**82**	**56.95**	**32 (13)**	–

Meat Processing 2

Sampling Site	Pre-Clean Sampling				Post-Clean Sampling			
Food Contact Surfaces	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**
SS Cooked Chicken Cutting Area	372798	111645.87	106105 (28)	$<10^2$	ND	–	ND	up to 10^2
Freezer Tunnel Conveyor	131651	40404.78	51096 (39)	10^3 - 10^4	NS	–	–	–
SS Table (Cooked Chicken	O/L	–	–	10^3	ND	–	ND	$<10^2$
Hot Strip Conveyor (Plastic)	25915	3934.34	8790 (34)	$<10^2$	915	1084.13	617 (67)	10^4
Hot Strip Conveyor Board (Plastic)	2274	1110.09	804 (35)	$<10^2$	158	82.47	32 (20)	$<10^2$
Hot Strip Conveyor SS Table	286	270.82	101 (35)	$<10^2$	ND	–	ND	$<10^2$
Mean of all surfaces	**106585**	**31473.2**	**333792 (34)**	–	**215**	–	**130 (17)**	–
Environmental Surfaces	**Mean Total ATP/100c (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Estimate Colony Count/100cm² Plate Count Agar**
Hold Chill Wall	ND	–	ND	$<10^2$	ND	–	ND	$<10^2$
Hold Chill Floor	42066	12648.22	10388 (25)	10^3	10133	4167.15	2687 (27)	10^3 - 10^4
Sink Area Handwash	3273	3242.24	1169 (36)	10^2 - 10^3	215	325.92	14 (7)	up to10^2
Freeze Conveyor Leg Area	1429	335.76	1093 (76)	NS	3857	5011.92	3306 (86)	NS
Freezer Tunnel Floor	18993	22572.16	6564 (35)	$<10^2$	495	344.05	289 (58)	NS
Blast Freezer Floor	39217	27468.88	10473 (27)	10^2 - 10^3	56679	86861.89	25472 (50)	10^4
Blast Freezer Wall	ND	–	ND	$<10^2$	139	80.62	41 (29)	10^2
Mean of all surfaces	**14997**	**9466.8**	**4241 (28)**	–	**10216.8**	**13827.4**	**4544 (37)**	–

Meat Processing 3

Sampling Site	Pre-Clean Sampling				Post-Clean Sampling			
Food Contact Surfaces	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Mean Colony Count/100cm² Plate Count Agar**	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Mean Colony Count/100cm² Plate Count Agar**
Rack Saveloys	29032	10584.34	6369 (22)	5.3×10^1	1156	316.83	299 (26)	1.2×10^3
Hot Dog Skinning Machine	120	17.67	20 (17)	NG	122	82.77	ND	1.4×10^1
Mean of all surfaces	**14576**	**5301.00**	**3195 (20)**	**2.7×10^1**	**639**	**199.8**	**150 (13)**	**6.0×10^2**
Environmental Surfaces	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Mean Colony Count/100cm² Plate Count Agar**	**Mean Total ATP/100cm² (RLU's)**	**Standard Deviation**	**Mean Microbial ATP/100cm² (RLU's) + (%)**	**Mean Colony Count/100cm² Plate Count Agar**
Ambient Chiller Floor	232019	41376.45	230622 (99)	5.2×10^7	66552	22374.28	62849 (94)	8.7×10^6
Blast Chiller Floor	385148	116072.94	381873 (99)	4.9×10^7	19670	28533.74	19083 (97)	6.0×10^5
Underneath Black Pudding Conveyor	ND	–	ND	2.6×10^2	ND	–	ND	9.4×10^1
Main Production Area Floor	6426	6634.64	5752 (90)	8.6×10^5	382576	168778.72	37644 (99)	7.0×10^7
Floor outside oven	153425	28700.84	152600 (99)	8.5×10^6	137776	45356.61	135854 (99)	2.7×10^7
Mean of all surfaces	**155404**	**38556.97**	**154169 (77)**	**2.2×10^7**	**121315**	**53008.67**	**51086 (78)**	**2.1×10^7**