The retention testing of sterilising grade membranes with Pseudomonas diminuta

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The Retention Testing of Sterilising Grade Membranes with
*Pseudomonas diminuta*

by

Sara Waterhouse

A Doctoral Thesis

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The Retention Testing of Sterilising Grade Membranes with 
*Pseudomonas diminuta*.

Sara Waterhouse May 1994

**Key words:** Microfiltration, cross-flow, sterilisation, *Pseudomonas diminuta*, ATP luminescence, impedance, DEFT, bioluminescence.

**ABSTRACT.**

Membranes with a pore size rating of 0.2 μm are recommended for the sterilisation of liquids by filtration and are validated for this purpose by a retention test with *Pseudomonas diminuta*. Practices for retention testing were found to vary among the membrane manufacturers and only one type of commercial 0.2 μm rated membrane was found to reliably retain *P. diminuta*. The retention for *P. diminuta* given by experimental grafted membranes was studied and was sometimes higher than that given by non-grafted membranes due to obstruction of the pores by graft material. The dimensions for individual cells of *P. diminuta* was studied by scanning electron microscopy and a rapid electronic method. Bacteria of larger dimensions than the pore size rating of experimental membranes were found in test permeates. It was shown that cells from an aerated *P. diminuta* culture were larger than cells from a similar but stationary culture.

A retention test procedure for 0.2 μm rated membranes using cross-flow filtration was developed. The procedure simulated process conditions and enabled tubular ceramic monolithic membranes and flat-sheet membranes to be retention tested with *P. diminuta*. It is feasible that a standard retention test using cross-flow filtration can be developed. The time needed for results from current retention test procedures to become available is a consequence of using traditional cultural techniques for permeate analysis. Test procedure were developed using three popular methods for the rapid detection and enumeration of bacteria (ATP luminescence, impedance microbiology and the DEFT) for the detection and enumeration of *P. diminuta* in retention test permeates. The method using ATP luminescence was found to be the most applicable.

The development of a bioluminescent strain of *P. diminuta* through genetic engineering will enable the rapid, sensitive and straightforward retention testing of 0.2 μm rated membranes. Retention tests using a bioluminescent strain of *Escherichia coli* containing the structural genes for bacterial luciferase indicated that the proposed test is feasible. Developments were made towards cloning the same genes into *P. diminuta*. The use of all bioluminescent micro-organisms for membrane retention testing is the subject of a patent application and a proposal for a three year SERC research grant.
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Abbreviations used throughout the text.

ac Available chlorine
AC Alternating current
AU Arbitrary units (for bioluminescence)
ASTM American Society for Testing and Materials
ATCC American Type Culture Center
ATP Adenosine triphosphate
BPU Bactometer processing unit
BSA Bovine serum albumin
CEN Comite European de Normalisation
cfu Colony forming units
DAPI 4,6-diamino-2-phenylindole
DEFT Direct epifluorescence filter technique
d.H₂O Distilled water
DMSO Dimethyl sulphoxide
DNA Deoxyribonucleic acid
DOP Diocylphthalate
DT/IDT Detection time/impedance detection time
EDTA Ethylenediaminetetra acetic acid
ELISA Enzyme-linked immunosorbent assay
FDA Food and Drug Administration
FMN₇ Reduced flavin mononucleotide
HEPES N-(2-hydroxyethyl)piperazine-N’-(2-ethane)sulphonic acid
HIMA Health Industry Manufacturing Association
kb Kilobase-pairs
kd Kilodalton
L-broth Luria broth
L-agar Luria agar
LAL Limulus amoebocyte lysate
LRV Log. Reduction value
MPN Most probable number
NADH₂ Reduced nicotinamide dinucleotide
NMWCN Nominal molecular weight cut-off
NCIMB National Centre for
ATCC American Type Culture Center Industrial and Marine Bacteria
NRB A commercial extractant for bacterial ATP
PCB Plate count broth
ppm Parts per million
PCR Polymerase chain reaction
PPLO Pleuropneumonia-like organism
PTFE Polytetrafluoroethylene
PVDF Polyvinylidenedifluoride
PWP Pure water permeate rate
RLU Relative light units (for ATP luminescence)
RNA Ribonucleic acid
SDS Sodium dodecyl sulphate
SEM Scanning electron microscopy
SLB Saline lactose broth
TCA Trichoroacetic acid
TMP Trans-membrane pressure
Tris Tris(hydroxymethyl)aminomethane
TSB Tryptone soy broth
USP United States Pharmacopoeia
UF Ultrafiltration
1. INTRODUCTION.

Microfiltration is a pressure-driven membrane separation process utilising rigid, porous membranes with pore size ratings from 0.1 to 10 μm capable of retaining particles such as paint pigment, smoke, bacteria, yeasts and fat globules (Davis 1992). Sterilising filtration is the complete removal of viable organisms from liquids and gases by filtration with 0.2 μm rated membranes (Meltzer 1987a). Examples of liquids routinely sterilised by filtration include those used for injection (parenterals) such as heat-sensitive drugs, vitamins, antibiotics, other intravenous infusions and many reagents used in biotechnology (Goel et al 1992). There is a long history of the satisfactory use of 0.2 μm rated membranes for sterilisation filtration (Robinson 1984).

The numerical pore size rating of a membrane is determined by a physical integrity test such as the bubble-point test (ASTM F 316 1984). However, a more accurate indication of the retention ability of sterilising grade membranes is achieved by an additional bacterial retention test. The membrane is challenged with large numbers of a small, nonpathogenic bacterium of the species Pseudomonas diminuta which must be retained. The retention test strain of P. diminuta was first isolated by its ability to consistently penetrate 0.45 μm rated membranes and has been accepted as the standard retention test organism for 0.2 μm rated membranes (Bowman et al 1967, Leahy and Sullivan 1978). Current test procedures using P. diminuta involve determining the sterility of the permeate by culture techniques which require a long time before results become available (HIMA 1982, ASTM D 3862 1992).

The call for the improved characterisation of membranes by the manufacturers means that the retention testing of 0.2 μm rated membranes with P. diminuta should be included into all routine quality control procedures. A major programme for standardisation in biotechnology for the European Community (EC) is underway and is controlled by the Comité Européen de Normalisation (CEN). Routine test permeate sterility testing can only decrease the probability that a final product produced using membranes will contain micro-organisms (Robinson 1984) and thus membrane users and regulatory agencies will welcome any assurance of the retention ability of the membrane. Robinson (1984) states that any improvement in the precision of the pore rating will give a competitive edge to the membrane manufacturers and also recommends that the manufacturers provide a clear description of how their membranes are rated. He suggests that research should be directed at improving the accuracy with which sterilising grade membranes are characterised.

Current bacterial retention test procedures could be improved by firstly, creating a test that will give results within a short period of time and secondly, by developing a
test that permits membranes to be retention tested not only using traditional dead-end filtration but also during cross-flow filtration. This study will initiate research into both of these areas. With cross-flow filtration, commonly used for the filtration of feedstocks with a high particulate load, the feed is continually recirculated parallel to the membrane surface to decrease the rate of membrane fouling. Fouling involves irreversible interactions between retained material and the membrane that decreases productivity and the membrane life-time. (Davis and Birdsell 1987). All current retention test procedures use dead-end filtration. The generation of a rapid test procedure would improve the efficiency of the retention testing process whilst the development of a test using cross-flow filtration would enable the accurate retention testing of membranes designed for use in this mode under simulated process conditions.

The research from October 1989 to October 1991 was carried out under an EC funded BRITE EURAM contract RI 1B 0292 C (CD) entitled: Improvement of tangential microfiltration for the bio-industries. (Partner 0 - Tech-Sep, St. Maurice de Beynost, France; partner 1 - Advanced Protein Products Ltd., Brierly Hill, UK and partner 2 - Department of Chemical Engineering, Loughborough University of Technology (LUT), UK). Dead-end filtration was traditionally used for filtration but research into and applications for cross-flow filtration are increasing (Mir et al 1992). However, membranes used for dead-end filtration are not suitable for use in the cross-flow mode. Membranes intended for cross-flow filtration with biological materials have to be specially designed with a low fouling potential and a high permeate rate. Important areas of concern for the membrane user are the often irreversible decrease in permeate rate and the increased retention for species expected to penetrate the membrane. These are both due to the accumulation of rejected material on the membrane surface which leads to membrane fouling. Experimental polymeric microfiltration membranes for cross-flow filtration were produced and modified by Tech-Sep in order to decrease the rate of fouling by protein during filtration. The pore size rating of the membranes was determined at Tech-Sep. The retention efficiency for \textit{P. diminuta} given by sterilising grade membranes was determined at LUT.

Chapter 2 introduces membrane separations, microfiltration and procedures for the pore size characterisation of microfiltration membranes. Methods for the detection and enumeration of viable \textit{P. diminuta} in retention test permeates are discussed. The concept of developing a light-emitting strain of \textit{P. diminuta} through genetic engineering for the rapid and sensitive retention testing of membranes is introduced. The aims of the study are discussed in detail at the end of the chapter and are compared with the results in the conclusions (chapter 6). Additional material for chapters 2, 3 and 4 can be found in the appendices situated after the references (chapter 7).
2. LITERATURE REVIEW.

An introduction to membrane separations, dead-end and cross-flow filtration and membrane fouling follows. Applications for microfiltration and the structure of microfiltration membranes are considered. Integrity tests and bacterial retention tests used for the pore size characterisation of 0.2 μm rated membranes are described. The suitability of popular rapid methods for the detection of bacteria to be applied to the detection of \( P. \) diminuta in retention test permeates is considered, and \textit{in vivo} bacterial luminescence is introduced.

2.1. Membrane operations.

A membrane operation employs a permselective membrane for separation, concentration or fractionation of a solution or mixture (Gekas 1986). The membrane itself can be a rigid film, or take the form of a liquid supported in a porous structure or as an aqueous or oil phase sandwiched between two oil or aqueous phases respectively (Ho and Li 1992). Liquid membrane technology is a relatively new field and is not within the scope of this study. The fluid feed to be separated can be a liquid or a gas, and the driving force for the separation can be either pressure or the difference in chemical potential between the feed and the permeate. Feed is defined as the fluid entering a membrane module or plant, permeate as that portion of feed passing through the membrane and retentate as that portion of the feed not passing through the membrane (Gekas 1986). Microfiltration, ultrafiltration, reverse osmosis and some gas separations are membrane operations that use porous membranes with pressure as the driving force for separation. The relationship between the separation processes of microfiltration, ultrafiltration and reverse osmosis can be seen in \textit{figure 2.1.a}.

2.1.1. Principles of membrane separations.

A permselective membrane is defined as an intervening phase separating two phases acting as an active or passive barrier to the transport of matter between phases adjacent to it (Gekas 1986). The membrane will separate components of a fluid by differences in one or more properties such as size, shape, electrical charge, solubility or diffusion rate. A membrane may be biological in origin, for example the plasma membrane and
Figure 2.1.a. Range of application for pressure-based separation processes (from Rautenbach & Albrecht 1989).

![Diagram showing the range of application for pressure-based separation processes.

Figure 2.1.b. Sizes of several natural and industrial particles (from Davis 1992).

![Diagram showing the sizes of various particles, including urban aerosols, smoke, dust, smog, mist/fog, spray, colloidal silica, clay, silt, sand, carbon black, paint pigment, pulverized coal, flexible long-chain macromolecule, coiled, extended, viruses, bacteria, synthetic polymer spheres, red blood cell, yeast cells.]

4
organelle membranes of eukaryotic cells typically consisting of glycoproteins, glycolipids and lipopolysaccharides (Zubay 1984), or it may be artificial. Artificial membranes can be manufactured from organic polymers or inorganic materials such as ceramics, metal or glass.

Artificial membranes can be porous or non-porous and dense in nature. Porous membranes have a static network of open voids that represents the pore system of the membrane. Nominal pore diameters can range from 2 nm to 10 μm and separation is determined mainly by the difference between the size of the pores and the feed components. Porous membranes have been defined as those where the fraction of void volume becomes comparable to that occupied by the membrane material (Kesting 1971). Non-porous, dense membranes do not have this void structure. Separation can be between molecules of the same size and is determined by the degree of solubility of feed components in the membrane material and their relative rate of diffusion across the membrane. This theory is not universally accepted and the alternative theory is that all membranes are porous (Sourirajan and Matsuura 1985). A pore can be defined as a space between two molecules and therefore components do not dissolve in the membrane but simply pass between the molecules. The first theory will be adopted for this study. An example of an operation using a non-porous, dense membrane is pervaporation. Here separation is achieved by the reduction of partial pressure on the permeate side of the membrane and a phase transformation takes place in connection with mass transport through the membrane (Rautenbach and Albrecht 1989). Only artificial, porous membranes will be considered in this study.

2.1.1.1. Overview of pressure-driven membrane separations.

Microfiltration and ultrafiltration are operations that separate components of a liquid predominantly according to their size and shape. Microfiltration membranes will retain fine particles with linear dimensions in the range of approximately 0.02 to 10 μm and uses a trans-membrane pressure (TMP) of between 0.5 and 5 bar (Davis 1992). Ultrafiltration will retain particles with a molecular weight of approximately 300 to 500,000 daltons or a solvated particle size range from 2 to 20 nm (Gekas 1986) and uses a TMP of between 2 and 10 bar or higher (Kulkarni et al. 1992a). Typical retained species with microfiltration can be seen in figure 2.1.2. and include bacteria, yeasts, paint pigment and smoke particles (Davis 1992). Ultrafiltration membranes will retain smaller entities such as proteins and starches and, for example, have been used for the concentration of gelatin and the recovery of lactalbumin and lactoglobulin from cheese whey (Kulkarni et al. 1992b).
Microfiltration membranes are usually classified according to the nominal pore size determined in micrometers (section 2.3.) whilst it is conventional to refer to ultrafiltration membranes by their nominal molecular weight cut-off value (NMWC) (Cooper and Van Deerver 1979, Smolders and Vugteveen 1985). The membrane is challenged with a series of chemically similar species of a globular morphology (such as purified proteins or dextrans) with different molecular weights. The NMWC is defined as the molecular weight of the species for which the membrane has more than 90% rejection (Porter 1992a). This cut-off value can not always be correlated with physical pore size as rejection is characterised by the molecular conformation of the species used.

Reverse osmosis will retain small solute molecules of dimensions between 1-10 Ångstroms and requires a high TMP of up to 100 bar (Rautenbach and Albrecht 1989). Osmosis is a spontaneously occurring phenomenon where solvent will flow through a membrane from an area of low solute concentration to an area of high solute concentration until equilibrium is reached. Here the pressure difference between the two sides of the membrane is equal to the osmotic pressure difference. Osmotic pressure is defined as that particular pressure that causes the nett flow to be reduced to zero (Laidler and Meiser 1982). To reverse the direction of the flow of solvent (for example water) a pressure difference greater than the osmotic pressure is applied and the separation of water from ionic solutes, for example, is made possible.

There is no single accepted theory for the mechanism of separation by reverse osmosis and suggestions for this involve porous and non-porous membrane models (Bhattacharyya and Williams 1992). The porous membrane model explains that separation is realised not only by the difference in sizes between the solute and pore diameter but is also affected by surface forces between the solute and the inner pore wall (Sourirajan and Matsuura 1985). Reverse osmosis membranes are often characterised by the extent of salt (as NaCl) rejection (Gekas 1986) or by the rejection of selected organic solvents. Reverse osmosis has been used for the desalination of salt waters (Meares 1966) and production of potable water form waste waters, as well as for concentration operations such as the treatment of egg white prior to spray drying (Sudak 1990) the concentration of caffeine (Pancuska and Mlynarczyk 1974) and the concentration of green tea juice (Zhang et al 1992).

Gas separation using porous membranes uses the pressure difference across the membrane to provide the driving force for the diffusion of gas across the membrane (Zolandz and Fleming 1992). This technique is the only membrane-based method used to separate isotopes of a gaseous element as separation is solely on the basis of mass ratios, and it was used during World War II to enrich uranium in the fissionable isotope of mass 235 in the development of the atomic bomb.
2.1.1.2. Dead-end and cross-flow filtration.

The volume or mass of permeate passing through a membrane per unit area and unit time is termed the flux (Gekas 1986). With traditional dead-end filtration the feed is directed perpendicular to the membrane surface and the permeate stream is in the same direction. During dead-end filtration the retained solute forms a continually increasing cake on the membrane surface. In comparison, the feed in cross-flow filtration is continuously recirculated parallel to the membrane surface and the permeate stream is perpendicular to the direction of feed flow (Zydney and Colton 1986, Murkes and Carlsson 1988, Rios et al 1989, Davis 1992). Schematic diagrams of dead-end and cross-flow filtration can be seen in figure 2.1.c.

The flux rate during membrane filtration can decrease due to solute concentration polarisation and fouling. The rate of development of concentration polarisation and subsequent membrane fouling can be controlled by the use of cross-flow filtration. Concentration polarisation is brought about by the solvent passing through the membrane bringing the solute close to the membrane surface (Van den Berg and Smolders 1990). Consequently, the concentration of solute increases towards that surface. A gel layer is formed on the membrane surface when the solute concentration reaches its solubility limit. The convection of solute to the gel layer is balanced by the diffusion of solute from this back into the bulk feed.

Flux reduction can be ascribed to the decrease in hydraulic permeability due to formation of a gel layer (Kulkarni et al 1992a). The popular boundary layer model for cross-flow filtration is shown in figure 2.1.d. and is one of several models for flux decline in ultrafiltration processes (Van den Berg and Smolders 1990). The boundary layer is represented by $\delta$ where the solute concentration changes from $C_f$ (bulk feed concentration) to $C_w$ (concentration at membrane surface). As $C_w$ reaches the solubility limit, further increases will cause the formation of the gel layer on the membrane surface. Further increases in pressure will not increase the flux rate. The formation of $\delta$ and the gel layer are controlled by the hydrodynamic flow conditions for filtration. The deposition of substances onto a membrane surface due to concentration polarisation is reversible. The continuous recirculation of feed parallel to the membrane surface during cross-flow filtration decreases the rate of boundary and gel layer development. The gel layer can also be removed by cleaning.

Fouling can be defined as the deposition of material on the membrane surface and/or pores as a result of concentration polarisation. This can be caused either by contaminating substances in the fluid to be filtered or actual retained components (Matthiasson et al 1984, Gekas 1986, Hanemaaijer et al 1989). Fouling is characterised by membrane-solute interactions (such as the adsorption of proteins during
Figure 2.1.c. Dead-end and cross-flow filtration (from Davis 1992).

DEAD END

CROSSFLOW

Figure 2.1.d. Boundary layer model for concentration polarisation during cross-flow filtration (from Kulkarni et al. 1992).
ultra and microfiltration) and consequently is less easily reversed than concentration polarisation. Fouling leads to an irreversible change in the performance of the membrane. Some membranes which have become badly fouled can be cleaned either chemically or mechanically to regain some of the initial flux rate and prolong membrane life. However, rigorous cleaning is difficult due to the deposition of fouling material actually inside the membrane structure (Mir et al 1992). Three types of foulant are recognised, organic precipitates such as biological materials as colloids or macromolecules, inorganic precipitates such as metal hydroxides or calcium salts and particulates including micro-organisms (Mulder 1991). The permeate rate is decreased and the retention characteristics of the membrane are permanently altered due to pore constriction by the fouling layers. The use of cross-flow filtration decreases the rate of membrane fouling through decreasing the rate of development of boundary and gel layers as described above.

Microfiltration membranes have been used successfully in the dead-end mode for the clarification of beverages and the sterilisation of pharmaceuticals (Goel et al 1992). The use of these membranes in the cross-flow mode highlighted the need to develop specific membranes with a lower fouling potential. It is known that the amount of protein adsorbed depends on the membrane material, the solute type, and the concentration and ionic strength of the protein and that the attachment of hydrophilic chains to a hydrophobic membrane will increase permeate flux during protein ultrafiltration (Vanden Berg and Smolders 1990, Toyomoto and Higuchi 1992). Hydrophobic membranes such as polypropylene and polytetrafluoroethylene (PTFE) will adsorb more protein than hydrophilic membranes (such as cellulose acetate and polyacrylonitrile) due to hydrophobic interactions. The structures of some polymers used for microfiltration membranes can be seen in figure A.1, appendix A and the structure and manufacture of polymeric membranes is discussed in section 2.2.1.

Cross-flow micro and ultrafiltration are methods of increasing importance with the biotechnology industries for large-scale filtration and concentration processes (Cueille et al 1987). These operations often involve filtration of proteinaceous solutions that have a high fouling potential. For example, the removal of antibiotics from microbiological fermentation broths (Davis and Birdsell 1987) and the processing of milk and whey where the deposition of poorly soluble salts such as calcium phosphate and the adsorption of proteins are the major causes of fouling (Hanemaaijer et al 1989). A decreased rate of fouling is concomitant with longer membrane lifetimes and an increased productivity.
2.1.1.3. Membrane configuration.

A filtration module can be defined as the smallest practical unit containing one or more membranes (Gekas 1986). A high membrane packing density (the effective membrane area per unit volume) is recommended to give a good production rate. Other prerequisites for good module design are cost-effective manufacture, easy access for cleaning and cost-effective membrane replacement (Rautenbach and Albrecht 1989) although these may be contrary to a high packing density. The configuration used for a particular membrane operation is important in determining the efficiency of the process.

Dead-end filtration is often used where the volume to be filtered is small and the particulate load and the fouling capacity of the liquid is low, such as the sterile filtration of injectable liquids and other reagents used in biotechnology (Goel et al 1992). Some modules used for dead-end filtration can be seen in figure 2.1.e. Membranes for dead-end filtration are available in flat disc, disposable syringe-end unit (figure 2.1.e.(i).), pleated-sheet cartridge (figure 2.1.e.(ii).) and stacked flat disc (figure 2.1.e.(iii).) configurations. A pleated-sheet module consists of a pleated flat sheet membrane contained within a pressurised housing. The feed is pumped into the housing through the membrane and the permeate is drained through the centre channel of the cartridge. This design has the advantage over traditional disc filters in that a large surface area is presented for filtration. Pre-sterilised, disposable syringe-end filters are convenient for the rapid processing of small volumes.

Some modules used for cross-flow filtration can be seen in figure 2.1.f. Plate-and-frame and spiral-wound modules incorporate flat sheet membranes (Porter 1990b). A plate-and-frame module (figure 2.1.f.(i).) consists of two membranes sealed onto a porous, plastic support plate. The feed is recirculated across the membrane surfaces and the permeate drains via the porous back-plate to be collected separately. Multiple stacks of spaced plates can be used operating with either a serial or parallel filtration mode.

High membrane packing densities are possible using the spiral-wound configuration (figure 2.1.f.(ii).) originally developed for reverse osmosis. Two membranes are sealed together on three sides with a porous spacer material in the middle to form a sandwich. The unsealed edge of this is connected to the centre of a core tube, and several such sandwiches are spirally-wound around the core with feed-side spacers placed in between. The whole structure is assembled into a pressure housing. The retentate is pumped across the surfaces of the membranes in a direction perpendicular to the core and is drained parallel to this to be recirculated. The permeate passes into the middle of the membrane sandwich and drains into the core to be collected separately.
Figure 2.1.e. Module configurations for dead-end filtration (from Goel et al 1992).

(i) Disposable syringe filters.

(ii) Pleated sheet cartridges.

(iii) Stacked disc cartridges.
Figure 2.1.f. Module configurations for cross-flow filtration (from Mir et al 1992).

(i) Plate-and-frame (flat-sheet).

(ii) Spiral-wound (flat-sheet).

(iii) Tubular.

(iv) Ceramic multichannel monolith.
Cylindrical membrane filters are available in a capillary, hollow-fibre or tubular conformation (figure 2.1 f.(iii).) (Kulkarni et al 1992c, Mir et al 1992). Usually, the membrane lines the lumen of the channel. Capillary membranes with an internal diameter of 0.2-3.0 mm are arranged in a bundle in a shell tube. The feed is pumped through the channel lumen and permeate passing through the capillary walls is collected from the shell.

Hollow-fibres with an internal diameter of 50-100 µm are also used in bundles assembled into a pressure vessel in the same way, or the fibres can be U-shaped and with all the open bores fixed to an end-plate at one end of the vessel. Here, the feed is pumped into the pressure vessel (sometimes by a centrally placed porous distribution tube), the retentate is collected and recirculated and the permeate passes into the tubes to be collected at the free ends. Tubular membranes have an internal diameter of 1-2.5 cm and are used inserted into a sleeve of porous material then into an impermeable pressurised tube support. Feed is pumped through the lumen and the permeate is collected by holes situated at stages along the support housing. Capillary and hollow-fibre configurations have a higher packing density than the tubular arrangement, for which this can be increased by assembling several membranes into a common support block.

Inorganic membranes are also available in a tubular multichannel monolith configuration (figure 2.1 f.(iv).) (Hsieh 1991). The ceramic multichannel monolith has several channels to increase the packing density compared with conventional tubular membranes. The monolith is assembled into a pressure housing, retentate is pumped through the channels and the permeate that passes through the membrane walls is collected from the pressure housing.

2.1.2. Microfiltration and sterilising filtration.

Membranes account for a growing portion of the world-wide biotechnology market. Data collated by Tech-Sep indicate that equipment for dead-end filtration accounted for about 70% of the microfiltration section (value estimated at $100 million) of the downstream biotechnology separation and purification market in 1990. Equipment for cross-flow microfiltration accounted for about 30% of the same section. About 20% of this cross-flow market was for the disposable cassette type membrane often used for sterile filtration. The other 10% was for filtration using plate-and-frame modules with flat-sheet membranes, also filtration with tubular and hollow fibre configurations. The first major application for microfiltration membranes was for the sterilisation and microbiological analysis of water supplies during World War II (Porter 1992b). Since then a variety of organic polymers (figure A.1., appendix A) such as cellulose acetates,
ethyl cellulose, aromatic polyamides such as polysulphone and polyethersulphone, polyvinylidene fluoride (PVDF), polyvinylchloride, polypropylene and PTFE as well as inorganic materials (Burggraaf and Keizer 1991) have been widely used to manufacture membranes.

Microfiltration has applications in the food and beverages industry (Nielsen 1992), for sterile filtration of pharmaceutical and biotechnological products (Meltzer 1987a) and cell harvesting, for the treatment of effluent waste-waters (Murkes and Carlsson 1988) and the production of particle-free water for the micro-electronics industry (Grant and Zahka 1990).

2.1.2.1. Sterilising filtration.

Sterile filtration is the absolute removal of micro-organisms from a fluid (either liquid or gas) that can not be sterilised by heat and is widely used for both pharmaceutical and other biotechnological applications. The relevant technology for gas sterilisation is beyond the immediate scope of this study (Blakie 1987). Microfiltration has several applications in the pharmaceutical industry in the processing of parenterals in order to achieve three main goals (Goel et al 1992):

(a) Final product sterilisation.
(b) The reduction of bacterial load to maintain low pyrogen (section 2.4.1.) levels in parenterals that will be sterilised by autoclaving.
(c) The removal of organic and inorganic particulate matter from both the feedstock fluids and final parenteral solutions.

Liquids routinely sterilised by filtration include heat-sensitive synthetic large (100-1000 ml) and small (<100 ml) volume parenterals, ophthalmic solutions, the nutrient feed to and liquid products from fermentors including antibiotics, vitamins, vaccines and bioengineered proteins, other intravenous infusions such as electrolytes and also serum (Denyer 1982, Meltzer 1987a, Goel et al 1992). Aseptic processing involves the filling of presterilised containers with sterile drugs produced by filtration, which are then sealed with presterilised closures.

Non-infectious particulate material in parenterals is highly undesirable since this could cause a direct blockage of the finer blood vessels, precipitate clot formation to cause emboli, cause local granulomas where the particle becomes embedded in a tissue or give rise to antigenic reactions with an allergic response (Fifield and Leahy 1983). The results could include blindness, strokes, pyrogenic reactions, abscesses and malignant tumours. Membrane filters are advantageous over fibrous filters made from asbestos, felt, paper or glass fibres used previous to membrane development because they do not shed particulate matter.
The American Society for Testing and Materials (ASTM D 3862 1992), the Heath Industry Manufacturing Association (HIMA 1982) and the US Food and Drug Administration (FDA 1987) recommend that a membrane with a pore size of no more than 0.2 μm (the United States Pharmacopeia, USP XXII 1990, recommend 0.22 μm) should be used for the sterilisation of liquids including parenterals. Membrane filters with a pore size of 0.1 μm or less may be needed for the sterilising filtration of foetal calf serum and other media and reagents used for tissue culture which are prone to contamination with Mycoplasma species. These are small, pleomorphic bacteria that lack a rigid cell wall and are bounded by a single triple-layered membrane (Woese et al 1980, Maniloff and Morowitz 1982). The cells are deformable and will pass through a 0.2 μm rated membrane. Mycoplasmas were originally termed pleuropneumonia-like organisms (PPLO) when it was recognised that one species belonging to the group was the aetiologic agent for atypical pleuropneumonia (Chanock et al 1962). Mycoplasmas are destructive contaminants of tissue culture (Rottem and Barile 1993) and have been dubbed "membrane parasites" (Razin et al 1981) due to their ability to adhere tenaciously to animal membranes.

Use of a membrane with a smaller pore size rating than 0.2 μm is concomitant with a reduction in flux rate. Suggested requirements for general final sterilisation by a 0.1 μm pore membrane in the pharmaceutical industry were not justified (Robinson 1984). The 0.2 μm pore size membrane is considered wholly adequate for sterilisation purposes unless organisms that consistently pass through these membranes are regularly encountered.

2.1.2.2. Other applications for microfiltration.

Cross-flow microfiltration using inorganic membranes to remove bacteria, lipids and casein has been applied to the treatment of whey prior to ultrafiltration to produce whey protein concentrate (Bhave et al 1991b). Skimmed milk has been concentrated by microfiltration prior for the production of cheese and other dairy products using membranes with pore sizes from 0.1-0.4 μm (Woychik et al 1992). Membranes with pore sizes from 1.0 to 1.5 μm have been used to remove bacteria from milk without significant decrease in the level of casein micelles which range from 0.025-0.3 μm in size (Kosikowski and Mistry 1990). Studies using tubular ceramic multichannel monoliths found that a pore size rating of 1.4 μm gave retention rates between 99.93-99.99% for seven species of bacteria representing the contaminating flora of raw milk (Trouvé et al 1991). Similar membranes with a pore size rating of 1.8 μm have been used for the production of skimmed milk from raw milk. These membranes were
able to retain 98% of the milk fat without significantly affecting the level of casein and gave a logarithmic reduction value (section 2.3.2.) of around 2.0 for the bacterial flora (Piot et al 1987).

Evaporation is commonly used for the concentration of foods and beverages for further processing. However, this can have a detrimental effect on the compounds responsible for flavour and aroma, sugars could caramelise and proteins could denature and precipitate at the elevated temperatures used (Bhave et al 1991b). Cross-flow microfiltration does not produce these disadvantages. Wines and beers can be filtered before bottling or canning to remove haze or potential spoilage organisms such as pediococci, lactobacilli or wild yeasts without affecting flavour (Reed 1986). Beer can be separated from yeast in fermentation tank bottoms (Le 1987). Cross-flow microfiltration has been used for the concentration of fruit purees and for clarifying fruit juices by removing suspended solids and bacteria (Mohr et al 1989).

The products of microbial batch fermentations can be separated from the cells using cross-flow microfiltration, for example with the production of vinegar by acetic bacteria (Ripperger and Schultz 1986). Membranes with pore sizes ranging from 0.2 to 1.5 μm can be used depending on the size of micro-organism to be separated. Cross-flow microfiltration has been used to remove dyes, latex, cottonseed protein and soy-bean protein from wastewater, as well as oily residues such as cutting-oil and lubricating oil, and potable water is produced using microfiltration to remove bacteria (Bhave et al 1991a). Particulate-free water can be produced for the microelectronics industry as microcircuits are sensitive to particulate contamination greater than 0.1 μm in diameter (Couture and Capaccio 1984, Grant and Zahka 1990).

2.2. Structure and manufacture of microfiltration membranes.

Porous, artificial membranes can be classified by material as organic or inorganic and by morphology as isotropic or anisotropic. Membranes can be classified further morphologically by pore conformation. Sintering, stretching and phase-inversion methodologies all produce an interconnecting tortuous network of pores whilst membranes manufactured by track-etching and anodisation have separate, cylindrical pores.

Some membrane structures may be seen in figure 2.2. The porosity of a membrane is defined as the ratio of void volume to bulk volume (Johnston 1992). For an isotropic membrane (figure 2.2.(i).), the porosity is the same from the feed side to the permeate side and separation is realised by the whole membrane. For an anisotropic membrane (figure 2.2.(ii).) the porosity of the feed side membrane surface is less than for the rest
of the membrane (Gekas 1986). The top surface is the effector of separation whilst the
more porous layer acts as a support. The separation layer is thinner than the support layer
typically, 0.5 and 50-200 μm respectively. The flux rate is inversely related to the
thickness of the actual barrier layer and thus is higher for anisotropic membranes
compared to isotropic membranes of the same overall thickness. For this reason
anisotropic membranes are used for cross-flow filtration. Anisotropic membranes can
be manufactured in one stage or the membrane and support layer can be made in separate
operations and out of different materials. These are called composite membranes.
Production is a two-step process but is advantageous as each layer can be optimised, the
support layer for strength and the barrier layer for flux and solute rejection characteristics.

2.2.1. Organic membranes.

Organic membranes are manufactured by sintering, stretching, track-etching or by
a phase-inversion process. The choice of polymer will determine the chemical, thermal
and mechanical stabilities as well as characterising the surface effects such as the
adsorptive nature and wettability of the membrane (Toyomoto and Higuchi 1992).
Membranes can be made by sintering suitable polymers. Sintering refers to a change in
shape undergone by a cluster of particles when held at a high temperature (Mulder 1991).
A polymer powder is compressed at a high temperature, the interface between contacting
particles disappears and the pore size of the resulting isotropic membrane is determined
by the particle size of the powder used. Polyethylene, polypropylene (figure A.1.,
appendix A) and PTFE are examples of suitable polymers for the manufacture of sintered
membranes and the production of membranes with pores size ratings from 0.1-10 μm
are possible. Stretched membranes (figure 2.2.(iii).) are made by stretching an extruded
polymer film to form slit-like pores 0.1-3.0 μm wide (Strathman 1990). Many polymers
are semi-crystalline and consist of a amorphous, unordered phase and an crystalline,
ordered phase where the nature of the polymer sub-units allows such a regular
arrangement. Polymer films with a high degree of crystallinity, such as PTFE, can be
stretched perpendicular to the direction of extrusion and ruptures between the areas of
crystallinity occur.

2.2.1.1. Phase-inversion membranes.

Most polymeric membranes used today are made by the phase-inversion process
(Kesting 1971). This process was developed by Zsigmondy to manufacture cellulose
nitrate membranes (Zsigmondy and Bachmann 1922). A polymer solution,
Figure 2.2. Membrane structures (from Mulder 1991 and Goel et al 1992).

(i) Isotropic polymeric phase inversion.

(ii) Anisotropic polymeric phase inversion.

(iii) Polymeric stretched.

(iv) Polymeric track-etched.

(v) Anisotropic composite ceramic.

(vi) Anodised aluminium oxide 0.2 μm rated (Anopore).
(for example cellulose acetate in acetone), is precipitated into two phases, a solid, polymer-rich phase that forms the membrane matrix and a liquid, polymer-poor phase that forms the membrane pores. Precipitation of the polymer can be achieved by cooling if the polymer is not readily soluble at room temperature, solvent evaporation or by the addition of water. For precipitation by solvent evaporation, the polymer is dissolved in a volatile good solvent and a non-volatile poor solvent is added. The mixture is spread out (or cast) onto a glass plate. The volatile good solvent evaporates leaving the poor solvent rich in polymer which precipitates to form the membrane structure. The polymer must be incompatible with the non-volatile solvent or the resulting membrane is less structured and becomes non-porous. The slow addition of water from the vapour phase can also be used to realise precipitation. Isotropic membranes (figure 2.2.(i).) with pore sizes from \(<0.1\) to \(>20\,\mu\text{m}\) are manufactured this way, originally from cellulose polymers and later from Nylon, polysulphone or PVDF (Kunst and Sourirajan 1974). A combination of precipitation by solvent evaporation with imbibition of water from the vapour phase is often used. As a general rule, the faster the precipitation the smaller the pore sizes of the membrane.

Anisotropic phase-inversion membranes (figure 2.2.(ii).) are produced by first casting a solution of the solvent then quenching it by immersion in a non-solvent such as water (Baker 1991). Again, the solution separates into a polymer-rich solid phase to form the membrane structure and a solvent-rich liquid phase to form the pores. The rapid introduction of precipitating agent causes the pores at the surface of the cast film to be smaller than those towards the casting surface and the anisotropic structure develops. Loeb and Sourirajan (1964) were the first to produce anisotropic phase inversion membrane filters. These were used originally for reverse osmosis but the process has been adapted for the production of ultra and microfiltration membranes. Capillary and hollow-fibre membranes are generally manufactured by casting the polymer solution through a double-bore nozzle. The solution is always cast through the outside bore and a precipitant is fed down the inside bore to create an anisotropic membrane with the barrier on the inside. If the barrier is needed on the outside for hollow-fibre membranes, inert gas is fed down the middle and the solution is cast into a precipitant bath.

Tubular phase-inversion membranes can be made by ultrasonic welding of flat sheet membranes into the desired conformation or by casting the solution onto the inside of a support tube using an implement slightly smaller than the inner tube diameter and immersing the whole tube into a precipitation bath. Anisotropic composite membranes can be made by first casting a thick support membrane, then casting another film on top
of this. It is important for this support layer to be finely microporous so that the second solution does not seep in, and the permselective layer often takes the form of a non-porous membrane for gas separation or pervaporation.

2.2.1.2. Track-etched membranes.

Membranes with pores produced by nucleation track-etching have non-interconnecting cylindrical pores perpendicular to the feed surface and a low porosity (figure 2.2.(iv)). The technique was originally applied to thin sheets of mica to produce molecular sieves (Price and Walker 1962). It is important that pores do not overlap to produce oversize pores that will decrease the retentivity of the membrane. The possibility of this increases with the number of pores per unit membrane area. The track-etched membrane can be observed using scanning electron microscopy (SEM) and the diameter of the pores measured directly.

For commercial membranes, the surface of thin polymer film (usually polycarbonate) is bombarded with a massive charged particle such as a heavy positive ion supplied by uranium or californium (Kesting 1971). Parallel tracks are produced by using a collimated beam of particles, evacuating the space between the source and the film and exposing the assembly to thermal neutrons. The particles pass perpendicularly through the membrane leaving straight tracks of broken chemical bonds. These tracks, termed "damage tracks", are etched using an acid or base to form the pores. The porosity of the membrane is controlled by degree of ionic bombardment whilst the pore size is determined by time exposure to the etching liquid. The thickness of these membranes is limited to a maximum of 15-20 μm due to the limited depth of penetration possible by the fission particles. Whereas tortuous pore membranes rely partially on the depth of the interconnecting pore network to entrap particles, the track-etched membranes rely on the size ratio of particle to pore diameter for separation. Due to the low porosity however, flux rate is lower for a track-etched membrane than for a tortuous-pore membrane of the same pore size rating.

2.2.2. Inorganic membranes.

Inorganic membranes are generally more stable chemically, thermally and mechanically than organic membranes yet are not ductile and are thus susceptible to fractures (Cueille and Ferreira 1991). The lifetime of inorganic membranes is greater than that of polymeric membranes and they are not susceptible to an irreversible decrease in flux rates due to compaction. This is known to affect polymeric membranes used in
pressurised systems (Bert 1969). Inorganic membranes can be rigorously cleaned, used at high pressures and may be used with organic solvents which will dissolve organic polymeric membranes.

2.2.2.1. Ceramic membranes.

Ceramics are formed by the combination of a metal with a non-metal in the form of an oxide, nitride or carbide with aluminium or zirconium oxide being commonly used (Meares 1987, Burggraaf and Keizer 1991). The membrane structure is pre-formed then stabilised by heat. Tubular or multichannel monolithic conformations (figure 2.1.f) are popular for ceramic membranes. The pore size is dependant on the particle size of the material used and ceramic membranes for microfiltration with pore sizes from 0.1-20.0 μm or even as low as 4.0 nm can be made. A porous support is dipped into a colloidal suspension of the ceramic material, the dispersant soaks into the support leaving a gel-layer of material on the surface which is stabilised to form the membrane by heating to 400°C. Composite ceramic membranes can be made by adding a barrier layer with much smaller particle size of the same or different material to the pre-formed support (figure 2.2.(v)).

Ceramesh composite ceramic membranes are made by suspending a slip of zirconium oxide from a metal mesh which is then heated to stabilise the structure (Cowieson 1992). The slip meniscii form thin tortuous-pore membranes with a 0.2 μm pore size. The membrane is not brittle or easily damaged like the anodic oxidisation membranes described below and may be bent up to 90° without cracking. Ceramesh membranes are available in flat discs or in a conformation suitable for use in cross-flow modules.

2.2.2.2. Anodic oxidation membranes.

Inorganic aluminium oxide membranes with a cylindrical pore structure and a high porosity can be produced by anodic oxidation (figure 2.2.(vi)) (Smith 1974). One side of a thin aluminium foil is anodically oxidised in an acid electrolyte and the oxide develops into a regular hollow honey-comb pattern. By altering the process conditions, isotropic or anisotropic membranes of 60 μm thickness can be produced. Once the required thickness of membrane filter is reached, the oxidation is stopped. Pore sizes of 0.02 μm are possible and are proportional to the applied voltage. These membranes (Anopore, marketed by Whatman Scientific Ltd. Maidstone U.K.) are available in filter disc and syringe-end filter form and have also recently been produced in a form suitable for use in a cross-flow module. Anopore membranes produce a good flux rate but have the disadvantage that they are brittle.
2.2.2.3. Other inorganic membranes.

Porous metal microfiltration membranes can be made by sintering a metal powder such as stainless steel, tungsten or molybdenum (Mulder 1991). These have excellent chemical, temperature and mechanical resistance and are used for the filtration of aggressive, corrosive liquids. Porous glass membranes also exhibit similar resistances and can be made by cooling a mix of silica and metallic oxides to allow this to separate into a soluble and insoluble phase. The soluble phase is leached out with an acid or base to leave the silicon-rich porous insoluble phase. Sintering of a glass powder can also be used. Pore sizes of 0.05 µm can be achieved by leaching whilst sintering produces pore sizes from 0.1-10 µm (Burggraaf and Keizer 1991).

2.3. Membrane pore size characterisation.

The bubble-point method is a popular test used both before and after filtration to determine the integrity of a system with the membrane in situ, and is also used to estimate the diameter of the largest pore of a membrane. Determination of integrity ensures that the membrane has no tears or holes and that it is sufficiently sealed into the housing. Membranes with a pore size rating of 0.2 µm can also be retention tested with *P. diminuta* in order to confirm the ability to retain bacteria.

2.3.1. Integrity tests.

The bubble-point test will represent the maximum limiting pore diameter of membranes with maximum pore size rating of 0.1 to 15.0 µm (ASTM F316 1984). The apparatus used for the test and the interpretation of results from the test can be seen in figures 2.3.(i). and 2.3.(ii). The limiting pore diameter is defined as the diameter of the circle having the same area as the smallest section of a given pore. The bubble-point test is non-destructive as the membrane can be used for filtration after the test.

A 47 mm diameter membrane is thoroughly soaked in water (after using a wetting agent such as 70% isopropanol for hydrophobic membranes) and is assembled into the filter holder. The holder is closed and slight gas pressure is applied to the membrane to eliminate the possibility of liquid back-flow. The upper membrane surface is covered with 2-3 mm of the test fluid (usually water) and the gas pressure to the non-submerged surface is increased slowly. The lowest pressure that causes a steady stream of bubbles from the central area of the liquid is recorded and termed the bubble-point (figure 2.3.(ii).). Bubbles arising from the edge of the membrane signify a leak in the seal around the membrane.
Figure 2.3.a. The bubble-point test (from ASTM F 316 1984).

(i) Basic bubble-point apparatus.

(ii) Interpretation of bubble-point test results.
The maximum pore diameter can be calculated using eqn.1:

\[ r = \frac{2\gamma \cos \theta}{p} \]  

where \( r \) is the pore radius in \( \mu \text{m} \), \( \gamma \) the surface tension of the test fluid used in N/m, (70 \( \times \) \( 10^3 \) N/m for pure water) \( p \) the applied pressure in psi, and \( \cos \theta \) is a function of the contact angle for the test fluid with the inner membrane surface. The contact angle for water is usually assumed to be zero thus a value for \( \cos \theta \) of 1.0 is generally accepted.

The column of water in each pore is held in place by surface tension and the test assumes all pores to be non-interconnecting and cylindrical in shape (Hofmann 1984). The bubble-point is that particular pressure needed to overcome the surface tension and displace the water column from the pore with the largest diameter. Water will be displaced from the largest pore first as pressure is inversely related to the diameter of the theoretical cylinder. The bubble-point pore size rating is thus a function of the surface tension of the fluid used and must be re-determined if a different fluid to that used for the rating is to be used in the filtration. Alcohols or hydrocarbons are sometimes used and will give the bubble-point at a lower pressure than if water was used due to the lower surface tension. The retention of water inside the pores obeys the laws of capillarity where, for a capillary brought into contact with a wetting liquid, the liquid will rise to a certain height in that capillary determined by the surface tension of the liquid, the cosine of the contact angle between the liquid and the inside surface of the capillary, and is inversely related to the diameter of that capillary.

The bubble-point test can be carried out on flat sheet and pleated-sheet cartridges (Johnston 1992). The integrity of a membrane filtration system can be determined \textit{in situ} by first ensuring the system is sterile, allowing some of the process fluid to flow through the membrane to wet it, drain off excess fluid leaving a reservoir on top of the membrane and applying gas pressure as usual. If the results are not satisfactory, it must be possible for the permeate to be drained from the downside without contaminating the rest of the system. The test is often carried out after the filtration process to ensure no leaks or perforations developed during the operation.

The pore size ratings of most ceramic membranes are determined by mercury porosimetry (Mulder 1991), a variation of the bubble-point test. The volume of mercury forced into the pores of a dry membrane with each increment of pressure can be quantified and related to pore diameter. The same equation (eqn.1.) can be applied, remembering that since mercury does not wet the membrane \( \cos \theta \) will have a negative value. The pore size rating is determined from the pore size distribution generated which is not accurate.
since mercury taken up into blind-ended pores will be included in the results. This technique is used for membranes with pore sizes from 5 nm to 10 μm, and is not recommended for use with polymeric membranes as the high pressures required will deform the pore structure.

2.3.2. Bacterial retention tests.

It is important that a membrane used for sterile filtration is shown to be capable of removing all bacteria from solution. Bacteria present the most suitable "worst case" challenge to the retention efficiency of sterilising grade membranes as they naturally exist in a wider range of sizes than non-biological particles such as latex spheres. Standard retention test procedures using dead-end filtration exist for flat disc and pleated-sheet cartridge membranes (ASTM D 3862 1992, HIMA 1982, ASTM F 838 1992). A brief description of the history concerning the development of retention tests using P. diminuta and a description of current retention test methods follows.

Bowman et al (1967) developed test methods to determine the efficiency of retention given by 0.45 μm and 0.22 μm rated membranes using Serratia marcescens and a small, unnamed pseudomonad respectively. The pseudomonad was found contaminating a broth culture of Bacillus cereus filtered to 0.45 μm that was used to produce penicillinase. The species was found to have a stable size and phenotype when sub-cultured and consistently passed through a 0.45 μm rated membrane filter. The test for both types of membrane involved filtering a suspension containing large numbers of the relevant organism through a sterile membrane under vacuum into a flask which was incubated for 5 days. The test membrane was placed onto a nutrient agar plate and incubated for at least 48 hours. Turbidity in the flask indicated that the membrane had failed to retain the challenge. The test itself was validated by the absence of bacterial growth on the edge of the incubated filter that laid underneath the seal during the test.

Rogers and Rossmore (1970) developed a method for determining the pore size of an unknown membrane filter. They used a series of challenges with different species of micro-organism of decreasing mean cell sizes, including the small pseudomonad used by Bowman et al (1967). The number of bacteria in the filtrate was estimated using a most probable number (MPN) method (section 2.4.1.) and the retention efficiency given by the membrane for each species was calculated. It was assumed that when the retention was 100%, the difference between the mean cell size and the mean pore diameter was less than 0.1 μm. The pseudomonad isolated by Bowman (1967) was identified as a small, mutant strain of the species P. diminuta discovered by Leifson and Hugh (1954). This mutant strain was filed as the American Type Culture Center (ATCC) strain 19146.
and became the standard organism for the retention testing of 0.2 \( \mu m \) rated membranes. 

*P. diminuta* satisfies the prerequisites for a standard retention test organism of being nonpathogenic, easy to culture and of not having an inconveniently long generation time (Meltzer 1987a). Pseudomonads are indigenous to water based systems and their presence is not tolerated in pharmaceutical preparations due to the presence of pyrogens thus the relevance of using *P. diminuta* for the retention testing of such sterilising grade membranes is reinforced.

The ASTM produced a standard retention test for 0.2 \( \mu m \) rated membrane filters using *P. diminuta* (ASTM D 3862 1992) by using a method similar to that developed by Bowman et al (1967). The FDA (1987), USP (XXII 1990) and the HIMA (1982) have also published guidelines to retention testing with *P. diminuta*. The HIMA (1982) states that a sterilising grade membrane filter should be capable of retaining \( 1 \times 10^7 \) *P. diminuta* per cm\(^2\) of available membrane surface area. It is important to give an adequate challenge to the retention capabilities of the membrane filter whilst avoiding the build up of a thick cake layer on the membrane surface which will obscure the pores from the challenge.

It is essential that the retention test methodology is capable of detecting one viable bacterium in the whole of the test permeate. Test permeate analysis may be by either of two methods. The entire permeate can be incubated to give a simple and straightforward method for ascertaining sterility (ASTM D 3862 1992) and bacterial growth is indicated by turbidity. Alternatively, the entire permeate is analysed by membrane filtration (HIMA 1982). The permeate is filtered through a second membrane which is incubated on agar until retained bacteria form visible, individual colonies. This permits a numeric indication of retention ability to be calculated as the logarithmic reduction value (LRV, \( \log_{10} \text{cfu challenge/cfu permeate} \), where cfu is colony forming units) given by that membrane for that organism. A 0.45 \( \mu m \) rated membrane is used for this purpose (HIMA 1982, MacDonald et al 1989, ASTM F 838 1992). It is considered that although *P. diminuta* will penetrate membranes of this pore size rating, the degree of retention given to the low numbers of bacteria that will be encountered in test permeates is adequate (Meltzer 1987a).

### 2.3.2.1. Retention of *P. diminuta* by membranes.

It has been found that the retention capacity of 0.2 \( \mu m \) polymeric membrane filters decreases with an increase in applied TMP and also that *P. diminuta* can be retained to a degree by polymeric membranes with pore size ratings greater than 0.2 \( \mu m \) (Tanny et al 1979). This suggests that the mechanism of particulate retention by membrane filters is not solely by sieve retention, where particles are retained on the surface of the
membrane by pores with a smaller diameter than the smallest bacterium, but also by adsorptive retention where the pore size distribution presents pores that are larger than the bacteria. Here particles enter the membrane through the larger pores and are adsorbed to the pore walls by a combination of Van der Waals' forces and hydrodynamic attraction. An increase in TMP will tend to overcome these forces and cause less bacteria to be adsorbed.

Adsorptive retention has been demonstrated by comparing retention of latex spheres by membranes before and after the addition of a surfactant (Wrasidlo and Mysels 1984). There was a greater decrease in retention by adsorption retentive membranes than for sieve retentive membranes due to the disruption in adsorptive forces by the surfactant. Membranes with an isotropic, tortuous pore structure will retain bacteria by sieve and adsorptive retention. Anisotropic membranes will retain to a greater extent by sieve retention in the barrier layer presented to the feed due to the presence of a support layer of high porosity. Those membranes with a cylindrical pore conformation such as the track-etched and Anopore membranes are close to a purely sieve retentive model and thus depend heavily on the relationship between pore size and cell dimensions for the retention of bacteria.

2.3.2.2. Penetration of sterilising grade membranes by bacteria.

It is known that other species of bacteria apart from the mycoplasmas will pass through a 0.2 μm rated membrane. Wallhäuser (1979) reported that some Pseudomonas species isolated from demineralised water consistently penetrate these membranes in greater number than P. diminuta. Some bacteria will exhibit miniaturisation when grown in a nutritionally limiting medium (Shirey and Bissonnette 1991). The cells are decreased in size as a survival technique in response to a diminished carbon source. Howard and Duberstein (1980) reported that twelve species of bacteria isolated from well water will penetrate a 0.2 μm rated membrane that will retain 100% of a P. diminuta challenge, among them several Pseudomonas species. The microbiological analysis of water samples and the filtration of serum and other tissue culture reagents (section 2.1.2.1.) both represent cases where bacteria that consistently pass through 0.2 μm are encountered and 0.1 μm rated membranes should be used.

2.3.3. Pseudomonas diminuta.

The genus Pseudomonas was described in 1894 as "cells with polar organs of motility, formation of spores in some species but rarely." (Palleroni 1986). Pseudomonads are currently recognised as a large heterogenous group of rod-shaped,
Gram-negative, montrichous, aerobic bacteria typified by their ability to utilise a wide range of carbon sources. *P. diminuta* is small, motile, nonpathogenic Gram-negative rod of dimensions by 1.0-4.0 μm. The species has a single polar flagellum with an unusually short wavelength of slightly over 0.6 μm compared to the value of 2.0 μm typical for pseudomonads (Leifson and Hugh 1954). The general phenotypic characteristics for *P. diminuta* are described below (Ballard *et al* 1968, Buchanan and Gibbons 1974):

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>P. diminuta</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Spore production</td>
<td>not present.</td>
</tr>
<tr>
<td>(b) Accumulation of endogenous poly-hydroxybutyrate (PHB)</td>
<td>present.</td>
</tr>
<tr>
<td>(c) Metabolism</td>
<td>obligate aerobe.</td>
</tr>
<tr>
<td>(d) Optimal growth temperature</td>
<td>30°C.</td>
</tr>
<tr>
<td>(e) Exogenous nutritional requirements</td>
<td>pantothenate, biotin and cyanocobalamin.</td>
</tr>
<tr>
<td>(f) Denitrification</td>
<td>not present.</td>
</tr>
<tr>
<td>(g) Oxidase reaction</td>
<td>positive (variable intensity).</td>
</tr>
<tr>
<td>(h) Carbon sources</td>
<td>limited. All strains were unable to hydrolyse starch, gelatin, Tween-80 or exogenous PHB.</td>
</tr>
<tr>
<td>(i) Other</td>
<td>acid produced from primary alcohols by those strains capable of utilising alcohol.</td>
</tr>
</tbody>
</table>

The degree of homology between the base sequences in ribosomal ribonucleic acid (rRNA) of different species of *Pseudomonas* has been used to assign species to groups, termed rRNA homology groups (Palleroni *et al* 1972, Whitaker *et al* 1981). *P. diminuta* and *Pseudomonas vesiculaire* belong to homology group IV, the diminuta group. These species characteristically lack prephenate dehydrogenase and possess arogenate dehydratase which are regarded as enzymological markers for the group. One strain of the *P. diminuta* species carries the plasmid-borne property of enzymatic parathion hydrolysis (Serdar and Gibson 1985). Parathion is a broad-spectrum organophosphate insecticide and hydrolysis of this reduces the toxicity nearly 100-fold. This is of interest considering that enzymatic hydrolysis is much faster than traditional chemical hydrolysis.

*P. diminuta* will produce a creamy turbidity with a characteristic pellicle when
grown as an agitated, aerated tryptone soy broth (TSB) culture. When examined microscopically the rod-shaped bacteria are seen to aggregate in clusters. For the retention test *P. diminuta* is cultured in a nutritionally limiting saline lactose broth (SLB). The cells produced are not only smaller and shorter in response to the diminished carbon source but do not tend to form aggregates. The aspect ratio (length:diameter) for *P. diminuta* cultured in SLB is 1.0-2.5 compared to 2.0-5.0 when cultured in TSB (Leahy and Sullivan 1978). These cells produce a rigorous challenge to the retention ability of test membranes. It is important that cells for the membrane challenge are taken from an early stationary phase culture. A sample from this phase will contain bacteria with a narrower range of sizes than from the logarithmic phase (where bacteria experience a rapid increase in size) yet will still contain a high percentage of viable cells.

### 2.3.4. Problems with current procedures for pore size characterisation

The bubble-point test will not accurately determine the maximum pore diameter of microfiltration membranes. Retention by membrane filters has already been shown to be due to adsorptive as well as sieve retentive mechanisms (Lukaszewicz and Meltzer 1979, Wrasidlo and Mysels 1984). Thus a numerical pore size rating obtained by the bubble-point test alone is not a sufficient indication of retention capability. Integrity tests however, are invaluable when used to simply detect damage or defects for a membrane assembly either before or after filtration.

The actual bubble-point determined by eye is subject to inaccuracies due to operator variation and is known to vary with the area, thickness and material of the test membrane, and with the wetting fluid used (Johnston 1992). Most membranes used today have a tortuous pore structure whilst the bubble-point test assumes all pores to be cylindrical and non-connected as described earlier. Also, it is known that gas can pass through the membrane at pressures below the bubble-point due to diffusion (Hofmann 1984, Meltzer 1987b). This diffusion is caused by the differential partial pressure across the membrane. The solubility of gas on the pressurised side increases with pressure to give a higher concentration of dissolved gas on the up-stream side of the fluid-filled membrane filter. This results in diffusion of dissolved gas to the down-stream side with the lower dissolved gas concentration. This side is not pressurised and the gas comes out of solution. The rate of diffusional gas transport is less than the rate of bulk gas transport that occurs at the bubble-point, but increases with the area and porosity of membrane tested. Thus there is the possibility of diffusional gas flow masking the true bubble-point gas flow. Inaccuracies in maximum pore size determination increases with the area of membrane
used. The inadequacies of the standard bubble-point test have been recognised and the related forward-flow test was developed in an attempt to overcome some of them, but is not widely used. If the pressure to the membrane is slowly increased, a slow rate of diffusional air flow through the membrane is seen until the bubble-point pressure is reached (seen as a sharp increase in air flow). Airflow downstream of the test membrane is quantified at 80% of the theoretical bubble-point. If this airflow is less than the specified value then the membrane is integral and may be used.

Bacterial retention tests involve either incubation of the whole permeate or membrane filtration analysis as described earlier. It takes 48 hours for *P. diminuta* to form visible colonies and test procedures have been described that require up to 14 days for results to become available (HIMA 1982, ASTM F 838 1992). Clearly this method of testing, although presenting a rigorous "worst-case" challenge to the sterilising efficiency of the membrane, is disadvantaged by the time period required. There is need for a rapid test method to make the process of validating 0.2 μm rated membranes more efficient which would be a direct benefit to membrane manufacturers. Criteria for such a test to be readily accepted into routine use are described below:

<table>
<thead>
<tr>
<th>Test requirement</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Rapidity</td>
<td>To be of an advantage over current culture techniques, the test will be capable of confirming sterility within a few hours, or at least within one working day (taken as 8 hours).</td>
</tr>
<tr>
<td>(b) Sensitivity</td>
<td>The test will be capable of analysing an entire retention test permeate with a sensitivity of detection of 1 viable cfu.</td>
</tr>
<tr>
<td>(c) Specificity</td>
<td>Ideally, the test will be capable of identifying any bacteria detected as <em>P. diminuta</em> to eliminate erroneous test results due to contamination of the permeate.</td>
</tr>
<tr>
<td>(d) Skill</td>
<td>The test methodology should be straightforward and should not require an undue level of technical skill or involve an excessive amount of labour.</td>
</tr>
<tr>
<td>(e) Cost</td>
<td>The cost of carrying out the test on a routine basis, including initial instrumentation required and test reagents, should not be prohibitive.</td>
</tr>
</tbody>
</table>
Methods for the detection and enumeration of bacteria are described in the next section and are considered for the analysis of retention test permeates.

2.4. The enumeration of bacteria.

The methodology for the enumeration of bacteria can be classified into two groups, direct and indirect (Jarvis 1989). With a direct count the micro-organisms themselves are observed by methods such as microscopic examination or by allowing viable cells to grow into colonies visible with the naked eye. All indirect estimations are based on measurements of physical or chemical properties of cellular components or of cell metabolism such as adenosine triphosphate (ATP) luminescence, the Limulus amoebocyte lysate (LAL) test and electrometric methods.

The traditional method for the enumeration of viable bacteria is the plate count. The need for more rapid and less labour intensive procedures for the quantification of bacteria arose from the limitations of culture techniques. A small aliquot (usually less than 1 ml) of a sample is spread onto an agar plate or mixed with molten agar and plates are then incubated until colonies of bacteria are visible (Swanson et al 1992). The viable count of that sample is determined in cfu per ml using the average number of colonies for triplicate plates, taking into account the volume of the sample plated and the sample dilution factor. There are variations to improve the efficiency of the method, agar plates can be automatically poured, an automatic spiral plater can be used to inoculate plates with test samples and colonies can be automatically counted (Messer et al 1984). The time period required for the development of results however, cannot be decreased.

The plate count remains in many cases the most popular way to quantify viable bacteria, yeasts and moulds and the used of selective media allows tentative identification of the species. Methodology and the necessary instrumentation is technically straightforward. However, the plate count will not accurately determine the individual cell count. A colony could arise from an aggregate of any number of cells. Also, the method is not rapid due to the incubation period of one or more days needed for the colonies to become visible. Growth depends on the ability of the organism to replicate and form a colony on the agar medium and at the incubation temperature used. All colony count procedures are subject to various errors including those in sampling and dilution, pipetting, distribution, counting and recording colony numbers and errors of calculation (Jarvis 1989). Counting errors occur when plates become crowded and colonies overlap and it is recommended that only plates with between 25 and 250 colonies are counted (Tomasiewicz et al 1980). The quality of results is dependent upon the skill of the operator.
and the method is laborious due to the requirement for numerous replicate dilutions. The search to find an alternative method that is faster, more sensitive, accurate and convenient continues.

2.4.1. An overview of popular methods for the detection and enumeration of bacteria.

Membrane filtration and MPN estimations are not rapid techniques and have already been applied to bacterial retention tests (section 2.3.2.). The advantage of membrane filtration is that bacteria in the entire permeate can be captured on a single membrane and consequently the sensitivity of detection is high (theoretically one viable organism) (Sharpe and Peterkin 1988), although this relies upon the ability of the analytical membrane to retain *P. diminuta*. This method is useful when analysing aqueous samples that contain low numbers of bacteria and has been used to enumerate *Legionella* spp. in water (Smith *et al* 1993). Although sensitive, time is again needed for the development of visible colonies and errors arise through the number of bacteria forming single colonies and with colony crowding as with the plate count.

Multiple tube MPN estimations involve inoculating aliquots of 10-fold serial dilutions of the sample into replicate tubes (usually 3 or 5) of broth medium which are then incubated until bacterial growth is confirmed by the presence of turbidity (Harrigan and McCanna 1976, Barnard and McClure 1984). The number of tubes for each dilution that are positive for growth is noted. That particular combination of positives is referred to in a set of MPN index tables that give an estimate of the most probable number of viable bacteria per unit weight (or volume) of the sample, within certain confidence limits. The technique is useful where a quantitative estimate is needed for bacteria that are present in low numbers. The technique is simple yet results are again dependent on the time taken for visible growth to appear in all potentially positive tubes. The sample size is small, species identification is not possible and scope for error is high as the results are estimations only.

Several other popular methods for the quantification of bacteria are described. The main points making them unsuitable for the analysis of retention test permeates are stated.

Reduction-oxidation (redox) responsive organic dyes (usually indigo or indophenol derivatives) can be used as indicators of redox potential if the reduced and oxidised dye forms are coloured differently (Kroll 1989). A colour change can be elicited by the generation of reducing power primarily as reduced nicotinamide dinucleotide (NADH₂) by actively metabolising bacteria. The rate of reduction for such a dye is indicative only of the degree of metabolic activity in that sample, will depend on the species of bacteria.
present and should not be interpreted in terms of microbial numbers. Dye reduction tests using methylene blue or resazurin have been widely used by the dairy industry to rapidly determine the quality of raw milk (Luck 1982), however a 10 minute test will only specify samples with greater than $1 \times 10^6$ cfu per ml. Such tests are simple and quick to perform. However, due to the lack of sensitivity, accuracy and the small sample size needed dye reduction tests are not suitable for the analysis of retention test permeates.

The LAL test exploits the clotting reaction observed between the blood of the horseshoe crab *Limulus polyphemus* and endotoxins (pyrogens) shed from Gram-negative bacteria (Westphal 1975, Devleeshouwer *et al* 1985). Bacterial endotoxins are lipopolysaccharide moieties arising from the outer cell membrane that, for mammals, cause the release of endotoxins from white blood cells that travels to the hypothalamus to cause a rise in body temperature. The LAL test is often used in the pharmaceutical industry for the analysis of parenterals and is also used for the analysis of foods (Hansen *et al* 1982). Purified lysate prepared from amoebocytes from the crab haemolymph can be used to detect bacteria by positive gelation of the sample within 60 minutes by being mixed in equal proportions with the sample. Species identification of the detected bacteria is not possible. The standard gelation test has a sensitivity of $1 \times 10^2$ to $1 \times 10^3$ cfu per ml for various *Pseudomonas* species, although the general sensitivity has been increased 50 times by the introduction of a colorimetric assay using the chromogenic substrate p-nitroaniline that undergoes cleavage with a concomitant colour change (Jay 1989). This improved test delivers results in approximately 45 minutes. Although rapid and sensitive, disadvantages of this method include the small sample size required, the high cost of the amoebocyte lysate, the insufficient sensitivity of detection and the fact that it is very susceptible to extraneous contamination. Also, the test will detect endotoxin not only from non-viable cells, but also from cell wall fragments that will inevitably arise from the filtration of bacteria.

Species-specific nucleic acid probes directed to deoxyribonucleic acid (DNA) or RNA, often in combination with the polymerase chain reaction (PCR, a method where a piece of target DNA can replicated many thousands of times in order to increase the sensitivity of detection for this), have been used most successfully to detect and identify low numbers of bacteria (Steffan and Atlas 1988, Bej *et al* 1990, Dodd *et al* 1990, Koide *et al* 1993, Wegmüller *et al* 1993). The probe can be tailored for each application (Wolcott 1991, Grant and Kroll 1993) and although the test is sensitive, probes will detect nucleic acid from non-viable cells and cell fragments as described for the LAL test. Nucleic acid probe techniques can be rapid, sensitive and highly specific, but require expensive reagents, a certain amount of time for the development of the most suitable probe and the procedure demands a degree of specialised skill.

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Bacteria can be detected and identified by immunological techniques, the most popular of which being the sandwich enzyme-linked immunosorbent assay, or ELISA (Wyatt et al 1993) using either polyclonal or monoclonal antibodies (Köhler and Milstein 1975, Rose and Stringer 1989, Candlish 1991). The ELISA is a straightforward method once the antibodies have been raised and results are available in a few hours. Sensitivity of detection is usually around $1 \times 10^5$ cfu per ml but with preincubation (unfortunately detracting from the rapidity of the method) this can be as low as $5 \times 10^2$ per ml. Again, cell fragments can be detected rather than whole cells, only small samples can be analysed and production of the optimum antibody preparation can be a lengthy process.

In this study, ATP luminescence and electrometric microbiology (both indirect methods) and the Direct Epifluorescence Filter Technique (DEFT) methodologies will be applied to the rapid enumeration of *P. diminuta* the test permeates produced using the ASTM retention test procedure for 0.2 μm rated membranes (ASTM D 3862 1992). Each of these popular techniques has the advantage mainly in that large sample volumes can be analysed and that each method can be applied immediately to retention testing without major changes in methodology. Equipment and reagents are widely available commercially. The retention test procedures incorporating the rapid methods are described in section 3.4. and are compared and contrasted in section 5.3. according to all of the ideal test criteria detailed at the end of section 2.3.4..

### 2.4.2. ATP luminescence.

All living cells contain ATP. The light-producing enzymatic reaction between the luciferase enzyme from the firefly *Photinus pyralis* and the substrate luciferin is stoichiometric for ATP with a quantum yield of 0.88 (Gregg 1991). Strehler and Totter (1952) were the first to determine the linear relation between ATP and light output and to realise the potential of the firefly luciferin-luciferase system for the assay of ATP.

#### 2.4.2.1. Adenosine triphosphate.

ATP serves as the principal immediate donor of energy in biological systems rather than a long-term storage form of energy (Stryer 1988). ATP is a nucleotide (unit composed of a nitrogenous base, a sugar and one or more phosphate groups) consisting of adenine, ribose and triphosphate and is shown in figure 2.4.a. It is able to act as an energy donor due to the large amount of energy liberated with hydrolysis of each of the phosphoanhydride bonds in the triphosphate unit. Energy from the hydrolysis of ATP is used to drive reactions that require an input of energy such as active transport whilst ATP can be reformed from ADP and phosphate by the energy released by glycolysis.
Hydrolysis of ATP produces adenosine diphosphate (ADP) and orthophosphate (P\textsubscript{i}) or adenosine monophosphate (AMP) and pyrophosphate (PP\textsubscript{i}) as shown in eqn.2a. and eqn.2b. where \( \Delta G^{\circ} \) is the Gibbs free energy change at pH 7.0 where all the reactants are present at a concentration of 1.0 M.

\[
\begin{align*}
ATP + H_2O & = ADP + P_i + H^+ \quad \Delta G^{\circ} = -7.3 \text{ kcal/mol} \quad \ldots(2a) \\
ATP + H_2O & = AMP + PP_i + H^+ \quad \Delta G^{\circ} = -7.3 \text{ kcal/mol} \quad \ldots(2b)
\end{align*}
\]

ATP can be regenerated from ADP and P\textsubscript{i} by the enzyme adenylate kinase. Measurements of ATP are a good indication for viable microbial numbers since the cellular level of ATP will remain approximately constant under defined conditions due to the role of ATP in the energy status of the cell (Stanley 1989). ATP levels change during cell division however, any sample of bacteria will contain cells dividing in an asynchronous manner and consequently it is always the average level of ATP for a sample of cells that is measured. Bacteria deprived of oxygen will show a decrease in ATP levels that can be increased simply by resupplying air and maybe adding glucose. A change in nutrient source or concentration, temperature, pH and a change in light intensity for photosynthetic organisms will all alter the intracellular ATP level (Stanley 1986). Provided experimental conditions are regulated then fluctuation of ATP levels should not be a problem.

2.4.2.2. Firefly luciferase.

The reaction between the enzyme luciferase (E) and luciferin (L) takes place in the tail (lantern) of the firefly in two stages (eqn.3. and eqn.4.) and requires magnesium and oxygen (Neufeld et al 1985, Stannard 1989).

\[
\begin{align*}
LH_2 + ATP + E & \xrightarrow{Mg^{2+}} E - LH_2 - AMP + PP_i \\
E - LH_2 - AMP + O_2 & \rightarrow (E - L - AMP)^+ + H_2O \\
\downarrow & \\
(E - L - AMP)^+ + hf(\lambda = 562nm) & \quad \ldots(4)
\end{align*}
\]

The structures of oxidised and reduced luciferase are shown in figure 2.4.b.. The electronically excited intermediary complex of luciferase-luciferin-AMP (eqn.4., represented by E-L-AMP\textsuperscript{+}) returns to the ground state by the emission of a photon of
Figure 2.4.a. The structure of ATP.

* = phosphoanhydride bond

Figure 2.4.b. The structure of firefly luciferase.

(a) Reduced

(b) Oxidised
light to give luminescence that is yellow-green in colour. The reaction is affected by interfering (or quenching) factors that cause a reduction in measureable photons. Turbidity and colour absorb photons to cause optical quenching whilst enzyme inhibitors and high ion concentrations (over 0.1 M) produce chemical quenching. The optimal pH for the reaction is 7.75 and deviations from this affect the efficiency of the reaction (Vanstaen 1980).

2.4.2.3. ATP measurements as a rapid method for the enumeration of bacteria.

Traditional culture methods for enumerating bacteria rely on the amplification of cell numbers by growth and division to give a visible result. The analysis of ATP is a rapid method for quantifying viable bacteria and can give results in a matter of minutes rather than days. There is a disadvantage in that the source of the ATP can not be determined, there can be no discrimination between species of bacteria or between bacterial ATP and extraneous ATP. The technique is most efficient where a pure bacterial culture is used without a source of non-microbial ATP. When the sample contains eukaryotic somatic cells (for example if the bacterial content of meat is being assayed) an agent that lyses these cells only is used first. It is possible to differentially lyse eukaryotic and bacterial cells due to the more robust nature of microbial cell walls and membranes (Stanley 1989). The nonmicrobial ATP is extracted and hydrolysed with an ATP-ase enzyme or is removed by centrifugation or filtration. A nonionic detergent such as Triton X-100 (octylphenoxypolyethoxyethanol, Rohn and Haas USA) is often used as such an extractant. The microbial cells are then specifically lysed and luminescence is quantified after the addition of a luciferin-luciferase preparation as usual.

The amount of ATP present in a single average bacterial cell of a certain species can be calculated by first determining the correlation between emitted light and a dilution series of ATP. The amount of ATP per bacterium can then be calculated by measuring light emitted from a dilution series of that species for which the viable cell count is also determined. The viable count for a sample containing an unknown number of bacteria can then be determined by measuring the ATP content of the cell suspension per unit volume and dividing this by the calculated quantity of ATP per cell (Vanstaen 1980).

2.4.2.4. Bacterial ATP extractants.

It is important to use an agent to lyse bacteria that will not only give the maximum release of ATP but one that will not interfere with the luciferase reaction (Jago and Sidorowicz 1989, Simpson and Hammond 1989). A reliable extractant fulfils three requirements (Lundin 1984):
(a) to release the entire intracellular pool of the metabolite.
(b) to give complete, rapid and irreversible inactivation of all the enzyme systems in the extracted cells that may affect the metabolite levels during extraction, storage or analysis of extracts.
(c) the extractant must not inactivate the metabolite or the enzyme system involved in the analysis.

For example, trichloroacetic acid (TCA) or dimethylsulphoxide (DMSO) are good extractants for bacterial ATP but the sample needs to be diluted before the assay as they both will reduce the activity of luciferase. There are four classes of bacterial ATP extractant:

(a) organic compounds such as ethanol, acetone and DMSO.
(b) acids such as TCA, sulphuric and nitric acids.
(c) boiling buffers and steam.
(d) various cationic detergents.

and it is best to compare the efficiency of a variety of extractants for a new application (Stanley 1989).

2.4.2.5. Instrumentation.

Photons emitted during bioluminescence are reflected to gather upon a photomultiplier to ensure detection. The entire instrument is enclosed in a light-tight case to ensure the only photons detected are those emitted from the reaction mixture. Many commercial luminometers have an automatic time controlled reagent injection system to allow the sequential analysis of 50 or more samples (Berthold 1991). This is beneficial as sample volume is usually less than 1 ml. Most instruments can be linked to a computer to permit automated data collection and processing. It is generally the efficiency of the photomultiplier that dictates the sensitivity for detecting the photons and consequently the ability to detect small numbers of bacteria. The performance of several commercial luminometers has been studied (Jago et al. 1989a). The sensitivity of detection is usually approximately 1 x 10^3 cfu (Stanley and McCarthy 1989, Stannard 1989) assuming that one bacterial cell contains one femtogram (fg) of ATP (Vanstaen 1980, Wood and Gibbs 1982).

2.4.2.6. Applications.

ATP luminescence has been used to determine bacterial numbers in a large variety of foods and beverages including beer (Avis and Smith 1989), milk (Griffiths and Phillips 1989) and other dairy products, fish (Ward et al. 1986) meat (Eriksen and Olsen 1989), mineral water (Baumgart et al. 1980) and fruit juice (Vanstaen 1980). The enumeration
of yeasts (that typically contain twenty times more ATP per cell than bacteria) can also be carried out (Wooldridge 1989). During the period 1980-1989 the detection of bacteriuria became the major clinical application of ATP assays (Lundin 1989, Gregg 1991) which were also used to detect bacteremia (Nilsson et al 1989). The effect of biocides on microbial growth can be monitored using ATP luminescence (Denyer 1989) and detection of bacterial contamination in intravenous fluids with an ATP assay has been described (Bopp and Wachsmuth 1981). ATP luminescence has been used to assess and monitor the number of micro-organisms and their activity in samples of water, soils, sediments and waste material in a shorter time than would be possible using traditional culture techniques (Van der Werf and Verstraete 1984). Hygiene monitoring in the food manufacturing industry can be implemented by ATP measurements where the maximum permissible level of ATP on a surface after cleaning is referred to as the acceptance threshold (Slater 1992). The structural gene (luc) for firefly luciferase has been cloned into *Escherichia coli* and with the addition of luciferin, light output has been used to monitor viable cell density in batch fermentations (Lasko and Wang 1992).

### 2.4.3. Electrometric microbiology.

The growth and multiplication of bacteria causes uncharged or weakly charged substrates such as proteins, carbohydrates and lipids to be metabolised into more highly charged end-products such as amino acids, lactate and acetate (Firstenberg-Eden and Eden 1984). The increase in the number of charged moieties in the growth medium produces a concomitant measurable change in certain electrical properties such as impedance, conductance or capacitance. The length of time taken for this change to reach a threshold level (equivalent to $1 \times 10^6$ cfu per ml) is inversely related to the initial number of viable bacteria present in the assayed sample (Easter and Gibson 1989). This time period is called the detection time (DT) (*figure 2.4.c.*) and by constructing a calibration curve of DT versus cfu per ml for known numbers of bacteria the viable count for a unknown sample can be determined in a matter of hours. A large number of samples can be processed concurrently and the most accurate results are obtained when pure cultures are assayed.

#### 2.4.3.1. Elementary electrical theory.

When an electrical field is put across an electrolyte solution such as broth medium, cations will migrate towards the cathode and anions towards the anode (Firstenburg-Eden and Eden 1984). This movement of ions produces the flow of current (I) measured in amperes within the medium. The flow of electrical current always encounters resistance
(R) resulting in a drop in potential difference (V) measured in volts and loss of electrical energy as heat. Thus electrical resistance measured in Ohms is defined by (eqn.5.):

\[ R = \frac{V}{I} \]  

...(5)

This assumes Ohms' Law which states that the current for an ohmic conductor (one that obeys Ohms' law) is proportional to the potential difference across it provided here is no change in the physical conditions such as temperature (Muncaster 1982). Conductance (G) measured in seimens is defined as the reciprocal of resistance (eqn.6.):

\[ G = \frac{1}{R} \]  

...(6)

Capacitance is the ability to store energy in an electrical field without dissipation. Ideally, a capacitor consists of a pair of conducting surfaces separated by an insulator, or dielectric. The measure of the extent to which a capacitor can store charge is designated the capacitance (C) (eqn.7.) measured in microfarads:

\[ C = \frac{Q}{V} \]  

...(7)

where Q is the magnitude of charge in coulombs on either plate and V is the potential difference between them. When alternating or time-varying current (AC) is applied, a current is produced proportional to the rate of voltage change (eqn.8.):

\[ I = C \frac{dv}{dt} \]  

...(8)

For two metal electrodes immersed in a conductive medium (an electrochemical well) each electrode-solution interface can be represented by a capacitor and the bulk of the medium by a conductive element (Firstenberg-Eden 1986). A schematic electrochemical well can be seen in figure 2.4. When an AC potential is applied to this system the resulting current will depend on the resistance to this AC flow termed impedance (Z) measured in Ohms which is a function of resistance, capacitance and the applied frequency in Hertz (eqn.9. and eqn.10.).

\[ Z = \sqrt{R^2 + \left(\frac{1}{2\pi fC}\right)^2} \]  

...(9)

or:

\[ Z = \sqrt{\left(\frac{1}{G}\right)^2 + \left(\frac{1}{2\pi fC}\right)^2} \]  

...(10)
An increase in conductance and/or capacitance will give a decrease of the impedance and an increase in current. Conductance will increase if the number of ions present increases, for example a non-ionised molecule of glucose is metabolised to two lactate ions which can be further metabolised with the addition of oxygen to carbonic acid. The number of ions has increased and the resulting smaller bicarbonate ions are more mobile and consequently better at conducting electricity than lactate ions. Even if the total charge remains the same, the charge carriers become more mobile and the conductance will increase.

Conductance measurements are representative of changes taking place in the bulk electrolyte solution, whilst the capacitative element is represented by those changes taking place around the electrodes themselves. Ions in the electrolyte next to an electrode will form a charged layer termed electrode polarisation. The arrangement resembles a capacitor where the layers of charge form the plates (figure 2.4.d). The effect of ions in solution outside the polarisation layer on this capacitance is negligible. Changes in the number, size and nature of the ions will affect the measurable capacitance. For example an increase in number will increase capacitance as will the presence of smaller ions that increase the concentration of charged particles and the effective surface area of the plate.

Metabolising bacteria consequently cause an increase in conductance and capacitance with an associated decrease in impedance. It is customary to monitor the inverse of impedance, the admittance (Y) (eqn.11.) in seimens when observing microbial growth as it is believed to be conventional for microbiologists to study an element that increases with time rather than decreases.

\[ Y = \frac{1}{Z} \]  

**(2.4.3.2. Test parameters.**)

It is important to be able to determine the threshold detection time as accurately as possible. Here, the electrical properties change rapidly and the increase in number of ions produced by bacterial metabolism is equal to the original level in the medium. A pure culture will always give the best curve. A good curve showing an increase in electrical property (signal) with time will indicate detection time as a sharp deflection from the baseline (figure 2.4.c.) (Firstenberg-Eden 1986). Some curves are compared in figure 2.4.e. and it can be seen that an irregular curve, or one with more than one deflection point, will give an inexact value for detection time.
Figure 2.4.c. Plot of electrical signal with time (from Firstenberg-Eden 1986).

Parameter definitions associated with signal curves.

Figure 2.4.d. Schematic representation of an electrochemical well (from Firstenberg-Eden and Eden 1984).

C represents the capacitative element associated with changes near the electrodes and G represents the conductive element associated with changes in the bulk ionic medium.
Figure 2.4.e. Comparison of signal curves (from Firstenberg-Eden & Eden 1984).

A good curve (centre) is compared to four curves in which it may be difficult to determine the DT. The graph axes are the same as in figure 2.4.c..

Figure 2.4.f. A sample calibration curve.

PRODUCT CODE: MILK35
* SAMPLES: 135
SPECIFIED CFU/ML: 0.10E+06
MULT. CORR.: 0.96
LOG CFU = 0.0419T2 - 1.06T + 8.77
CUTOFF TIME: 3.58
CAUTION TIME: 4.99
It has been found experimentally (Richards et al 1978) that capacitance was subject to fluctuations that did not correlate well with any measured variable, such as temperature, and was regarded as an unreliable method for observing bacterial growth. Impedance includes a capacitative element as described (eqn.9.) but is a more reliable signal to monitor due to the presence of the conductive factor. Conductance is often looked upon as being the signal of choice. Optimal conditions vary for each application and it is known that medium composition (Owens 1985), temperature, pH and the species of bacteria present will all contribute to the quality of curve.

2.4.3.3. Calibration curve.

The calibration curve is constructed using optimum test conditions by plotting the detection time versus \(10^{\log_{10} cfu\ per\ ml}\) (determined by the plate count) for a number of dilutions of the required species of bacteria. A sample calibration curve can be seen in figure 2.4f. Detection time and bacterial load are inversely related and the calibration curve has a negative slope. Regression analysis of the data produces a line equation and a correlation coefficient. A coefficient of -1.00 would indicate a calibration where all points lie on the same line with no scatter. Test conditions are optimised to give a coefficient as close to -1.00 as possible. It is recommended that to generate a reliable calibration curve, that at least 50 points should be used to construct the curve that represent samples with bacterial loads that cover 4-5 log. cycles. The line equation generated can be used to determine the number of bacteria per ml for unknown test samples.

2.4.3.4. Instrumentation.

The first electrical measurements of microbial growth were in 1898 where the increase in conductivity of putrefying blood was observed. This finding was verified in 1912, and in studies between 1926-1929 conductivity increases were correlated with ammonia production for Clostridia spp. inoculated into different broths (Hadley and Yaiko 1985). It was shown in 1938 that bacterial proteolysis was closely related to conductimetric measurements and in 1958 the relationship between acid production in Streptococcus lactis and culture conductivity was established. However, it was not until the 1970’s that such electrical measurements were considered as a useful tool in microbiology to rapidly enumerate bacteria or determine sterility and instrumentation began to be developed (Ur and Brown 1975). Practical components for such a growth analyser are a thermostatically controlled incubation unit, electrochemical wells for the samples (these vessels could be bottles or multi-well modules with electrodes), a computer to carry out the data manipulation and to control the incubator, a monitor to
display data and a printer. Commercial examples are the Bactometer Microbial Monitoring System, the Malthus Microbiological Growth Analyser and the Rapid Automated Bacterial Impedance Technique (RABIT).

2.4.3.5. Applications.

Electrometric measurements can be used to ascertain the microbiological load and hence the potential storage life of foods and beverages (Eckett 1986, Williams et al 1992). Using the Bactometer 64 model, a certain number of bacteria per ml of sample can be chosen as being acceptable for each application and the detection time corresponding to this can be designated the cut-off time. Samples with a higher bacterial load and a shorter DT than the cut-off are classed as unacceptable and results for such samples are displayed in red. A caution time can also be chosen whereby DT's longer than this are satisfactory and displayed in green, whilst results between the cut-off and caution times are regarded as marginal and displayed in yellow.

Electrical measurements have been used to determine bacterial numbers in many foods (Powell 1990) including raw milk (Firstenberg-Eden and Tricarico 1983), ground beef (Kahn and Firstenberg-Eden 1985), water (Irving et al 1989) and frozen vegetables and fish (Van Spreekens and Stekelenburg 1986) usually within a day. The use of selective media will enable the growth of specific bacteria or yeasts to be observed such as coliforms from meat (Firstenberg-Eden and Klein 1983) or salmonellae (Easter and Gibson 1989, Blackburn 1991). Growth media (which may be broth or agar) can be tailored for each new application to give the best signal curve and thus the most accurate calibration. Preincubation of the sample may increase the sensitivity of the method for which detection levels can be $1 \times 10^5$ cfu per ml. The efficiency of antimicrobials in hand lotion and other cosmetics has been determined impedimetrically (DePasquale et al 1985) and antibiotics can be detected in milk (Okigbo and Richardson 1985). Sterility tests usually take a longer time than estimation of bacterial numbers but are still often more rapid than traditional methods. Clinical applications include screening blood and urine for bacteremia and bacteriuria (Firstenberg-Eden and Eden 1984).

2.4.4. The DEFT.

The DEFT has the advantages of direct microscopic observation in that it is very rapid and some identification of the bacteria seen can be made. The technique was developed to rapidly quantify bacteria in raw milk before acceptance by the dairies and creameries (Pettipher 1983). The milk sample is filtered under vacuum through a track-etched membrane which is then stained with a highly specific fluorescent dye
(acridine orange) and viewed using an epifluorescence microscope. The number of fluorescing bacteria per field are counted and the total or viable bacterial load per ml of filtered sample can be determined in under an hour.

A high level of operator fatigue is associated with manual counting and only 30-40 slides per day can be inspected in this way. For semi-automated counting of DEFT slides, the epifluorescence microscope is fitted with a closed-circuit television camera linked to a television image analyser (Pettipher and Rodrigues 1982). The fluorescing bacteria are detected by virtue of their grey level (contrast) and the sensitivity of detection can be set accordingly. A microprocessor will perform the calculations to determine bacterial load per ml of sample. Up to 50 slides per hour can be analysed by this method and the image analyser can be controlled by a computer to manipulate and store data. The membrane staining, rinsing, drying and counting has been fully automated in a recent piece of equipment named COBRA (Pettipher et al 1992).

2.4.4.1. Fluorescent dyes.

Fluorescence occurs when a fluorophore molecule is electronically excited by the absorption of light energy and returns to a more stable lower state by the emission of light energy of a longer wavelength (Laidler and Meiser 1982). This process is very fast and occurs within $1 \times 10^9$ to $1 \times 10^4$ seconds and the change in wavelength is known as the Stokes' shift. Fluorescence is best brought about by illumination with light of a wavelength close to that of the peak of the absorption (excitation) spectrum for that fluorophore (figure 2.4.g.). The excitation spectrum is a plot of the total intensity of fluorescence when the specimen is irradiated with light of certain wavelength, whilst the emission spectrum shows the fluorescence intensity that results from excitation at certain wavelengths (Ploem and Tanke 1987). The two spectra usually overlap. If the fluorophore is pure then the wavelength of emitted light is independent of that used for excitation.

Fluorescent dyes used as stains are called fluorochromes as opposed to non-fluorescent stains or diachromes (Pearse 1972). Much smaller amounts of fluorochromes can be detected compared to diachromes, they are more specific in their staining and are more suitable for the visualisation and detection of micro-organisms. Acridine orange was found to give the brightest staining of micro-organisms. Some biological substances (such as collagen, elastin and vitamin A) fluoresce under light of a short wavelength without need of a stain and such fluorescence is known as primary or autofluorescence. The light emission from fluorochromes is termed secondary fluorescence.
Figure 2.4.g. A typical fluorophore excitation/emission spectrum (from Ploem & Tanke 1987).

Figure 2.4.h. Acridine orange.

Figure 2.4.i. Schematic arrangement of an epifluorescence microscope (from Pettipher 1983).

\[ \text{Excitation peak} \]
\[ \text{Emission peak} \]

\[ \text{rel. intensity} \]
\[ \text{Wavelength} \]

\( (\text{CH}_3)_2 \)

\( (\text{CH}_3)_2 \)

\( \text{DS Dichromatic splitter} \)
\( \text{EF Excitation filter} \)
\( \text{HL Light source} \)
\( \text{OBJ Objective} \)
\( \text{OC Eyepiece} \)
\( \text{SF Secondary filter} \)
2.4.4.2. Acridine orange.

Acridine orange (3,6-bis(dimethylamino acridinium hydrochloride hydrate, molecular weight 267) is a cationic acridine dye seen as a burnt orange coloured powder with a mean exciting wavelength of 470 nm (figure 2.4.h.) (Gurr 1971, Green 1990). Although the exact binding mechanism for this dye is not determined, it is agreed that the dye binds to nucleic acids in two ways (Pettipher 1983):

(a) by electrostatic binding between the basic dye and the acidic phosphate groups of an RNA polynucleotide chain.

(b) by intercalation between the neighbouring base pairs of the two polynucleotide stands in DNA.

Acridine orange exhibits metachromasia, a phenomenon where upon polymerisation of the dye molecules the wavelength of the emitted light lengthens and the colour of fluorescence changes. Metachromasia is thus indicative of a high dye concentration. When acridine orange binds to double-stranded DNA, the helical structure distorts to allow the dye molecules to be intercalated approximately every third base pair. The dye molecules are not brought close enough for polymerisation interactions and fluorescence is green in colour (525 nm) or orthochromatic. If instead acridine orange binds to the phosphate groups in single-stranded RNA then the structure is flexible enough to bring dye molecules close enough for polymerisation to occur. Fluorescence is red/orange in colour (650 nm) or metachromatic (Pearse 1972). For the DEFT, viable bacteria are seen to fluoresce red/orange (as the metachromatic fluorescence produced by the RNA tends to mask the orthochromatic fluorescence by the DNA) and isolated somatic nuclei surviving the enzyme/surfactant pretreatment fluoresce green.

2.4.4.3. Epifluorescent microscopy.

A schematic representation of an epifluorescent microscope is shown in figure 2.4.i. The exciting light is directed incident to the sample rather than through it (Ploem and Tanke 1987). Tungsten halogen lamps, high pressure mercury lamps and high pressure xenon lamps are suitable light sources and the choice of which depends on the excitation spectrum of the fluorochrome to be used. High pressure mercury lamps emit strongest in the region between 300 to 700 nm and are recommended if UV or general high energy excitation light is required. The exciting light is reflected to the sample and the emitted fluorescence is transmitted to the eyepiece by a chromatic beam splitter. These mirrors have an interference coating that will reflect light of a shorter wavelength than a certain value and transmit light of a longer wavelength. Since emitted light is of
a longer wavelength than exciting light the two can be separated. This technique of epi-illumination can be used with thick or opaque samples to give images of an even intensity and consequently is suited for the observation of membranes.

2.4.4.4. Development of the DEFT.

At the time of the DEFT development the number of bacteria in milk could only be determined by plate count methods, by microscopic examination or by dye reduction tests (section 2.4.1.). Four grades of milk are recognised and the farmer receives payment accordingly. Demand was for a rapid detection and quantification test for bacteria in raw milk that could be carried out by the processor looking at milk supplies and also in central milk testing labs (Pettipher 1983).

Bacteria in milk were first enumerated by microscopic examination of methylene blue stained films on glass slides and the sensitivity of the technique was improved by filtering the sample through a membrane to concentrate it (Erhlich 1955, Merrill 1963). Methylene blue only stained cells faintly especially if they had been heat treated and was replaced by highly specific fluorescent dyes that did not stain debris (Scholefield 1978). Direct microscopic examination has the advantages of allowing tentative identification of the bacteria on the basis of morphology, of being rapid and that the slides can be kept as a record. In reponse to the need expressed by the Scientific Consultative Panel of the Dairy Trade Federation for a rapid, sensitive, precise, convenient and economical test for quantifying bacteria in raw milk the project that led to the development of DEFT was set up at the National Institute for Research in Dairying at Reading in 1975 (Pettipher 1983).

2.4.4.5. Applications.

The DEFT has been used to enumerate bacteria in many different environments than the raw milk for which it was developed. It is important when applying the method to a new field for the first time that a good correlation between the DEFT count and the standard plate count is achieved and that enough of the test sample is filtered to attain adequate sensitivity (Pettipher 1989).

Various milks and milk products can be assayed using the DEFT (sometimes an increase in the volume of surfactant used is necessary for viscous samples such as cream or butter) (Pettipher 1983) as well as other food stuffs such as fish, minced pork, beef and bacon, frozen and fresh meats, poultry and comminuted meats (Shaw and Farr 1989), mayonnaise salads, vegetables and spices. For beverages, a technique resembling DEFT
was originally used to enumerate bacteria and yeasts in wines (Cootes and Johnston 1980) using a different fluorochrome similar to acridine orange (euchrysine 2GNX) and sensitivity was reported to be one viable bacterium per ml.

The DEFT has been applied to the assessment of urine samples for bacteriuria (Fernández 1994) and the microbiological analysis of intravenous fluids (Denyer and Ward 1983, Denyer et al 1989). An earlier method similar to DEFT using acridine orange and epifluorescent microscopy was used to observe freshwater bacteria (Jones and Simon 1975), whilst a similar technique using the stain DAPI (4,6-diamidino-2-phenylindole) and a 0.015 μm rated track-etched membrane was used to evaluate the abundance of viruses in sea water (Hara et al 1991). Yeasts can also be enumerated with DEFT (Pettipher 1987), an application of interest to those food and beverage industries that use sugar concentrates as contaminating yeasts surviving pasteurisation and processing result in spoilage. Inconsistencies have been reported for acridine orange staining of heat-treated cells (Betts et al 1988) and a modified aniline blue fluorochromic staining system (Viablue) has been tried instead with clear differentiation between viable and non-viable cells (Hutcheson et al 1988).

2.5. Bacterial bioluminescence.

The production of a light-emitting strain of P. diminuta by cloning the necessary genes from naturally occurring bioluminescent bacteria will create a simple and straightforward means of detecting viable bacteria in the test permeates of 0.2 μm rated membranes. The biochemistry and genetics for in vivo bacterial bioluminescence is described in detail and there is a summary of applications for recombinant bioluminescent bacteria.

Bacterial bioluminescence is an enzyme catalysed redox reaction involving the oxidation of long-chain aldehyde and FMNH₂ by molecular oxygen (Meighen 1988). Light-emitting species arise from a diverse range of organisms and include dinoflagellates, fungi, insects, shrimp and squid. Bioluminescent bacteria are by far the most abundant and widely distributed and are found in marine, freshwater and terrestrial environments although most bioluminescent bacteria originate from marine environments. Bioluminescent species have been classified into the genera Vibrio, Photobacterium, Alteromonas and Xenorhabdus, and are classed as motile, Gram-negative rods. Vibrio harveyi, Vibrio fischeri, Photobacterium phosphoreum, Photobacterium legionathi and Xenorhabdus luminescens have been studied in the greatest detail. Xenorhabdus spp. are the only species that can infect terrestrial organisms and most bioluminescent bacteria are associated with specific organs of luminescent
fish. The majority of marine fish are luminescent and suggested advantages for the relationship between the fish and such bacteria are the attraction of prey, diversion of predators and communication whilst the advantage to the bacteria remains unknown.

2.5.1. The bioluminescent reaction.

Bioluminescent enzymes are called luciferases which vary in nature between the different bioluminescent organisms. Luciferases of all bacterial species are heterodimeric enzymes of 77 kilodalton (kd) consisting of α and β subunits of 40 and 37 kd respectively (Hastings 1986). The two subunits are homologous with only one active site per dimer thus as a consequence individual subunits have no luciferase activity. The active site has been shown to be situated primarily on the α sub-unit although the β sub-unit is still necessary for light production. The luminescent reaction shown in eqn.12, is a mixed function oxidation of reduced flavin mononucleotide (FMNH₂) and a long chain aldehyde (R represents the aldehyde hydrocarbon chain) such as decyl aldehyde (decanal, C₁₀H₂₂O) by molecular oxygen (O₂). The return of the excited intermediary species flavin-4a-hydroxide to the ground state is accompanied by the emission of blue-green light (Baker et al 1992) that can be quantified using a photometer. The bacterial luminescent system is a branch of the electron transport pathway where electrons from reduced substrates are diverted to O₂ via FMN reductase and luciferase (Guerrero and Makemson 1989).

\[
\text{FMNH}_2 + RCHO + O_2 \xrightarrow{\text{Luciferase}} \text{FMN} + RCOOH + H_2O + hf(\lambda = 490\text{nm}). \quad \ldots(12)
\]

The reaction is specific for FMNH₂ (Meighen 1991). The specificity for aldehyde does vary among light-emitting species although decanal gives the brightest luminescence with Ph. phosphoreum, Ph. legionath, and V. fischeri luciferases and is known to cross the cell membrane much more readily than longer chain aldehydes.

2.5.2. Molecular biology of bacterial bioluminescence.

The α and β subunits of bacterial luciferase are encoded on adjacent genes, luxA and luxB respectively, that appear to have arisen by gene duplication as there is approximately 30% identity in amino acid sequence between α and β subunits of all bacterial luciferases (Meighen 1991). Both genes are approximately 1 kilobase-pairs (kb) in length and are transcribed in the same direction. Also present in the lux operon
are the genes *luxC*, *luxD* and *luxE* that encode the reductase, the transferase and the synthetase respectively for the multi-enzyme fatty acid reductase complex responsible for *in vivo* aldehyde synthesis in bioluminescent bacteria. The synthesis of aldehyde from fatty acid is shown in eqn 13:

\[
\text{RCOOH} + \text{ATP} + \text{NADPH} \rightarrow \text{NADP} + \text{AMP} + \text{PP} + \text{RCHO}
\]  

The function of the flavoprotein of most *Photobacterium* species encoded by *luxF* is unknown although it seems to have arisen through gene duplication due to some amino acid homology with the luciferase subunits. The order of the genes encoding the luciferase and the fatty acid reductase, *luxCDAB(F)E*, is the same in *lux* operons from all bioluminescent species. *LuxG* (present in all bioluminescent strains except *Xenorhabdus* spp.) and *luxH* (present only in *V. harveyi*) encode proteins of unknown function.

For a broth culture of a wild type bioluminescent strain, the development of light emission lags behind growth and maximum luminescence is usually seen just before a broth culture of the luminescent species enters stationary phase (Hastings *et al* 1978). This is due to the presence of autoinducer produced and excreted into the medium by the bacteria (Cao and Meighen 1993). Autoinducers for *V. fischeri* and *V. harveyi* are known to be composed of different fatty acid moieties. These control expression of the *luxAB* and *luxCDE* genes and are initially synthesised at a low rate. A threshold concentration is reached when they bind to a receptor to activate transcription of the *lux* operon. The proposed regulatory genes *luxI* and *luxR* identified for *V. fischeri* are located upstream from the *luxC* gene. The *luxI* gene product is believed to be responsible for autoinducer synthesis whilst the *luxR* gene product is believed to be the autoinducer receptor. Genes homologous to the *luxIR* genes of *V. fischeri* have not been found in *V. harveyi* where genes called *luxLMN* involved with the production of and response to the autoinducer molecule have been identified (Bassler *et al* 1993).

### 2.5.3. Applications.

The genes from the *lux* operon can be cloned into a suitable plasmid vector which is introduced into the recipient strain by transformation, conjugation and more rarely by transduction for expression in phenotypically dark species (Meighen 1991). To increase the stability of the recombinant organism the *lux* genes can be integrated into the bacterial genome (*section 5.5.*). The structural genes for bacterial luciferase have also been introduced into a plant expression vector and transferred into tobacco and carrot cells (Koncz *et al* 1987). Most bacterial species have sufficient intracellular levels of FMNH₂
and O$_2$ for $luxAB$ recombinant strains to emit light when supplied with aldehyde substrate. However, the addition of exogenous aldehyde is not necessary if the $luxCDE$ genes are also cloned to permit the endogenous synthesis of aldehyde from fatty acids. Genes from the $lux$ operons of $V. fischeri$ and $V. harveyi$ were the first to be cloned and most vector constructs include $lux$ genes from these species (Stewart and Williams 1992).

$Lux$ genes have been cloned into many different species of bacteria and the widest application for in vivo bioluminescence is in the field of molecular biology where it is used as a reporter gene in the study of gene expression (Nordeen 1988, Olsson et al 1988, Schauer et al 1988, Hill et al 1993). The luciferase system allows a simple, rapid, non-destructive and sensitive method for monitoring transcriptional activity. A bioluminescent strain of $Pseudomonas syringae$ has been used to study habitat-dependent gene expression. The light emitting bacteria were detected in situ by overlaying infected leaves with X-ray film (Cirvilleri and Lindow 1993). The behaviour of a bioluminescent strain of $Xanthomonas campestris$ released onto cabbage plants and soil in the environment (Shaw et al 1992), and of bioluminescent $P. syringae$ released into soil (Silcock et al 1992) has also been studied.

Wild-type or recombinant bioluminescent bacteria can be used to detect and quantify levels of growth inhibiting agents such as antibiotics, heavy metals, disinfectants and detergents, as light production is dependent on a continuing supply of FMNH$_2$ and thus on cell viability (Dodd et al 1990, Jassim et al 1990, Molders 1990, Stewart and Williams 1992, Andrew and Roberts 1993). $Lux$ genes have also been cloned into $Lactobacillus casei$ and $Streptococcus lactis$ for the detection of antibacterials in lactic acid starter cultures (Ahmad and Stewart 1991) and a bioluminescent strain of $Salmonella typhimurium$ has been used to study recovery of cells from freeze injury (Ellison et al 1991). The dependence of the bioluminescent reaction on oxygen has been exploited to develop a sensitive probe for this based on $V. fischeri$ covered by a gas permeable membrane (Lloyd et al 1981).

A novel concept for the detection of bacteria was introduced by Ulitzur and Kuhn (1987). Phenotypically dark bacteria could be detected rapidly by the introduction of $lux$ genes by bacteriophage. Phage reproduce by adsorption and injection of DNA into a specific host bacterium whose intracellular biochemistry is used to propagate the viral nucleic acid. The host range specificity of phage may be employed to present a sensitive detection assay for a particular species of bacteria in a mixed population. The phage carrying the $lux$ genes are dark as they lack the biochemistry for light production. However, luciferase activity can be detected within a few hours from the initial infection.
of the host bacterium. Phage containing luxAB genes have been used to detect enteric bacteria present at levels of $1 \times 10^4/g$ as early as 50 minutes post-infection and as few as ten E. coli cells can be detected after 100 minutes enrichment (Kodikara et al 1991).

The development of a bioluminescent strain of P. diminuta by cloning in the luxAB genes will enable the rapid detection of bacteria in test permeates upon the addition of aldehyde. The validation of 0.2 μm rated membranes will be carried out simply, sensitively and within real time. Other advantages for the proposed test are that detection will be specific for the bioluminescent P. diminuta. There will be no false negative retention test results due to contamination of the permeate. Viable cells only will be detected compared to ELISA and DNA probe methods (section 2.4.1) which may detect material from non-viable cells or cell fragments. By also cloning the luxCDE genes necessary for the synthesis of endogenous aldehyde, the need to add any exogenous reagent is circumvented providing that the intercellular production of large amounts of aldehyde does not prove toxic to the cell. A plasmid vector is needed that permits the stable inheritance and maintenance of lux genes in P. diminuta. Sufficient expression of the cloned genes is also needed to enable the sensitive detection of bioluminescent cells. These two areas present the most important challenges to the development of the required strain of P. diminuta.

For all recombinant micro-organisms used in biotechnology, genetic stability is an essential requirement before the strain can be used for large scale applications (Fleer 1993). Fleer described stability as the ability of the organism to constantly maintain an artificially introduced genetic trait during the cell lifetime. The instability of a recombinant system may result from segregational instability concerned with the transfer of genes from the mother to the daughter cell during cell multiplication, also a fluctuation in gene copy number may produce instability. Once the required recombinant strain of P. diminuta is obtained, rigorous testing will be required before it is considered suitable for release into routine use in industry.

2.6. Objectives.

There is a need to study current practices for the validation of 0.2 μm rated membranes by retention tests with P. diminuta and to investigate the requirement for a higher degree of standardisation concerning membrane pore size characterisation. There are already a number of standard procedures for retention testing with P. diminuta (section 2.3.2) and one will be applied to the retention testing of experimental 0.2 and 0.1 μm rated membranes designed for cross-flow filtration.
Hydrophilic materials were grafted onto hydrophobic membranes by the manufacturer in order to decrease the rate of membrane fouling as it is known that the potential for fouling decreases with the increasing hydrophilicity of membrane material (Goel et al 1992). Adsorption of product to the membrane surface is unwelcome, not only in terms of the high rate of fouling decreasing productivity and the life-span of the membrane but also in terms of product degradation. As well as the gel-layer of denatured proteins adsorbed to the membrane surface (section 2.1.1.2.), there is a boundary layer of unadsorbed proteins that are conformationally altered held reversibly on or near the membrane surface. The presence of conformationally altered therapeutic proteins in products is considered highly undesirable by the pharmaceutical industry.

It has been found that the adsorptive retention of a marine *Pseudomonas* spp. by solid substrata depended on the nature of the material (Fletcher and Loeb 1979). More bacteria became attached to uncharged hydrophobic plastics whilst very few attached to hydrophilic, negatively charged materials such as glass and oxidised plastics. The effect of grafting hydrophilic moieties onto native PVDF membranes on the retention for *P. diminuta* given by the experimental membranes will be determined. The same retention test procedure will also be applied to a selection of 0.2 and 0.1 µm rated commercial membranes in order to compare the ability to retain *P. diminuta*.

The range of cell sizes for *P. diminuta* in various broth cultures and in retention test permeates will be determined using scanning electron microscopy (SEM) and a rapid, electronic particle sizing technique. Sterilising grade membranes are retention tested with bacteria that naturally exist in a range of sizes to provide a realistic, rigorous, worst-case challenge to the membranes retention ability. However, the range of cell sizes for *P. diminuta* has not been determined. To have an idea of the size of the smallest cells in a challenge solution and also of the size of cells that may penetrate the test membrane will give more detailed information on the retention ability of that membrane. Also, details about the growth conditions for *P. diminuta* in retention test protocols are often scanty. *P. diminuta* is an obligate aerobe (Buchanan and Gibbons 1974) and the effect on the cell size due to the amount of aeration that the bacteria experience during growth will be studied. Differences in the culture method between establishments may mean that retention tests results are not comparable.

A new retention test procedure using cross-flow filtration will be developed. All current retention test procedures use dead-end filtration and it is not possible to test cross-flow membranes under process conditions. Results from filtration experiments carried out at Tech-Sep suggested that the retention capacity given by flat-sheet membranes designed for use with cross-flow filtration is lower when these membranes are used with dead-end filtration (results not shown). This discrepancy may be caused
by the flow of the feed sweeping the particles parallel to the membrane surface during cross-flow filtration. Thus there is need for a standard cross-flow retention test method for the validation of 0.2 μm rated membranes under simulated process conditions. Such a test would be capable of determining the retention for *P. diminuta* over a given period of time with defined values for the feed recirculation velocity and TMP. The feasibility for such a test will be studied. It is recognised that problems that may be encountered include maintaining the sterility of such a system over the entire test period whilst taking numerous feed and permeate samples and also the ability of the bacteria to withstand constant recirculation for prolonged periods of time.

The need for a more rapid and efficient retention test using *P. diminuta* has already been discussed in detail and criteria for the ideal rapid retention test has been laid down (section 2.3.4.). Although it is common practice to use 0.45 μm rated membranes for the analysis of test permeates, it can not be assumed that these will always retain *P. diminuta*, especially as bacterial cells in permeates will be smaller than those from a standard SLB culture. A rapid test method is required that will give accurate and reliable quantification of *P. diminuta* in test permeates. Rapid retention test procedures incorporating either an ATP luminescent assay, an electrometric technique or the DEFT to detect and enumerate *P. diminuta* in retention test permeates from sterilising grade membranes will be developed. All three rapid retention test procedures will be compared and the most suitable test method on the basis of all the ideal test criteria will be chosen.

The feasibility of using a bioluminescent strain of *P. diminuta* produced by genetic engineering for the retention testing of 0.2 μm rated membranes will be studied. Genes from naturally occurring bioluminescent bacteria have been cloned into many species of micro-organisms for specific applications (section 2.5.3.) but not for the purpose of retention testing membranes. Documentation concerning the genetic manipulation of *P. diminuta* is scarce and a bioluminescent strain of *P. diminuta* has not been produced to date. A bioluminescent strain of *E. coli* produced by genetic engineering containing genes cloned from the bioluminescent marine bacterium *V. harveyi* will be used for the retention testing of microfiltration membranes other than sterilising grade. Research concerned with cloning the same genes into *P. diminuta* will be carried out in order to produce a stable bioluminescent strain that will facilitate the rapid, sensitive and straightforward validation of sterilising grade membranes.
3. MATERIALS AND METHODS.

All chemicals used were analytical grade and were obtained from Fisons Scientific Equipment (Loughborough, Leics.) and all microbiological media used were obtained from Unipath Ltd. (Basingstoke, Hamps.) unless stated to the contrary.

3.1. Retention testing of experimental and commercial sterilising grade membranes.

Anisotropic phase-inversion PVDF membranes with pore size ratings ranging from 0.1-1.5 μm were grafted with hydrophilic material by Tech-Sep following established methods in an attempt to decrease the affinity for protein and thus to decrease the rate of membrane fouling. The procedure for grafting onto the native membrane will not be discussed in this study. The identity of the grafted material will not be divulged but referred to as A, B and C with a neutral, positive and negative charge respectively. Ungrafted PVDF membranes will be referred to as native. Scanning electron micrographs of a Tech-Sep anisotropic PVDF membrane can be seen in figure 3.1.a..

The amount of protein bound to the membrane feed surface after exposure to a solution of bovine serum albumin (BSA, Advanced Protein Products, Brierley Hill, West Mids.) was determined for each type of membrane using a modification of the Lowry method for quantification of protein (Lowry et al 1957, Peterson 1977, Lockley 1988) by personnel also working on the BRITE project.

The experimental membranes were cast onto a support backing which was either woven or non-woven in nature. Studies concerning the shrinkage of different backings during autoclaving were carried out by the manufacturers. All membranes were pre-wetted and packed in either a glycerol and formaldehyde (3 g/l) solution or a Triton X-100 (110 g/l) and formaldehyde (3 g/l) solution.

The ability to retain *P. diminuta* was determined for the 0.1 and 0.2 μm rated membranes using a modification of the ASTM (D 3862 1992) method for testing the retention capacity of 0.2 μm rated membranes for *P. diminuta*. It is recommended that the test permeate is incubated in its entirety for 7 days to confirm sterility without quantitation of any bacteria present. Quantitation of bacteria in test permeates is commonly achieved by membrane filtration analysis as previously discussed (section 2.3.4.). However, trial retention tests with the experimental membranes showed that the viable count of the test permeates was too high to be analysed in this way. Therefore,
Figure 3.1.a. Photomicrographs showing cross-sections of a Tech-Sep PVDF anisotropic membrane (from Tech-Sep.).

(i) Showing the anisotropic nature of the barrier and support layer.

(ii) Showing detail of the low-porosity barrier layer.
the viable count for the membrane challenge and the number of bacteria in the permeate were both calculated by performing a plate count. The remaining permeate after samples had been taken was always incubated in entirety for seven days to determine sterility. This method allowed the detection of one cfu of *P. diminuta* in the permeate and quantitation to a certain degree. Sampling in this manner is considered appropriate as a pure culture of bacteria that do not have a tendency to aggregate and which are present in low numbers are being analysed therefore conditions for the Poisson distribution are satisfied (Jarvis 1989). The same procedure was applied to the retention testing of commercial sterilising grade membranes.

3.1.1. Preparation of test membranes.

3.1.1.1. Experimental membranes.

Test membranes were cut into 47 mm diameter discs using a die, washed in three successive lots of distilled water (d.H₂O, 1 l) and were submerged in 500 ml sodium hypochlorite (Sigma Chemical Co. Ltd., Poole, Dorset) solution overnight at 5°C. A solution of sodium hypochlorite imparting 100 ppm of available chlorine (ac) was found to be the lowest concentration to effect sterility in this time. Immediately before the retention test, membranes were rinsed in two 100 ml lots of sterile d.H₂O then were assembled into funnel-type filtration holders and 20 ml of d.H₂O was filtered through each membrane. This was the standard procedure unless a comparison between hypochlorite-sterilised and autoclaved membranes was required.

For the autoclave, test membranes were washed as described then placed into a 90 mm diameter glass petri-dish interleaved with foil. The dish was filled with d.H₂O, a stainless steel ring was placed on top of the membranes which were then autoclaved submerged for 15 minutes at 121°C. This allowed the membranes to remain immersed in water during autoclaving without curling up which made them very difficult to handle. It was found that this method of autoclaving was more suitable than dry autoclaving as the need to re-wet membranes before the retention test may adversely affect the sterility of the membrane.

3.1.1.2. Commercial membranes.

The retention capacity of the experimental membranes was compared to that of a selection of commercial membranes which were prepared as follows:

(a) Nuclepore polycarbonate 0.2 and 0.1 μm rated track-etched membranes
(Costar UK Ltd, High Wycombe, Bucks) and Whatman cellulose nitrate 0.2 μm and 0.1 μm rated anisotropic membranes (Whatman International Ltd. Maidstone, Kent) were autoclaved dry interleaved in foil.

(b) Millipore Durapore (Millipore UK Ltd. Croxley Green, Watford) 0.22 μm rated PVDF membranes were wetted with 70% isopropanol, 20 ml d.H₂O was filtered through each and then the membranes were autoclaved submerged as described for the experimental membranes.

(c) Amicon (Amicon Ltd, Stonehouse, Gloucs.) Diaflow ultrafiltration (UF) membranes listed below:
   XM 300 and 100 (NMWC 300,000, and 100,000 daltons respectively)
   YM 100, 30, 10 and 2 (NMWC 100,000, 30,000 10,000 and 1,000 daltons respectively)
were washed to remove sodium azide preservative by being floated feed-side down in three successive lots of d.H₂O at 5°C for one hour per lot. The membranes were then sterilised as recommended by the manufacturer by either autoclaving submerged (YM) or cold-sterilisation with sodium hypochlorite (XM) as described for the experimental membranes. Sterile d.H₂O (5 ml) was filtered through each membrane before the retention test to remove residual sodium azide and hypochlorite.

(d) DDS-Niro (Niro Ltd. Abingdon, Oxon) anisotropic phase-inversion polysulphone and PVDF 0.2 and 0.1 μm rated membranes were washed and sterilised with sodium hypochlorite as described for the experimental membranes.

(e) Ceramesh (Ceramesh Ltd, Abingdon, Oxon.) 0.2 μm rated flat disc composite ceramic membranes were autoclaved wrapped in foil. The structure of the Ceramesh membrane can be seen in figure 3.1.b.(i). The woven structure of the metal wires supporting the ceramic material may be clearly seen. The square apertures between the wires are 150 μm across. A cross-section of the membrane is seen in figure 3.1.b.(ii). The ceramic at the centre of the meniscus is approximately 20 μm thick.

Ten of each of the commercial microfiltration membranes and five of each of the ultrafiltration membranes were retention tested with P. diminuta using the same procedure as for the experimental membranes.

3.1.2. Preparation of the P. diminuta challenge.

Lyophilised P. diminuta (catalogue no. 11090, ATCC 19146) was obtained from the National Centre for Industrial and Marine Bacteria (NCIMB, Torrey, Aberdeen). The bacteria were stored in skimmed milk as a cryoprotectant at -20°C for up to 2 years
Figure 3.1.b. The Ceramesh membrane (from Cowieson 1992).

(i) Membrane surface  (i) Membrane cross-section

Figure 3.1.c. Filtration equipment recommended by the ASTM for the bacterial retention testing of 0.2 μm rated membranes (from ASTM D 3862 1992).
(Lapage and Redway 1974) and were also stored on nutrient agar (recommended by the the NCIMB) at 5°C for up to 4 months. A pour-plate method was used throughout as the standard plate count method and plates were incubated at 30°C for 48 hours. Ringers solution was used as the diluent for *P. diminuta* and MacConkey agar was used where a solid growth medium was required throughout this study unless specified to the contrary.

A single colony from a stock agar plate stored at 5°C was used to inoculate 10 ml of TSB which was incubated for 24 hours at 30°C. After this time 1 ml was withdrawn and was used to inoculate 100 ml of SLB (7.6 g NaCl and 0.39 g lactose broth per litre, Leahy and Sullivan 1978) which was incubated for 24 hours at 30°C and 175 rpm in an orbital incubator. This is the standard SLB culture of *P. diminuta* and is referred to throughout the study. The membrane challenge was prepared according to ASTM (D 3862 1992) retention test specifications. An aliquot (1 ml) was withdrawn from the SLB culture and was added to 99 ml Ringers solution to give a challenge in the order of $1 \times 10^7$ cfu per cm² membrane area. Triplicate samples (100μl) were taken from the challenge and subjected to a plate count after appropriate dilution to determine the total viable count of the challenge.

### 3.1.3. The retention tests.

Filtration apparatus specified by the ASTM (D 3862 1992) was used and is shown in figure 3.1.c.. All filtration and manipulation of test permeates was carried out in a class two clean air cabinet (MDH, Andover, Hants.) to avoid air-borne contamination. Each type of test experimental membrane was tested twice in triplicate against a negative control. The challenge was filtered under a vacuum of 0.67-0.81 bar (500-600 mm Hg) into a receiving flask containing 140 ml of double-strength TSB. Ringers solution (40 ml) was used to wash out the vessel that had contained the challenge suspension and was filtered after the challenge until there was no liquid visible on the membrane surface. After filtration, ten 1 ml and ten 0.1 ml samples of the permeate were withdrawn and plated out. The test membrane was removed from the filtration funnel and placed feed-side upper-most on an agar plate. The plates and the permeate were incubated at 30°C for 48 hours and seven days respectively.

Bacterial colonies on MacConkey agar plates were identified as *P. diminuta* by morphology and chromogenesis, namely the presence of small, round, entire non-fermenting colonies that caused a yellowing of the agar. The neutral red dye in
MacConkey agar indicated the presence of acid produced by contaminating fermenting species by turning media and colonies deep red. Bacterial growth in the permeate itself was identified as *P. diminuta* by the characteristic pellicle produced and by a Gram-stain procedure (Pertel and Kazanas 1984). These tests could be further supported if required using the API 20 NE biochemical analysis strips (API Laboratory Products Ltd., Basingstoke, Hants.) for the identification of non-fermenting Gram-negative rods. These strips were regularly used to check the identity of long and short-term maintenance cultures of *P. diminuta*. The numbers of *P. diminuta* in the entire membrane challenge and in the entire test permeate were calculated and the LRV given by the membrane for *P. diminuta* was determined. The test membrane was validated as sterilising grade if all plates and permeate showed no growth of *P. diminuta* after the recommended incubation times indicating that the challenge had been fully retained. The retention test itself was validated if there were no colonies within that part of the membrane that laid underneath the seal area during the test.

### 3.2. Cell size analysis for *P. diminuta*.

Samples of *P. diminuta* prepared at LUT were subjected to critical point drying and were analysed by SEM by Leicester University Scanning Electron Microscope Unit, Department of Pre-clinical Sciences, Leicester University. The analysis of *P. diminuta* by electronic particle sizing was carried out by the Particle Sizing Lab., Department of Chemical Engineering, LUT.


All glassware was rinsed and all media was prepared with reagent grade high purity water filtered to 0.2 μm (from a Millipore OrganexQ system and from now on referred to as MilliQ high purity water) to reduce particulate debris. Duplicate shaken and still TSB and SLB cultures of *P. diminuta* were prepared as described in section 3.1.2. by incubation for 24 hours in either a stationary or an orbital incubator. Duplicate 2 and 4 ml aliquots of each TSB and SLB *P. diminuta* cultures respectively were suspended in 10 ml of sterile Ringers solution. All cells were fixed with 1% gluteraldehyde (electron microscopy grade, Sigma Chemical Co. Ltd.) for 1 hour at 5°C.

A 5 ml portion of each preparation was filtered through a 25 mm diameter 0.1 μm rated Nuclepore membrane (Costar UK Ltd.) under a vacuum of 0.74 bar. The sample vessel was then washed out with 2 ml of Ringers solution which was filtered also until there was no liquid visible on the membrane surface. After marking the feed side, each
membrane was quickly put into 3 ml of acetone (previously filtered to 0.2 μm) in a glass universal bottle that had been rinsed with 10 ml of the same. To study the cells that had pass through retention test membranes, the test permeate was collected into an empty side-arm flask the contents of which were then filtered through a Nuclepore membrane as described and placed into acetone also.

The membrane was placed in a folded envelope of vellum while submerged in 100% dry acetone. The envelope was then transferred to critical point dryer (CPD) boat full of acetone which was subsequently sealed into the dryer. The acetone was replaced with liquid carbon dioxide at 55.51 bar within the CPD. After extraction from this, the membrane was cut into small segments and a single segment was mounted on a top stage stub for the DS130 SEM (see below) using a small adhesive tab (Agar Scientifics) and silver colloid paint. The specimen was then coated with gold to a thickness of 5 nm in a Polaron E5150 sputter coater with cooled specimen stage. The specimen was then studied in an International Scientific Instruments DS 130 scanning electron microscope using the high resolution top stage. Various voltages were tried to produce the clearest results without specimen damage. Size analysis of the bacteria from the resulting photomicrographs was carried out by taking length and breadth (halfway along the length) measurements of individual cells.

3.2.2. Electronic particle sizing.

Samples of the same original TSB and SLB *P. diminuta* cultures described for the SEM experiments were analysed using the Coulter Counter model TA11 16 channel electronic particle size analyser (Coulter Electronics Ltd., Luton, Beds.) (figure F.1., appendix F). This apparatus was pre-calibrated using suspensions of standard spherical particles of polystyrene divinyl benzene. Aliquots of each 24 hour culture (3.33 x 10^2 ml of the TSB cultures and 1 ml of the SLB cultures) were added to 38 ml of electrolyte (MilliQ high purity water) and analysed using a 30 μm diameter aperture and a test volume of 5.00 x 10^2 ml. At least four sets of results were obtained for each type of *P. diminuta* culture.

3.3. The cross-flow retention tests.

The retention of *P. diminuta* given by experimental and commercial flat-sheet membranes, and by ceramic multichannel monoliths over a period of time during cross-flow filtration was studied. In each case SLB was chosen for the feed solution and was prepared using MilliQ high purity water. This medium is used to culture *P. diminuta*
for the retention test challenge and should cell multiplication occur during the test run time then the mean cell dimensions for *P. diminuta* should not be altered. It was not expected that the numbers of *P. diminuta* would not increase dramatically over the run period as early experiments showed the mean generation time for this species in SLB to be 1.62 hours (results not shown).

3.3.1. The Rayflow module.

The Rayflow is a compact plate-and-frame module of dimensions 240 mm x 150 mm x 50 mm designed by Tech-Sep for feasibility tests on ultrafiltration and microfiltration membranes using small scale membranes and pumps available in all laboratories (*figure 3.3.a.*). It is capable of filtration with a TMP range of 0-3 bar, a retentate recirculation rates of up to 500 l/h (3.5 m/s) (*eqn. 14.*) and a maximum working temperature of 50°C. During the design of the module, it was important to keep the feed channel cross-section as small as possible maximise the linear velocity of the feed and minimise fouling and it is recommended that the TMP should also be kept low for the same reason. The surface area of the test membrane available for filtration when assembled is 1.31 x 10^2 m². The linear velocity of the feed for all experiments using cross-flow filtration was determined by the equation described below:

\[
\text{Volumetric flow rate} = \text{linear velocity} \times \text{cross-sectional area of pipe} \quad \text{... (14)}
\]

\[
(m^3/s) \quad (m/s) \quad (m^2)
\]

The module end manifolds are manufactured from polycarbonate, the membrane support plate from polysulphone and the seals that hold the membrane onto the support (*figure 3.3.b.*) from butyl rubber. All materials are food-grade and withstand autoclaving conditions to comply with demands from the biotechnological industries. The original module used for microfiltration had the configuration seen in *figure B.1.* (*appendix B*) and could be used for filtration with either a series or parallel flow configuration. One membrane could be attached to each side of the support plate and the permeate would drain into the centre of the plate and exit through ports on each side of this.

When used for microfiltration, it was found that the permeate rates were too high compared to those obtained with ultrafiltration. The permeate could not exit the module fast enough and a back-pressure would build up on the permeate side of the membrane. Back pressure is undesirable as the membrane could be lifted from the support plate threatening the integrity of the seals. The support plate was modified at Tech-Sep by
sealing up the original permeate ports and by drilling a series of evenly spaced holes across the plate as shown in figure B.2. (appendix B) and figure 3.3.b. Now only one membrane could be attached to each support plate and the permeate could pass quickly from the feed side of the module to the permeate side through the plate itself to drain and exit without danger of producing back pressure.

The test rig is shown in figure 3.3.c. A Watson and Marlow (Watson and Marlow Ltd. Falmouth) 601S/R peristaltic pump was used to drive the filtration. At the time it was considered that the action of a centrifugal pump would be damaging to bacteria. The pulse from peristaltic pumps may cause compaction or deformation of the surface of polymeric membranes and could also cause motion of the membrane and to threaten the integrity of the seals. To remove the pulse the feed was pumped through the sealed vessel 2 before entering the module. All the tubing used was silicone and vessels were polypropylene to render the entire rig autoclavable.

3.3.1.1. Preparation.

The pure water permeate (PWP) rate was determined for each membrane before the retention tests were carried out using 10 l of MilliQ high purity water as the feed. The rig was assembled without the Rayflow module with 10 l of SLB in the feed vessel 1 (figure 3.3.c.) and was autoclaved for 30 minutes at 121°C. Earlier trials with the Rayflow had shown that the membrane seal integrity was adversely affected if the module was autoclaved with a test membrane already seated onto the support plate, possibly due to movement of the membrane during the autoclave cycle. The module itself was loosely assembled before autoclaving to allow for expansion due to the heat. Leakage around the membrane seal area would invalidate the test results and the membrane could not be re-seated after autoclaving without jeopardising the sterility of the system. Also it was mentioned earlier that the experimental membranes were best autoclaved totally submerged in water. It would be difficult to autoclave the membrane in situ as the loosely assembled Rayflow will not hold water.

A selection of membranes used previously for the dead-end retention test was used for the cross-flow retention test with the Rayflow. The test 0.2 and 0.1 μm rated experimental, 0.22 μm rated Durapore and 0.2 and 0.1 μm rated DDS membranes were cut to fit the Rayflow and washed as described in section 3.1.1. The Rayflow was assembled with the membrane (as described in appendix B) and was cold-sterilised with 100 ppm ac sodium hypochlorite. This sterilising solution was gently circulated around the module for 10 minutes using a small capacity peristaltic pump ("Masterflex" pump controller, Cole Palmer Instruments Co. Ltd. Bishop Stortford) with a low recirculation velocity of 330 ml/min. The filled module was then left overnight at 5°C. The challenge
Figure 3.3.a. The Rayflow cross-flow filtration module.

The fully autoclavable Rayflow module is shown where (a) is the retentate inlet from the pump, (b) is the retentate outlet and (c) is the permeate outlet.
Figure 3.3.b. The Rayflow membrane support plate.

Detail of the support backplate for the test membrane is shown where (a) is the seal holding the membrane to the retentate side of the plate, (b) is the seal for the permeate side of the plate and (c) indicates drainage holes for the permeate.
Figure 3.3.c. The Rayflow test rig.

Schematic diagram of the Rayflow test rig where (a) is the 10 l feed reservoir and (b) is the Rayflow module. The TMP was taken as the mean of P1 and P2 whilst permeate and retentate samples were taken via S1 and S2 respectively.
used for the retention test was a 100 ml standard SLB culture of *P. diminuta*. Considering the area of the membrane exposed for filtration mentioned earlier then the recommended challenge level of at least $1 \times 10^7$ cfu per square cm is achieved (HIMA 1982, FDA 1987).

3.2.1.2. The retention tests.

The test rig was assembled according to figure 3.3.c. All filtration was carried out in the class 2 clean air cabinet. Before the retention test the Rayflow module was emptied of the hypochlorite solution and 51 of sterile MilliQ high purity water was filtered without recirculation using the same small capacity peristaltic pump with a low feed velocity. The module was assembled into the test rig. The *P. diminuta* culture was added to the reservoir and filtration was started with permeate recirculation with a linear velocity of 3.5 m/s, and a TMP of 0.5 bar seen as an average of readings from P1 and P2, for a 1 hour period.

Samples (5 ml) of the permeate were taken via S1 and samples (5 ml) of the retentate were drawn up from the reservoir via a sterile syringe (S2) at timed intervals. A small volume was taken off from each outlet before the sample was collected. Permeate rates were determined by running off a certain volume into a sterile measuring cylinder which was then returned to the reservoir. The outlet S1 was left submerged in sterile d.H$_2$O in between periods of sampling. Five aliquots (1 ml) of both retentate and permeate samples were diluted accordingly and were subjected to a plate count. The LRV given by the membrane was calculated for the time period for which the sample was taken.

3.3.1.3. After the test.

After the retention test the feed was allowed to drain into the reservoir, the screw-nuts on the module were slackened off (appendix B) and the entire assembly was autoclaved. The module components, tubing and vessels were soaked in 4% Decon 90 (Decon Labs. Ltd. Hove) for 2 hours and then washed with a standard laboratory detergent.

3.3.2. The Microkerasep monoliths.

The Microkerasep is a tubular, ceramic, multichannel monolith available with pore sizes ratings of 0.1, 0.2 or 0.45 μm. Three types of Microkerasep monolith were used for retention tests and can be seen in figure 3.3.d. The monoliths are 400mm long, 20 mm wide and are available with either seven channels that are 4.5 mm in diameter or nineteen channels that are 2.5 mm in diameter. The total membrane area for these two
types of monolith are 3.30 x 10\(^2\) m\(^2\) or 5.10 x 10\(^2\) m\(^2\) respectively. The Kerasep monolith is 856 mm long and is also available with the same choice of pore size and channel specifications, with total membrane areas of 7.0 x 10\(^2\) m\(^2\) and 0.11 m\(^2\). The membranes are composite in nature consisting of a macroporous aluminium oxide and titanium oxide network, and each channel is coated with either a layer of finely divided titanium oxide (for the 0.45 and 0.20 \(\mu\)m rated models) or a layer of finely divided zirconium oxide (for the 0.1 \(\mu\)m rated models). A SEM photomicrograph of a 0.2 \(\mu\)m rated monolith can be seen in figure 3.3.4. displaying the composite structure. The Microkerasep is autoclavable and can be used with a TMP of up to 6 bar, a temperature of up to 380°C and a retentate recirculation velocity of up to 2,000 I/h. Four 0.2 and 0.1 \(\mu\)m rated monoliths in total (listed in section 4.3.2.) were retention tested with \textit{P. diminuta} using cross-flow filtration.

A stainless steel housing with dual permeate outlets was used for the Microkerasep monolith which was held in place by silicone rubber seals. The test rig is shown in figure 3.3.f.. A Grundfos JP5 (Grundfos Pumps Ltd., Leighton Buzzard) centrifugal jet pump was used to drive filtration after studies to determine the survival of \textit{P. diminuta} in such an environment. The sources of physical stress to the bacteria were the high shear forces delivered by the action of the pump and the increase in retentate temperature caused by the heat given out by the pump motor. The sensitivity of the bacteria to an increase in temperature was studied as described in section 3.3.2.1.. A cooling loop was included in to the test rig to compensate for the heat emitted by the pump motor and it was found that \textit{P. diminuta} were not adversely affected by the shear forces, possibly due to their small size. It was decided to use this pump for further work as a higher feed rate could be delivered compared to the peristaltic pump used earlier.

### 3.3.2.1. Temperature sensitivity of \textit{P. diminuta}.

This experiment was carried out in triplicate. The effect of a change in temperature on the viable count for \textit{P. diminuta} over a five hour period was studied. Each of five standard SLB cultures of \textit{P. diminuta} was placed in either a 10, 20, 30, 40 or 50°C environment provided by a refrigerated incubator, room temperature, an incubator and two thermostatically heated water baths respectively. Samples (1 ml) were withdrawn in triplicate from each culture at 0, 1, 2, 3 and 4 hours, diluted accordingly and subjected to a plate count. It can be seen from the results in figure C.1., appendix C that at 10 and 20°C the viable count remained stable for the entire period. At 40°C the count remains stable for 1 hour before decreasing steadily, whilst at 50°C the count decreases dramatically for 1 hour before continuing to decrease steadily. The abrupt fall in growth rate at high temperatures is caused by the thermal denaturation of proteins and hence
Figure 3.3.d. MicroKerasep tubular multichannel monoliths.
Figure 3.3.e. Photomicrographs of the MicroKerasep membrane.

(i) Showing detail of the anisotropic composite barrier and support layer.

(ii) Showing detail of the membrane feed surface.
Figure 3.3.f. The Microkerasep test rig.

Schematic diagram of the Microkerasep test rig where (a) is the feed reservoir, (b) is the cooling loop and the test monolith is contained within housing (c). The TMP was taken as the mean of P1 and P2 whilst permeate and retentate samples were taken via S2 and S3 respectively.
cellular function (Stanier et al 1987). Thus the run retentate temperature was maintained at approximately 25°C for the duration of the run allowing for 15°C rise for greater than 1 hour before the viability of the bacteria was affected.

3.3.2.2. Preparation.

The PWP rate for each monolith was determined before the retention tests using 10 l of MilliQ high purity water as the feed. The rig was assembled according to figure 3.3 f. with the housing in place but without the monolith. Sodium hypochlorite (100 ppm ac) solution was recirculated for 20 minutes at 0.5 bar and 500 l/h making sure that some solution had been run through all permeate and retentate outlets. The outlet from permeate port S1 was continually returned to the reservoir. The end of sample taps S2 and S3 were immersed in the sodium hypochlorite solution and the rig was left overnight. The test monolith was wrapped in foil with the silicone seals, and autoclaved along with the 10 l of SLB for the feed solution for 30 minutes at 121°C. It was felt that the ceramic nature of the monolith would retain hypochlorite that would be difficult to wash out and that retained hypochlorite may give a falsely high LRV. The P. diminuta culture was prepared as described in section 3.3.1.1.

3.3.2.3. The retention tests.

Each monolith was retention tested in duplicate. The refrigeration unit was switched on 1 hour before start of the experiment to maintain a retentate temperature of 25±3°C during the experiment. The hypochlorite solution drained from the rig and two successive 10 l lots of sterile d.H2O was run without recirculation through the entire rig including all permeate and retentate taps. This d.H2O was drained away, the monolith was aseptically assembled into the housing and the P. diminuta culture was added to the feed reservoir.

Filtration was carried out for up to 6 hours using a 0.2 μm rated 19 channel monolith with a range of TMP’s from 0.5 to 3.5 bar (measured as the mean of P1 and P2) and a variety of recirculation rates from 500 to 2,000 l/h. Retentate recirculation rate and TMP were controlled by valves V1 and V2. Permeate from the S1 outlet was continually returned to the reservoir whilst permeate taken from S2 was solely for sampling. Earlier results showed no significant difference between viable counts of permeate samples taken from either outlet (results not shown).

The permeate rates were obtained by running an exact copy of the retention test without sterile conditions using an autoclaved culture of P. diminuta for the challenge. It was difficult to obtain reliable permeate rates during the course of the experiment without jeopardising the sterility of the system. The viable count of the culture was
determined via a plate count before autoclaving to confirm that the challenge level was correct. Numerous samples of the permeate could be taken and returned to the reservoir without affecting the sterility of the retentate. The procedure for permeate rate determination will need to be studied during further developments of the test rig. The retention tests were repeated for three 0.2 and 0.1 μm rated monoliths over a period of two hours with a TMP of 0.5 bar and a linear velocity of 3.0 m/s.

Samples (5 ml) of the permeate and retentate were taken from S2 and S3 respectively at timed intervals after running off a small volume. The sample outlets were left immersed in sterile water between periods of sampling. The temperature of the run-off volume was checked using a digital thermometer. Retentate samples were diluted accordingly and all samples subjected to a plate count. The LRV given by the monolith for *P. diminuta* was calculated for each time interval.

### 3.3.2.4. After the test.

After the test, all the feed was drained into the reservoir, the monolith was aseptically removed into an autoclave bag and both were autoclaved. Sodium hypochlorite solution (400 ppm ac) was recirculated for 10 minutes at 0.5 bar and 500 l/h and the rig was then left overnight. It was convenient to use tap water for this stage which is not suitable for cleaning the membrane itself due to the high impurity content. The hypochlorite solution was drained from the rig which was washed through with 10 l tap water without recirculation. A 1% (w/v) solution of Ultrasil 50 (Henkle Chemicals Ltd. London) was recirculated for 1 hour at 30°C and was drained. Finally, the rig was washed through with 50 l tap water followed by 20 l of d.H₂O without recirculation.

The monolith was soaked in 1,000 ppm ac. sodium hypochlorite for 30 minutes. This was then assembled into the rig and 10 l MilliQ high purity water was filtered without permeate recirculation. The monolith was soaked in 0.5 M NaOH (general laboratory grade) at 80°C for 1 hour and rinsed as described above, and then was soaked in 0.33 M nitric acid for the same time and rinsed again. For each rinsing, the MilliQ water was filtered until the pH of the permeate reached neutral. The rig and the pump were drained and the test monolith was dried in a warm oven at 50°C.

### 3.4. Application of popular rapid methods for the enumeration of bacteria to retention testing.

All ATP luminometry (*figure 3.4.a.*) and electrometric experiments using the Bactometer (*figure 3.4.b.*) were carried out at the Department of Food, Fisheries and Environmental Studies, University of Humberside, Grimsby. Initial DEFT experiments
Figure 3.4.a. The Lumac M2010/A luminometer.

Figure 3.4.b. The Bactometer (model 64).
Figure 3.4.c. The epifluorescence microscope used for the DEFT.
were carried out using Biofoss equipment hired from Foss Electric, Bishopthorpe, York, whilst investigations concerning the DEFT microcolony method were also carried out using equipment at the University of Humberside (figure 3.4.c.).

The correlation between the viable count determined by each rapid method and the plate count was determined using a 10-fold dilution series of a standard SLB culture of \textit{P. diminuta}. Retention tests were then carried out against a negative control for experimental 0.1 and 0.2 \textmu m rated PVDF membranes. Membranes that had been intentionally damaged were also tested to provide a positive control for the detection of \textit{P. diminuta} by each of the rapid methods. The permeates were analysed by both the rapid method in question and the standard culture methodology used in this study described in section 3.1.3. The test membranes were washed and sterilised by autoclaving as described in section 3.1.1. and the viable count for the membrane challenge was determined by the both the rapid method in question and the plate count accordingly.

3.4.1. ATP luminescence.

3.4.1.1. Reagents and equipment.

Luminescence was measured in relative light units (RLU) using a Lumac M2010/A luminometer (Lumac Ltd. Batley, West Yorks.) with an integral manual/automatic reagent injection system, that was connected to a Epson LX-80 printer (figure 3.4.a.). Background luminescence was determined in triplicate each day. All reagents used for ATP luminescence assays were also obtained from Lumac. The ATP luminescence Water Test Kits were used throughout and contained NRB ATP extractant (a mixture of ionic surfactants), lyophilised Lumit-pm (d-luciferin, luciferase, BSA and dithiothreitol) which was reconstituted as recommended by the manufacturer with Lumit buffer (0.025 M HEPES buffer, MgSO\textsubscript{4}, 0.002 M EDTA to chelate metal ions that would interfere with the reaction of the enzyme and sodium azide, pH 7.75) and Lumacult ATP-free nutrient broth medium.

It was ensured that all reagents were at room temperature before use due to the enzymatic nature of the luminescence reaction. NRB and Lumacult were stored at 5°C whilst Lumit-pm was stored at -20°C immediately after reconstitution, as 1 ml aliquots dispensed into sterile polystyrene Lumacuvettes wrapped in foil. These cuvettes were used for storage and for all luminescence assays as plastic containers are less likely than glass to cause background light activation (Leach and Webster 1986). Any thawed, reconstituted Lumit-pm was not refrozen as this procedure causes a decrease in enzyme activity (Stanley 1989).
ATP for standardisation (10 μg ATP as the disodium salt with 3.6 μmol glycine, 0.18 mg dithiothreitol and 0.2 mg BSA) was dissolved and diluted with sterile distilled ATP-free water. A 1 μg/ml solution was stable for 8 hours at room temperature and 1 ml aliquots of the same dilution were stored at -20°C in foil-wrapped Lumacuvettes for up to 4 weeks. Bactowash disinfectant and sterile bidistilled ATP-free water was used to wash out the luminometer reagent injection system. Ringers' solution gave the lowest background luminescence compared to other media (TSB, SLB, 0.1% peptone water, maximal recovery diluent, results not shown) and was used as a diluent for *P. diminuta* throughout the ATP luminescence experiments.

ATP-free water was used instead of dH₂O for rinsing glassware and for the preparation of media and was prepared as follows: 1 litre of dH₂O was boiled with one drop of HCl (concentrated) for 5 minutes and was neutralised with 0.1 M NaOH when cool. This water was then divided between five polypropylene containers (250 ml capacity) which were autoclaved for 60 minutes at 121°C (Baumgart et al 1980). This was stored at 5°C until required.

3.4.1.2. ATP extractant.

The nucleotide releasing capabilities of TCA (Sigma Chemical Co. Ltd.) and boiling Tris-EDTA buffer were compared to that given by the commercial NRB extractant.

(a) TCA

A 1 ml aliquot of a 1.25, 2.5, 5, or 10% w/v TCA solution was pipetted into aliquots of the same volume from a 10-fold dilution series of *P. diminuta*. Each was agitated gently for 5 minutes when 200 μl aliquots were taken in triplicate, 100 μl Luminit-pm added via the luminometer injection system and the luminescence reading was taken after 10 seconds (Lundin 1984, Stanley 1986, Simpson and Hammond 1989). Dilutions (10-fold and 50-fold) of the extracted samples were made accordingly and assayed for luminescence following the same procedure.

(b) Boiling buffer

One ml of each dilution series was also pipetted into a small glass test tube with 1 ml of boiling buffer (50 mM/L Tris, with 4 mM/L EDTA pH 7.75). After 3 minutes, this was cooled rapidly to 0-4°C by plunging in ice and a correction was made for evaporation during boiling (McEntee et al 1989). Aliquots (200 μl) were assayed for luminescence as described above.
(c) NRB

The extraction procedure recommended by Lumac was carried out using 100 μl of NRB and 100 μl of sample with 100 μl Lumit-pm added after 30 seconds. The luminescence reading was taken after 10 seconds.

In order to ascertain the optimum conditions for the ATP assay, the effect of NRB extractant on the activity of luciferase was also studied. Luminescence was monitored over 5 minutes for both (a) 100 μl Lumit-pm, 10 x 10^10 g ATP in 100 μl ATP-free d.H2O with 100 μl NRB and (b) the same where 100μl of ATP-free d.H2O was substituted for the NRB.

3.4.1.3. Calibration.

Luminescence was quantified for triplicate samples from a 10-fold dilution series of *P. diminuta* in order to determine the relationship between emitted light in RLU and viable cell count. The SLB culture of *P. diminuta* was gently filtered through a 0.4 μm rated experimental PVDF membrane (prepared and autoclaved as described in section 3.1.1.1.) at 0.16 bar in order to separate cells, and was left at 30°C for 30 minutes before use to recover ATP levels. This was to represent bacteria that had penetrated a test membrane. Previous tests showed that ATP luminescence falls due to physical stress immediately after rigorous filtration but stabilises within a 30 minute recovery period. The experiment was carried out in triplicate NRB extractant (100 μl) was added to a 100 μl aliquot from the dilution, after 30 seconds the same amount of Lumit-pm was also added luminescence was quantified 10 seconds after the addition of luciferase. The viable count was determined for triplicate samples of each dilution using a plate count. The relationship between emitted light and ATP concentration was determined. Luminescence was quantified as described above for 100 μl aliquots from a 10-fold dilution series of ATP with 100 μl NRB and 100 μl Lumit PM. The ATP content of an average *P. diminuta* colony forming unit can then be determined knowing the relationship between viable count and luminescence and also ATP and luminescence. The luminescence given for a standard amount of ATP (1 x 10^10 g) was determined each day that ATP assays were carried out.

3.4.1.4. Correlation between the ATP assay and the plate count.

Membrane filtration was used to isolate *P. diminuta* from the sample prior to the luminescence assay. The experiment was carried out in triplicate. Samples from a 10-fold dilution series of *P. diminuta* were analysed in duplicate against a negative control in order to determine the sensitivity of the method. Samples (10 ml) were filtered through an autoclaved Amicon Diaflow YM 30 UF membrane (shown to retain *P. diminuta*,
section 4.1.2.) under a vacuum of 0.74 bar. The UF membranes were washed and autoclaved as described in section 3.1.1. A small amount (5 ml) of sterile Ringers solution was filtered after the sample. The viable count was determined for triplicate samples of each dilution using a plate count.

After filtration of the sample, the UF membrane was laid in a petri-dish and was overlaid with 1 ml of Lumacult. The petri-dish was sealed with self-sealing film and was incubated at 30°C for up to five hours. This prevented the membrane from drying out due to evaporation of the broth medium. After this time, the membrane was gently washed with 1 ml NRB extractant for 3 minutes using an automatic pipette. The entire volume of liquid was assayed for luminescence as 200 μl aliquots with 100 μl Lumit-pm. The viable count for the whole sample filtered may be determined knowing the amount of ATP contained within an average colony forming unit of *P. diminuta* and was compared to the corresponding viable count obtained from the plate count.

3.4.1.5. The retention tests.

The same filtration and incubation method was used to analyse retention test permeates. Each membrane type was assayed twice in triplicate using both the ATP method and the standard culture method. The bacterial challenge was filtered directly into an empty flask, 100 μl was withdrawn and streaked onto a MacConkey agar plate and incubated for the identification of any bacteria present (section 3.1.3.). The entire remaining contents of the flask were then immediately filtered through the UF membrane. The flask was rinsed out with Ringers solution (10 ml) which was filtered also. The methodology was continued as described previously.

3.4.2. Electrometric microbiology.

3.4.2.1. Equipment.

A Bactometer Microbial Monitoring System M64 (Biomerieux-Vitek Inc. Hazelwood, Missouri USA) comprising a single Bactometer Processing Unit (BPU) (as many as four BPU’s may be run with one microprocessor), a Nerve Centre II microprocessor, a colour terminal and a Fujitsu DX2100 printer was used throughout (figure 3.4.b.). The BPU will hold four sterile disposable modules with integral electrodes (also Biomerieux-Vitek) consisting of 16 wells each with a sample capacity of 2 ml. The BPU has two compartments capable of an independent temperature range of 55°C to 10°C below ambient. Each well is assayed sequentially every six minutes and data is
transferred to the microprocessor at the end of each cycle. The change in the total impedance, the conductive or capacitative elements of the medium may be monitored over several days.

3.4.2.2. Correlation between the impedance assay and the plate count.

The quality of signal curve given by *P. diminuta* suspended in the TSB broth traditionally used for *P. diminuta* was compared to that obtained when plate count broth (PCB, Difco Laboratories Ltd., East Molesey, Surrey) recommended for pseudomonads (Firstenberg-Eden and Eden 1984) was used. Total impedance, capacitance or conductance was monitored over 48 hours at 30°C for duplicate aliquots (2 ml) from 10-fold serial dilutions of a SLB culture of *P. diminuta*, prepared either in single- or double-strength TSB or PCB. For the calibration curve, 2 ml samples from a similar dilution series were similarly analysed, prepared with the most suitable broth medium and monitoring the most suitable signal determined by the preceding experiment. The viable count was determined for triplicate samples of each dilution using a plate count. The calibration curve was constructed using the Bactometer software. A comparison was made between calibration curves using a standard SLB culture or one that had been prefiltered as described in section 3.4.1.3.

3.4.2.3. The retention tests.

Each type of experimental membrane was tested twice in triplicate using both the impedimetric method and the standard cultural method. For the impedance assay, filtration was carried out as specified by the standard retention test method but double-strength PCB was substituted for TSB in the receiving flask. After the filtration, 16 aliquots (2 ml) of each test permeate were taken and used to fill the wells of a single module. As all of the permeate could not be monitored impedimetrically, the remaining permeate was analysed as described in section 3.1.3. for the culture test method. The modules were fitted into the BPU and total impedance was monitored for 48 hours at 30°C. The total count for *P. diminuta* of each test permeate was calculated for both the impedance assays and the control plate count assays and the LRV given by the test membrane was calculated. If results proved encouraging then large capacity sample holders could be obtained for the analysis of entire test permeates.

3.4.3. The DEFT.

The standard DEFT procedure developed for the dairy industry involves a short preincubation of the milk sample at 50°C with a crude trypsin preparation
(to digest somatic cells) and a surfactant (Triton X-100) as there were originally problems concerning the ability of the sample to pass through the membrane (Pettipher 1983). This heated preincubation and pre-warming of the vacuum filtration apparatus also helps to mobilise the milk fat to further aid filtration (Sharpe and Peterkin 1988). For the enumeration of bacteria in aqueous samples, the volume assayed can be larger than that recommended in standard methodology and there is usually no need for the preincubation step or prewarming of the filtration equipment (Denyer and Lynn 1987). Generally, 0.6 μm rated 25 mm diameter Nuclopeore track-etched membranes are recommended with the DEFT for the analysis of milk and other foodstuffs. However, a membrane with a smaller pore size rating is used for this application of the DEFT due to the small size of *P. diminuta*.

### 3.4.3.1. Reagents and equipment.

The BioFoss System 3 DEFT equipment (Foss Electric, Bishopthorpe, York) was used for the first section of work and comprised an Olympus BH-2 RFL epifluorescent microscope with automated stepping stage and mercury light source, a vacuum filtration manifold with a five tower (250 ml) capacity and vacuum gauge, an image analyser and a reagent filtration/dispenser unit. The AMS 40-10 image analyser with Biology Software and Chalnicon tube TV camera produced a viable count expressed as cfu per ml of sample.

Acridine orange concentrate, pH3 buffer concentrate and Triton X-100 concentrate were obtained from Difco and all were diluted according to the manufacturers recommendations. Acridine orange is sensitive to ultraviolet light and was stored in an amber bottle. All solutions could be kept for up to three weeks in the dark at 5°C.

Nuclopeore 0.1 μm rated 25 mm diameter membranes (already proven to give adequate retention of *P. diminuta*, section 4.1.2.) were obtained from Costar UK Ltd. and were sterilised as described in section 3.1.1. Non-fluorescent immersion oil (non-drying, non-silicone, refractive index 1.516) was obtained from Foss Electric Ltd.

### 3.4.3.2. Correlation between the DEFT and the plate count.

Samples (1ml) from 10-fold serial dilutions of a prefiltred (section 3.4.1.3.) SLB culture of *P. diminuta* were analysed in triplicate by manual and semi-automated DEFT methods against a negative control. The Ringers' solution used as the diluent for *P. diminuta* was prepared with MilliQ high purity water. Triplicate samples from each dilution were also subjected to a plate count. The filtration manifold towers and bases
were rinsed with MilliQ water before being either autoclaved or swabbed with 70% ethanol (filtered to 0.2 μm) respectively to reduce the amount of debris collected on the DEFT membrane.

All reagents were filtered to 0.2 μm directly before use using a disposable, sterile syringe-end filter unit to avoid the deposition of debris on the DEFT membrane. Each sample was filtered through the Nuclepore membrane under a vacuum of 0.50-0.67 bar. The standard method recommends washing out the sample vessel with 2 ml of 0.1% v/v Triton X-100 and filtering this also but it was found that for this application this had no beneficial effect on the quality of the final sample slide, if anything the fluorescence was brighter when the Triton rinse was omitted. Instead, the sample tube was rinsed out with 2 ml sterile Ringers’ solution which was then filtered also.

The membrane was overlaid for 2 minutes with 2 ml acridine orange (0.025% w/v) pH 6.6 which was then filtered. Citrate buffer pH 3.0 (2.5 ml) was filtered to rinse the membrane. Bacteria have been found to take up acridine orange best at a near neutral pH yet fluoresce more brightly at a low pH (Pettipher 1983). The membrane was rinsed finally by filtering 2.5 ml of isopropanol to reinforce the intensity of fluorescence. This was then allowed to air-dry for a few minutes before being mounted in non-fluorescent immersion oil on a microscope slide with a 40 x 24 mm coverslip. It was most important that there were no air-bubbles in the oil above or below the membrane to distort the plane of focus when viewing the membrane microscopically. Visible bubbles and excess oil were removed by gentle pressure. All DEFT slides were kept in the dark at 5°C, however the intensity of luminescence decreased and any difference in colour between stained bacteria became less distinguishable after one week. Therefore, the best results were obtained when the slides were observed within 24 hours.

DEFT slides were viewed microscopically with epifluorescence using a x100 oil immersion lens (using the non-fluorescent oil). The number of clumps (any cell or group of cells separated by a distance equal to or greater than twice the smallest diameter of the cells to each other) of viable, orange-fluorescing bacteria in randomly selected fields was counted. Manual counts were taken from 20 frames selected randomly across the membrane. Semi-automated counts were taken for the same number of frames using the automated stepping-stage, from the same slides using the full-frame image analyser conformation. It is recommended for the standard DEFT method that the number of fields counted depends on the number of clumps per field. The criteria for counting bacteria on DEFT slides is described on the following page (Pettipher 1983).
Standard procedure for counting bacteria on DEFT slides.

<table>
<thead>
<tr>
<th>Fields counted</th>
<th>Mean clumps per field</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0-10</td>
</tr>
<tr>
<td>10</td>
<td>11-25</td>
</tr>
<tr>
<td>6</td>
<td>26-50</td>
</tr>
<tr>
<td>3</td>
<td>51-75</td>
</tr>
<tr>
<td>2</td>
<td>76-100</td>
</tr>
<tr>
<td>dilute &amp; repeat</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

It is important that the evaluation of the DEFT applied to bacterial retention tests is accurate and 20 fields were counted for each slide independent of the density of bacteria. From the manual counts, the number of bacteria in the sample was calculated as cfu/ml using the following equations (MF represents the microscope factor):

\[
    DEFT\ count = \text{Mean number of clumps per field} \times MF \quad \ldots (15)
\]

where \[ MF = \frac{\text{Area of membrane used for filtration}}{\text{Microscope field area} \times \text{sample volume}} \quad \ldots (16) \]

3.4.3.3. The retention tests.

Each membrane type was assayed in twice in triplicate using the manual DEFT, the automated DEFT and the standard culture method. For the DEFT assays, retention tests were carried out as standard but filtration was into an empty flask that had been rinsed with MilliQ high purity water before autoclaving. To use TSB in the receiving flask would increase the amount of debris on the DEFT slide and the prolong the time taken for filtration of the test permeate due to the increased viscosity compared to Ringers solution. Each test permeate was poured from the receiving flask into a DEFT filtration tower and was filtered through a 0.1 μm rated Nuclepore membrane. A silicone bung swabbed with 70% ethanol and fitted with a 0.2 μm pore size syringe-end filter was inserted into the top of each tower to prevent airborne contamination of the sample. The DEFT membranes were stained, mounted and counted as described for the calibration.
3.4.3.4. The microcolony DEFT.

In the previous experiments, it was not easy to distinguish between individual cells and debris using the image analyser due to the small size of *P. diminuta*. Allowing viable cells captured on the DEFT membrane to multiply to form microcolonies may improve the sensitivity of detection. A microcolony is a clump or group of cells which are of at least four in number arising from a single cfu (Rodrigues and Kroll 1988, Newby 1991). Identification of the bacteria forming the microcolony can be made on the basis of cellular morphology and by the use of selective media where samples of mixed flora are encountered.

A Nikon Optiphot epifluorescence microscope with a Nikon mercury light source shown in figure 3.4.c. was used to study the ability of *P. diminuta* to form microcolonies. The correlation between DEFT count and plate count was redetermined. Photomicrographs were taken for the microcolony experiments with a Nikon camera/microscope adapter, a Nikon UFX-II exposure meter and Kodak Gold ASA 200 colour film.

3.4.3.5. Correlation between the microcolony DEFT and the plate count.

Samples from a dilution series of *P. diminuta* were analysed by a plate count and the manual DEFT count (section 3.4.3.2.), and also by a microcolony DEFT count. Considering the long generation time for *P. diminuta*, it may be of an advantage to develop a selective medium to prevent possible overgrowth of the incubated DEFT filters with contaminating microcolonies. A comparison was made between microcolony formation by *P. diminuta* incubated on L-agar (appendix D) and L-agar supplemented with streptomycin (Sigma Chemical Co. Ltd.) at 50 μg/ml to which *P. diminuta* is resistant.

The standard DEFT method was carried out but unstained DEFT membranes were laid onto agar plates (pre-warmed to 30°C) immediately after the sample filtration and were incubated to permit the formation of microcolonies. The membranes were incubated at 30°C for periods of time up to 12 hours. They were then reassembled back into the filtration manifold, stained with acridine orange following the standard method and DEFT slides were produced. Each DEFT slide was analysed by both a manual DEFT count (including all single cells and microcolonies) and a microcolony count (microcolonies only). The correlation between the viable count calculated from the microcolony DEFT (using either non-selective and selective media) and the plate count was determined.
3.4.3.6. The retention tests.

Each type of experimental membrane was tested twice in triplicate and permeates were analysed using the manual DEFT count, the microcolony DEFT count (after 6 hours incubation) and the standard culture method.

3.5. Bacterial bioluminescence.

3.5.1. Use of bioluminescent E. coli for retention testing.

3.5.1.1. Assay of bioluminescence.

The bioluminescence of individual colonies was determined by the addition of 1 μl 10% v/v decyl aldehyde (decanal, Sigma Chemical Co. Ltd.) in ethanol to the lids of inverted petri-dishes and was visible in the dark by the naked eye after 10 minutes. The bioluminescence of samples was measured in arbitrary units (AU) using a Lab-Line ATP photometer and was calibrated using [1-^14C] hexadecane as described by Hastings and Weber (1963). Samples (400 μl) were assayed in triplicate and bioluminescence was quantified over 6 seconds, 10 seconds after the addition of 10 μl decanal.

3.5.1.2. Bacteria.

E. coli JM101 (pPA3) (figure 3.5.a.) containing the luxAB genes and the Tn903-derived aph gene conferring kanamycin resistance (Andrew and Roberts 1993) was maintained on Luria agar (L-agar, appendix D) supplemented with kanamycin at 15 μg/ml and was grown in Luria broth (L-broth) similarly supplemented at 37°C and 200 rpm when required. The development of bioluminescence for such a broth culture was monitored over a 14 hour period. Samples (5.2 ml) were withdrawn at hourly intervals for bioluminescence assay and viable count determinations in triplicate, and to monitor the optical density at 600 nm. The correlation between viable count and bioluminescence was determined using triplicate samples from 10-fold serial dilutions of the same broth culture.

3.5.1.3. The retention tests.

Experimental 0.8 and 0.4 μm rated PVDF membranes were prepared and autoclaved as described in section 3.1.1.1. Each type of membrane was retention tested twice in duplicate against a negative control. The membrane challenge was taken from an early stationary phase culture of E. coli. A 100 μl aliquot of this was suspended in 40 ml Ringers solution, from which 1 ml was withdrawn and used to calculate the total viable count for the challenge by bioluminescence assay and by plate count. This
challenge preparation delivered the recommended level of approximately $1 \times 10^7$ cells per cm$^2$ of available membrane surface (HIMA 1982, FDA 1987). The challenge was filtered through the test membrane under a vacuum of 0.43 bar into a flask containing 50 ml double-strength L-broth. The vessel that had contained the challenge was washed out with 10 ml of Ringers which was filtered also and the final volume was supplemented with kanamycin at 15 µg/ml. Samples (4.2 ml) of the permeate were withdrawn for both bioluminescence assays and viable counts in triplicate 30 minutes after each filtration. The remaining permeate was then incubated at 37°C and similar samples were taken for analysis at intervals of one hour.

3.5.2. Development of a bioluminescent *P. diminuta*.

3.5.2.1. Bacteria and plasmids.

The broad host range RSF1010-based plasmid pNJR12 (figure 35.b.) containing the genes for tetracycline resistance (Tc$^\ast$) and kanamycin resistance (Km$^\ast$) was chosen as the vector, to be introduced into *P. diminuta* by conjugation mediated by the IncP-derived plasmid R751 (Maley et al 1992).

*P. diminuta* was maintained as described in section 3.1.2. and was cultured in L-broth at 30°C when required. *E. coli* JM101 was maintained on L-agar, *E. coli* JM101 (pNJR12) and *Mycobacterium smegmatis* MR155 (pPA3) (the positive control for bioluminescence) on L-agar supplemented with kanamycin at 50 µg/ml and *E. coli* JM101 (pNJR12/R751) on L-agar supplemented with kanamycin at 50 µg/ml and with trimethoprim at 100 µg/ml. All the above mentioned strains of *E. coli* were cultured at 37°C in L-broth appropriately supplemented when required.

The luxAB genes encoding the two structural subunits of *V. harveyi* luciferase were contained in the plasmid pSB226 (figure 45.a., section 4.5.2.1.) obtained as a gift from G.S.A.B. Stewart, Department of Applied Biochemistry and Food Science, University of Nottingham.

3.5.2.2. Manipulation of DNA.

Plasmid DNA was isolated using the miniprep. method (Sambrook *et al* 1989) and was analysed by restriction enzyme digestion, electrophoresis on agarose gel (0.7% agarose w/v in TAE buffer, *appendix D*) and visualisation with ethidium bromide (0.5 µg/ml) under UV light throughout the study. Ligation of DNA digested by the appropriate restriction enzymes was carried out by 1 µl T4 DNA ligase and 2 µl x10 ligase buffer (*appendix D*) in a total reaction volume of 20 µl overnight at 14°C. Cells were rendered
Figure 3.5.a. Plasmid pPA3.

Structure of pPA3 where Km' represents the Tn903-derived kanamycin resistance gene, E. coli R represents the E. coli origin of replication, mycobacterial R represents the mycobacterial origin of replication and Hsp60Pr represents the 5' regulatory region of the Mycobacterium bovis BCG hsp60 gene. The arrows indicate direction of transcription. LuxA and luxB represent the structural genes for luciferase.

Figure 3.5.b. Plasmid pPNJR12 (from Maley et al 1992).

Restriction endonuclease cleavage map of pPNJR12 where Km' and Tc' represent the tetracycline and kanamycin resistance genes. The filled box represents the Bacteroides plasmid pB8-51.
competent for transformation by the CaCl₂ method (Sambrook et al 1989) and bioluminescent transformants were to be identified by visual inspection after exposure to decanal after initial selection by antibiotic resistance.

3.5.2.3. Filter mating.

Recipient and donor strains were both grown to mid-log phase. One ml of each was pelleted by centrifugation at 9400 xg in a microfuge separately as controls, the supernatant removed and each pellet resuspended in 100 μl L-broth. Each suspension was spread onto two different 45 mm diameter cellulose acetate membranes each laid onto L-agar. One ml of the donor culture was pelleted in the same manner, not resuspended and the same volume of recipient pelleted also in the same vial. The supernatant was removed, the combined pellet resuspended in 100 μl L broth and the suspension was spread onto another membrane. All membranes were incubated at 30°C overnight after which each was washed with 1 ml Ringers solution. Triplicate aliquots (100 μl) of the wash, and 10⁻¹ and 10⁻² dilutions from it, were plated onto L-agar supplemented to be specific for the required transconjugate. The number of colonies arising from each plate after incubation was noted and as many as possible were isolated for identification.

3.5.2.4. Construction of radiolabelled DNA probe.

The required probe DNA was randomly labelled with ³²P in the following manner: DNA (18 μl or approximately 10 ng) was boiled for 5 minutes in a capped, vented ependorf after which 4 μl OLB buffer, 0.8 μl Klenow DNA polymerase I and 10 μCu dCT³²P were carefully added. This was incubated for 5 hours at room temperature and was boiled for 5 minutes before being added to the hybridisation solution.

3.5.2.5. Colony hybridisation.

Numerous colonies were dotted onto 82 mm diameter filters (DuPont, Colony/plaque screen) laid onto L-agar plates supplemented with the appropriate antibiotics and also onto duplicate plates for reference. A negative and positive control was included. The filters were incubated overnight whilst the reference plates were incubated until colonies were visible when they were stored at 5°C. Details of all solutions used can be found in *appendix D*. Each filter was then sequentially laid (colony side uppermost) onto 3MM paper (Whatman International Ltd.) soaked with 10% SDS (w/v) for three minutes, then denaturing solution, neutralising solution and 2x SSC, each for five minutes. No solution was allowed to contact the surface of the filters. The filters were allowed to air-dry at room temperature for 30 minutes when they were sandwiched
between 3MM paper and dried for 1.5 hours at 80°C. The cellular debris was removed by placing each filter in 50 ml 2x SSC, scrubbing the surface gently with polyamide wool and rinsing with the same volume of fresh 2x SSC.

The filters were placed in a hybridisation tube containing 25 ml pre-hybridisation solution with 200 μg/ml denatured herring sperm DNA and were rotated gently for 2 hours at 65°C. The pre-hyb. solution was poured off and was replaced with 25 ml of hybridisation solution and 200 μg/ml denatured herring sperm DNA at 65°C, the ^32^P-labelled probe was carefully added and hybridisation was carried out overnight at 65°C. The hyb. solution was replaced with 50 ml wash 1 (10% 2x SSC, 0.1% SDS) and the filters were washed for 15 minutes at 65°C, this was replaced with the same volume of wash 2 (2.5% 2x SSC, 0.1% SDS) then several changes of wash 3 (0.5% 2x SSC, 0.1% SDS) until the washes indicated the absence of radioactive material. The filters were allowed to air-dry for 1 hour before being exposed to X-ray film (DuPont, Corex 4) overnight.
4. RESULTS.

The results fall into five categories; the application of a retention test method using *P. diminuta* to the pore size characterisation of experimental and also of commercial 0.2 and 0.1 μm rated membranes (*section 4.1.*), detailed cell size analysis of *P. diminuta* (*section 4.2.*), the retention of *P. diminuta* given by flat sheet and tubular configuration membranes during cross-flow filtration (*section 4.3.*), the development of rapid retention test procedures by applying popular techniques for the detection of bacteria (*section 4.4.*) and also research concerning the potential use of a bioluminescent strain of *P. diminuta* to be developed through genetic engineering (*section 4.5.*) for the rapid and straightforward retention testing of 0.2 μm rated membranes.

4.1. Retention testing of experimental and commercial sterilising grade membranes.

4.1.1. Experimental membranes.

PVDF is a popular material for microfiltration membranes as it is resistant to a number of commonly used chemicals such as ammonia, hydrogen peroxide, sulphuric, nitric and hydrofluoric acids and also produces a membrane with a good degree of thermal and mechanical stability (Toyomoto and Higuchi 1992). Hydrophobic PVDF membranes have a high fouling potential for protein due to the hydrophobic interactions between regions of hydrophobic amino acids in the protein and the membrane surface. The polymers used to make low-protein binding hydrophilic membranes, such as cellulose acetate and polyacrylonitrile, do not have thermal stability and are susceptible to attack from certain chemicals and antimicrobial agents. The introduction of hydrophilic polymers to the surface of the hydrophobic membrane will theoretically produce a stable membrane with the advantages of hydrophilicity.

The retention test results for native and grafted experimental sterilising grade membranes can be seen in *table 4.1.a.*. The membranes had been packed in a glycerol/formaldehyde solution and were sterilised by hypochlorite. Each result shown was calculated from six individual filtration experiments. Filtration was carried out at both 0.67 and 0.81 bar to demonstrate the retention capabilities of the test membranes at the upper and lower limits for the vacuum specified by the ASTM (D 3862 1992). Not one of the 0.2 or the 0.1 μm rated membranes were seen to retain the recommended challenge.
Table 4.1.a. Comparison of retention efficiency (as LRV) given by hypochlorite-sterilised native and surface modified membranes (packed in glycerol).

<table>
<thead>
<tr>
<th>Membrane material</th>
<th>Pore size rating (in µm)</th>
<th>LRV 0.67 bar</th>
<th>LRV 0.81 bar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>0.2</td>
<td>3.84</td>
<td>3.19</td>
</tr>
<tr>
<td>A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>B</td>
<td>4.32</td>
<td>3.83</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>4.25</td>
<td>3.52</td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>0.1</td>
<td>4.26</td>
<td>4.35</td>
</tr>
<tr>
<td>A</td>
<td>4.33</td>
<td>4.05</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>4.34</td>
<td>3.95</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>4.46</td>
<td>4.16</td>
<td></td>
</tr>
</tbody>
</table>

Where N/A refers to that type of membrane being not available.

Table 4.1.b. Comparison of retention efficiency (LRV) given by hypochlorite-sterilised and autoclaved native and surface modified membranes (packed in Triton X-100).

<table>
<thead>
<tr>
<th>Membrane material</th>
<th>Pore size rating (in µm)</th>
<th>LRV Hypochlorite</th>
<th>LRV Autoclave</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>0.2</td>
<td>3.15</td>
<td>3.19</td>
</tr>
<tr>
<td>A</td>
<td>3.17</td>
<td>3.39</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>3.37</td>
<td>3.38</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>3.39</td>
<td>3.34</td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>0.1</td>
<td>4.25</td>
<td>4.19</td>
</tr>
<tr>
<td>A</td>
<td>4.29</td>
<td>4.38</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>4.41</td>
<td>4.45</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>4.44</td>
<td>4.19</td>
<td></td>
</tr>
</tbody>
</table>
level of *P. diminuta* although a larger LRV was given by the 0.1 μm rated membranes. The LRV's obtained given after filtration under the higher vacuum were consistently lower than those given with the lower vacuum.

One aim for this section of work was determine whether grafting hydrophilic material onto PVDF membranes decreased the retention capacity for *P. diminuta*. The results were tested statistically (Bhattacharyya and Johnson 1977).

The null hypothesis (*H₀*) was that the LRV for grafted membranes (μ₂) is not less than the LRV for native membranes (μ₁).

The alternative hypothesis (*H₁*) was that the LRV for grafted membranes is less than the LRV for native membranes, due to decreased adsorption of *P. diminuta*.

By application of the following equations the significance of the difference between mean LRV's given by grafted and ungrafted membranes can be determined. The term *t* represents the Students' *t* distribution, *x* and *y* represent the mean value of the samples to be compared whilst *n* represents the number of samples in each group. The term *s²*<sub>pooled</sub> represents the pooled estimator of the common variance, *σ²*.

\[
t = \frac{x - y}{s_{pooled} \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}
\]  

... (17)

where

\[
s_{pooled}^2 = \frac{\sum (x_i - \bar{x})^2 + \sum (y_i - \bar{y})^2}{n_1 + n_2 - 2}
\]  

... (18)

The results for the 0.2 and the 0.1 μm rated membranes can be seen in tables 4.1.c and 4.1.d. The level of significance *α* in each case and the degrees of freedom (d.f.) are given. It was shown that the retention capacity for *P. diminuta* given by grafted membranes was not found to be less than that given by the native membranes. If anything, the LRV's given by the grafted membranes appeared to be the greater. A decrease in pore size for some of the grafted membranes due to steric hindrance by the graft material was reported by the manufacturers. To test the theory that the graft was causing an increase in retention capacity for these membranes, new null and the alternative hypotheses were applied and the statistical analysis was repeated.

The null hypothesis (*H₀*) was that the LRV for grafted membranes (μ₂) is not greater than the LRV for native membranes (μ₁).

The alternative hypothesis (*H₁*) was that the LRV for grafted membranes is greater than the LRV for native membranes, due to obstruction of the pores.
Table 4.1.c. Testing of the statistical hypothesis concerning the retention given by native and grafted 0.2 \( \mu m \) rated membranes.

\[ H_0: \mu_1 \leq \mu_2 \quad \alpha = 0.05 \]
\[ H_I: \mu_1 > \mu_2 \quad \text{D.f.} = (n_1+n_2-2) = 10 \]

<table>
<thead>
<tr>
<th>Membrane material</th>
<th>Mean LRV</th>
<th>( t_{0.5} )</th>
<th>( t_{\text{observed}} )</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>(3.84)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>4.32</td>
<td>1.812</td>
<td>-7.037</td>
<td>( H_0 ) is not rejected</td>
</tr>
<tr>
<td>B</td>
<td>4.25</td>
<td>1.812</td>
<td>-6.882</td>
<td>( H_0 ) is not rejected</td>
</tr>
</tbody>
</table>

Table 4.1.d. Testing of the statistical hypothesis concerning the retention given by grafted and native 0.1 \( \mu m \) rated membranes.

\[ H_0: \mu_1 \leq \mu_2 \quad \alpha = 0.05 \]
\[ H_I: \mu_1 > \mu_2 \quad \text{D.f.} = (n_1+n_2-2) = 10 \]

<table>
<thead>
<tr>
<th>Membrane material</th>
<th>Mean LRV</th>
<th>( t_{0.5} )</th>
<th>( t_{\text{observed}} )</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>(4.26)</td>
<td>1.812</td>
<td>-0.284</td>
<td>( H_0 ) is not rejected</td>
</tr>
<tr>
<td>A</td>
<td>4.33</td>
<td>1.812</td>
<td>-0.411</td>
<td>( H_0 ) is not rejected</td>
</tr>
<tr>
<td>B</td>
<td>4.34</td>
<td>1.812</td>
<td>-0.908</td>
<td>( H_0 ) is not rejected</td>
</tr>
<tr>
<td>C</td>
<td>4.46</td>
<td>1.812</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1.e. Testing of the statistical hypothesis concerning the retention given by grafted and native 0.2 \( \mu m \) rated membranes.

\[ H_0: \mu_1 \geq \mu_2 \quad \alpha = 0.05 \]
\[ H_I: \mu_1 < \mu_2 \quad \text{D.f.} = (n_1+n_2-2) = 10 \]

<table>
<thead>
<tr>
<th>Membrane material</th>
<th>Mean LRV</th>
<th>( t_{0.5} )</th>
<th>( t_{\text{observed}} )</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>(3.84)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>4.32</td>
<td>&quot;</td>
<td>7.037</td>
<td>( H_0 ) is rejected</td>
</tr>
<tr>
<td>B</td>
<td>4.25</td>
<td>&quot;</td>
<td>6.882</td>
<td>( H_0 ) is rejected</td>
</tr>
</tbody>
</table>

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Table 4.1.f. Testing of the statistical hypothesis concerning the retention given by grafted and native 0.1 \( \mu m \) rated membranes.

\[ H_0: \mu_1 \geq \mu_2 \quad \alpha = 0.05 \]

\[ H_1: \mu_1 < \mu_2 \quad D.f. = (n_1+n_2-2) = 10 \]

<table>
<thead>
<tr>
<th>Membrane material</th>
<th>Mean LRV</th>
<th>( t_{0.5} )</th>
<th>( t_{\text{observed}} )</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>(4.26)</td>
<td>1.812</td>
<td>0.284</td>
<td>( H_0 ) is not rejected</td>
</tr>
<tr>
<td>A</td>
<td>4.33</td>
<td>1.812</td>
<td>0.411</td>
<td>( H_0 ) is not rejected</td>
</tr>
<tr>
<td>B</td>
<td>4.34</td>
<td>1.812</td>
<td>0.908</td>
<td>( H_0 ) is not rejected</td>
</tr>
<tr>
<td>C</td>
<td>4.46</td>
<td>1.812</td>
<td>1.812</td>
<td>( H_0 ) is not rejected</td>
</tr>
</tbody>
</table>

Table 4.1.g. Testing of the statistical hypothesis concerning the retention given by hypochlorite-sterilised (H) and autoclaved (A) 0.2 \( \mu m \) rated membranes.

\[ H_0: \mu_1 \leq \mu_2 \quad \alpha = 0.05 \]

\[ H_1: \mu_1 > \mu_2 \quad D.f. = (n_1+n_2-2) = 10 \]

<table>
<thead>
<tr>
<th>Membrane material</th>
<th>LRV H</th>
<th>LRV A</th>
<th>( t_{0.5} )</th>
<th>( t_{\text{observed}} )</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>3.15</td>
<td>3.19</td>
<td>1.812</td>
<td>-1.099</td>
<td>( H_0 ) is not rejected</td>
</tr>
<tr>
<td>A</td>
<td>3.17</td>
<td>3.39</td>
<td>1.812</td>
<td>-3.694</td>
<td>( H_0 ) is not rejected</td>
</tr>
<tr>
<td>B</td>
<td>3.37</td>
<td>3.38</td>
<td>1.812</td>
<td>-0.177</td>
<td>( H_0 ) is not rejected</td>
</tr>
<tr>
<td>C</td>
<td>3.39</td>
<td>3.34</td>
<td>1.812</td>
<td>0.501</td>
<td>( H_0 ) is not rejected</td>
</tr>
</tbody>
</table>

Table 4.1.h. Testing of the statistical hypothesis concerning the retention given by hypochlorite-sterilised (H) and autoclaved (A) 0.1 \( \mu m \) rated membranes.

\[ H_0: \mu_1 \leq \mu_2 \quad \alpha = 0.05 \]

\[ H_1: \mu_1 > \mu_2 \quad D.f. = (n_1+n_2-2) = 10 \]

<table>
<thead>
<tr>
<th>Membrane material</th>
<th>LRV H</th>
<th>LRV A</th>
<th>( t_{0.5} )</th>
<th>( t_{\text{observed}} )</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>4.25</td>
<td>4.19</td>
<td>1.812</td>
<td>0.9547</td>
<td>( H_0 ) is not rejected</td>
</tr>
<tr>
<td>A</td>
<td>4.29</td>
<td>4.38</td>
<td>1.812</td>
<td>-1.7934</td>
<td>( H_0 ) is not rejected</td>
</tr>
<tr>
<td>B</td>
<td>4.41</td>
<td>4.45</td>
<td>1.812</td>
<td>-0.3999</td>
<td>( H_0 ) is not rejected</td>
</tr>
<tr>
<td>C</td>
<td>4.44</td>
<td>4.19</td>
<td>1.812</td>
<td>3.0531</td>
<td>( H_0 ) is rejected</td>
</tr>
</tbody>
</table>

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The results can be seen in tables 4.1.e. and 4.1.f. For the 0.2 μm rated membranes, the null hypothesis was rejected in each case inferring that the retention ability of the grafted membranes was greater than that of the native membranes. For the 0.1 μm rated membranes however, the null hypothesis was not rejected in each case. The study of protein adsorption by native and grafted membranes by other researchers on the BRITE project (results not shown) showed that this was not decreased by the presence of graft material A or B but was decreased by graft C. Graft B (with the highest molecular weight) was seen by the manufacturers to cause the greatest amount of steric hindrance of the pores, which was not seen for graft A (with the lowest molecular weight).

It can be concluded for these results that grafting hydrophilic material to native hydrophobic membranes did not decrease the retention ability but rather retention was increased due to obstruction of the membrane pores with graft material. This effect was seen to be applicable to the membranes with larger pore size (0.2 μm rated membranes). The increased steric hindrance caused by graft B was reflected by 0.2 μm rated membranes (table 4.1.a.) grafted with this material having a higher retention ability for *P. diminuta* than did other grafted and native membranes of the same pore size.

Table 4.1.b. shows the LRV's for *P. diminuta* given by membranes cast onto an experimental support backing that was designed not to shrink or deform upon autoclaving. The ability to steam sterilise or autoclave equipment is very important for the bio-industries. Filtration was carried out under a vacuum of 0.74 bar. The glycerol used to impregnate previous experimental membranes was found by the manufacturers to damage the membrane pore structure when autoclaved. Glycerol could not be fully washed out from the membranes before autoclaving and the membranes used for this section of work were impregnated with a Triton X-100/formaldehyde solution which was easily washed out. The retention for *P. diminuta* given by autoclaved membranes was compared with that given by hypochlorite-sterilised membranes using the same statistical analysis described earlier. It is important to be able to show that the retention ability of a sterilising grade membrane will not decrease when autoclaved due to damage caused by heat. The null and the alternative hypotheses were as follows:

The null hypothesis ($H_0$) was that the LRV for autoclaved membranes ($\mu_2$) is not less than the LRV for hypochlorite-sterilised membranes ($\mu_1$).

The alternative hypothesis ($H_1$) was that the LRV for autoclaved membranes is less than the LRV for hypochlorite-sterilised membranes, due to damage during autoclaving.
The results can be seen in tables 4.1.g. and 4.1.h.. For both the 0.2 and the 0.1 μm rated membranes, the null hypothesis was not rejected in all cases but one. No importance was attached to this single result and further studies would be needed to determine its’ significance. It was concluded that these experimental sterilising grade membranes were suitable to be used after autoclaving.

4.1.2. Commercial membranes.

The efficiency of retention given by a variety of commercial 0.2, 0.22, and 0.1 μm pore size rated membranes with different pore conformations (capillary pore, isotropic and anisotropic) and manufactured from different materials was studied and found to vary widely (table 4.1.i.). All of the commercial membranes examined proved more capable of retaining the recommended challenge of *P. diminuta* than the experimental membranes that were designed solely for cross-flow filtration. Each result for the microfiltration membranes was calculated using the results from 10 individual filtration experiments whilst the results for the UF membranes were calculated from 5 individual filtration experiments.

It was found that of the 0.2 and 0.22 μm rated membranes, only the Ceramesh composite ceramic membranes consistently produced a sterile filtrate and were validated as sterilising grade. Fewer of the 0.22 μm rated Durapore membranes than any of the 0.2 μm rated membranes were seen to pass the retention test. The anisotropic Whatman cellulose nitrate 0.2 μm rated membranes gave the second best test pass rate with those membranes failing to impart sterility giving a mean LRV of 5.52.

Of the 0.1 μm rated membranes, Nuclepore 0.1 μm track-etched membranes gave a 100% test pass rate with both the Whatman cellulose nitrate and DDS Niro polysulphone membranes giving a test pass rate of 90%. Amicon UF membranes with a NMCW of less than 100,000 daltons were seen to retain *P. diminuta*. However, some of the 300,000 and 100,000 dalton NMWC membranes failed to retain the same level of challenge. This suggests the presence of very small bacterial cells at the extreme of the cell size distribution range.

It can also be seen from the results in table 4.1.i. that although the Whatman and Nuclepore membranes exhibit a high test pass rate, sometimes the LRV given by those that fail the test are quite low. This could be explained by either damage to the membrane or, taking into account the thin nature of the two types of membrane involved, a loss of seal integrity due to movement of the membrane during filtration. A study of the bacterial retention capacities of the Durapore, DDS-Niro and the experimental membranes when used in a cross-flow configuration follows in section 4.3.

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Table 4.1.i.  Retention of *P. diminuta* given by a range of commercial membranes.

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Membrane</th>
<th>Retention test pass rate</th>
<th>Mean LRV by failed membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whatman</td>
<td>Cellulose nitrate 0.2 μm</td>
<td>70%</td>
<td>5.52</td>
</tr>
<tr>
<td></td>
<td>Cellulose nitrate 0.1 μm</td>
<td>90%</td>
<td>4.49</td>
</tr>
<tr>
<td>Millipore</td>
<td>Durapore PVDF 0.22 μm</td>
<td>40%</td>
<td>4.26</td>
</tr>
<tr>
<td>Nuclepore</td>
<td>Polycarbonate 0.2 μm</td>
<td>60%</td>
<td>3.43</td>
</tr>
<tr>
<td></td>
<td>Polycarbonate 0.1 μm</td>
<td>100%</td>
<td>N/A *</td>
</tr>
<tr>
<td>DDS Niro</td>
<td>PVDF 0.2 μm</td>
<td>30%</td>
<td>4.67</td>
</tr>
<tr>
<td></td>
<td>Polysulphone 0.2 μm</td>
<td>60%</td>
<td>4.98</td>
</tr>
<tr>
<td></td>
<td>PVDF 0.1 μm</td>
<td>80%</td>
<td>5.76</td>
</tr>
<tr>
<td></td>
<td>Polysulphone 0.1 μm</td>
<td>90%</td>
<td>5.36</td>
</tr>
<tr>
<td>Ceramesh</td>
<td>Ceramic 0.2 μm</td>
<td>100%</td>
<td>N/A</td>
</tr>
<tr>
<td>Amicon UF</td>
<td>XM 300</td>
<td>60%</td>
<td>5.11</td>
</tr>
<tr>
<td></td>
<td>XM 100</td>
<td>80%</td>
<td>5.32</td>
</tr>
<tr>
<td></td>
<td>YM 100</td>
<td>100%</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>YM 30</td>
<td>100%</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>YM 10</td>
<td>100%</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>YM 2</td>
<td>100%</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* Where N/A = not applicable.
4.2. Cell size analysis for \textit{P. diminuta}.

The individual cell sizes for cultures of \textit{P. diminuta} were studied either directly by SEM or by a rapid, electronic particle sizing technique. The dimensions of bacteria are not constant and are dependant upon the age and nutritional status of the cells (Stanier \textit{et al} 1987). The range of cell dimensions for TSB and SLB cultures of \textit{P. diminuta} that were either shaken or stationary during incubation were determined. No standard retention test methodology studied during the research (including ASTM D 3862 1992) mentioned whether the SLB culture should be shaken or stationary. The dimensions of \textit{P. diminuta} found in test permeates from triplicate 0.1 and 0.2 \textmu m rated membranes were determined.


A selection of the many SEM photomicrographs produced during the cell size analysis of \textit{P. diminuta} can be seen in appendix E. Width and breadth measurements were taken from as many fully visible cells as possible. It was clearly seen from both the numerical results (\textit{tables 4.2.a} and \textit{4.2.b}) and from the photomicrographs that growth of \textit{P. diminuta} in SLB (rather than TSB) caused the decrease in cell dimensions and the change in cell morphology towards a more coccoidal rod shape described by Leahy and Sullivan (1978). A decrease in the overall mean dimensions was seen for those cells grown in the stationary rather than the orbital incubator. This effect was observed for both the TSB and the SLB cultures of \textit{P. diminuta}. There were also some bacterial cells present in both the still and shaken SLB cultures of dimensions much smaller than the mean values for each type of culture. This may be why some of the less retentive UF membranes (NMWC 300,000 and 100,000 dalton) failed to produce a sterile permeate (\textit{table 4.1.b}).

The mean dimensions of cells that had passed through 0.2 \textmu m rated membranes were similar although slightly larger than those that had penetrated the 0.1 \textmu m membranes. It was noted that neither of the length or breadth measurements were less than 0.2 or 0.1 \textmu m. This indicates that the failure to retain (for these membranes only) was due to either the presence of oversize pores or to inaccurate pore size rating, and not by the presence of bacteria smaller than the pore size rating.

In conclusion, the conditions of growth for \textit{P. diminuta} are seen to be important in determining the range of cell sizes and should be clearly stated in retention test procedures. The differences between stationary and shaken cultures was studied in this
Table 4.2.a. Cell size analysis by SEM for TSB cultures of *P. diminuta*.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Number of samples</th>
<th>Property Measure-</th>
<th>Standard</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Standard deviation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSB still</td>
<td>n = 35</td>
<td>Length min.</td>
<td>0.833</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>max.</td>
<td>1.935</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mean</td>
<td>1.190</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Width min.</td>
<td>0.268</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>max.</td>
<td>0.673</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mean</td>
<td>0.407</td>
<td>0.472</td>
</tr>
<tr>
<td>TSB shake</td>
<td>n = 40</td>
<td>Length min.</td>
<td>1.059</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>max.</td>
<td>1.766</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mean</td>
<td>1.303</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Width min.</td>
<td>0.203</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>max.</td>
<td>0.374</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mean</td>
<td>0.346</td>
<td>0.213</td>
</tr>
</tbody>
</table>

Table 4.2.b. Cell size analysis by SEM for SLB cultures of *P. diminuta*.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Number of samples</th>
<th>Property Measure-</th>
<th>Standard</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Standard deviation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLB still</td>
<td>n = 41</td>
<td>Length min.</td>
<td>0.236</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>max.</td>
<td>1.475</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mean</td>
<td>0.851</td>
<td>0.388</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Width min.</td>
<td>0.082</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>max.</td>
<td>0.833</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mean</td>
<td>0.451</td>
<td>0.295</td>
</tr>
<tr>
<td>SLB shake</td>
<td>n = 37</td>
<td>Length min.</td>
<td>0.224</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>max.</td>
<td>1.574</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mean</td>
<td>0.893</td>
<td>0.511</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Width min.</td>
<td>0.183</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>max.</td>
<td>0.844</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mean</td>
<td>0.523</td>
<td>0.312</td>
</tr>
</tbody>
</table>
Table 4.2.c. Cell size analysis by SEM for *P. diminuta* in retention test permeates.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Number of samples</th>
<th>Property</th>
<th>Measurement (μm)</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLB shake from PVDF 0.2 μm</td>
<td>n = 29</td>
<td>Length</td>
<td>0.220</td>
<td>0.982</td>
</tr>
<tr>
<td></td>
<td></td>
<td>min. max. mean Width</td>
<td>0.271</td>
<td>0.475</td>
</tr>
<tr>
<td>SLB shake from PVDF 0.1 μm</td>
<td>n = 28</td>
<td>Length</td>
<td>0.358</td>
<td>0.676</td>
</tr>
<tr>
<td></td>
<td></td>
<td>min. max. mean Width</td>
<td>0.237</td>
<td>0.437</td>
</tr>
</tbody>
</table>

Table 4.2.d. Cell size analysis by electronic particle sizing for *P. diminuta*.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Number distribution</th>
<th>Weight/volume distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak channel (μm)</td>
<td>Cells per ml</td>
</tr>
<tr>
<td>1 TSB still</td>
<td>Not peaked</td>
<td>3.07×10^8 at cut-off</td>
</tr>
<tr>
<td>2 TSB shake</td>
<td>1.00 to 0.79</td>
<td>1.79×10^9</td>
</tr>
<tr>
<td>3 SLB still</td>
<td>1.00 to 0.79</td>
<td>2.29×10^7 at cut-off</td>
</tr>
<tr>
<td>4 SLB shake</td>
<td>Not peaked at cut-off</td>
<td>5.76×10^7</td>
</tr>
</tbody>
</table>

*Results for 3 inaccurate due to the abnormally high background count.*

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study, but the differences between cultures shaken at different speeds may need to be
determined to develop a truly standardised retention test procedure. The ability of
sterilising grade membranes to allow the penetration of bacteria with larger mean
dimensions than the pore size rating indicates the value of such retention tests and
highlights the inaccuracies of physical pore size determinations.

4.2.2. Electronic particle sizing.

A schematic diagram of the Coulter Counter equipment for the analysis of particle
size and a brief description of its method of operation can be found in appendix F. The
equipment was originally developed for haematological uses to measure and count red
blood corpuscles but has been superseded in this area by more sensitive techniques. The
Coulter equipment is now is used for general particle sizing in a great range of
applications.

Detailed results from the electronic particle size analysis of *P. diminuta* can also
be found in appendix F and a summary of these is shown in table 4.2.d. Each result is
the mean of four consecutive size distribution measurements corrected for background
noise. For the shaken TSB culture, the distribution of cell sizes was seen to peak in the
particle size range 1.00-0.79 µm. For all of the other cultures, this distribution was not
seen to peak within the size detection limits of the equipment. For these cultures, the
number of cells per ml at the cut-off point for the lower limits of detection is displayed
(table 4.2.d.). The results for the still SLB culture were distorted probably due to the
presence of particulate contamination. The results for weight/volume distribution
reflected those seen for the numerical distribution, and although the distribution was not
always seen to peak in either case, a decrease in cell size with growth in SLB broth and
by stationary incubation as shown by the SEM analysis was indicated. The lower particle
size detection limit throughout these experiments was consistently 0.63 µm. Although
the use of the Coulter Counter produced results quickly and easily, the technique was
not sensitive enough to adequately analyse the still TSB culture, either of the SLB cultures
or to analyse any retention test permeates at all.

The procedure for individual cell size analysis by SEM was labour intensive but
produced detailed information about the dimensions for *P. diminuta* and was more
suitable for this purpose than rapid analysis by the Coulter Counter. The use of image
analysis for the measurement of cell size would greatly increase the efficiency of the
SEM technique. The direct microscopic measurement also has the advantage of giving
knowledge about the cell morphology as well as the size. The Coulter counter does not
permit specific discernment of particle shape and results are expressed in spherical
equivalents. It is also not possible to discriminate between bacterial and non-bacterial particles. A greater sensitivity of detection and more accurate analysis of bacterial dimensions may be achieved if a smaller aperture for use with the Coulter particle size analysis equipment could be obtained.

4.3. The cross-flow retention tests.

The retention testing of sterilising grade membranes with *P. diminuta* during cross-flow filtration has not previously been possible. A selection of commercial and experimental flat-sheet polymeric membranes were retention tested with *P. diminuta* over a one hour period using cross-flow filtration. A similar cross-flow retention test was also developed to determine the retention characteristics given by ceramic multichannel monoliths over periods of time up to 6 hours.

4.3.1. The Rayflow module.

All filtration experiments were carried out with a TMP of 0.5 bar and a linear velocity of 3.5 m/s acceptable for many applications of microfiltration in the bioindustries to minimise the fouling rate for the membrane. For the results shown in this section and also in appendix G, each test membrane was given a reference number. The membranes and numbers are listed below:

**Membrane number:**

1 - 0.2 μm experimental PVDF
2 - 0.1 μm experimental PVDF
3 - 0.2 μm PVDF Durapore
4 - 0.2 μm PVDF DDS
5 - 0.2 μm Polysulphone DDS
6 - 0.1 μm PVDF DDS
7 - 0.1 μm Polysulphone DDS

The LRV was determined at 15 minute intervals during the 1 hour filtration experiment. It can be seen from the results shown in figures 4.3.a. and 4.3.b. that the Durapore membrane gave a greater LRV for *P. diminuta* than the 0.2 μm rated experimental membrane which in turn gave a greater LRV than the 0.1 μm rated experimental membrane. This is the same trend that was seen in the results for dead-end
filtration with the same types of membranes (section 4.1.2.). The isotropic Durapore membrane also gave a lower permeate rate than anisotropic membranes of the same pore size rating (figure G.1., appendix G). The DDS-Niro membranes gave greater LRV's than the experimental membranes of the same pore size and the 0.22 μm rated Durapore membrane. Permeate samples from both 0.1 μm rated DDS membranes and the polysulphone 0.2 μm rated DDS membrane analysed 15 minutes after the start were sterile. Permeate samples from both 0.1 μm rated DDS membranes analysed 30 minutes after the start were sterile. From then on, the permeate samples were not sterile and in general the LRV tended to decrease with time. Not one of the membranes tested fully retained P. diminuta over the entire test period but the possibilities for using membranes in series for cross-flow filtration must be taken into account and will be discussed more thoroughly in section 5.2..

The cross-flow retention test was able to give information about the efficiency of the membranes as well as of the module itself. It would be expected that the LRV would be seen to increase from the start of the experiment as the pores become blocked with bacteria. Only the Durapore membrane exhibited this trend. The SLB used as the feed fluid consists mainly of saline with a very low organic content (section 3.1.2) and will not foul the membrane to a great extent, and the decrease in permeate rate can be assumed to be due mainly to bacteria blocking the pores. However, on the whole the LRV tended to decrease during these experiments (figures 4.3.a and 4.3.b.) whilst the permeate rates tended to decrease (table G.2, appendix G.).

There did not appear to be a clear pattern to the change in LRV with time for these experiments. The decrease in LRV may be due to failure of the membrane seals to maintain integrity. The seals would quite often leak slightly during the experiment without showing a concomitant increase in permeate rate. When the membrane and seal was inspected after the filtration experiment a slight lifting of the seal from the membrane could be seen and it was clear that retentate had been able to pass into the permeate at this point. This was reflected by the high viable count seen for the permeate sample. The design of the seal (appendix B) could allow feed to pass around and under the edge of the membrane without actually moving the seal from its seating in the backplate. A suggestion for future designs is that the membrane might protrude from underneath the seal to make leakage of the feed around the edge of the membrane more difficult. It is quite possible that the decrease in LRV with time may be due to a small leak under the membrane seal. The effect on the permeate rate caused by the feed passing into the permeate may be masked by the overall decrease in permeate rate with time as the membrane pores become blocked with bacteria. It would only require the leakage of a
Figure 4.3.a. Retention efficiency (as LRV) for *P. diminuta* given by flat-sheet organic membranes 1-3 (page 105) during cross-flow filtration with a linear velocity of 3.5 m/s and a TMP of 0.5 bar. Legends refer to filtration time in minutes.

Figure 4.3.b. Retention efficiency (as LRV) for *P. diminuta* given by flat-sheet organic membranes 4-7 during cross-flow filtration with a linear velocity of 3.5 m/s and a TMP of 0.5 bar.
very small volume of feed into the permeate to cause the observed decrease in LRV. Therefore the design of a module to be used for sterilising filtration needs careful consideration. Overall, the Rayflow module had the advantages that it was simple to use and that the materials from which it was manufactured rendered it light and easy to handle, inexpensive and fairly robust. The transparent nature of the end manifolds meant that any major problems with the seating of the membrane *in situ* or with the progress of filtration in general could be easily observed.

### 4.3.2. The Microkerasep monoliths.

Although flat-sheet sterilising grade membranes may be retention tested using dead-end filtration, this option is not applicable to tubular configuration membranes. Four Microkerasep ceramic multichannel monoliths listed below were retention tested with *P. diminuta* using cross-flow filtration.

**Monolith number:**

1. 0.2 μm 19 channel (used for trials)
2. 0.2 μm 7 channel
3. 0.2 μm 19 channel
4. 0.1 μm 19 channel

Trial filtration experiments were carried out to determine the retention efficiency given by a 0.2 μm rated 19 channel monolith (monolith 1) with a variety of recirculation velocities and TMP's. The results of these experiments can be seen in *figures G.3. and G.4. (appendix G)* and *figure 4.3.f.* Retention tests using monoliths 2, 3 and 4 were also carried out using a linear recirculation velocity of 3.0 m/s and a TMP of 0.5 bar. These conditions were requested by the manufacturers, Tech-Sep.

For all the trial experiments and the retention tests using monoliths 2, 3 and 4 (*figures 4.3.c and 4.3.e*), the LRV was seen to increase during the first hour of run time with a concomitant decrease in permeate rate (*figure 4.3.d.*) as the membrane pores becomes blocked with bacteria. At no time during any experiments did the seal integrity for the monolith fail although the silicone seals may possibly loose integrity towards the end of their lifetime after repeated autoclaving. A decrease in LRV was given in response to an increase in feed linear velocity and also to an increase in TMP (*figures G.3. and G.4., appendix G*), *figure 4.3.e.*. The retention test results for monoliths 2 and 3 (*figure 4.3.c.*) showed that a LRV of approximately 3.30 to 3.50 was obtained after a run time
The permeate rates were in the order of $3-5 \times 10^2 \text{ l/h/m}^2$ (table G.6., appendix G). These results were obtained using the optimal operating parameters recommended to decrease the rate of membrane fouling. Although the monoliths did not retain the recommended challenge level of \textit{P. diminuta} the possibility exists of using two or more monoliths in series as mentioned in section 4.3.1.

Comparing the retention test results for monoliths 2 and 3, (figure 4.3.c.), it can be seen that during the filtration experiment, the LRV increased more rapidly for monolith 2 that has the fewer number of channels. The trend is also reflected by the permeate rates for the monolith 2 (figure 4.3.d.) which are seen to decrease more rapidly than the permeate rates for monolith 3. Remembering that both monoliths were exposed to approximately the same number of bacteria in the feed, this may be explained by the membrane pores for monolith 2 becoming blocked with bacteria more rapidly as there was a smaller area available for filtration. The 0.1 \text{ \mu m} rated monolith 4 consistently gave lower LRV's and higher permeate rates than the 0.2 \text{ \mu m} rated monolith 3 with the same number of channels. The increase in retention capacity with time follows the same pattern shown by the monolith with the larger pore size but with lower LRV's. This could be assumed to be due to some defect in the pore structure of the monolith.

Comparing the retention test results from trial experiments using monolith 1 (figure G.3., appendix G) with those for monolith 3 (the same model of monolith) (figure 4.3.e.) under the same experimental conditions, it was found that the LRV's determined for monolith 1 were higher than those for monolith 3. Considering that the pore size rating of these monoliths are the same and that monolith 1 was used nine times before these results were obtained and monolith 3 only twice, it could be that the cleaning methods used are not sufficient to restore original performance characteristics and a fouling layer of bacteria gradually built up inside the porous ceramic structure. The problems of restoring permeate rates to used membranes is well-known and further studies may need to be undertaken to develop a more efficient means of cleaning and regenerating these monoliths. It can also be seen from figure 4.3.e. that the LRV’s given by monolith 3 can be increased by using a slower recirculation speed of 0.65 m/s. This would however only be practicable if the feed fluid had a low fouling capacity. A decrease in the LRV given by monolith 1 was seen after 4 hours run time for experiments using TMP's of 1.5 and 2.5 bar with a linear velocity of 3.0 m/s (figure 4.3.f.). This may be due to grow-through, where multiplication of bacteria entrapped by the membrane permits the small daughter cells to pass through pores that previously retained the larger parent cells. Grow-through is particulary undesirable during sterilising filtration (Denyer 1982).
Figure 4.3.c. Retention efficiency (as LRV) for *P. diminuta* given by monoliths 2, 3 and 4 (page 108) during cross-flow filtration with a linear velocity of 3.0 m/s and a TMP of 0.5 bar.

![Graph showing retention efficiency over time for monoliths 2, 3, and 4.]

Figure 4.3.d. Permeate rates (as l/h/m²) given by monoliths 2, 3 and 4 relevant to the results shown in figure 4.3.c.

![Graph showing permeate rates over time for monoliths 2, 3, and 4.]

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Figure 4.3.e. Retention efficiency (as LRV) for *P. diminuta* by given by monolith 1 (after 10 filtration experiments) during cross-flow filtration with a linear velocity of 3.0 m/s and a TMP of 0.5 bar, by monolith 3 with 0.65 m/s and 0.5 bar and also monolith 3 with 3.0 m/s and 0.5 bar.

![Graph showing retention efficiency for different monoliths and conditions](image)

Figure 4.3.f. Grow-through of *P. diminuta* seen using monolith 1 during six hour cross-flow filtration experiments with a linear velocity of 3.0 m/s and a TMP of either 1.5 bar or 2.5 bar.

![Graph showing grow-through for different TMPs](image)
The presence of grow-through could be proved by carrying out similar retention tests using a monolith with a larger pore-size rating and \textit{E. coli}. This species of bacteria has a much shorter generation time of approximately 20 minutes. A decrease in LRV after a shorter period of time than that seen for \textit{P. diminuta} would indicate grow-through. If grow-through is not responsible (and taking into account there was no concomitant increase in the number of bacteria in the retentate for these experiment) it must be assumed that there was a loss of integrity in the system. The probability of a defect occurring in the membrane structure itself during filtration to permit the penetration of an increased number of bacteria is slim due to the robust nature of the ceramic monolith.

In conclusion, retention test procedures were developed in order to determine the retention efficiency of flat-sheet and polymeric membranes over periods of time up to several hours. \textit{P. diminuta} in the feed was not adversely affected by being continually recirculated using a centrifugal pump. Samples of retentate and permeate were taken at timed intervals and a LRV was determined using a plate count method. These procedures were adequate for the retention testing of those membranes used and the development of a standard cross-flow retention test method are considered more fully in \textit{section 5.2}.

\textbf{4.4. Application of popular rapid methods for the enumeration of bacteria to retention testing.}

The results from membrane retention tests incorporating each of three rapid methods for the detection and enumeration of bacteria are discussed separately in detail. First of all, the correlation between the rapid method and the plate count for the enumeration of \textit{P. diminuta} was determined. Retention tests were then carried out for 0.2 and 0.1 \textmu m rated membranes and the permeates were analysed by each rapid method and a cultural retention test method. The efficiency of all three rapid retention test methods are compared and discussed together in the next chapter (\textit{section 5.3}.)

\textbf{4.4.1. ATP luminescence.}

The efficiency of popular agents for extracting bacterial ATP was studied in order to obtain a high sensitivity of detection for \textit{P. diminuta}. The correlation between viable count and ATP luminescence was determined and the ATP content of a single average cfu of \textit{P. diminuta} was calculated. A membrane filtration and incubation method was
used to isolate *P. diminuta* from entire test permeates. ATP was extracted from the retained bacteria to permit the rapid and sensitive enumeration of viable cells from test permeates.

4.4.1.1. ATP extractant.

The sensitivity of detection for bacteria increases as the efficiency with which ATP is released from bacterial cells becomes greater. TCA is known to be an excellent nucleotide extracting agent and the efficiency of new extractants is often compared with this as a standard (Lundin 1984, Stanley 1986, Simpson and Hammond 1989). Although TCA is a good extractant it is known to inhibit the action of the luciferase enzyme by denaturation (Ludin and Thore 1975). The use of boiling buffer has also been proved to be an efficacious nucleotide extractant without affecting luciferase activity (Prioli and Brown 1984, McEntee et al 1989, Nilsson et al 1989).

It can be seen from the results in table 4.4.a. that the commercial NRB extractant produced the highest level of luminescence and is considered to be the most suitable ATP extractant for *P. diminuta*. For the same dilutions of *P. diminuta*, the use of boiling buffer as an extractant gave levels of luminescence lower than those given by NRB. The use of higher ratios of sample to buffer than described in this study have been applied (Lundin and Thore 1975, Stanley 1986) but further dilution of the sample will decrease the sensitivity of detection for low numbers of bacteria. The inhibition of luciferase by TCA was demonstrated when increasing amounts of TCA were used to extract ATP from *P. diminuta*. This denaturing effect of TCA was seen to be overcome by further diluting the sample but the sensitivity of detection for low numbers of bacteria was compromised and levels of luminescence comparable to those seen when NRB was used were not seen. NRB was used as the ATP extractant for all following bioluminescence determinations.

Internal standardisation may be used as a means of determining the correlation between luminescence and the amount of ATP present. After the unknown sample is analysed and the first reading for luminescence taken, a known amount of ATP is added to the cuvette and a second luminescence reading taken. The amount of ATP present in the sample before the standard ATP is added can be determined by:

\[
\frac{ATP_{added}}{X_b - X_a} = k
\]

where

\[
SampleATP = k(X_a - b)
\]
Table 4.4.a. Comparison of the efficiency of bacterial ATP extractants for *P. diminuta* (results in RLU).

**ATP extractant**

<table>
<thead>
<tr>
<th>Sample</th>
<th>TCA solution</th>
<th>NRB</th>
<th>Tris-EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.25% 2.50% 5.00% 10.00%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.42x10^2 4.66 4.66 5.00</td>
<td>2.54x10^4</td>
<td>1.44x10^4</td>
</tr>
<tr>
<td>2</td>
<td>2.75x10^2 2.33 3.33 4.33</td>
<td>2.73x10^3</td>
<td>2.07x10^2</td>
</tr>
<tr>
<td>3</td>
<td>3.03x10^1 1.66 2.33 2.33</td>
<td>2.64x10^3</td>
<td>2.43x10^1</td>
</tr>
<tr>
<td>4</td>
<td>0 0 0 0</td>
<td>3.30x10^1</td>
<td>4.33</td>
</tr>
<tr>
<td>5</td>
<td>0 0 0 0</td>
<td>8.33</td>
<td>0</td>
</tr>
</tbody>
</table>

| 10x dil.n. | | | |
| 1 | 1.35x10^2 2.43x10^2 5.03x10^1 5.33 | N/A† | N/A |
| 2 | 5.21x10^1 1.14x10^2 4.18x10^1 6.00 |
| 3 | 6.55 6.76x10^1 2.21 2.33 |
| 4 | 0 2.39x10^1 0 0 |
| 5 | 0 7.57 0 0 |

| 50x dil.n. | | | |
| 1 | 3.10x10^1 9.42x10^1 1.20x10^2 1.09x10^2 | N/A | N/A |
| 2 | 1.01x10^1 4.13x10^1 7.81x10^1 3.87x10^1 |
| 3 | 3.33 2.66 7.02 9.66 |
| 4 | 0 0 3.33 3.00 |
| 5 | 0 0 0 0 |

* Viable counts for 10-fold serial dilutions of *P. diminuta*:

<table>
<thead>
<tr>
<th>Sample</th>
<th>cfu/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.97x10^6</td>
</tr>
<tr>
<td>2</td>
<td>9.53x10^5</td>
</tr>
<tr>
<td>3</td>
<td>9.78x10^4</td>
</tr>
<tr>
<td>4</td>
<td>1.01x10^4</td>
</tr>
<tr>
<td>5</td>
<td>1.24x10^3</td>
</tr>
</tbody>
</table>

† N/A = not applicable
Where  
\[ X_a = \text{RLU of the sample} \]
\[ X_b = \text{RLU from the known amount of ATP added} \]
\[ k = \text{amount of ATP per RLU} \]
\[ b = \text{RLU from the negative control} \]

However, it is now realised that some nucleotide extractants such as NRB inhibit luciferase progressively (Lundin 1984, Jago et al 1989a) and thus the first luminescence reading only can be accepted. The effect of NRB on the activity luciferase enzyme is demonstrated in figure 4.4.b. Luminescence given in response a standard amount of ATP (1 x 10^{10} g) was monitored over a period of time both with and without NRB present. NRB caused a rapid increase in light output that peaked after approximately 10 seconds and then decayed rapidly with time. In the absence of NRB, light emission was stably maintained at a lower level over the same period of time. This initial stimulation of luciferase activity by NRB has been reproduced by ethoxylated amines, polyethylene glycol and certain ionic detergents and is postulated to be a result of the enzyme assuming a conformation closer to that adopted in vivo (Jago et al 1989a). The addition of d-luciferin or Mg^{2+} is known not to restore luciferase activity as light emission decreases with time from the peak and it is assumed that this is probably due to enzyme denaturation by NRB. The emission profile given with NRB is thus suited to a rapid, sensitive assay for ATP where luminescence is quantified at the high peak 10 seconds after the addition of luciferase enzyme.

4.4.1.2. Calibration.

Internal standardisation was tried for this application and the results proved to be very varied (results not shown). The alternative method used in this study involved determining the relationship between luminescence and ATP concentration using a dilution series of ATP and also the relationship between luminescence and viable count for a dilution series of P. diminuta. The ATP standard curve can be seen in figure H.1. (appendix H) and using linear regression analysis a good correlation (\( r^2 = 0.99 \)) was determined between the amount of ATP present and luminescence. By quantifying luminescence given when analysing samples from a 10-fold dilution series of P. diminuta also subjected to a plate count, a good correlation (\( r^2 = 0.98 \), figure H.2., appendix H) was achieved. However, the sensitivity of the luminometer limited the practical level of detection to between 1 x 10^{2} and 1 x 10^{3} cfu/ml. It was calculated that 1 RLU was given in response to 3.31 x 10^{-14} g ATP and that the ATP content of a single average colony
Figure 4.4.b. The luminescent reaction between firefly luciferase, luciferin and ATP in the presence and absence of NRB.

![Graph showing ATP luminescence over time with and without NRB]

Figure 4.4.c. Results of rapid retention tests where test permeates were analysed by an ATP assay after membrane filtration and incubation for 5 hours.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>LRV ATP assay</th>
<th>LRV standard test</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 µm</td>
<td>3.87</td>
<td>3.56</td>
</tr>
<tr>
<td>0.2 µm*</td>
<td>1.59</td>
<td>1.33</td>
</tr>
<tr>
<td>0.1 µm</td>
<td>4.70</td>
<td>4.66</td>
</tr>
<tr>
<td>0.1 µm*</td>
<td>1.81</td>
<td>1.74</td>
</tr>
</tbody>
</table>

Where * = damaged membrane.
forming unit of *P. diminuta* was $5.74 \times 10^{-16}$ g or 0.57 fg ATP. This compares favourably to the value of one femtogram of ATP per colony forming unit often quoted for a typical or general species of bacteria (*section 2.4.2.3*).

### 4.4.1.3. Correlation between the ATP assay and the plate count.

Using the membrane filtration and incubation method to analyse a dilution series of *P. diminuta*, a good correlation ($r^2 = 0.99$) was determined between the total number of bacteria captured by the UF membrane and the total RLU given after 5 hours incubation at 30°C (*figure H.3., appendix H*). The sensitivity of detection was 10-100 cfu from the initial sample filtered through the UF membrane. Earlier trials to investigate how concentration by this method represented the bacterial load of the sample that followed the same basic method but without any incubation showed an equally good correlation of $r^2 = 0.98$ (results not shown). In this case, the individual calculated number of bacteria was comparable but slightly less than the number obtained from the plate count. This is presumably due to not being able to wash off or extract ATP from all the bacteria on the UF membrane. The initial total number of bacteria isolated from an unknown sample was calculated by quantifying the total luminescence given in response to all the ATP extracted from the membrane after 5 hours incubation.

### 4.4.1.4. The retention tests.

A LRV could be calculated within a time period of 6 hours for all the experimental 0.1 and 0.2 μm rated PVDF membranes using the rapid retention test with ATP assay. The number of bacteria in the membrane challenge was sufficient to permit enumeration by the standard ATP luminescence assay. Each single retention test result shown in *table 4.4.c.* was calculated using the results from six individual filtrations. The results show that a distinction was made between test permeates from membranes of different pore size ratings and also between those from intact and damaged membranes. The LRV's calculated by the ATP luminescence method were slightly higher than those obtained by the plate count. As mentioned above, the membrane filtration and incubation method gave slightly lower individual values for the viable count than did the plate count which could explain the higher LRV values remembering that the challenge viable count was determined by the standard ATP assay.

Trial retention tests using the ATP assay were carried out for membranes of a type already known to have a good retention test pass rate (three Whatman 0.1 and three 0.2 μm rated cellulose nitrate membranes, *section 4.1.2*). After 5 hours incubation, a LRV of 1.93 was calculated for one out of the three 0.2 μm rated membranes tested at that
time whilst no evidence of viable bacteria was detected for the rest. Unfortunately, even though the first sample from an incubated membrane may test negative for luminescence the membrane cannot be incubated further and tested again at a later date as the bacteria have already been lysed. It can only be concluded that there were either no viable bacteria present or that bacteria were present but such low numbers that the ATP content was too small to be quantified by the luminometer used.

The sensitivity of detection can be increased by longer incubation times and also by decreasing the volume of Lumacult broth and NRB extractant used to wash the UF membrane. The other membranes were left to incubate for a total of 21 hours overnight. Again, the ATP assay did not indicate the presence of viable bacteria. These test membranes produced a sterile permeate. A trial correlation using a dilution series of \textit{P. diminuta} confirmed that the sterility of a sample could be confirmed after this period of incubation. Further investigation would study more closely the correlation between luminescence after extended periods of incubation and the viable count of the initial sample.

In conclusion, a rapid retention test method was developed incorporating an ATP assay for the determination of sterility for the permeate. This test proved adequate for retention testing the experimental 0.2 and 0.1 \textmu m rated membranes used in this study after an incubation period of 5 hours. The main drawback of this retention test method is the inability to rapidly identify the source of ATP for a sample testing positive for luminescence. In this study, a small aliquot was taken from the membrane after incubation but before lysis and was streaked onto a MacConkey agar plate to study the occurrence of contamination. All bacterial growth seen during the experiments was identified as \textit{P. diminuta} and microbiological contamination of the permeate was not seen.

4.4.2. Electrometric microbiology.

The most suitable test conditions for the electrometric analysis of samples containing \textit{P. diminuta} using the Bactometer were determined. These were then were applied to a rapid impedimetric method for the analysis of permeates from sterilising grade membranes.

4.4.2.1. Correlation between the impedance assay and the plate count.

It was found that the use of PCB as the diluent for \textit{P. diminuta} gave a greater percentage change in electronic signal and a better quality signal curve than did TSB (results not shown) and was used throughout for further electrometric assays. Capacitance, conductance and impedance signals were then monitored for samples from
a dilution series of *P. diminuta*. Either single- or double-strength PCB was used as the
diluent. It can be seen from figure 4.4.d. that the quality of curve obtained using
single-strength PCB whilst monitoring impedance is superior to the others. Those curves
obtained when capacitance was monitored are not included and are far inferior to those
displayed. The best results are often seen when the medium has been custom made for
a specific situation (Owens 1985, Gatti and Neviani 1993) but the use of the commercially
available PCB was found to be entirely satisfactory.

A slight double-hump in the curve can be seen when double-strength PCB was
used for the diluent (figure 4.4.d., signal curve number 3). This tendency to deviate
towards a double-hump conformation is often observed when samples containing
pseudomonads are analysed (Firstenberg-Eden and Eden 1984). The extra inflection is
undesirable as this can be mistaken for the DT and thus the value for the viable count
will be inaccurate.

A comparison was made between calibration curves constructed using dilutions
from either standard SLB cultures of *P. diminuta* or one that had been gently filtered to
0.4 μm as described in section 3.4.1.3. Both calibration curves can be found in appendix
H (figures H.4. and H.5., $r^2 = 0.98$ and 0.97 respectively). The number of cfu per ml for
a given value of IDT was seen to increase with this prefiltration. This demonstrates that
small aggregates of bacteria are being broken up as expected and confirms that such a
culture will represent bacteria in retention test permeates as accurately as possible. It is
known that growing *P. diminuta* in SLB rather than TSB inhibits the tendency to
aggregate (Leahy and Sullivan 1978) and using direct microscope observation with the
DEFT (section 4.4.3.) existing clusters were seen to be of less than five cells, the most
common formation being pairs. A theoretical sensitivity of detection of one cfu per sample in approximately 34 hours can be obtained from figure H.5. (appendix H) but it
is known that the inaccuracy of results increases for extremely high and low viable
counts. The experimental sensitivity of detection determined when constructing the
calibration curve was approximately 10 cfu/ml.

4.4.2.2. The retention tests.

IDT's of between 22 and 29 hours were obtained for aliquots of test permeates
from experimental 0.2 and 0.1 μm rated membranes and the LRV given by each test
membrane could be determined within 2 days (table 4.4.e.). Each single retention test
result shown was calculated using the results from six individual tests. Again, there was
a clear distinction between the two types of membrane with different pore sizes and the
damaged and intact membranes. This impedimetric method was adequate for assaying
the sterilising grade experimental membranes but is not suitable as implemented
Figure 4.4.d. Comparison of the quality of electrical signal obtained when analysing samples containing *P. diminuta*. Either total impedance or conductance was monitored and *P. diminuta* was suspended in either single-strength or double-strength PCB.

Table 4.4.e. Results of rapid retention tests where test permeates were analysed by impedimetric assay.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>LRV impedance assay</th>
<th>LRV standard test</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 µm</td>
<td>3.43</td>
<td>3.37</td>
</tr>
<tr>
<td>0.2 µm*</td>
<td>1.15</td>
<td>1.17</td>
</tr>
<tr>
<td>0.1 µm</td>
<td>4.38</td>
<td>4.40</td>
</tr>
<tr>
<td>0.1 µm*</td>
<td>1.22</td>
<td>1.34</td>
</tr>
</tbody>
</table>

Where * = damaged membrane.
for retention testing other membranes as the entire test permeate could not be analysed. It is possible to obtain large capacity sample vessels (Irving et al 1989) for the Bactometer and these would be necessary if a standard retention test method using the impedimetric assay was to be developed. Comparing the LRV's calculated with results from the Bactometer with those given by the plate count, it can be seen that those obtained from the Bactometer tend to be slightly lower. This may be due to a decrease in accuracy for detecting small numbers of bacteria at the extreme limit of detection for the Bactometer.

Trial retention tests were carried out for three Whatman 0.1 and three 0.2 μm rated cellulose nitrate membranes using the same procedure. The remaining permeate from the 0.1 μm rated membranes could be confirmed as sterile after the recommended incubation period and no viable bacteria were detected by either the plate count or the impedance assay. The results obtained after analysing test permeates from the 0.2 μm rated membranes showed that *P. diminuta* was detected to a mean level of 27 cfu/ml by the plate counts in all replicate samples from one of the test permeates. The incubated filtrate was positive for *P. diminuta* but the results from the Bactometer were negative. The other two permeates were sterile. This reinforced the view that the impedimetric test has a lower sensitivity of detection for small numbers of viable bacteria than the plate count method mentioned above.

In conclusion, the application of an impedimetric assay using the Bactometer to the analysis of retention test permeates from experimental sterilising grade membranes provides a simple and straightforward means of determining the viable count for up to 64 samples concurrently, but does not offer a great time advantage over the standard retention test method implemented in this study. The use of single-strength PCB with impedance monitoring gave the best quality signal curve when assaying samples containing *P. diminuta*. The LRV's for the experimental membranes could be determined within two days with a sensitivity of detection of approximately 10 cfu/ml. The method provided no mean of identifying the species of bacteria present in the sample but general contamination of a pure culture can sometimes be detected by the presence of an irregular signal curve. There is also evidence to suggest that the accuracy of the impedance assay is somewhat less than that of the traditional plate count when samples containing low numbers of bacteria are being analysed. The test procedure enabled the rapid determination of retention efficiency for the experimental membranes. However, the use of large sample bottles would be required to permit the analysis of entire test permeates for a standard retention test method using an impedimetric assay. The detection of sterility for entire test permeates in this manner will undoubtedly require a longer period of run time than that used in this study.
4.4.3. The DEFT.

The efficiency of the manual and the semi-automated DEFT method for the enumeration of viable *P. diminuta* from retention test permeates was determined and compared. The microcolony DEFT was then applied in an attempt to increase the sensitivity of detection for these small bacteria and the both the standard DEFT count and microcolony DEFT count were then used to analyse further retention test permeates.

4.4.3.1. Correlation between the DEFT and the plate count.

When viewing DEFT slides, viable *P. diminuta* were identified as small, short rods that were often present in pairs exhibiting an orange/yellow fluorescence. Non-viable *P. diminuta* were distinguished by green fluorescence and were not included in the viable count. A photomicrograph showing *P. diminuta* stained with acridine orange can be seen in figure 1.1., appendix I. Contaminating species were recognised by their larger size and brighter orange fluorescence whilst angular particles fluorescing an extremely bright orange were identified as acridine orange dye particles.

A good correlation \((r^2 = 0.98)\) for manual and automated DEFT counts compared with the plate count was determined in both cases (figures H.6. and H.7., appendix H). However, the individual manual counts were always lower than those obtained by the plate count and the automated counts were always lower than those obtained by manual counts. The lack of sensitivity with the manual count is due to the small size of *P. diminuta* and the difficulty when first using the DEFT in distinguishing such small bacteria from debris. There was also a problem with the membranes sometimes being sucked-in around the edge of the wire mesh support during vacuum filtration that causes a loss in seal integrity and will lower the viable count due to loss of sample. The thickness of the track-etched membranes tends to decrease in accordance with the pore-size rating and this sucking-in is not a problem when the recommended 0.6 \(\mu m\) membranes are used.

The further lack of sensitivity with the automated count is again due to the small size and thus relatively low intensity of fluorescence of *P. diminuta*. Most of the debris was of a larger size and brighter fluorescence than *P. diminuta* and whereas a distinction can often be made between these by eye, the level of detection using the image analyser could not be set to pick up all the bacteria without including all the debris. The problem of the pores of the Nuclepore membrane taking up dye to give spots of fluorescence similar to those seen by fluorescing bacteria was not encountered, probably due to the smaller 0.1 \(\mu m\) pore size membrane being used. It has been recommended that black
Nuclepore membranes might be used to increase the contrast between bacteria and background (Jones and Simon 1975) but the standard white ones were found to be entirely satisfactory.

4.4.3.2. The retention tests.

Retention tests for grafted and non-grafted 0.2 and 0.1 μm rated experimental membranes were carried out to compare the efficiency of manual DEFT counts with semi-automated DEFT counts for the analysis of test permeates. The viable count for the membrane challenge was determined by the either the manual or automated DEFT as required after filtering a single 0.5 ml aliquot. The LRV for each membrane could be calculated within 1-2 hours with a sensitivity of detection of approximately 100 cfu per ml of sample. The LRV’s calculated by the manual DEFT count were very similar to those obtained by the plate count whilst those obtained using the automated count are slightly higher for the 0.2 μm rated PVDF and graft A membranes (table 4.4f.). Each single retention test result shown was calculated using the results from six individual tests. Although the calculated LRV’s in all cases were similar, the individual viable counts determined by a manual count were always lower than those determined by the plate count. The individual viable counts determined by the automated count especially were usually approximately one logarithmic cycle lower than those given by the plate counts as discussed in the previous section.

Preincubation of the sample before using the DEFT has been used to increase the sensitivity of the method to six viable bacteria per sample after an incubation period of several hours (Denyer and Lynn 1987). This method was not applied as the major problem encountered with the DEFT is not detecting low numbers of *P. diminuta* but that the cells are too small to be easily detected. Although the application of the DEFT made retention test results available within a very short period of time, the preparation of reagents and the construction and analysis of DEFT slides was found to be very laborious.

4.4.3.3. The microcolony DEFT.

The microcolony DEFT was employed in an attempt to increase the sensitivity of detection for *P. diminuta* by automated equipment. It has been reported that sensitivity of detection of 10 cfu per 100 ml has been achieved using the DEFT microcolony technique with non-selective agar to detect *Pseudomonas cepacia* artificially inoculated into pharmaceutical grade water (Newby 1991). To achieve this high sensitivity a fully automated counting system was essential although the system used had a problem differentiating between microcolonies and some fluorescing debris.
Table 4.4.f. Results of rapid retention tests where test permeates were analysed by a manual or a semi-automated DEFT method.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>LRV DEFT manual</th>
<th>LRV DEFT auto</th>
<th>LRV standard test</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 μm</td>
<td>3.36</td>
<td>3.47</td>
<td>3.36</td>
</tr>
<tr>
<td>PVDF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2 μm</td>
<td>3.18</td>
<td>3.34</td>
<td>3.32</td>
</tr>
<tr>
<td>Graft A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 μm</td>
<td>3.86</td>
<td>3.88</td>
<td>3.90</td>
</tr>
<tr>
<td>PVDF</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.4.g. Results of rapid retention tests where test permeates were analysed by a standard DEFT count (single cells and microcolonies) and a microcolony count (microcolonies only).

<table>
<thead>
<tr>
<th>Membrane</th>
<th>LRV DEFT manual</th>
<th>LRV DEFT micro-colony</th>
<th>LRV standard test</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 μm</td>
<td>3.33</td>
<td>3.64</td>
<td>3.36</td>
</tr>
<tr>
<td>0.2 μm*</td>
<td>1.94</td>
<td>1.74</td>
<td>1.11</td>
</tr>
<tr>
<td>0.1 μm</td>
<td>4.29</td>
<td>4.54</td>
<td>4.27</td>
</tr>
<tr>
<td>0.1 μm*</td>
<td>2.00</td>
<td>2.03</td>
<td>1.90</td>
</tr>
</tbody>
</table>

Where * = damaged membrane.
Figure 4.4.h. (i) *P. diminuta* microcolony formation on L-agar. The number of microcolonies is expressed as the percent of microcolonies from the standard DEFT count (eqn. 21.).

(ii) *P. diminuta* microcolony formation on L-agar supplemented with streptomycin.

Where: dilution 2 = $9.77 \times 10^5$ cfu/ml  
dilution 4 = $1.22 \times 10^6$ cfu/ml  
dilution 3 = $8.84 \times 10^4$ cfu/ml  
dilution 5 = $1.29 \times 10^3$ cfu/ml
Different equipment was used for these experiments so the correlation between the manual DEFT count and the plate count was redetermined and found to be good ($r^2 = 0.97$) (results not shown). Initially, the formation of microcolonies from viable bacteria retained on DEFT membranes after the filtration of samples from a serial dilution of *P. diminuta* was studied. A selection of photomicrographs showing microcolonies of *P. diminuta* stained with acridine orange can be seen in figures 1.1, 1.2., and 1.3., appendix I. The DEFT membranes were incubated on either L-agar or L-agar supplemented with streptomycin for up to 12 hours. No difference in the appearance of the DEFT membranes when viewed microscopically was seen after two hours of incubation compared to non-incubated membranes.

Both the standard DEFT count (including all single cells and microcolonies) and the microcolony count (microcolonies only) was determined for each slide. For the results shown in figures 4.4.h.(i). and 4.4.h.(ii)., the number of microcolonies is expressed as percent microcolonies of the standard count and was calculated using the following equation:

$$\text{Percent Microcolonies} = \left( \frac{\text{microcolony count}}{\text{standard DEFT count}} \right) \times 100 \ldots (21)$$

The results for those DEFT membranes incubated on L-agar can be seen in figure 4.4.h.(i). Not all viable cells of *P. diminuta* formed microcolonies after incubation. The percentage of microcolonies for each dilution tended to increase between 4 to 12 hours incubation. For the dilution containing the highest bacterial load (dilution 1) the microcolonies were seen to become confluent after an 8 hour incubation period. The number of cells forming the microcolonies were 6-10, 10-12, 15-18, 20-30 and 20-40 after 4, 6, 8, 10 and 12 hours of incubation respectively. The percentage of microcolonies was also seen to increase as the bacterial load of sample decreased and microcolonies of other species of bacteria than *P. diminuta* were not seen.

Microcolony formation on DEFT membranes that were incubated on L-agar supplemented with streptomycin (at 50 µg/ml) exhibited the same trends but all the percentage microcolony counts were lower than those seen for the same dilutions with the non-selective agar (figure 4.4.h.(ii).). Injury in response to stress can result in a failure of some portion of a bacterial population to multiply and form colonies on selective media (Hoadley 1981). The decrease in the occurrence of microcolony formation with the higher concentration of bacteria could be due to competition for nutrients or by inhibition of growth by other bacteria in close proximation. The lag before microcolony formation and increase in microcolony count with time seen in all results could also be
due to the bacteria recovering from physical stress imposed by the DEFT process. A poor correlation ($r^2 = 0.92$ and 0.93) between the DEFT microcolony count using either a non-selective or selective medium respectively was obtained (figures H.8. and H.9., appendix H). Microcolony formation by contaminating species was not seen during these experiments and it was concluded that the use of a selective agar medium was not necessary for this application.

Retention tests were carried out for 0.2 and 0.1 μm rated experimental PVDF membranes and the test permeates were analysed by a manual DEFT count, a manual DEFT microcolony count after a 6 hour incubation period and the standard culture method used throughout this study. Each single retention test result shown was calculated using the results from six individual tests. The results can be seen in table 4.4.g. and the LRV's obtained for the standard DEFT count are similar to those obtained by the plate count. However, the LRV's obtained by the microcolony count after 6 hours are higher. Again, there were differences between the individual viable counts obtained by the plate count and the manual DEFT count as discussed in section 4.4.3.1. The individual viable counts determined by microcolony counts were much lower than those determined by the manual DEFT counts.

The formation of microcolonies permitted the easy detection of each colony forming unit but due to the observations concerning microcolony formation discussed above, it was considered that the enumeration of microcolonies is not an accurate enough means by which to determine the viable count of retention test permeates. The manual DEFT count remains the most suitable means of quantifying *P. diminuta* compared to the automated and the microcolony count. Trial retention tests for three Whatman 0.2 and three 0.1 μm rated membranes were carried out using both the manual DEFT count and the standard cultural method used in this study to analyse the permeates. No viable *P. diminuta* were detected in any of the test permeates. Inspection of the entire DEFT membrane surface was attempted in order to ascertain the sterility of the sample but this was found to be a most tedious task.

In conclusion, application of the DEFT to the analysis of test permeates created the most rapid retention test method that was unfortunately the most laborious to carry out. This method was also advantaged in that identification of the bacteria could be made on the basis of morphology. The manual DEFT count was the best method for the analysis of the DEFT slides as the individual cells of *P. diminuta* were too small to be detected reliably by the semi-automated method. The microcolony technique was applied to the DEFT membranes before staining with acridine orange but microcolony formation by *P. diminuta* proved too irregular to be relied upon to give accurate values for viable count. The retention test method using the manual DEFT count was adequate for the
determination of retention efficiency for the experimental membranes with a sensitivity of detection of approximately 100 cfu per sample. However, although an entire test permeate can be filtered through the DEFT membrane, the attempt to reliably detect a single cfu on the whole of this membrane viewed microscopically involved extensive analysis of the whole membrane that requires a long time.

4.5. Bacterial bioluminescence.

A bioluminescent strain of *E. coli* was applied to the retention testing of non-sterilising grade membranes in order to demonstrate the feasibility of using bioluminescent micro-organisms for membrane retention tests. Progress was made towards the development of a bioluminescent strain of *P. diminuta* in that a suitable plasmid vector for the luxAB genes was chosen and was seen to be introduced into *P. diminuta* by conjugation.

4.5.1. Use of bioluminescent *E. coli* for retention testing.

Trials were carried out to study the possibility of using bioluminescent bacteria for the rapid retention testing of membranes. *E. coli* is included in the range of microbiological species used industrially for the retention testing of 0.65 and 0.8 μm rated membranes. The bacterial retention testing of membrane with pore size ratings other than sterilising grade is discussed more fully in section 5.5. The retention testing of experimental 0.8 and 0.4 μm rated PVDF membranes with a bioluminescent strain of *E. coli* was seen to produce test permeates with a range of bacterial loads.

The bioluminescent *E. coli* JM101 (pPA3) used in this study was developed during the generation of a bioluminescent strain of *M. smegmatis* (Andrew and Roberts 1993). The development of bioluminescence for a broth culture of *E. coli* (pPA3) can be seen in figure J.1., appendix J. A good correlation ($r^2 = 0.98$) between viable count and bioluminescence was determined (figure J.2., appendix J). A specific bioluminescence of $4.61 \times 10^3$ quanta/sec/cfu was calculated for cells taken from an early stationary phase culture (reached after 5.5 hours incubation) that was used for the membrane challenge. The linear relationship between viable count and emitted light indicates the suitability of using bioluminescent bacteria for membrane retention testing.

The retention test results are shown in figures 4.5.a. and 4.5.b.. The result shown for each type of membrane was calculated using the results from four individual filtration experiments. Mean LRV's for the 0.8 μm rated membranes of 2.57 and for the 0.4 μm rated membranes of 3.81 could be calculated using the bioluminescence assay with no
Figure 4.5.a. Rapid retention test results for 0.8 μm rated membranes challenged with bioluminescent *E. coli*.

![Graph for 0.8 μm rated membranes](image)

Figure 4.5.b. Rapid retention test results for 0.4 μm rated membranes challenged with bioluminescent *E. coli*.

![Graph for 0.4 μm rated membranes](image)
permeate incubation. The lower limit of detection for the bioluminescent *E. coli* after filtration was found to be $9.5 \times 10^3$ cfu/ml compared to $1.0 \times 10^4$ cfu/ml before filtration. This reduction in sensitivity is assumed to be due to the physical stress imposed on the cells during filtration. However, the specific bioluminescence for the bacteria in test permeates immediately after filtration was found to be only slightly lower than usual at $3.96 \times 10^3$ quanta/sec/cfu rising to $4.35 \times 10^3$ quanta/sec/cfu after 1 hours incubation at 37°C. It must also be taken into account that any cells in the permeate will have smaller dimensions than those in the challenge and consequently produce less bioluminescence.

The lower detection limit for the bioluminescence assay was determined by the sensitivity of detection for light given by the luminometer used. There are two ways by which the detection for bioluminescent *E. coli* can be made more sensitive. Firstly, the bacteria can either be concentrated from the permeate into a smaller volume effectively raising the numbers to above the lower limit of detection dictated by the equipment. This was demonstrated by the membrane filtration method described for the ATP assay used in this study. Secondly, the numbers of bacteria in the sample itself can be increased so that again the lower limit of detection is reached. This can be brought about by an incubation period before the sample is assayed. It was more convenient for this study to use the second method. The remaining test permeates were incubated at 37°C for several hours. The viable count and bioluminescence was seen to increase rapidly after an initial lag period of about one hour (figures 4.5.a. and 4.5.b.) and the sensitivity of detection for *E. coli* in test permeates was increased approximately 30-fold by incubation.

Exponential growth for all the test permeates was seen to commence approximately one hour after filtration. It is assumed that the lag period was due to the recovery of physically stressed cells after filtration as mentioned earlier. The mean generation times calculated during the period of exponential growth (determined graphically, results not shown) for permeates from the 0.8 and 0.4 μm rated membranes were 38.33 and 41.09 minutes respectively. These times are slightly longer than the generation time of 31.47 minutes calculated for a standard broth culture of *E. coli*. The test permeates from membranes with the greatest retention capacity will contain the lowest numbers of bacteria of the smallest size. Small cells tend to have longer generation times (Stanier *et al* 1987) and accordingly, longer generation times were seen for bacteria in the permeates from the 0.4 μm rated membranes.

It is agreed that the required sensitivity of detection for a membrane retention test is one viable cfu per entire test permeate (*section 2.3.2.*). It can be calculated (by rearranging eqn. 21. for $t-t_0$) that for an initial inoculum of one viable *E. coli* per permeate (100 ml) it would require an incubation time of 10.56 hours for the number of bacteria present to reach the lower limits for reliable detection by the photometer (taken as
\[ Z = Z_0 10^{k(T - T_0)2.303} \] 

In conclusion, the use of a bioluminescent strain of *E. coli* to determine the retention efficiency of membrane filters was both rapid and straightforward. A lower detection limit of \(9.5 \times 10^1\) cfu per ml of permeate was obtained for this strain after filtration and it was calculated that a sensitivity of detection of 1 cfu per permeate could be obtained if the permeate was incubated for 10.56 hours. The feasibility of using a bioluminescent strain of *P. diminuta* for the retention testing of 0.2 µm rated membranes was shown. However, the specific bioluminescence for this strain would need to be high enough to ensure the reliable detection of at least one viable bacterium in the test permeate and it must be remembered that the entire test permeate must be analysed. These results and the possibilities for developing a standard retention test method using bioluminescent bacteria are discussed more thoroughly in sections 5.4. and 5.5.

4.5.2. Development of bioluminescent *P. diminuta*.

4.5.2.1. Conjugal transfer of pNJR12 to *P. diminuta*.

Filter mating between *P. diminuta* and *E. coli* JM101 (pPNJR12/R751) was carried out overnight. Kanamycin resistant *P. diminuta* transconjugants were selected for on L-agar supplemented with kanamycin and streptomycin each at 50 µg/ml. Numerous colonies were seen after 48 hours incubation. A mean conjugal transfer frequency of \(7.41 \times 10^6\) (expressed as transconjugants per recipient at the end of mating) was obtained with a mean donor:recipient ratio for of 30:1. Whilst most colonies were comparable in size to typical *P. diminuta* colonies grown on non-selective L-agar there were also small colonies present in a ratio of 15:1 (large:small). There were many small more colonies present after 96 hours of incubation. All colonies were identified as *P. diminuta* by the appearance of colonies grown on MacConkey agar (section 3.1.2), by the Gram-stain and by morphology when examined microscopically. No colonies were seen on either the control donor or control recipient plates.
Construction of plasmid pISR600 for *P. diminuta*. Plasmid pNJR12 can also be seen in figure 4.5.b. and plasmid pSB226 contains the luxAB genes from *V. harveyi.*
Both types of colony were screened for the presence of pNJR12 by colony hybridisation with $^{32}$P-labelled pNJR12 and it was found that 85% of the colonies seen to give rise to a positive signal after autoradiography were large. Although pNJR12 contains the gene for tetracycline resistance (figure 4.5.c.), no resistance to tetracycline at 25 μg/ml was exhibited by any of the colonies containing pNJR12 indicating that this gene was not expressed. The smaller satellite colonies may have arisen through spontaneous resistance to kanamycin. However, the lack of such large numbers of the characteristically small colonies on the control plates suggests that the catabolism of antibiotic by the larger colonies could reduce the level in the agar to allow the break-through growth seen.

4.5.2.2. Construction of plasmid pISR600.

The plasmid pNJR12 was digested with SstI and allowed to ligate overnight with pSB226 digested with EcoRI and Smal (figure 4.5.c.). This DNA was then used to transform E. coli JM101 and the resulting transformants containing the plasmid pISR600 were selected for on L-agar supplemented with kanamycin at 50 μg/ml. Not one of the transformants obtained were seen to be bioluminescent and the presence of the luxAB genes could not be detected by colony hybridisation using a $^{32}$P-labelled EcoRI/Smal digest of pSB226.

4.5.2.3. Future objectives.

It is planned that the experiments concerning the development of a bioluminescent P. diminuta detailed in this study will be repeated. On the successful transformation of E. coli with pISR600 this will then be transferred to P. diminuta via the same filter mating method already described. The stability and expression of the luxAB genes in the recombinant P. diminuta is to be studied and a number of well-characterised promoters from both E. coli and P. diminuta are to be tested for their ability to provide high level expression of the cloned genes. The plans for further work are discussed more fully in section 5.5.
5. DISCUSSION OF THE RESULTS.

The use of *P. diminuta* for the retention testing of sterilising grade membranes is increasing and those membranes validated by such a test possess a distinct market advantage, as it has been shown that they are capable of producing a sterile permeate. A representative sample from the manufactured membrane batch is selected for pore size characterisation. An integrity test such as the bubble-point test is used to detect holes or tears in the membrane and also to determine the numerical pore size rating. Membranes with a pore size rating of 0.2 μm can then be validated as sterilising grade by a retention test with high numbers of *P. diminuta* which must be retained.

Current practices for the characterisation of sterilising grade membranes by membrane manufacturers and pharmaceutical companies were studied, and practices for using bacterial retention tests were found to differ between membrane manufacturers. Some membrane manufacturers make correlations between bubble-point test results for 0.2 μm rated polymeric membranes and the ability to retain *P. diminuta*. The pore size characteristics for that type of membrane is determined solely by bubble-point from then on. The bubble-point pressure given by the test membrane must correspond to a pressure at which the membrane from the initial test was shown to produce a sterile filtrate. These correlations are not absolute, and inexact correlations between two different characteristics such as bubble-point test results and retention ability cannot be relied upon to predict the retention ability of sterilising grade membranes. The inaccuracies pertaining to the use of bubble-point test results alone to predict the sterilising ability of membranes have already been discussed in section 2.3.4. and it is quite clear that only an additional retention test with *P. diminuta* can confirm the ability to retain bacteria.

Another practice carried out by membrane manufacturers is to corroborate the bubble-point results for each batch of membranes by retention tests with *P. diminuta*. This procedure will give a more accurate indication of the retention ability of a membrane than will the correlation method described above. Some commercial sterilising grade membranes are not retention tested with *P. diminuta* at all but are characterised solely by numerical pore size determinations obtained by the bubble-point test, the problems with which have been discussed thoroughly in this study as mentioned earlier. The individual, cylindrical conformation of the pores found in track-etched membranes allow the exact pore sizes for these membranes to be determined by direct inspection using scanning electron microscopy. Although accurate measurements of the pore diameter can be made, it will be difficult using microscopic observation to obtain the range of pore sizes for the whole membrane. The pore size distribution obtained by mercury
Porosimetry is relied upon to determine the pore size rating of ceramic membranes. The pore sizes of tubular ceramic membranes are determined solely by physical integrity tests and such membranes have not to date been retention tested with *P. diminuta*.

As well as the differences in application of the retention test with *P. diminuta* there are the variations in actual methodology as detailed in section 2.3.4.. Although the challenge level of $1 \times 10^7$ cfu/cm$^2$ recommended by the HIMA (1982) is generally adhered to, it has been suggested that this level is too high and is not representative of a realistic bacterial load that is actually experienced during product filtration (Mouwen and Meltzer 1993). There have been reports of lower challenge levels of $1 \times 10^4$ or $1 \times 10^2$ cfu/cm$^2$ being used (Meltzer 1987a) but membranes validated with a lower challenge level will theoretically have an inferior retention ability compared to those validated according to the HIMA (1982). Different challenge levels should not be used and one retention test methodology should be maintained between all test users. A standard test method should strive to meet three primary criteria: the suitability of the methodology, the clarity of purpose to minimise ambiguities and exact clarity of procedure. The development of rapid, straightforward standard procedures concerning the retention testing of 0.2 μm rated membranes for dead-end and cross-flow filtration using *P. diminuta* will result in a more accurate representation of membrane sterilising ability.

Although retention testing with *P. diminuta* is often carried out by the membrane manufacturer, it has been recommended that validation of 0.2 μm rated membranes should also be undertaken by the membrane users (Meltzer 1987a). Not all sterilising grade membranes are currently validated by the manufacturer as mentioned earlier. Meltzer (1987a) recommended that several membrane types should be tested at the same time to allow flexibility in the purchasing decision and to eliminate price, delivery and technical problems that can be associated with a single source qualification. Several manufacturers lots and several membranes per lot should be validated to define the variation between membranes and membrane systems. Unfortunately, validation by the membrane user is often perceived as being unnecessary and is not carried out. Pharmaceutical companies were found to rely upon the pore size characterisation undertaken by the manufacturers and often, current retention test procedures were perceived to be more complex and time-consuming than they actually are. The development of a rapid and improved test method will be advantageous primarily to current test users to increase the efficiency of their pore size characterisation procedures but will also encourage the practice of validation of 0.2 μm membranes to become more widespread.
5.1. Determination of membrane retention ability using \textit{P. diminuta}.

A retention test procedure based on the standard ASTM method (ASTM D 3862 1992) for the retention testing of 0.2 μm rated membranes with \textit{P. diminuta} was used to effectively characterise new experimental 0.1 and 0.2 μm rated membranes. Samples from each test permeate were withdrawn and analysed using a plate count method and the remaining permeate was incubated to determine sterility. This method was found to be adequate for the analysis of those experimental and commercial membranes tested.

Differences in the ability to retain \textit{P. diminuta} were found between some grafted and native membranes of the same pore size rating, and were presumed to be attributable to steric hindrance caused by the hydrophilic material grafted to hydrophobic membranes in an attempt to reduce fouling. Overall, both the 0.2 and the 0.1 μm rated membranes were seen to retain \textit{P. diminuta} poorly but both types of membrane were capable of producing good flux rates when used in the Rayflow module. The recommended challenge of $1 \times 10^7$ cfu/cm$^2$ (HIMA 1982) was not retained by the 0.2 μm rated membranes and LRV's of between 3.0-4.5 were given for \textit{P. diminuta}. These membranes could not be validated as sterilising grade. However, the membranes were designed for use in a cross-flow plate and frame module and it might be assumed that the use of two such membranes with a serial mode of filtration would produce the required retention characteristics. It is widely believed that LRV's are additive although this has not been proven and should not be assumed (Meltzer 1987a). Meltzer also reported that although it has been proposed by the FDA that the required LRV might be produced by a combination of membranes rather than singly, this was rejected on the grounds that such a move would weaken the significance of a final, sterilising membrane.

The retention ability given by the experimental membranes was seen to decrease with an increase in applied TMP. It has been stated that the retention given by 0.2 μm rated membranes is purely by a sieve retentive mechanisms and not by adsorptive sequestration (Goel et al 1992). If this was the case for the experimental membranes, then the retention ability would be independent of the TMP, and clearly retention was seen to be brought about by adsorption also as discussed in section 2.3.2.1..

The suitability of those membranes preserved in a wetting solution of Triton-X 100 and formaldehyde rather than in a glycerol solution for sterilisation by autoclaving was confirmed. The non-woven autoclavable support material used for the membranes provided a good degree of rigidity and rendered them easy to handle, whilst decreasing the tendency to curl up during autoclaving. Although the use of a preservation solution containing Triton X-100 meant that the membranes could be autoclaved, those
membranes packed in glycerol were less susceptible to drying out in the package and leakage due to the greater viscosity of glycerol and are altogether more suitable for packing in re-sealable plastic bags.

The retention ability for *P. diminuta* given by commercial 0.2 and 0.1 μm rated membranes, that were subjected to the same retention test procedure as the experimental membranes, was seen to vary. The pore sizes of the commercial membranes had been characterised in differing ways and not all had been retention tested by the manufacturer. The only 0.2 μm rated membrane tested that consistently retained 100% of the recommended *P. diminuta* challenge was of a ceramic flat sheet configuration. This type of membrane was not characterised by bubble-point but by mercury intrusion. All phase-inversion membranes solely or partially characterised by bubble-point failed to retain *P. diminuta* to varying degrees. On the other hand, the track-etched membranes not routinely validated with *P. diminuta* showed good retention ability. For this type of membrane, the accurate determination of largest pore diameter by direct microscopic inspection and the application of the bubble-point test as an integrity test only was seen to give an accurate indication of retention ability. These thin membranes have cylindrical pores and are close to a purely sieve retentive model thus the efficiency of retention can be predicted quite accurately by knowing the diameter of the pores. The retention given by tortuous pore membranes can not be ascertained accurately by the pore size rating alone. Adsorptive retention is not dependent on the differences in size between particle and the pore and can not be determined solely by pore diameter measurements as discussed in section 2.3.4.

Neither of the retention test protocols (section 2.3.2.) contained information about the specific growth conditions for *P. diminuta* whilst it was shown using SEM analysis that the degree of aeration this obligate aerobe receives during the preparation of the membrane challenge affects the cell dimensions. It was also shown that the experimental 0.2 and 0.1 μm rated phase-inversion membranes not routinely retention tested by the manufacturer failed to retain *P. diminuta* with minimum cell sizes greater than the pore size rating of the membrane. Not one of the experimental membranes retained *P. diminuta* as described earlier in this section and it can be suggested that the actual pore size rating for these membranes should be larger than the 0.2 and 0.1 μm rating given. Size analysis by SEM was found to be more suitable for the cell size analysis of *P. diminuta* than was the method using the Coulter Counter electronic particle size analyser. A rapid electronic cell sizing method would be useful to membrane manufacturers for the rapid analysis of *P. diminuta* in the retention test permeates in order to ascertain whether retention failure was due to oversize pores or undersize bacteria. However, the Coulter Counter was not sensitive enough to permit the accurate size analysis of individual cells.
The need for all 0.2 μm rated membranes to be retention tested with *P. diminuta* was highlighted and there was seen to be need for a clearly defined standard test method. Concerns have been expressed that the concentration of *P. diminuta* per unit volume of challenge diluent will affect the retention ability of the test membrane (Mouwen and Meltzer 1993). Thus as well as stating the required bacterial challenge per cm² of available filter surface, the standard tests should also use a volume of diluent that maintains the same concentration of *P. diminuta*. It is believed that a simple, rapid test procedure that is comparable in sensitivity with current culture techniques will encourage the more widespread use of bacterial retention tests and will result in a more accurate characterisation of the bacterial retention ability of sterilising grade membranes.

5.2. Development of retention tests with *P. diminuta* using cross-flow filtration.

All present bacterial retention test methodologies employ dead-end filtration. Whilst variations on the basic test procedure for flat sheet membrane discs have been developed to allow the retention testing of pleated-sheet membrane cartridges (HIMA 1982, ASTM F 838 1992) there are no current means of retention testing membranes using cross-flow filtration. The ASTM (F 838 1992) recognised the need to develop a retention test for pleated-sheet membrane cartridges utilising positive pressure to drive filtration rather than a vacuum to reflect in-use practices. The test also used a challenge volume that represents in-use feed volumes for the test membrane. The results obtained in this study indicate that it is possible to develop a standard retention test for cross-flow membranes involving the filtration of a certain volume of feed for a pre-determined period of operation under fixed parameters such TMP and linear velocity. The bacterial load of the feed will coincide with current recommended challenge levels. Samples of the retentate and permeate will be taken at timed intervals and the test membrane will be shown to be capable of producing a sterile permeate during the run time under the recommended conditions.

Trial experiments for such a test were able to demonstrate the effect of varying linear flow rates and TMP's on the retention efficiency given by a 0.2 μm rated ceramic multichannel monolith. An increase in either linear velocity of the feed or the TMP caused a reduction in LRV. A retention test procedure was developed that proved successful in comparing the ability of 0.2 and 0.1 μm rated monoliths to retain *P. diminuta* under the same conditions. A similar test was also used to ascertain the suitability of a small, plate-and-frame Rayflow module for sterilising filtration and to determine the retention efficiencies of a selection of membranes during cross-flow filtration. A decrease
in LRV was seen during six hour filtration experiments with the ceramic monoliths. This was presumed to be attributable to the grow-through of \textit{P. diminuta} retained by the membrane. However, the feed used for these experiments had a very high bacterial load which will not be generally encountered during sterilisation filtration. The extent of grow-through seen in these experiments was much magnified compared to that which will occur during process filtration.

Keeping the filtration system sterile considering the constant sampling during the test was not a problem even when working in an engineering pilot lab. Preparation of the rig before and after the test was labour intensive although not particularly difficult, as was the plating-out of retentate and permeate samples that required large amounts of media. The rigs were hypochlorite-sterilised in accordance with the procedure used for the dead-end retention tests carried out in this study. The application of steam-sterilisation (currently used for some process sterilisation) would decrease the amount of preparative and post-test work needed for those systems containing membranes suitable for steam-sterilisation. It is impossible to recommend at the moment the use of one sterilisation method for the proposed standard cross-flow retention test due to the variation in membrane materials available. Whilst taking small aliquots of the permeate and retentate was a sufficient means of analysis for the test membranes used, it is realised that larger sample volumes may be needed for a standard test. These could be analysed by membrane filtration using a membrane known to effectively retain \textit{P. diminuta} or by using a rapid method for the detection of bacteria (discussed in the next section) that is capable of assaying large volumes.

The development of the current test methodology will include the determination of suitable test parameters and run time, and a possibly a standard module for flat sheet membranes somewhat like the Rayflow in design. This will standardise the area of membrane used for the test. It was requested by the manufacturers that the Microkerasep monoliths be tested at a linear velocity of 3 m/s and a TMP of 0.5 bar which are ideal conditions for the cross-flow filtration of biological feeds with a high fouling rate. The retention efficiency of \textit{P. diminuta} was seen to increase from the start of the test and stabilised after about one hour of run time so a test duration of two hours was chosen in this study. Longer run times could be implemented in order to study the effect of grow-through on the microbial load of the permeate. The retention ability given by the test membrane could also be determined with two different values for TMP, for example 0.5-1.5 bar, instead of a single value. This would demonstrate that the test membrane is capable of producing a sterile permeate even if the TMP deviates from its required value during process filtration. Comparisons between the retention profiles of many different types of membrane, and information about actual process conditions need to be studied.
in order to determine standard test parameters. It is important that the apparatus needed for the standard test method is neither too expensive nor specialist that it is out of reach of membrane manufacturers. The recirculation pump and cooling unit used for the Microkerasep rig do not fall into these categories and the rest of the fittings were easily and cheaply available.

5.3. Development of a rapid retention test using *P. diminuta*.

The three rapid methods used for the detection and enumeration of *P. diminuta* in retention test permeates are the most popular of those in use today and have already been accepted into routine testing and standards methodology (Dutka 1992). Comparisons between each method and the standard plate count were used in order to determine the suitability of the rapid method for the accurate enumeration of bacteria (Anderson *et al* 1986, Connolly *et al* 1993).

For a rapid method for the enumeration of viable bacteria to be considered suitable for replacing the traditional cultural methodology for analysing test permeates it must above all be capable of achieving the same sensitivity of detection of one cfu in the whole permeate. This goal presented the greatest challenge and although all three of the methods were capable or potentially capable of analysing the entire permeate, the required level of sensitivity was not seen.

The criteria for the rapid retention test method to be considered suitable for superseding current traditional methodology were detailed in section 2.3.4.. Neither of the rapid test procedures as implemented fully satisfied all the criteria. When required to choose between them however, the test using the ATP luminescence assay would seem the most applicable overall. This method could be applied to the generation of a standard rapid procedure for the routine retention testing of membranes. The ATP assay could also be applied to the generation of a rapid cross-flow retention test. Large sample volumes of the retentate and permeate could be analysed by the filtration and ATP luminescence assay described in this study to give a rapid test with a high sensitivity of detection for viable *P. diminuta*. The greatest disadvantage associated with DEFT analysis was the amount of time, labour and skill needed in the preparation of the DEFT slide and counting the viable bacteria. Use of the Bactometer was the most unlikely method to analyse retention test permeates and took longer than both the ATP or DEFT assays to deliver results.
The method used for the analysis of retention test permeates should be rapid. The ATP assay method allowed concentration of the permeate from 140 ml to 1 ml. A theoretical sensitivity of detection of 10-100 viable cfu per permeate was given after 5 hours incubation of the UF membrane used to concentrate bacteria from the permeate. The DEFT was by far the most rapid of the three methods by giving results in under an hour but is not capable of giving an adequate sensitivity of detection. The impedimetric assay using Bactometer gave results in over 30 hours for small sample volumes and would not offer a great time advantage over current culture procedures.

With increased incubation times the sensitivity of detection for the ATP assay increased, however this involved incubation overnight and detracted from the overall rapidity of the method. The sensitivity could also theoretically be increased by using a smaller diameter analytical membrane and smaller volumes of broth medium for incubation of the membrane and ATP extractant. For the DEFT a sensitivity of detection of not less than 100 cfu per sample was achieved and a detection level of 1 cfu per permeate would involve the accurate inspection of the entire membrane surface which would be very laborious. Again, the use of a smaller diameter membrane would increase the sensitivity of detection. The Bactometer is not capable of analysing the entire test permeate without the modifications in procedure described in the results, and a sensitivity of detection of not less than 10 cfu per ml of sample was achieved. If the analysis was carried out using large capacity sample bottles then it can be estimated that a few days would be needed for results to become available with the required sensitivity of 1 cfu per permeate.

The procedure for the retention test using the ATP assay was straightforward compared to the DEFT procedure which was very laborious. The most tedious aspects for the ATP assay were the reconstitution and storage of the luciferin-luciferase reagent which could be prepared in bulk. The labour aspect for the DEFT has been improved for other DEFT applications by the use of automated equipment for counting and analysis (Pettipher and Rodrigues 1982, Pettipher et al 1992) or by using the microcolony technique (Rodrigues and Kroll 1988, Newby 1991). However, these are not possible for the application of analysing retention test permeates due to the lack of sensitivity of detection for P. diminuta and the irregularities seen with microcolony formation. The methodology for the Bactometer was the most simple and straightforward of the three methods which constitutes the only point of advantage for this technique.

For the ATP assay, there were no means for the identification of bacteria on the incubated membrane without culture procedures requiring at least 48 hours. However, microbiological contamination of the permeate was not encountered during both the development and use of the procedure. The DEFT gave the advantage that the direct
observation involved permitted the identification of bacteria on the basis of morphology. Accurate species identification with the Bactometer was not possible although general contamination of a pure culture can sometimes be indicated by the presence of an irregular signal curve.

The cost of retention testing 0.2 μm rated membranes with _P. diminuta_ by:

(a) The standard ASTM (D 3862 1992) procedure.
(b) The ASTM procedure with the incubation and plate count method developed in this study.
(c) The ASTM procedure with the ATP assay.
(d) The ASTM procedure with the impedance assay using the Bactometer.
(e) The ASTM procedure with the DEFT.

was determined. The cost of each individual retention test and permeate analysis was calculated using a spreadsheet constructed for this application. The results may be seen in appendix K. The cost analysis was based on the procedures used in this study being applied to routine retention testing in industry. Overheads were put in at 115%. Using the ASTM procedure, the ASTM and plate count and ASTM and the ATP assay, six individual filtration experiments were carried out by one person per day. Using the ASTM and the DEFT technique, two persons were required to uphold the same rate of testing. With the Bactometer M64 model used in this study for the impedance assays, it was only possible to analyse four permeates every two days which has been accounted for in the analysis.

It can be seen from the analysis that the cost of the ASTM with plate count procedure is only slightly more expensive than the basic ASTM procedure at £25.30 (table K.1., appendix K) and £28.73 (table K.2., appendix K) per analysis respectively. Unfortunately neither of these procedures are rapid. At £32.28 (table K.3., appendix K) per individual analysis, the cost of the procedure using the ATP assay is more expensive than either of the purely cultural procedures but less expensive than analysis using the DEFT or the Bactometer at £52.04 (table K.5., appendix K) or £109.61 (table K.4., appendix K) respectively. As well as being the slowest of all the rapid procedures to yield results, the impedimetric assay is the most expensive. The procedure using the ATP assay required the lowest capital expenditure of all the rapid methods. The high initial cash requirement associated with high capital expenditure is undesirable both for existing companies wanting to expand their quality control facilities and for new companies. The need for obtaining a large lump sum for equipment either in total or via a lease which requires long-term commitment is a major disadvantage for an analytical technique. The need
for a large workforce is also a disadvantage due not only to the payment of salaries but also to the need for commitment to staff. Thus a method such as the DEFT which is relatively laborious is not suitable for the routine analysis of retention test permeates.

Dissatisfaction with the amount of time required for culture techniques to yield results from microbiological analyses has lead to the continued search for alternative rapid methods. This is particularly true for clinical and food hygiene analysis to which the ATP luminescence assay has been applied so successfully (section 2.4.2.6.). However, an important requirement for a rapid method is to produce a sensitivity of detection for viable bacteria that is on a par with culture techniques. As a consequence, the sterility testing of large volumes produces the greatest challenge to any rapid technique. Research concerning the rapid sterility testing of test permeates by the membrane manufacturers has not been reported to date. Yet this study, and in particular the next section, shows that a rapid and sensitive standard retention test procedure that is neither laborious or unduly expensive is within reach.

5.4. Application of bioluminescent bacteria to retention testing.

The use of a bioluminescent strain of *P. diminuta* for retention testing membranes will have advantages in rapidity and sensitivity compared to those rapid procedures discussed in the previous section. Through genetic engineering, it should be possible to optimise the specific bioluminescence for *P. diminuta* to enable the rapid and sensitive detection of viable cells within an hour without the need for expensive reagents or apparatus. It is not possible to discuss detailed cost implications as the exact methodology for the proposed test has not been determined.

The use of nucleic acid probes coupled with the PCR technique is currently popular for detecting low numbers of specific bacteria as discussed in section 2.4.1.. A probe has been developed that will detect *P. diminuta* (the type strain was used) specifically (Schleifer *et al* 1993). This probe consists of an short oligonucleotide sequence labelled with fluorescein that is complimentary to a sequence of *P. diminuta* 16S rRNA. Single-cell detection limits were possible using either a microscopic method or by colony hybridisation. Hybridisation of the probe to single cells and detection by microscopic examination may be rapid, but offers no advantages over the DEFT procedure carried out in this study. Colony hybridisation requires the formation of colonies from individual cells before the probe is hybridised and will offer no great time advantages over current culture retention test methods. Schleifer and colleagues (1993) reported that single cell detection limits (without the use of PCR) were only possible if the probe was directed at amplified target sequences. This is achieved with probes directed towards rRNA since
rRNA s are present in growing cultures in very high copy numbers of up to \(1 \times 10^5\) copies per cell. When a specific fluorescent probe was hybridised to \(E.\ coli\), bacteria proliferating on a nutritionally poor medium exhibited fluorescence that was considerably dimmer than those grown on a rich medium. These bacteria had a higher rRNA content and showed a very strong fluorescence. Since \(P.\ diminuta\) are prepared for the retention test by starvation in SLB medium then a pre-enrichment phase would be required if fluorescent rRNA probes were to be used for detection.

Dead-end retention tests for non-sterilising grade microfiltration membranes using a bioluminescent strain of \(E.\ coli\) demonstrated that the use of light-emitting bacteria for the rapid and sensitive retention testing of membranes is possible. Plasmid pPA3 containing the luxAB genes from \(V.\ harveyi\) (figure 3.5.a.) was constructed and transformed into \(E.\ coli\) in order to develop the bioluminescent strain of \(M.\ smegmatis\) used to assay the antimycobacterial effect of antibiotics and biocides (Andrew and Roberts 1993). Although transcription of the lux genes for \(E.\ coli\) JM101 (pPA3) was under the control of a mycobacterial promoter and the specific bioluminescence was not optimal, a lower limit of detection of approximately \(1 \times 10^2\) cfu/ml for this strain in retention test permeates was obtained. This is already more sensitive than the lower detection levels of \(10^3-10^4\) cfu/ml quoted for the assay of ATP using firefly luciferase and luciferin (Bopp and Wachsmuth 1981, Neufeld et al 1985, Ward et al 1986, Stanley and MacCarthy 1989). The sensitivity of detection for viable bioluminescent \(E.\ coli\) in test permeates was seen to increase with post-test permeate incubation. However, this incubation technique would not be as useful for permeates containing bioluminescent \(P.\ diminuta\) as the generation time for \(P.\ diminuta\) (section 3.3.1.) is approximately 4-5 times longer than that of \(E.\ coli\), which is typically about 20 minutes (Stanier et al 1987). These experiments highlighted the need to produce a bioluminescent strain of \(P.\ diminuta\) with high specific bioluminescence to give the required sensitivity of detection without the need for lengthy incubation procedures.

Developments were made towards cloning the luxAB genes from \(V.\ harveyi\) into \(P.\ diminuta\). The broad-host-range IncQ RSF1010-based plasmid pNJR12 (Maley et al 1992) was mobilised into \(P.\ diminuta\) by conjugation mediated by the IncP plasmid R751. The introduction and stable maintenance of an IncQ plasmid also mobilised by a helper plasmid into a different strain of \(P.\ diminuta\) has been described for an unrelated purpose (Serdar and Gibson 1985). The first attempt at transforming \(P.\ diminuta\) with the vector containing luxAB genes did not produce the required transformant. Details of the approaches planned for the production of a strain of \(P.\ diminuta\) with high specific bioluminescence are given in section 5.5.
The exact procedure for the proposed dead-end retention test using bioluminescent *P. diminuta* is yet to be determined. The methodology should be kept as straightforward as possible bearing in mind the criteria for the ideal rapid retention test laid out in section 2.3.4. It must be remembered that cloning the *luxCDE* genes into *P. diminuta* as well as the *luxAB* genes will remove the need to add exogenous substrate, and that use of such a strain where light emission does not have to be artificially induced will create a more simple test procedure. This option and the development of a cross-flow retention test using bioluminescent *P. diminuta* are discussed more fully in section 5.5.

There are two methods considered suitable for the analysis of retention test permeates. Both of these options require the development of a luminometer specially designed to analyse either the permeate or isolated bacteria.

Firstly, viable bioluminescent *P. diminuta* in the entire test permeate stream could be detected and enumerated using an on-line system. On-line detection of bioluminescence in real time is recognised as being a useful tool for monitoring both naturally occurring and recombinant bioluminescent micro-organisms (Sheintuch et al 1992, Huang et al 1993, Konstantinov et al 1993, Lasko and Wang 1993). The test permeate stream could be analysed using a luminometer fitted with a flow-through cell. The use of a fibre-optic probe coupled to a luminometer would present a non-invasive constant monitoring system that does not jeopardise the sterility of the process. An on-line monitor for bioluminescence consisting of a photomultiplier tube juxtaposed with a flow-through glass cuvette has already been described for monitoring the biomass of growing cultures of recombinant bioluminescent *E. coli* (Huang et al 1993).

Secondly, bacteria from the permeate could be collected using membrane filtration using an ultrafiltration membrane already shown to reliably retain *P. diminuta*. Viable bioluminescent *P. diminuta* on the membrane could be studied initially using photon-imaging technology. The small amount of aldehyde substrate required for the initiation of bioluminesence could be introduced to the membrane in the vapour phase via an injection system similar to that already seen on some models of luminometer. In both cases, the lower limit for detection of the bioluminescent bacteria will be determined partially by the sensitivity of the light detection system. However, the development of a strongly bioluminescent *P. diminuta* is preferable to the requirement for highly sensitive and expensive light detection equipment if the test method is to replace current routine retention test methods using traditional culture techniques.

A patent application has been filed encompassing the potential use of all bioluminescent micro-organisms for the retention testing of microfiltration membranes with a variety of pore sizes. Also a three year research proposal for approximately £150,000 entitled "The Retention Testing of 0.2 μm rated Membranes using
Bioluminescent *Pseudomonas diminuta* has been submitted to the SERC Separations Initiative. This is concerned initially with the construction of a bioluminescent strain of *P. diminuta* and the application of this strain to the dead-end and cross-flow retention testing of 0.2 μm rated membranes. The proposed research also includes the retention testing of non-sterilising grade membranes with other bioluminescent micro-organisms described in the next section.

5.5. Further work.

It is intended to continue with the research into the development of a bioluminescent strain of *P. diminuta* and the use of bioluminescent micro-organisms for the retention testing of membranes. Genes from the *lux* operon have been successfully cloned into several species of *Pseudomonas* (De Weger et al 1991, Silcock et al 1992, Cirvilleri and Lindow 1993). However, the genetics of *P. diminuta* are not well characterised. Apart from some strains being useful in certain areas of biodegradation (Serdar and Gibson 1985) or the hydrolysis of glutaryl-7-cephalosporanic acid (Binder et al 1993), *P. diminuta* is only regarded as a tool for membrane retention testing. There are several possible approaches concerning cloning the *lux* genes into *P. diminuta*, which are described below. It is not possible at present to comment upon which will be the most successful.

Further work will repeat the cloning experiments detailed in the study (section 4.5.2.). On obtaining the required recombinant *P. diminuta* (pISR600), well-characterised promoters from *Pseudomonas* species and *E. coli* will be cloned upstream from the *luxAB* genes and assayed for their ability to confer high level expression and bioluminescence. Single cell and microcolony detection levels have been reported for a bioluminescent strain of *Pseudomonas syringae* pv. *phaseolica* where transcription of the *luxAB* genes is driven by a phage promoter (Waterhouse et al 1993). It may be that high level constitutive production of luciferase will be detrimental to the cell. In this case, the use of a powerful, inducible promoter would permit expression of the cloned genes in *P. diminuta* only when required for the retention test.

Alternatively, the *luxAB* genes could be introduced directly into the chromosome via transposon mutagenesis with a mini-Tn5 *luxAB* transposon (De Lorenzo et al 1990). This would create a mutant with a chromosomal gene fusion between a *P. diminuta* promoter and the *lux* genes. Use of this transposon proved successful in generating a bioluminescent *luxAB* gene fusions in *Pseudomonas putida*. The need for constant selective pressure with antibiotics required when using plasmid vectors is removed. A mutant exhibiting consistent high level bioluminescence will be suitable for retention
testing provided that there is no difference in mean cell dimensions and growth characteristics compared to the wild-type *P. diminuta*. However, it must be considered that the presence of one copy of the *lux* genes in the chromosome (compared to multiple copies using high copy number plasmid vectors) may give a lower level of bioluminescence.

It has been reported that for strains of the fish pathogenic bacterium *Yersinia ruckeri*, the presence of up to six genomes per cell has been detected by flow cytometry during the initial phase of starvation (Thorsen *et al* 1992). This was accompanied by the reduction in cell size and tendency towards a more coccoidal morphology usually associated with starvation survival (Shirey and Bissonnette 1991, Herman and Costerton 1993). Considering that the dimensions of *P. diminuta* are deliberately reduced by growth in a nutritionally limiting medium (Leahy and Sullivan 1978), it may be possible to increase the number of copies of *lux* genes cloned into the *P. diminuta* chromosome. Multigenomic cells have also been observed in starving cells of the bacterium Alteromonas denitrificans (Thorsen *et al* 1992).

If these approaches do not prove entirely satisfactory, a gene library of *P. diminuta* chromosomal DNA will be constructed in a broad-host-range promoter probe vector using the *luxAB* as a reporter of gene expression (Farinha and Kropinski 1990). This library will be introduced into *P. diminuta* by conjugation and those promoters conferring high level expression of the cloned genes will be indentified by strongly bioluminescent clones. In all cases, the stability of the bioluminescent strain when subjected to cryopreservation and lyophilisation will need to be determined (Janda and Opekarska 1989). As mentioned earlier, the growth characteristics (Waterhouse *et al* 1993) and cell dimensions of the bioluminescent strain will each be compared to that for the original strain to confirm the suitability for use as a standard retention test micro-organism.

The bioluminescent strain of *P. diminuta* will applied first to a rapid retention test using dead-end filtration. The two methods described in the preceding section for the analysis of retention test permeates will be studied. This strain will then be applied to the retention testing of membranes using cross-flow filtration. It was indicated earlier (section 5.2.) that although this retention testing as implemented was straightforward, the analysis of numerous samples using culture methods was tedious, time consuming and required a large amount of media and disposables. The use of bioluminescent bacteria that could be enumerated in real-time would greatly decrease the test time and expense. It was also mentioned that although the sampling technique used in this study was adequate for those membranes analysed, larger volumes will have to be taken when
testing a wider variety of membranes. These could be analysed by membrane filtration and incubation to give an unlimited sample size, but several days will still be needed to determine the sterility of that sample.

The use of bioluminescent *P. diminuta* for a standard cross-flow retention test would create a rapid and efficient means of determining the retention ability of 0.2 µm rated membranes under simulated process conditions over a set filtration time. Detection of bioluminescent bacteria in retentate and permeate either on-line by use of a flow-cell or by isolation with membrane filtration described in section 5.4. would permit the detection and enumeration of bioluminescent bacteria in large sample volumes.

The bioluminescent strain of *P. diminuta* could be developed further by cloning in the *luxCDE* genes to permit the production of endogenous aldehyde substrate from fatty acid (section 2.5.2.). The production of a strain that does not require the addition of exogenous reagent to initiate light emission is particularly applicable to the cross-flow retention test where numerous samples of retentate and permeate have to be assayed over a period of time. A method for the on-line determination of viable cell number has been presented using a strain of *E. coli* cloned with the *luc* gene encoding firefly luciferase (Lasko and Wang 1993). This method was disadvantaged in that the substrate luciferin had to be added to the fermentation vessel. Luciferin is expensive and required storage at defined temperatures and away from light. The development of a cross-flow retention test requiring either the addition of a simple aldehyde substrate or no exogenous reagent at all will create a straightforward test procedure. However, cloning the additional *luxCDE* genes may be regarded as a refinement to the original objective of cloning the *luxAB* genes as the accumulation of high levels of endogenous aldehyde may prove toxic to the cell as mentioned earlier.

It is expected that the successful test will be marketed mainly to membrane manufacturers for rapid validation of sterilising grade membranes but will also be available to the users of such membranes for the recommended in-house tests (Meltzer 1987a). The cloning of genes from the *lux* operon into micro-organisms for specific applications is a relatively new field, but already the use of recombinant bioluminescent bacteria to detect contaminating mercury in the environment has been patented (Molders 1990). The use of a bioluminescent *P. diminuta* for the rapid retention testing of 0.2 µm rated membranes should be readily accepted considering that *P. diminuta* has been the standard test organism for these membranes for many years, although genetically engineered organisms require rigorous testing before released into routine industrial use as mentioned in section 2.5.3.148
The development of bioluminescent strains of the other species of bacteria used for membrane retention testing will also be considered. Listed below are those species currently used by membrane manufacturers:

<table>
<thead>
<tr>
<th>Pore size rating (µm)</th>
<th>Micro-organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>Acholeplasma laidlawii</td>
</tr>
<tr>
<td>0.2</td>
<td>P. diminuta</td>
</tr>
<tr>
<td>0.3</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td>0.45</td>
<td>S. marcescens</td>
</tr>
<tr>
<td>0.65</td>
<td>E. coli and Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>0.8</td>
<td>S. cerevisiae</td>
</tr>
</tbody>
</table>

All the species of bacteria listed above are Gram-negative and this is convenient as it is known that light emission from recombinant bioluminescent Gram-positive bacteria is some orders of magnitude less than that from Gram-negative bacteria (Karp 1989, Cook et al 1993). S. marcescens is already accepted as the standard retention test organism for 0.45 µm rated membranes recommended for general membrane filtration analysis (Bowman et al 1967, ASTM D 3863 1992). Species of micro-organisms similar to those listed above have been used to determine the retention characteristics of membranes with a variety of pore sizes (Rogers and Rossmore 1970, Bower 1986). P. diminuta, Streptococcus faecalis, Staphylococcus epidermidis, S. marcescens and Candida albicans have been used to challenge 0.22 µm rated peritoneal dialysis membranes over a period of 4 weeks (Leahy et al 1980).

The development of a standard retention test for 0.1 µm rated membranes using a bioluminescent strain of mycoplasma is considered the most important goal after the successful production of a bioluminescent P. diminuta and rapid retention test method. There is no standard method for the bacterial retention testing of 0.1 µm rated membranes although Acholeplasma laidlawii is often used, due to the practice of using 0.1 µm rated membranes to remove contaminating destructive mycoplasma from tissue culture reagents (Meltzer 1987a). The mycoplasma genome is the smallest of those recorded for prokaryotes and is considered a good potential cloning host (Rottem and Barile 1993). A retention test procedure using A. laidlawii has been described (Bower 1986) that involved simple incubation of the permeate for 21 days to permit the detection of sterility, without means for enumerating viable bacteria. A. laidlawii is a facultative anaerobe and all culturing and permeate incubation was carried out under anaerobic conditions. The
application of a rapid technique involving bioluminescence would create an improved, straightforward and quantitative test that is applicable to the routine validation of retention ability.

The role of bacterial retention tests for the validation of sterilising grade membranes has been confirmed in this study but the retention testing of non-sterilising grade membranes with the species of micro-organisms listed earlier is also considered by the membrane manufacturers to be very important for the characterisation of new, experimental membranes. The generation of a series of bioluminescent micro-organisms would permit the rapid retention testing of 0.1-0.8 μm rated membranes.

A different approach to producing a rapid retention test procedure would be to introduce lux genes into wild-type P. diminuta in retention test permeates via bacteriophage specific for that species. The phage are inherently dark but the lux genes will be expressed in the host to give bioluminescence after a short incubation period. The concept of using lux+ recombinant phage in this manner was introduced by Ulitzur and Khun (1987) who describe a sensitive assay for E. coli using specific phage containing the luxCDABE genes. A large sample volume containing suspended E. coli was filtered through a membrane which was incubated with 1 ml of medium containing the phage. Bioluminescence could be detected after 100 minutes from samples that contained as few as 10 cfu per ml. Kodikara et al (1991) used lux+ recombinant phage to detect enteric indicator bacteria (E. coli, Enterobacter, Citrobacter and Klebsiella) present at levels of 1 x 10^4/g without pre-enrichment, and as few as 10/g after an incubation period of four hours. Bioluminescent phage have also been used to detect and enumerate low numbers of S. typhimurium (Turpin et al 1993).

A phage assay for the other micro-organisms used for retention testing is also possible. Phage specific for P. diminuta have not been identified but those specific for P. aeruginosa have been discussed (Holloway and Krishnapillai 1975) and phage for S. marcescens and E. coli are listed in the NCIMB catalogue. The method for a retention test using phage in this way would be slightly more complicated than one using a recombinant bioluminescent bacterium as host bacteria are required for the replication and expression of the lux genes.

The development of a standard method for the validation of sterilising grade membranes for gases using lux+ recombinant phage is possible. Air-borne phage are destructive contaminants of bulk fermentation vessels (Meltzer 1987c). The retention ability of a 0.2 μm rated membrane used to sterilise liquids is known to be higher when used to filter air although membranes of this pore size are considered the standard for
sterilising gases. These membranes are used for the sterilisation of incoming gases such as air or pure gases for batch fermentors, and are also used as sterilising vent filters for fermentors.

There is no standard retention test method for the retention testing of membranes used for sterilising gases. Non-bacterial challenges, such as aerosols of dioctylphthalate (DOP) (Perkowski 1983) or latex spheres (ASTM F 1215 1992) have been used. Aerosol preparations of P. diminuta have also been used (Duberstein and Howard 1978, Conway 1984), although the logic of using vegetative bacteria to represent the bacterial load of air has been questioned as these would rapidly desiccate in nature. A retention test employing spores of Bacillus subtilis has been described (Robertson and Frieben 1984) but the most commonly used challenge for 0.2 μm rated membranes used to sterilise gases is an aerosol preparation of T1 phage (Conway 1984, Meltzer 1987c). In all cases, micro-organisms in test air-flow permeates are usually collected by impingement onto suitable media. Packing genes from the lux operon into T1 phage for expression in the host E. coli will enable detection of penetrating viruses more rapidly than by using traditional culture techniques.

This discussion so far has dealt only with microfiltration membranes. However, it is recognised that there is a need for a standardised method for the characterisation of the retention ability of ultrafiltration membranes (Gekas 1986). The NMWC values currently used to grade these membranes will differ between manufacturers as different reference solutes (eg Dextran, polyethylene glycol) are used sometimes with different retention coefficients (section 2.1.1.1.).

Viruses have been used to retention test membranes (Morowitz et al 1962) although this is not standard practice. It may be feasible that standard retention test procedures for UF membranes using a range of lux* recombinant phage could be developed. These might be carried out by challenging the membrane with a known number of phage, then filtering the permeate through a secondary filter to which is added a small volume of media (say 1 ml) containing the host bacterium. This volume can be assayed for bioluminescence after a suitable but short period of incubation. When defining a sterilising grade membrane as one that will remove all viable micro-organisms from solution (Meltzer 1987a), viruses are not included and apparently do not appear to pose a great health risk. If the removal of viruses from solution is required then UF membranes will be used, thus retention testing such membranes with viruses seems applicable.

Ultrafiltration is also used for the 100-fold concentration of viruses such as the rabies virus produced for vaccines (Meltzer 1987d). In fact, ultrafiltration membranes are used throughout the production of vaccines as sterilising vent membranes, for the removal of whole cells and cell debris in the production of bacterial toxoid and for the
final filtration of virus preparations to remove viral aggregates. As Meltzer (1987d) recommends that the choice of membranes should be defined by the validation process it seems logical that the ultrafiltration membranes should be routinely retention tested by viruses, the generation of \( \text{lux}^+ \) recombinant forms of which will provide a rapid and efficient means of effecting this.
6. CONCLUSIONS.

The first objective described in section 2.6. was to study current practices for the retention testing of 0.2 μm rated membranes. The practices for the determination of numerical pore size and for the validation of sterilising ability for 0.2 μm rated membranes using \textit{P. diminuta} were seen to vary between the membrane manufacturers. There were also variations in the procedure adopted for each of these tests by different establishments. The recommendation that 0.2 μm rated membranes should be retention tested with \textit{P. diminuta} to a level of $1 \times 10^7$ cfu/cm$^2$ of filterable area for the membrane (HIMA 1982)) is the most maintained feature among retention test procedures (Meltzer 1987a), although not all manufacturers of 0.2 μm rated membranes and not one of the pharmaceutical companies carried out routine retention testing. The general view offered by the pharmaceutical industry was that the process of retention testing with \textit{P. diminuta} is too lengthy to be considered worthwhile for routine use.

The sterilising ability of experimental, sterilising grade membranes was studied. A modification of the ASTM procedure for the validation of 0.2 μm rated membranes (ASTM D 3862 1992) was developed in order to test the retention ability of experimental 0.2 and 0.1 μm rated membranes, not one of these membranes retained the required number of \textit{P. diminuta} and could not be validated as sterilising grade. SEM analysis of \textit{P. diminuta} in test permeates from native 0.2 and 0.1 μm rated experimental membranes showed that cells with larger dimensions than the numerical pore size rating of the membranes were present. Thus it was inaccuracies in the pore size rating of the membrane, rather than the presence of very small bacteria in the challenge that caused the lack of retention ability. It was concluded that the grafting of hydrophilic material to native PVDF 0.1 and 0.2 μm rated membranes did not decrease the ability of the membrane to retain \textit{P. diminuta}. However, an increase in retention ability by some membranes grafted with material of a high molecular weight was seen and the pore size rating determined by the manufacturer for such membranes compared to the native membrane was seen to decrease after grafting. Clearly, steric hindrance caused by the graft as well as the affect of membrane material on the tendency for bacteria to adsorb to the pore walls must be taken into account when considering the retention ability of grafted sterilising grade membranes.

The retention efficiency for \textit{P. diminuta} given by commercial sterilising grade membranes was compared to that given by the experimental membranes. A selection of commercial 0.2, 0.22 and 0.1 μm rated membranes was retention tested with \textit{P. diminuta} using the same procedure that was developed for the experimental membranes. Only one type of 0.2 and 0.22 μm rated membranes was seen to reliably retain the
recommended challenge level of *P. diminuta* and was validated as sterilising grade. Of the 0.1 μm rated membranes, again only one type was seen to reliably retain *P. diminuta*. The retention test pass rate for these membranes was higher than for the 0.2 μm rated membranes and for those membranes that failed the retention test, a higher LRV for *P. diminuta* was given. A selection of ultrafiltration membranes was retention tested also and *P. diminuta* was found in some permeates from UF membranes with a NMWC of 300,000 and 100,000 dalton. The cell size analysis of bacteria by SEM revealed that cells with dimensions much less than the mean were occasionally found in *P. diminuta* cultures. Generally, the commercial membranes were more effective at retaining *P. diminuta* than were experimental membranes of the same pore size rating.

The size of individual cells of *P. diminuta* was studied using either SEM or the Coulter counter electronic particle sizing equipment. SEM was found to be the most suitable method for this application and the range of width and breadth measurements for cells from four different types of *P. diminuta* broth culture was determined. The Coulter Counter was able to produce results very rapidly but was not sensitive or accurate enough to analyse cultures of *P. diminuta*. Using SEM, it was found that the mean cell dimensions for *P. diminuta* were dependent on the amount of aeration the culture received during growth. A standard retention test procedure should include full and clear information concerning the growth conditions for *P. diminuta*. The size analysis of *P. diminuta* in the test permeates of 0.1 and 0.2 μm rated experimental membranes by SEM showed that the failure to effect sterility in this case was due to the presence of pores larger than the pore size rating of the membrane as mentioned earlier.

The development of a new, standard cross-flow retention test was undertaken. Retention test procedures using *P. diminuta* were developed in order to determine the retention efficiency of 0.2 and 0.1 μm rated membranes during cross-flow filtration simulating process conditions. Information concerning the efficiency of the module as well as the test membrane was obtained. The retention efficiencies of experimental and commercial flat-sheet organic membranes, and also of inorganic membranes in a tubular monolithic conformation were determined using a simple, small-scale test rig that could be set up in any laboratory. The test used for the monoliths involved recirculating a feed volume of 10 l containing *P. diminuta* around a simple test rig for periods of time up to six hours using a retentate linear velocity of 3 m/s and a TMP of 0.5 bar. Samples of the retentate and permeate were withdrawn at timed intervals and the retention efficiency given by the membrane at that time was calculated. Neither of the flat-sheet membranes or monoliths tested reliably retained *P. diminuta* although filtration using two or more
membranes in series will increase the overall retention capacity. These experiments demonstrated the feasibility for developing a standard retention test procedure for those membranes designed to be used for cross-flow filtration.

Rapid retention test procedures were developed. Retention test methods were developed by applying each of three popular rapid methods for the detection of bacteria to the enumeration of *P. diminuta* in test permeates. The method that used a ATP luminescence assay to detect and quantify *P. diminuta* isolated from test permeates using membrane filtration was the most suitable although all methods (as implemented) did not achieve the required sensitivity of detection. The ATP content for *P. diminuta* has not previously been determined and one average colony forming unit of *P. diminuta* was found to contain 0.57 fg of ATP. The total number of viable bacteria retained by the analytical membrane could be determined after five hours incubation by extracting all the bacterial ATP from the membrane and quantifying the ATP luminescence. The DEFT produced retention test results within the shortest time, just over an hour, but was the most laborious of the three methods. The application of an impedimentic assay (using the Bactometer) produced the most straightforward rapid procedure but was the most expensive means of analysing test permeates that was considered in this study and took the longest time to produce results.

Developments were made towards the generation of a bioluminescent strain of *P. diminuta* for the rapid retention testing of 0.2 µm rated membranes. The feasibility for such a test was demonstrated by using a bioluminescent strain of *E. coli* for retention testing 0.8 and 0.4 µm rated membranes. Bioluminescent *E. coli* were detected in test permeates on the addition of a simple reagent and the retention ability of the membranes was determined in about an hour. Test permeates were incubated to demonstrate the increased sensitivity of detection but membrane filtration could also be used to harvest the bacteria for the bioluminescence assay. The RSFl010-based broad-host-range plasmid pNJR12 (Maley et al 1992) was chosen as the vector for the *luxAB* genes to be cloned into *P. diminuta*. This plasmid was seen to be mobilised into *P. diminuta* by the IncP plasmid R751. Plasmid pISR600 containing the *luxAB* genes will be constructed and transformed into *E. coli* for subsequent introduction in to *P. diminuta*. The development of a rapid retention test procedure using bioluminescent *P. diminuta* will have advantages over current tests procedures and over the rapid retention test procedures developed already in this study.

It is planned to continue research concerning the generation of bioluminescent *P. diminuta* and the development of rapid retention test procedures using bioluminescent micro-organisms. These areas are covered by a patent application and are the subject of a SERC research grant proposal. The use of bioluminescent bacteria for retention testing
is applicable not only to 0.2 µm rated membranes used for sterilising liquids, but also to microfiltration membranes with pore size ratings from 0.1 to 0.8 µm, 0.2 µm rated membranes for sterilising gases and ultrafiltration membranes.

Overall, the area of more accurately and efficiently characterising the bacterial retention efficiencies of 0.2 µm rated membranes has been addressed in this study. The value of using retention tests for the characterisation of new sterilising grade membranes was demonstrated, indicating that retention testing should be part of the routine pore size characterisation process for all membrane manufacturers. The main aim has been to consider the development of rapid, well-defined and straightforward standard retention test procedures to be used universally by membrane manufacturers for the generation of a truly standardised product. The research in this study established and investigated the need for creating a rapid and accurate dead-end retention test, for creating a cross-flow retention test and discussed the possibility for combining the two to generate a rapid, cross-flow test without the need for extensive analysis of the many samples by culture techniques. Needs were also established for clarifying and standardising the exact procedures concerning the growth conditions for _P. diminuta_ used to challenge the membrane, and from the literature collated for the discussion it can be concluded that both the challenge level and the volume of challenge diluent are susceptible to variation and should be maintained at a set value. Until the procedure for the retention testing of 0.2 µm rated membranes becomes sufficiently standardised (and this procedure is used and adhered to by all membrane manufacturers) then the failure of these membranes and those of a smaller pore size rating to retain _P. diminuta_ (and the variation between the makes of membrane) will continue.

Basic retention test procedures using culture techniques give an acceptable sensitivity of detection for viable bacteria and have not been developed further for nearly 30 years. Reticence concerning the development of a rapid retention test procedure is based on the belief that the required sensitivity of detection will not be achieved but as mentioned at the end of section 5.3., this study has initiated the research that will lead towards the development of more efficient standard retention test procedures.

It has been shown quite clearly that not only does the method by which viable _P. diminuta_ are enumerated in test permeates need careful judgement, but also that the choice of method is dependent on a number of factors which should all be taken into consideration. It is not sufficient that the method of detecting viable bacteria is merely rapid or straight-forward. The DEFT produced the most rapid analysis of test permeates but did not give the required sensitivity of detection and was laborious whilst the impedance assay was the most straightforward but was the most expensive of all the methods used to analyse permeates. The most important factor is the accuracy and
sensitivity with which viable bacteria in the test-permeates are detected. The cost, labour and speed associated with the test are all be instrumental in determining the suitability of the new test procedure for routine use in industry.

For a rapid method for the enumeration of bacteria to be applied to the proposed cross-flow retention test, then the cost and labour aspect associated with the method becomes more important as numerous samples will be taken at intervals during the test, compared with the dead-end test which only generates one sample per test membrane. The ATP luminescence assay could be applied to the rapid cross-flow test but a more suitable alternative would be the proposed assay using a highly bioluminescent strain of *P. diminuta*. Although it is not possible to determine the exact details for this proposed assay, providing that the required strain of *P. diminuta* can be developed and that the detection of luminescence may be carried out without the need for unduly expensive equipment, it can be expected that the proposed test will be more rapid and less expensive than the application of the ATP luminescence assay.

It became clear that the validation of 0.2 μm rated microfiltration membranes used for sterilising liquids is not the only area that requires standardisation. The validation of 0.1 μm rated membranes with *A. laidlawii* is not yet a standard retention test and is only carried out by a few membrane manufactures. Only a few brands of 0.3-0.8 μm rated membranes are retention tested with micro-organisms by the manufacturers. The case for initiating research concerning the routine retention testing of ultrafiltration membranes with viruses was discussed in section 5.5. The retention testing of sterilising grade membranes used for gases with bacteriophage (section 5.5.) is not a standard procedure, and although it is more representative of in-use conditions for the membranes than is a retention test using latex spheres, this second test procedure is a listed standard test (ASTMF 1215 1992). It seems that the use of a test that requires expensive particle light-scattering counters for the detection of latex spheres downstream from the membrane is preferable to the use of a microbiological test due to the ease and speed with which results are made available.

The perception of a lengthy and tedious microbiological retention test for membranes is one that is especially evident amongst the pharmaceutical industry, and is fostered by reliance upon traditional culture techniques. When standard retention test procedures are generated that break away from this traditional image of microbiological analysis, such as tests using bioluminescent micro-organisms which permit the rapid and accurate detection of viable organisms in test permeates, then this perception of microbiological retention tests will be dispelled and the incidence of use for routine microbiological retention tests will expand.
7. REFERENCES.


M.C. Porter (ed.) 136-259 Noyes.


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APPENDICES

A - Materials for organic membranes.

Figure A.1. Structures of some common polymers used for the manufacture of organic membranes (from Toyomoto & Higuchi 1992).

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyethylene (PE)</td>
<td>((-\text{CH}_2\text{)}_n)</td>
</tr>
<tr>
<td>Polyvinylidenefluoride (PVDF)</td>
<td>((-\text{CH}_2\text{CH}_2\text{)}_n)</td>
</tr>
<tr>
<td>Polypropylene (PP)</td>
<td>((-\text{CH}_2\text{CH}_3\text{)}_n)</td>
</tr>
<tr>
<td>Polycarbonate (PC)</td>
<td>((-\text{O}\text{C}=-\text{O}\text{)}_n)</td>
</tr>
<tr>
<td>Teflon</td>
<td>((-\text{CF}_2\text{)}_n)</td>
</tr>
<tr>
<td>Cellulose Acetate (CA)</td>
<td>((-\text{OAc})_n)</td>
</tr>
<tr>
<td>Polyethersulfone (PES)</td>
<td>((-\text{S}=-\text{O})_n)</td>
</tr>
<tr>
<td>Polysulfone</td>
<td>((-\text{S}=-\text{O})_n)</td>
</tr>
<tr>
<td>Polyvinylalcohol (PVA)</td>
<td>((-\text{CH}_2\text{CH}_2\text{OH})_n)</td>
</tr>
<tr>
<td>Polyacrylonitrile (PAN)</td>
<td>((-\text{CH}_2\text{CH}_2\text{CN})_n)</td>
</tr>
<tr>
<td>Polyphenylenesulfide (PPS)</td>
<td>((-\text{S})_n)</td>
</tr>
</tbody>
</table>
B - The Rayflow module.

Figure B1 shows the original module whilst Figure B2 shows the modifications made to increase the permeate flow rate. B1 depicts a configuration using two membrane support plate whilst the modified version used one plate only. The modified module was used in all cases and the part nomenclature from B1 remains valid.

Detail of membrane seal:

The screw-nuts are fitted to the protruding bolts and tightened first by hand in the following order before being torqued down to 3 Nm in the same order.

Order for tightening screw-nuts.

Recommended cleaning agents for the autoclavable polycarbonate module are sodium hypochlorite (0.3%) solution recirculated for 20 minutes at 20-40°C followed by distilled water not recirculated for 10 minutes. If necessary, before this standard procedure the module could be cleaned with nitric acid solution (0.6%) for 20 minutes with recirculation. After washing the module is disassembled by slackening off the screw-nuts in reverse order.
Figure B.1. The original Rayflow module (taken from the Tech-Sep Rayflow manual.)
Figure B.2. The Rayflow module modified for microfiltration (from Tech-Sep).
C - Tolerance for non-optimal temperatures exhibited by *P. diminuta*

Figure C.1. The effect of changing temperature on the viable count for *P. diminuta*.

The effect of an increase or decrease in temperature from ambient (20°C) for SLB cultures of *P. diminuta* introduced to environments with five different temperatures is shown. The viable count (as log$_{10}$ cfu/ml) was determined at one hour intervals and is expressed as the percent increase or decrease from the viable count determined at the start of the experiment.
D - Solutions used for molecular biology.

(i) Luria broth (L-broth).
NaCl \hspace{1cm} 5 \text{ g/l} \\
Yeast extract \hspace{1cm} 5 \text{ g/l} \\
Tryptone \hspace{1cm} 10 \text{ g/l}

(For L-agar add 6g/l bacteriological agar)

(ii) TAE buffer.
Tris acetate \hspace{1cm} 0.04 \text{ M} \\
EDTA \hspace{1cm} 0.001 \text{ M}

(iii) x10 Ligation buffer
10 \mu l Tris pH 7.5 \hspace{1cm} 1.0 \text{ M} \\
2 \mu l MgCl$_2$ \hspace{1cm} 1.0 \text{ M} \\
2 \mu l ATP \hspace{1cm} 0.1 \text{ M} \\
6 \mu l high-purity D$_2$O

(iv) SSC.
NaCl \hspace{1cm} 0.15 \text{ M} \\
Trisodium citrate \hspace{1cm} 0.015 \text{ M}

(v) Denaturing solution.
NaOH \hspace{1cm} 0.5 \text{ M} \\
NaCl \hspace{1cm} 1.5 \text{ M}

(vi) Neutralising solution.
NaCl \hspace{1cm} 1.5 \text{ M} \\
Tris.HCl \hspace{1cm} 0.5 \text{ M}

(vii) Prehybridisation solution.
SSC \hspace{1cm} 3x \\
Denhardts’ \hspace{1cm} 5x \\
SDS \hspace{1cm} 0.1\% \\
PEG 8000 \hspace{1cm} 6\%

(viii) Hybridisation solution.
SSC \hspace{1cm} 3x \\
Denhardts’ \hspace{1cm} 2x \\
SDS \hspace{1cm} 0.1\% \\
PEG 8000 \hspace{1cm} 6\%

(ix) Denhardts’ 1x solution.
Ficoll 400 \hspace{1cm} 2\% \\
BSA \hspace{1cm} 2\% \\
PVP \hspace{1cm} 2\%
E - A sample of SEM photomicrographs used for the cell size analysis of *P. diminuta*.

Figure E.1. Shaken TSB culture of *P. diminuta*.

Figure E.2. Still TSB culture of *P. diminuta*.
Figure E.3. Shaken SLB culture of *P. diminuta*.

Figure E.4. Still SLB culture of *P. diminuta*.
Figure E.5. Test permeate containing *P. diminuta* from a 0.2 \( \mu \text{m} \) rated experimental membrane.

Figure E.6. Test permeate containing *P. diminuta* from a 0.1 \( \mu \text{m} \) rated experimental membrane.
F - Results for the electronic cell size analysis of *P. diminuta*.

Figure F.1. Schematic diagram showing the mode of operation for the Coulter Counter (from the instruction manual for the model TA11).

The Coulter Counter instrument will determine the number and size of particles suspended in an electrically conductive liquid. The test suspension (a) is forced through a small aperture between immersed electrodes (b). The resistance between the electrodes is changed as a particle passes between them to produce a short voltage pulse proportional to the size of the particle. The series of pulses is then electronically scaled and counted. Samples of controlled volumes are sucked through the aperture using a vacuum. The following results are from the particle size analysis of different cultures of *P. diminuta* using the Coulter Counter.
Table F.2. Still TSB culture of *P. diminuta*.

**LOUGHBOROUGH UNIVERSITY OF TECHNOLOGY**
**DEPARTMENT OF CHEMICAL ENGINEERING**

**PARTICLE SIZE ANALYSIS BY COULTER COUNTER MODEL TAII**

<table>
<thead>
<tr>
<th>Material .................</th>
<th>bacteria</th>
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<tbody>
<tr>
<td>Calibration .............</td>
<td>Standard latex</td>
</tr>
<tr>
<td>Lab ref ..................</td>
<td>93/</td>
</tr>
<tr>
<td>Client ...................</td>
<td>sw</td>
</tr>
<tr>
<td>Client's ref ............</td>
<td>1 tsb still</td>
</tr>
<tr>
<td>Coulter orifice diameter</td>
<td>30μm</td>
</tr>
<tr>
<td>Filename ..................</td>
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</tr>
<tr>
<td>Operator I.D .............</td>
<td>mrk</td>
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</table>

**Number distribution**

Table below shows counts per .05 ml of beaker suspension.

<table>
<thead>
<tr>
<th>Channel Size</th>
<th>B.G. Run 1</th>
<th>Run 2</th>
<th>Run 3</th>
<th>Run 4</th>
<th>Av-BG</th>
<th>Stats</th>
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<td>0</td>
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<td>0.00</td>
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<td>1</td>
<td>2</td>
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<td>-1</td>
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**Distribution by weight / volume**

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<thead>
<tr>
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<th>Percent weight in band</th>
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<td>8.00 - 10.08</td>
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<td>6.35 - 8.00</td>
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<td>2.52 - 3.17</td>
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<td>0.00</td>
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</table>

90 percentile size = 5.87um
50 percentile size = 1.07um
10 percentile size = 0.71um
Table F.3. Shaken TSB culture of *P. diminuta*.

<table>
<thead>
<tr>
<th>Material</th>
<th>bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration</td>
<td>Standard latex</td>
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<tr>
<td>Lab ref</td>
<td>93/</td>
</tr>
<tr>
<td>Client</td>
<td>sw</td>
</tr>
<tr>
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<td>2 tsb shake</td>
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<td>Operator I.D.</td>
<td>mrt</td>
</tr>
</tbody>
</table>

```
Number distribution
```

Table below shows counts per .05 ml of beaker suspension.

<table>
<thead>
<tr>
<th>Channel Size</th>
<th>B.G.</th>
<th>Run 1</th>
<th>Run 2</th>
<th>Run 3</th>
<th>Run 4</th>
<th>Run 5</th>
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<th>Stats</th>
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<tr>
<td>6.35 - 8.00</td>
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<td>5</td>
<td>3</td>
<td>1.15</td>
</tr>
<tr>
<td>5.04 - 6.35</td>
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<td>0</td>
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<td>0.00</td>
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</tbody>
</table>

```
Distribution by weight / volume
```

<table>
<thead>
<tr>
<th>Particle Size (um)</th>
<th>Cumulative</th>
<th>Channel Range (um)</th>
<th>Percent weight in band</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.00</td>
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<td>1.00</td>
<td>44.88</td>
<td>1.00 - 1.26</td>
<td>19.78</td>
</tr>
<tr>
<td>0.79</td>
<td>13.24</td>
<td>0.79 - 1.00</td>
<td>31.64</td>
</tr>
<tr>
<td>0.63</td>
<td>-0.00</td>
<td>0.63 - 0.79</td>
<td>13.24</td>
</tr>
<tr>
<td>0.50</td>
<td>0.00</td>
<td>0.50 - 0.63</td>
<td>0.00</td>
</tr>
</tbody>
</table>

90 percentile size = 3.39um
50 percentile size = 1.07um
10 percentile size = 0.75um
Table F.4. Still SLB culture of *P. diminuta*.

Material ....................... bacteria
Calibration ..................... Standard latex
Client .......................... sw
Client's ref .................... 3 slb still
Coulter orifice diameter ....... 30μm
Filename ........................ 93.dat
Operator I.D .................... mrk

<table>
<thead>
<tr>
<th>Table below shows counts per .05 ml of beaker suspension.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Channel Size</td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td>16.00 - 20.16</td>
</tr>
<tr>
<td>12.70 - 16.00</td>
</tr>
<tr>
<td>10.08 - 12.70</td>
</tr>
<tr>
<td>8.00  - 10.08</td>
</tr>
<tr>
<td>6.35  - 8.00</td>
</tr>
<tr>
<td>5.04  - 6.35</td>
</tr>
<tr>
<td>4.00  - 5.04</td>
</tr>
<tr>
<td>3.17  - 4.00</td>
</tr>
<tr>
<td>2.52  - 3.17</td>
</tr>
<tr>
<td>2.00  - 2.52</td>
</tr>
<tr>
<td>1.59  - 2.00</td>
</tr>
<tr>
<td>1.26  - 1.59</td>
</tr>
<tr>
<td>1.00  - 1.26</td>
</tr>
<tr>
<td>0.79  - 1.00</td>
</tr>
<tr>
<td>0.63  - 0.79</td>
</tr>
<tr>
<td>0.50  - 0.63</td>
</tr>
</tbody>
</table>

Distribution by weight / volume
---------------------------------
<table>
<thead>
<tr>
<th>Particle Size (μm)</th>
<th>Cumulative % wt under</th>
<th>Channel Range(μm)</th>
<th>Percent weight in band</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.00</td>
<td>84.40</td>
<td>16.00 - 20.16</td>
<td>15.60</td>
</tr>
<tr>
<td>12.70</td>
<td>79.58</td>
<td>12.70 - 16.00</td>
<td>4.82</td>
</tr>
<tr>
<td>10.08</td>
<td>74.75</td>
<td>10.08 - 12.70</td>
<td>4.82</td>
</tr>
<tr>
<td>8.00</td>
<td>74.06</td>
<td>8.00 - 10.08</td>
<td>0.20</td>
</tr>
<tr>
<td>6.35</td>
<td>74.46</td>
<td>6.35 - 8.00</td>
<td>0.50</td>
</tr>
<tr>
<td>5.04</td>
<td>74.21</td>
<td>5.04 - 6.35</td>
<td>0.25</td>
</tr>
<tr>
<td>4.00</td>
<td>73.28</td>
<td>4.00 - 5.04</td>
<td>0.92</td>
</tr>
<tr>
<td>3.17</td>
<td>72.02</td>
<td>3.17 - 4.00</td>
<td>1.26</td>
</tr>
<tr>
<td>2.52</td>
<td>70.49</td>
<td>2.52 - 3.17</td>
<td>1.53</td>
</tr>
<tr>
<td>2.00</td>
<td>67.30</td>
<td>2.00 - 2.52</td>
<td>3.19</td>
</tr>
<tr>
<td>1.59</td>
<td>61.29</td>
<td>1.59 - 2.00</td>
<td>6.01</td>
</tr>
<tr>
<td>1.26</td>
<td>48.60</td>
<td>1.26 - 1.59</td>
<td>12.59</td>
</tr>
<tr>
<td>1.00</td>
<td>30.66</td>
<td>1.00 - 1.26</td>
<td>17.94</td>
</tr>
<tr>
<td>0.79</td>
<td>6.92</td>
<td>0.79 - 1.00</td>
<td>23.74</td>
</tr>
<tr>
<td>0.63</td>
<td>-0.00</td>
<td>0.63 - 0.79</td>
<td>6.92</td>
</tr>
<tr>
<td>0.50</td>
<td>0.00</td>
<td>0.50 - 0.63</td>
<td>0.00</td>
</tr>
</tbody>
</table>

50 percentile size = 1.30μm
10 percentile size = 0.82μm
Table F.5.  Shaken SLB culture of *P. diminuta*.

<table>
<thead>
<tr>
<th>Material</th>
<th>bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration</td>
<td>Standard latex</td>
</tr>
<tr>
<td>Lab ref</td>
<td>93/</td>
</tr>
<tr>
<td>Client</td>
<td>sw</td>
</tr>
<tr>
<td>Client's ref</td>
<td>4 slb shake</td>
</tr>
<tr>
<td>Coulter orifice diameter</td>
<td>30 um</td>
</tr>
<tr>
<td>Filename</td>
<td>93.dat</td>
</tr>
<tr>
<td>Operator I.D.</td>
<td>mrk</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number distribution</th>
</tr>
</thead>
</table>

Table below shows counts per .05 ml of beaker suspension.

<table>
<thead>
<tr>
<th>Channel Size</th>
<th>B.G.</th>
<th>Run 1</th>
<th>Run 2</th>
<th>Run 3</th>
<th>Run 4</th>
<th>Run 5</th>
<th>Run 6</th>
<th>Run 7</th>
<th>Av-BG</th>
<th>Stats</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.00 - 20.16</td>
<td>0</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>6</td>
<td>5</td>
<td>12</td>
<td>11</td>
<td>7</td>
<td>1.23</td>
</tr>
<tr>
<td>12.70 - 16.00</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>6</td>
<td>7</td>
<td>13</td>
<td>10</td>
<td>12</td>
<td>7</td>
<td>1.63</td>
</tr>
<tr>
<td>10.08 - 12.70</td>
<td>0</td>
<td>6</td>
<td>8</td>
<td>11</td>
<td>9</td>
<td>8</td>
<td>17</td>
<td>5</td>
<td>9</td>
<td>1.31</td>
</tr>
<tr>
<td>8.00 - 10.08</td>
<td>0</td>
<td>13</td>
<td>12</td>
<td>10</td>
<td>12</td>
<td>15</td>
<td>14</td>
<td>12</td>
<td>13</td>
<td>0.48</td>
</tr>
<tr>
<td>6.35 - 8.00</td>
<td>0</td>
<td>12</td>
<td>19</td>
<td>18</td>
<td>9</td>
<td>11</td>
<td>14</td>
<td>13</td>
<td>14</td>
<td>0.98</td>
</tr>
<tr>
<td>5.04 - 5.35</td>
<td>1</td>
<td>13</td>
<td>20</td>
<td>21</td>
<td>21</td>
<td>20</td>
<td>24</td>
<td>24</td>
<td>19</td>
<td>0.84</td>
</tr>
<tr>
<td>4.00 - 5.04</td>
<td>1</td>
<td>27</td>
<td>28</td>
<td>16</td>
<td>23</td>
<td>19</td>
<td>38</td>
<td>23</td>
<td>24</td>
<td>1.46</td>
</tr>
<tr>
<td>3.17 - 4.00</td>
<td>6</td>
<td>44</td>
<td>41</td>
<td>41</td>
<td>30</td>
<td>38</td>
<td>31</td>
<td>40</td>
<td>32</td>
<td>0.95</td>
</tr>
<tr>
<td>2.52 - 3.17</td>
<td>19</td>
<td>66</td>
<td>56</td>
<td>52</td>
<td>76</td>
<td>57</td>
<td>62</td>
<td>83</td>
<td>46</td>
<td>1.88</td>
</tr>
<tr>
<td>2.00 - 2.52</td>
<td>64</td>
<td>177</td>
<td>136</td>
<td>164</td>
<td>158</td>
<td>160</td>
<td>189</td>
<td>166</td>
<td>100</td>
<td>1.55</td>
</tr>
<tr>
<td>1.59 - 2.00</td>
<td>167</td>
<td>513</td>
<td>471</td>
<td>477</td>
<td>493</td>
<td>456</td>
<td>448</td>
<td>470</td>
<td>308</td>
<td>1.25</td>
</tr>
<tr>
<td>1.26 - 1.59</td>
<td>328</td>
<td>1398</td>
<td>1377</td>
<td>1422</td>
<td>1376</td>
<td>1422</td>
<td>1369</td>
<td>1065</td>
<td>96</td>
<td>0.68</td>
</tr>
<tr>
<td>1.00 - 1.26</td>
<td>550</td>
<td>3827</td>
<td>3915</td>
<td>3882</td>
<td>3841</td>
<td>3704</td>
<td>3831</td>
<td>3741</td>
<td>3270</td>
<td>1.30</td>
</tr>
<tr>
<td>0.79 - 1.00</td>
<td>1160</td>
<td>27111</td>
<td>27601</td>
<td>27704</td>
<td>27027</td>
<td>26766</td>
<td>25324</td>
<td>26539</td>
<td>25850</td>
<td>3.20</td>
</tr>
<tr>
<td>0.63 - 0.79</td>
<td>6709</td>
<td>83348</td>
<td>82280</td>
<td>81771</td>
<td>83014</td>
<td>82807</td>
<td>82602</td>
<td>82485</td>
<td>75906</td>
<td>1.86</td>
</tr>
<tr>
<td>0.50 - 0.63</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Distribution by weight / volume

<table>
<thead>
<tr>
<th>Particle Size (um)</th>
<th>Cumulative % wt under</th>
<th>Channel Range(um)</th>
<th>Percent weight in band</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.00</td>
<td>65.84</td>
<td>15.00 - 20.16</td>
<td>34.16</td>
</tr>
<tr>
<td>12.70</td>
<td>54.25</td>
<td>12.70 - 16.00</td>
<td>11.56</td>
</tr>
<tr>
<td>10.08</td>
<td>46.37</td>
<td>10.08 - 12.70</td>
<td>7.89</td>
</tr>
<tr>
<td>8.00</td>
<td>40.95</td>
<td>8.00 - 10.08</td>
<td>5.42</td>
</tr>
<tr>
<td>6.35</td>
<td>37.99</td>
<td>6.35 - 8.00</td>
<td>2.96</td>
</tr>
<tr>
<td>5.04</td>
<td>35.89</td>
<td>5.04 - 6.35</td>
<td>2.09</td>
</tr>
<tr>
<td>4.00</td>
<td>34.81</td>
<td>4.00 - 5.04</td>
<td>1.29</td>
</tr>
<tr>
<td>3.17</td>
<td>33.75</td>
<td>3.17 - 4.00</td>
<td>0.86</td>
</tr>
<tr>
<td>2.52</td>
<td>33.14</td>
<td>2.52 - 3.17</td>
<td>0.61</td>
</tr>
<tr>
<td>2.00</td>
<td>32.46</td>
<td>2.00 - 2.52</td>
<td>0.68</td>
</tr>
<tr>
<td>1.59</td>
<td>31.42</td>
<td>1.59 - 2.00</td>
<td>1.04</td>
</tr>
<tr>
<td>1.26</td>
<td>29.63</td>
<td>1.26 - 1.59</td>
<td>1.73</td>
</tr>
<tr>
<td>1.00</td>
<td>26.87</td>
<td>1.00 - 1.26</td>
<td>2.75</td>
</tr>
<tr>
<td>0.79</td>
<td>15.98</td>
<td>0.79 - 1.00</td>
<td>10.89</td>
</tr>
<tr>
<td>0.63</td>
<td>-0.00</td>
<td>0.63 - 0.79</td>
<td>15.98</td>
</tr>
<tr>
<td>0.50</td>
<td>0.00</td>
<td>0.50 - 0.63</td>
<td>0.00</td>
</tr>
</tbody>
</table>

50 percentile size = 11.29um
10 percentile size = 0.73um
G - Results for the cross-flow retention tests.

Table G.1. Comparison of retention efficiency (as LRV) for *P. diminuta* given by flat-sheet organic membranes 1-7 (pages) during cross-flow filtration experiments with a linear velocity of 3.5 m/s and a TMP of 0.5 bar.

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>LRV 1</th>
<th>LRV 2</th>
<th>LRV 3</th>
<th>LRV 4</th>
<th>LRV 5</th>
<th>LRV 6</th>
<th>LRV 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>4.11</td>
<td>4.47</td>
<td>5.47</td>
<td>5.60</td>
<td>6.60</td>
<td>6.80</td>
<td>6.67</td>
</tr>
<tr>
<td>30</td>
<td>4.22</td>
<td>4.72</td>
<td>5.57</td>
<td>5.58</td>
<td>5.31</td>
<td>6.21</td>
<td>6.62</td>
</tr>
<tr>
<td>45</td>
<td>3.68</td>
<td>4.80</td>
<td>5.70</td>
<td>5.91</td>
<td>5.26</td>
<td>5.64</td>
<td>5.72</td>
</tr>
<tr>
<td>60</td>
<td>4.02</td>
<td>4.55</td>
<td>*</td>
<td>5.57</td>
<td>5.24</td>
<td>5.83</td>
<td>*</td>
</tr>
</tbody>
</table>

Where * = colonies too numerous to count.

Table G.2. Permeate rates (as l/h/m²) for the results shown in table G.1.

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Permeate 1</th>
<th>Permeate 2</th>
<th>Permeate 3</th>
<th>Permeate 4</th>
<th>Permeate 5</th>
<th>Permeate 6</th>
<th>Permeate 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.79x10³</td>
<td>1.47x10³</td>
<td>8.23x10²</td>
<td>7.99x10²</td>
<td>6.21x10²</td>
<td>6.04x10²</td>
<td>5.34x10²</td>
</tr>
<tr>
<td>15</td>
<td>1.55x10³</td>
<td>1.31x10³</td>
<td>4.89x10³</td>
<td>5.48x10²</td>
<td>4.03x10²</td>
<td>3.58x10²</td>
<td>2.59x10²</td>
</tr>
<tr>
<td>30</td>
<td>1.43x10³</td>
<td>1.24x10³</td>
<td>9.01x10³</td>
<td>4.26x10²</td>
<td>3.73x10²</td>
<td>3.04x10²</td>
<td>2.42x10²</td>
</tr>
<tr>
<td>45</td>
<td>1.38x10³</td>
<td>1.25x10³</td>
<td>8.27x10³</td>
<td>4.49x10²</td>
<td>2.89x10²</td>
<td>2.97x10²</td>
<td>2.43x10²</td>
</tr>
<tr>
<td>60</td>
<td>1.31x10³</td>
<td>1.18x10³</td>
<td>9.75x10³</td>
<td>3.20x10²</td>
<td>3.20x10²</td>
<td>2.97x10²</td>
<td>2.41x10²</td>
</tr>
<tr>
<td>PWP</td>
<td>2.02x10³</td>
<td>1.77x10³</td>
<td>1.44x10³</td>
<td>1.41x10³</td>
<td>9.73x10²</td>
<td>1.00x10²</td>
<td>7.39x10²</td>
</tr>
</tbody>
</table>
Figure G.3. Effect of variation in linear velocity and TMP on the retention efficiency (as LRV) for *P. diminuta* given by monolith 1 (page 18) during cross-flow filtration. The legends refer to the experimental values for TMP and linear velocity respectively.

![Graph](image.png)

Figure G.4. Effect of an increase in linear velocity on the retention efficiency (as LRV) for *P. diminuta* given by monolith 1 during cross-flow filtration with a TMP of 0.5 bar.

![Graph](image.png)
Table G.5. Comparison of retention efficiency (as LRV) for *P. diminuta* given by monoliths 2, 3, & 4 during cross-flow filtration with a feed velocity of 3 m/s and a TMP of 0.5 bar.

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>LRV 2</th>
<th>LRV 3</th>
<th>LRV 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>2.80</td>
<td>2.34</td>
<td>2.12</td>
</tr>
<tr>
<td>30</td>
<td>2.79</td>
<td>2.66</td>
<td>2.31</td>
</tr>
<tr>
<td>45</td>
<td>2.85</td>
<td>2.65</td>
<td>2.36</td>
</tr>
<tr>
<td>60</td>
<td>3.04</td>
<td>2.86</td>
<td>2.70</td>
</tr>
<tr>
<td>120</td>
<td>3.49</td>
<td>3.24</td>
<td>3.17</td>
</tr>
</tbody>
</table>

Table G.6. Permeate rates (as l/h/m²) for the experiments shown in table G.5.

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Permeate 2</th>
<th>Permeate 3</th>
<th>Permeate 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.31x10³</td>
<td>7.64x10²</td>
<td>8.50x10²</td>
</tr>
<tr>
<td>15</td>
<td>5.56x10²</td>
<td>5.90x10²</td>
<td>7.80x10²</td>
</tr>
<tr>
<td>30</td>
<td>4.22x10²</td>
<td>5.10x10²</td>
<td>6.82x10²</td>
</tr>
<tr>
<td>45</td>
<td>3.88x10²</td>
<td>4.46x10²</td>
<td>6.48x10²</td>
</tr>
<tr>
<td>60</td>
<td>3.52x10²</td>
<td>4.40x10²</td>
<td>6.25x10²</td>
</tr>
<tr>
<td>120</td>
<td>3.27x10²</td>
<td>4.30x10²</td>
<td>6.17x10²</td>
</tr>
<tr>
<td>PWP</td>
<td>1.84x10³</td>
<td>1.28x10³</td>
<td>1.33x10³</td>
</tr>
</tbody>
</table>
Table G.7. Retention efficiency (as LRV) for *P. diminuta* given by monolith 1 (after 10 filtration experiments) during cross-flow filtration with a linear velocity of 3.0 m/s and a TMP of 0.5 bar, monolith 3(i) with 0.65 m/s and 0.5 bar and monolith 3 (ii) with 3.0 m/s and 0.5 bar.

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>LRV 1</th>
<th>LRV 3(i)</th>
<th>LRV 3(ii)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>3.76</td>
<td>2.45</td>
<td>2.34</td>
</tr>
<tr>
<td>30</td>
<td>3.64</td>
<td>2.90</td>
<td>2.66</td>
</tr>
<tr>
<td>45</td>
<td>4.13</td>
<td>3.43</td>
<td>2.65</td>
</tr>
<tr>
<td>60</td>
<td>4.52</td>
<td>4.00</td>
<td>2.86</td>
</tr>
<tr>
<td>120</td>
<td>4.54</td>
<td>4.14</td>
<td>3.24</td>
</tr>
</tbody>
</table>

Table G.8. Grow-through of *P. diminuta* seen using monolith 1 during six hour cross-flow filtration experiments with a linear velocity of 3 m/s and a TMP of either (i) 1.5 bar or (ii) 2.5 bar.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>LRV 1(i)</th>
<th>LRV 1(ii)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>2.75</td>
<td>1.74</td>
</tr>
<tr>
<td>2</td>
<td>2.97</td>
<td>1.87</td>
</tr>
<tr>
<td>3</td>
<td>3.32</td>
<td>1.92</td>
</tr>
<tr>
<td>4</td>
<td>3.41</td>
<td>2.13</td>
</tr>
<tr>
<td>5</td>
<td>3.17</td>
<td>2.09</td>
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<tr>
<td>6</td>
<td>2.44</td>
<td>1.58</td>
</tr>
</tbody>
</table>
H - Correlation between rapid methods for enumerating bacteria and the plate count.

Figure H.1. Correlation between ATP luminescence (in RLU) and amount of ATP present.

Figure H.2. Correlation between ATP luminescence (in RLU) and plate count for *P. diminuta*.
Figure H.3. Correlation between ATP luminescence (in total RLU) after membrane filtration of sample and 5 hour incubation with plate count (total cfu) for *P. diminuta*.

![Graph showing correlation between ATP luminescence and plate count for *P. diminuta*](image)

Figure H.4. Correlation between IDT and plate count for *P. diminuta* (unfiltered culture) using the Bactometer. Total impedance was monitored and PCB was used as the growth medium.

![Graph showing correlation between IDT and plate count for *P. diminuta*](image)
Figure H.5. Correlation between IDT and plate count for *P. diminuta* (filtered culture) using the Bactometer. Total impedance was monitored and PCB was used as the growth medium.

Figure H.6. Correlation between manual DEFT count and plate count for *P. diminuta*. 
Figure H.7. Correlation between automated DEFT count and plate count for *P. diminuta*.

![Graph showing correlation between automated DEFT count and plate count for P. diminuta.](image)

Figure H.8. Correlation between the DEFT microcolony count (incubated on L-agar) and plate count for *P. diminuta*.

![Graph showing correlation between DEFT microcolony count and plate count for P. diminuta.](image)
Figure H.9. Correlation between the DEFT microcolony count (incubated on L-agar with streptomycin 50 µg/ml) and plate count for *P. diminuta*. 

\[ r^2 = 0.91 \]
I - Photomicrographs of *P. diminuta* taken during DEFT experiments.

Figure I.1. Differential staining of *P. diminuta* by acridine orange. Viable cells are orange.

Figure I.2.(i). Microcolonies of *P. diminuta* after 6 hours incubation.
Figure I.2.(ii). Microcolonies of *P. diminuta* after 6 hours incubation.

Figure I.3. Mature microcolonies of *P. diminuta* after 12 hours incubation.
J - Characterisation of *in vivo* bioluminescence for *E. coli* JM101 (pPA3).

Figure J.1. Development of bioluminescence for a broth culture of *E. coli* JM101 (pPA3). Bioluminescence is expressed in arbitrary units (AU) and is given for a $10^{-3}$ dilution of the culture.

![Graph showing bioluminescence over time](image)

Figure J.2. Correlation of bioluminescence with plate count for the enumeration of *E. coli* JM101 (pPA3).

![Graph showing correlation between viable count and bioluminescence](image)
K - Cost analysis for retention tests using traditional culture techniques and also rapid methods for the enumeration of bacteria.

Table K.1. Cost per permeate analysis using ASTM (D 3862 1992) procedure.

<table>
<thead>
<tr>
<th>Assay</th>
<th>ASTM D3862 1992</th>
<th>Cost/Yr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable Costs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Labour</td>
<td>No.</td>
<td>Salary pa +NI</td>
</tr>
<tr>
<td>Technician</td>
<td>1</td>
<td>£10,398</td>
</tr>
<tr>
<td>Manager</td>
<td>0.17</td>
<td>£21,408</td>
</tr>
<tr>
<td>Consumables</td>
<td>Media</td>
<td>Per litre</td>
</tr>
<tr>
<td>TSB</td>
<td>£0.97</td>
<td>0.28</td>
</tr>
<tr>
<td>SLB</td>
<td>£0.51</td>
<td>0.106</td>
</tr>
<tr>
<td>Each No./assay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disp.tips</td>
<td>£0.02</td>
<td>20</td>
</tr>
<tr>
<td>Fixed Costs</td>
<td>Building Rental</td>
<td>180</td>
</tr>
<tr>
<td>Rates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site services</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maintenance</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class 2 cabinet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autoclave</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total overheads</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Associated overheads</td>
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<td></td>
</tr>
<tr>
<td>Total fixed costs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capital Expenditure</td>
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<td></td>
</tr>
<tr>
<td>Class II Containment</td>
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<tr>
<td>Autoclave</td>
<td></td>
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<tr>
<td>Vacuum pump</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flasks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Funnels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pipettors (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orbital incubator</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stationary incubators (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depreciation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Cost/Year</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assays/technician</td>
<td></td>
<td>Days/wk</td>
</tr>
<tr>
<td>Per day</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Cost per Assay</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table K.2. Cost per permeate analysis using ASTM procedure with the plate count.

<table>
<thead>
<tr>
<th>Variable Costs</th>
<th>Labour</th>
<th>No.</th>
<th>ASTM/plate-count</th>
<th>Cost/Yr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Technician</td>
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<td>£10,398</td>
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<td>Manager</td>
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<td>£21,408</td>
<td>£3,566.01</td>
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<table>
<thead>
<tr>
<th>Consumables</th>
<th>Media</th>
<th>Per litre</th>
<th>Vol/assay</th>
<th>Cost/assay</th>
<th>Assay/yr</th>
<th>Cost/Yr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TSB</td>
<td>£0.97</td>
<td>0.26</td>
<td>£0.27</td>
<td>1362</td>
<td>£369.92</td>
</tr>
<tr>
<td></td>
<td>SLB</td>
<td>£0.51</td>
<td>0.006</td>
<td>£0.00</td>
<td>1362</td>
<td>£4.17</td>
</tr>
<tr>
<td></td>
<td>Agar</td>
<td>£1.31</td>
<td>0.2</td>
<td>£0.26</td>
<td>1362</td>
<td>£356.84</td>
</tr>
</tbody>
</table>

| Fixed Costs    | Building Rental      | 180 | £4.00     | £720.00    | £720.00 |
|                | Rates                |     | £1.00     | £180.00    | £180.00 |
|                | Site services        |     | £0.50     | £90.00     | £90.00  |

| Maintenance    | Class 2 cabinet      |     |           | £446.00    | £446.00 |
|                | Autoclave            |     |           | £800.00    | £800.00 |

<table>
<thead>
<tr>
<th>Associated overheads</th>
<th>Total overheads</th>
<th>Cost/Yr</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>£13,943.81</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Capital Expenditure</th>
<th>Per item</th>
<th>Per tech.</th>
<th>Life (yrs)</th>
<th>Cost/Yr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class II Containment</td>
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<td>£4,848.00</td>
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<td>£511.21</td>
<td>£511.21</td>
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<tr>
<td>Flasks</td>
<td>£11.44</td>
<td>£68.64</td>
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<td>£34.32</td>
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<tr>
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<tr>
<td>Pipettors (2)</td>
<td>£226.18</td>
<td>£226.18</td>
<td>3</td>
<td>£75.39</td>
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<td>Orbital incubator</td>
<td>£4,371.00</td>
<td>£4,371.00</td>
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<td>£874.20</td>
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<td></td>
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<td>£3,331.40</td>
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| Total Cost/Year     | £39,134.53   |         |

<table>
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<tr>
<th>Assays/technician</th>
<th>Per day</th>
<th>Days/Wk</th>
<th>Wks/yr</th>
<th>Assays/yr</th>
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<tr>
<td></td>
<td>6</td>
<td>5</td>
<td>45.4</td>
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| Cost per Assay      | £28.73 |         |


Table K.3: Cost per permeate analysis using ASTM procedure with the ATP luminescence assay.

<table>
<thead>
<tr>
<th>Assay</th>
<th>ASTM/ATP luminescence</th>
<th>Cost/Yr</th>
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</thead>
<tbody>
<tr>
<td>Variable Costs</td>
<td>Labour</td>
<td>No.</td>
</tr>
<tr>
<td></td>
<td>Technician</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Manager</td>
<td>0.17</td>
</tr>
<tr>
<td>Consumables</td>
<td>Media</td>
<td>Per litre</td>
</tr>
<tr>
<td></td>
<td>SLB</td>
<td>£0.51</td>
</tr>
<tr>
<td></td>
<td>Ringers</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>Petri-dishes</td>
<td>£0.11</td>
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<tr>
<td></td>
<td>disp. tips</td>
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<td></td>
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<td></td>
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<td></td>
<td>ATP water</td>
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<td></td>
<td>Lumacuvettes</td>
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<td>UF membranes</td>
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<tr>
<td>Fixed Costs</td>
<td>Building Rental</td>
<td>180</td>
</tr>
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<td></td>
<td>Rates</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Site services</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Maintenance</td>
<td>Per year</td>
</tr>
<tr>
<td></td>
<td>Class 2 cabinet</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Autoclave</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Luminometer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bacteria</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total overheads</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Associated overheads</td>
<td></td>
</tr>
<tr>
<td>Capital Expenditure</td>
<td>Class II Containment</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Autoclave</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vacuum pump</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flasks</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Funnels</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pippetors (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Orbital incubator</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stationary incubators (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Luminometer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Printer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Depreciation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total Cost/Year</td>
<td></td>
</tr>
<tr>
<td>Assays/technician</td>
<td>Per day</td>
<td>Days/wk</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Cost per Assay</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table K.4. Cost per permeate analysis using ASTM procedure with the Bactometer.

<table>
<thead>
<tr>
<th>Sara Waterhouse</th>
<th>Assay</th>
<th>ASTM/bactometer</th>
<th>Cost/Yr</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Variable Costs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Labour</td>
<td>No.</td>
<td>Salary pa + NI</td>
<td>Per tech.</td>
</tr>
<tr>
<td>Technician</td>
<td>1</td>
<td>£10,398</td>
<td></td>
</tr>
<tr>
<td>Manager</td>
<td>0.17</td>
<td>£21,408</td>
<td></td>
</tr>
<tr>
<td><strong>Consumables</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Media</td>
<td>Per litre</td>
<td>Vol/assay</td>
<td>Cost/assay</td>
</tr>
<tr>
<td>SLB</td>
<td>£0.51</td>
<td>£0.02</td>
<td>£0.01</td>
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<td>PCB</td>
<td>£2.01</td>
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<td>Each</td>
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<td></td>
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<tr>
<td>Modules</td>
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<td>£15.65</td>
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<td>Disp. tips</td>
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<td>30</td>
<td>£0.57</td>
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<td><strong>Fixed Costs</strong></td>
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</tr>
<tr>
<td>Building Rental</td>
<td>180</td>
<td>£4.00</td>
<td>£720.00</td>
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<td>Rates</td>
<td>£1.00</td>
<td>£180.00</td>
<td>£180.00</td>
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<td>Site services</td>
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<td>£90.00</td>
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<td><strong>Maintenance</strong></td>
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<tr>
<td>Class 2 cabinet</td>
<td></td>
<td>£446.00</td>
<td>£446.00</td>
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<td>Autoclave</td>
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<td>£800.00</td>
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<td>£1,860.00</td>
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<tr>
<td><strong>Bacteria</strong></td>
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</tr>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total overheads</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Associated overheads</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total fixed costs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Capital Expenditure</strong></td>
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<tr>
<td>Class II Containment</td>
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<td>£4,848.00</td>
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</tr>
<tr>
<td>Autoclave</td>
<td>£4,495.00</td>
<td>£4,495.00</td>
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</tr>
<tr>
<td>Vacuum pump</td>
<td>£511.21</td>
<td>£511.21</td>
<td>5</td>
</tr>
<tr>
<td>Flasks</td>
<td>£11.44</td>
<td>£22.88</td>
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<tr>
<td>Funnels</td>
<td>£33.28</td>
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<td>2</td>
</tr>
<tr>
<td>Pipettors (2)</td>
<td>£226.18</td>
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<td>2</td>
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<tr>
<td>Orbital incubator</td>
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<td>£4,371.00</td>
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</tr>
<tr>
<td>Stationary incubators (2)</td>
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<td>£1,384.00</td>
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<td>£224.95</td>
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</tr>
<tr>
<td><strong>Total Cost/Year</strong></td>
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<td></td>
</tr>
<tr>
<td><strong>Assays/technician</strong></td>
<td>Per day</td>
<td>Days/wk</td>
<td>Wks/yr</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5</td>
<td>45.4</td>
</tr>
<tr>
<td><strong>Cost per Assay</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table K.5. Cost per permeate analysis using ASTM procedure with the manual DEFT.

<table>
<thead>
<tr>
<th>Assay</th>
<th>ASTM/DEFT</th>
<th>Cost/Yr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Variable Costs

<table>
<thead>
<tr>
<th>Labour</th>
<th>No.</th>
<th>Salary pa</th>
<th>Per tech.</th>
<th>Total salary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Technician</td>
<td>2</td>
<td>£10,398</td>
<td></td>
<td>£10,398.49</td>
</tr>
<tr>
<td>Manager</td>
<td>0.33</td>
<td>£21,408</td>
<td></td>
<td>£3,568.01</td>
</tr>
</tbody>
</table>

### Consumables

<table>
<thead>
<tr>
<th>Media</th>
<th>Per litre</th>
<th>Vol/assay</th>
<th>Cost/assay</th>
<th>Assay/yr</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLB</td>
<td>£0.51</td>
<td>0.005</td>
<td>£0.00</td>
<td>1362</td>
</tr>
<tr>
<td>Ringers</td>
<td>£0.13</td>
<td>0.1</td>
<td>£0.01</td>
<td>1362</td>
</tr>
</tbody>
</table>

### Fixed Costs

| Building Rental | £4.00 | £720.00 | £1,080.00 |
| Rates           | £1.00 | £180.00 | £270.00   |
| Site services   | £0.50 | £50.00  | £135.00   |
| Maintenance     |       |         |           |
| Class 2 cabinet | £46.00 | £46.00 |
| Autoclave       | £80.00 | £80.00  |
| Microscope      | £100.00 | £100.00  |

### Capital Expenditure

| Class II Containment | £4,848.00 | £4,848.00 | 5 | £968.80 |
|                      |           |           |   |        |
| Autoclave            | £4,455.00 | £4,455.00 | 5 | £899.00 |
| Vacuum pump          | £511.21  | £511.21   | 5 | £102.24 |
| Flasks               | £111.44  | £68.64    | 2 | £34.32  |
| Funnels              | £33.78   | £19.99    | 2 | £39.94  |
| Pipettors (2)        | £226.18  | £226.18   | 3 | £75.39  |
| Orbital Incubator    | £4,371.00| £4,371.00 | 2 | £874.20 |
| Stationary Incubators (2) | £1,384.00 | 5 | £276.80 |
| Microscope           | £19,422.00| £19,422.00 | 5 | £3,884.40 |
| Manifold             | £1,224.00| £1,224.00 | 5 | £244.80 |
|                     |          |           |   | £7,460.60 |

### Depreciation

|               | £1,224.00 | £1,224.00 | 5 | £244.80 |

### Depreciation

|               | £7,460.60 |

### Total Cost/Year

| £70,872.18 |

### Assays/technician

<table>
<thead>
<tr>
<th>Per day</th>
<th>Days/wk</th>
<th>Wks/yr</th>
<th>Assays/yr</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>5</td>
<td>45.4</td>
<td>1362</td>
</tr>
</tbody>
</table>

### Cost per Assay

| £52.04 |