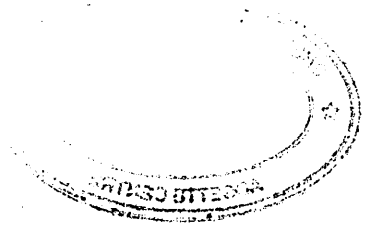
A COMPARISON OF FLOW CYTOMETRY AND CONVENTIONAL MICROBIOLOGY IN THE STUDY OF BIOFILMS

JIAN, X

PhD 2002



A Comparison of Flow Cytometry and Conventional Microbiology in the Study of Biofilms

By

Xiangrong Jian

A thesis submitted in partial fulfilment of the requirements of
Sheffield Hallam University
for the degree of Doctor of Philosophy

June 2002

Collaborating Organisations: Yorkshire Water.
Alcontrol Laboratories, UK.
Centre for Research in Environment
and Health (CREH).

Advanced Studies

April 1993 to June 2001.
Attended the Yorkshire Water and Alcontrol Laboratories seminars.

17th July of 1993.
Flow cytometry and environmental microbiology, Summer Conference. Society of Applied Bacteriology. University of Nottingham.

September 1993 to June 1998.
Training and using the Flow cytometer and Environmental microbiology and ecotoxic methods for detecting of the Pathogenic and indicator microorganisms; including *Legionella, Salmonella, E.coli, Staphylococcus, Amoeba, Cryptosoridium,* and *Giardia, Algae* at Yorkshire Water and Alcontrol laboratories.

October 1993 to February 1995.
Attended ''Rolling programme'' of seminars covering topics such as assertiveness, time management, preparing your transfer report and preparation of the thesis.

October 1993 to June 1998.
Attended School of Sciences seminars on a regular basis.

7 th –11 th November of 1994.
Visiting to Immunodiagnostics Department of CAMR (Porton Down, Salisbury) with regard to the development of monoantibodies, and biofilms studies.

10 th September 1996.
Visiting the Institute of Fresh Water with Adviser Dr Pickup for Flow cytometry and Environmental microbiology.

24th-29th February of 1997.
Visiting Air Products for novel waste water bio-chemical treatment project.

30 June 2001.
Attended the Annual Meeting (2001) of Chinese Association for Resources and Environment (CARE), University of Cambridge, England.

Acknowledgements

My sincere thanks are extended to my supervisors, Dr. J. Mills and Dr. J. Watkins for their helpful advice, encouragement and constructive criticisms during the course of this study and thesis preparation.

I would also like to thank Professor Clive Thompson, Dr. Mowthorpe, Dr. J. Barker, Dr. B. Davis, Dr R. W. Pickup and Professor D. Allen.

I gratefully acknowledge Yorkshire Water and Alcontrol Laboratories UK for the funding and for the laboratory facilities, particularly flow cytometry.

Finally, I am most grateful for the helps from my family, my parents, my sisters and brothers, and my wife Yingxin , my daughter Jiaan, and my son Che.

ABBREVIATIONS

ORGANISMS

A. castellanii	*Acanthamoeba castellanii*
A. haloplanktis	*Acanthamoeba haloplanktis*
A. polyphaga	*Acanthamoeba polythaga*
A. palestinensis	*Acanthamoeba palestinesis*
C. psittaci	*Chalmydia psittaci*
E. coli	*Escherichia coli*
FLA	free living *Amoeba*
H. vermiformis	*Hartmannella vermiformis*
L. micdadei	*Legionella micdadei*
L. longbeachae	*Legionella longbeachae*
L. pneumophila	*Legionella pneumophila*
L. monocytogenes	*Legionella monocytogenes*
M. tuberculosis	*Mycobacterium tuberculosis*
M. avium	*Mycobacterium avium*
P. aeruginosa	*Pseudomonas aeruginosa*
P. putida	*Pseudomonas putida*
S. enteritidis	*Salmonella enteritidis*
S. aureus	*Staphylococcus aureus*
S. typhimurium	*Salmonella typhimurium*
V. cholerae	*Vibrio cholerae*
V. vulnificus	*Vibrio vulnificus*
Spp.	*Species*

CHEMICALS BIOCHEMICALS AND MICROBIOLOGY

ABTS	2,2-aziono-bis-3-ethyl benzthiazoline-6 sulphonic acid
AO	acridine orange
ATCC	American type culture cellection
ATP	adenosine triphosphate

BCA	bicinchoninic acid
BCYE	buffered charcoal yeast extract
BIT	benzisothiazolone
BOD	biological oxygen demand
BDOC	biodegradable dissolved organic carbon
$CaCl_2.7H_2O$	calcium chloride (hydrous)
CMIT	5-chloro-N-methylisothiazolone
COD	chemical oxygen demand
CTC	5-cyano-2,3-ditolyltetrazolium chloride
$CuSO_4$	copper sulphate
ddH_2O	double distilled water
DAPI	4'6-diamidino-2-phenylindole
DC	direct count
DNA	deoxyribonucleic acid
EB	ethidium bromide
EDTA	ethylene diamine-tetra-acetic acid (Di-sodium salt)
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
FDA	Fluorescein diacetate
$Fe_4 (P_2O_7)_3.SO_4$	ferric pyrophosphate
FG	Feeley Gorman
FISH	Fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
GFP	green fluorescence protein
HCl	hydrochloric acid
HI	hexidium iodide
Hoechst 33342	HOE342
HPC	heterotrophic plate counts
KH_2PO_4	potassium orthophosphate
K_2HPO_4	di-potassium hydrogen phosphate

LBA	latex bead agglutination
mAb	monoclonal antibody
$MgCl_2$	magnesium chloride
$MgSO_4$ $7H_2O$	magnesium sulphate (hydrous)
NaCl	sodium chloride
Na_2CO_3	sodium carbonate
$NaHCO_3$	sodium bi-carbonate
NaOH	sodium hydroxide
NCTC	national collection of type cultures
NNA	no-nutrient agar
PAS	Page's amoeba saline
PBS	phosphate buffered saline
PC	plate count (colony count on plates)
PCR	polymerase chain reaction
PE	phycoerythrin
PI	propidium iodide
PMA	phorbal myristate acid
PHMB	polyhexamethylene biguanide
PYG	peptone yeast extract glucose
Rh123	Rhodamine 123
RNA	ribonucleic acid
RO	reverse osmosis
RPMI	Roswell Park Memorial Institute
TOC	Total organic carbon
Tris	tris (hydroxymethyl)- aminomethane
VNC	viable but can not cultured
VC	viable and can be cultured
YE	yeast extract
YEA	yeast extract agar

FLOW CYTOMETRY

AFM	atomic force microscopy

ANN	Artificial Neural Networks
CV	coefficients of variation
EFM	epifluorescence microscopy
FCM	flow cytometry (flow cytometer)
FCM -TC	flow cytometry total count
FCM – Target – C	flow cytometry, bacterial counts in sort region
FACS	fluorescence activated cell sorter
FL1	green fluorenscent detector (530nm)
FL2	orange fluorescent detector (550 nm)
FL3	red fluorescent detector (590nm)
FL4	uv fluorescent detector (<390nm)
FSC	forward scatter
HAS	high angle scatter
DIC	differential interference contrast
LAS	low angle scatter
PMT	photomultiple tube
SCLM	scanning confocal laser microscopy
SEM	scanning electron microscopy
SSC	side scatter
TEM	transmission electron microscopy
UV	ultra violet

UNITS

CFU	colony forming units
cm^2	centi metre-squared
d.	day
g	gram
x g	force due to gravity
h	hour
In	natural log
kDa	kilo Dalton
kHz	kilohertz
M	molar

mM	millimolar
mW	milliwatt
mg	milligram
min	minute
ml	millilitre
mm^2	millimetre-squared
µm	micro metre
nm	nano metre
OD	optical density
ppm	parts per million
rpm	revolutions per minute
sec	second
µl	microlitre
µg	microgram
V	Volt
V/v	volume per volume
wk	week
w/v	weight per volume
yr	years
>	greater than
<	less than

List of Tables

List of Figures

List of Appendices

Appendix A page

Appendix B

List of plates

Plate

Appendix C

Abstract

A Comparison of Flow Cytometry and Conventional Microbiology in the Study of Biofilms

Xiangrong Jian.

A comprehensive study on the application of flow cytometry (FCM) for the analysis of biofilms has been undertaken and the results presented in this thesis have shown that flow cytometry can been successfully used to enumerate, sort and image the bacteria and amoebae in biofilms and water distribution systems as a rapid and sensitive semi-automated technique compared with conventional microbiology.

It has been shown that the results of flow cytometric analysis of total *Legionella pneumophila* cells have a strong statistical correlation with the numbers of *Legionella* cfu by BCYE plate counting (BCYE PC) methods for biofilms and planktonic phases. There are also strong statistical correlations between flow cytometric analysis and epifluorescent microscopic (EFM) analysis (direct counting) for determination of bacteria, including *Legionella*, *Escherichia coli, Salmonella, Pseudomona*s and amoebae, and total and viable cells in pure cultures, water distribution systems and biofilms.

The flow cytometric protocols have been set up and optimised for the analysis of environmental microorganisms. The novel fluorescent dyes and immunofluorescence antibodies from the most current commercial dyes also have been screened and the staining protocols have been optimised and adopted for flow cytometric analysis and direct counting by epifluorescent microscopy. The tap water biofilms and river water biofilms were analysed by the flow cytometer and direct counting methods as well as by conventional microbiological methods (plate counting). The bacterial populations in real water distribution systems have been fully investigated and the total, viable bacteria were determined by the above methods.

The findings of this work have practical implications with respect to the rapid and automatic detection and predictions of *Legionella* spp. and the risk assessment from biofilms and water environments.

CONTENTS

1.0 INTRODUCTION

1.1 THE CULTURAL PROBLEM

The standard methods for counting bacteria in aquatic environmental samples involve plating with nutrient media in order to produce colonies for counting. The cultural methods will however only detect a proportion of the total population or obtain a "total culturable count" with the growth medium used. Many bacteria once in water become stressed or injured and fail to replicate in nutrient media although they may still be viable (Roszak and Colwell, 1987). Others become adapted to a low nutrient and low temperature environment and find culture media too nutrient rich or the incubation temperature too high, e.g. the aquatic pathogens such as *Vibrio cholerae* 01 that have adapted to their environment and associated conditions of stress (Huq *et al.,* 1990). Traditional culture detection methods may involve relatively high incubation temperatures (35-37 °C), plus the use of selective agents. Indirect measurements of metabolic activity such as respiration, photosynthesis, and enzyme activity have shown large differences in numbers of bacteria capable of growing on solid media compared with those actually present and metabolically active (Fry and Zia, 1982; Sleightholme and Roberts., 1994; McKay, 1992). Xu and colleagues (1982) suggested that the conventional view of bacterial growth and death should be re-evaluated.

The total population of environmental samples will consist of bacteria which are viable and can be cultured (VC) and bacteria which are viable but cannot be cultured (VNC) together with bacteria which are not viable (NV). The VNC state has been defined as a state in which bacterial cells are intact and alive when tested by one or more of the specific methods of metabolic activity, but do not grow on routinely employed bacteriological media (Sleightholme and Roberts, 1994).

There has been considerable interest recently in the possible existence of organisms that cannot form colonies on laboratory media but which are believed to be viable by other criteria. This so-called viable but not culturable state (VNC) has been described for a range of human pathogens, including some Gram-negative

species such as *Escherichia coli*, *Salmonella enteritidis* and *Legionella pneumophila* (McKay, 1992; Bovill *et al.*, 1994). Colbourne and Dennis (1989) were able to demonstrate the presence and viability of *L. pneumophila* in drinking waters despite not being able to culture the organism. Xu, *et al.*, (1982) have done a substantial amount of work on the existence of viable but non-culturable bacteria. Initially, cells of *E. coli* and *V. cholerae* suspended in water rapidly became non-culturable. Their continued presence was demonstrated by staining with acridine orange and their viability demonstrated by the ability of cells to elongate in a nutrient medium containing a DNA gyrase inhibitor (nalidixic acid). The combination of this work with bacteria-specific monoclonal antibodies enabled Huq *et al.*, (1990) to demonstrate viable *V. cholerae* in waters where traditional culture failed to detect them. This type of work has done much to establish the understanding of the survival of bacteria (particularly the Gram-negative bacteria) in water. The survival strategy (Roszak and Colwell, 1987) has improved the understanding of endemic cholera in certain areas of the world. Similar techniques have been used to study *Salmonella* spp. (Roszak, *et. al.*, 1984) and *Aeromonas salmonicida* (Pickup and Rhodes, 1997). Bogosian *et al.*, (1998) reported that the non-culturable bacteria do not enter the viable but non-culturable state and were dead by using the novel mixed culture recovery method.

Once in a hostile environment, the bacterial cell shrinks in size to become coccoid. Rapid transcription of DNA produces proteins which are designed to protect the cell against external factors such as salinity, pH, and temperature. Exposure of *S. typhimurium* to reduced levels of pH induces the production of proteins which protect the cell against low pH (Hickey and Hirshfield, 1990, Foster and Hall, 1991). These allow the cell to tolerate much lower level of acidity through the production of forty-three acid shock proteins and outer membrane proteins. Recovery of these bacteria may fail through cultural techniques because the medium may be too nutritionally rich or it may not contain the correct co-factors to allow recovery to take place. This theme will be enlarged later in the thesis. Culture will only identify a proportion of the total microbial population. It will not give any indication of the total numbers of cells present in a water sample nor will it give any

information about their state, for example whether they are dead, actively respiring or whether their membrane is intact.

A number of culture media are used for the general isolation of bacteria from the water environment e.g. yeast extract agar (Anon, 1982) is the standard method for counting bacteria from water samples in the United Kingdom; colony count agar (APHA, 1989) and diluted colony count agar are also standard methods and have been used for the detection of bacteria in mineral waters (Mavridon, 1992). A low - nutrient medium (R2A) was specifically developed for counting water bacteria (Reasoner and Geldrich, 1985). These media can be used in the pour plate method where the water is added to the plate together with molten agar medium. Alternatively, the agar may be poured first, allowed to set and the water spread on the surface of the plate. The second method does not expose the bacteria to elevated temperatures needed to keep agar molten and therefore does not run the risk of killing them by heat shock.

There have been some significant changes in cultural techniques in recent years and these have been reviewed by Watkins and Jian, (1997). The need for a more gentle recussitation through buffered media and lower incubation temperatures are well documented (Anon 1994). Such conditions help to recover environmentally stressed or damaged organisms. Media can also be adapted to help recovery. Addition of a small amount of sodium pyruvate was shown by Sartory, (1995) to enhance the recovery of chlorine-stressed organisms from water samples. The reduction of nutrient together with a more appropriate nutrient balance and lower temperature of incubation gave improved recoveries with water bacteria (Reasoner and Geldreich, 1985). Specific enzymes can also be targeted. The glycosidase enzymes of the enterobacteriacae have been used to create media that will identify coliforms and *E. coli* specifically (Edberg and Allen, 1988; Sartory and Howard, 1992). Many of these chromogenic or fluorogenic substrates were first designed for histological purposes but these have now been extended to produce commercial media for use in the water industry as well as having applications in food and clinical analysis.

1.2 BIOFILMS

Most waters contain planktonic bacteria. Some will be derived from the normal water flora whereas others will be present as contaminants from discharge of sewage and farm effluents, soil and surface run-off. Many of these bacteria are able to attach to solid surfaces and rapidly form biofilms. Clean surfaces, when placed into water containing planktonic bacteria can become covered within a matter of hours. Gradually, depending upon the nutrient levels available, (usually organic carbon derived from surface waters), microcolonies form held together in a matrix of excreted polymer glycocalyces. Biofilms are a complex diversity of microbial populations including aerobic and anaerobic bacteria, fungi, yeasts, algae and protozoa. With thin biofilms, there is no diffusion gradient and nutrient and oxygen can penetrate easily. As the biofilm becomes thicker, a diffusion gradient develops and availability of nutrient and particularly oxygen will be poor and microaerobic or anaerobic conditions will exist. This permits the growth of specific anaerobes such as sulphate reducing bacteria. Biofilms form readily in water distribution systems from bacteria supplied in the treated water. As they accumulate, they may cause taste and odour complaints as well as dirty water problems. They also provide a source of nutrient for animals such as *Asellus aquaticus.* The anaerobic bacteria can also cause corrosion problems. Biofilms by their nature protect microorganisms from the effects of biocides such as chlorine. They also have amoebae as a proportion of their population. Amoebae graze on the biofilm and ingest bacterial cells by a process of endocytosis. Ingested cells may be able to survive and even replicate. Once protected inside amoebal trophozoites, coliform bacteria were shown to be protected from chlorine levels as high as 50 mg/l (Schotts and Wooley, 1990). Barker *et al.*, (1992, 1995) reported that when *L. pneumophila* is grown in amoebae or in human monocytic cells, the activities of biocides such as polyhexamethylene (PHMB), benzisothiazolone (BIT), and 5-chloro-N-methylisothiazolone(CMIT) and rifampin were greatly reduced against the bacterium.

Analysis of biofilm formation by conventional culture is limited. Extraction of biofilms from solid surfaces is often difficult. Once extracted, the numbers counted will only be a proportion of the total population. In addition, as

shown by Watkins and Jian, (1997) the numbers counted by conventional microbiology will be a rapidly decreasing proportion of the total population as the biofilm develops. Most researchers have studied the formation of biofilms using small fermentation systems and recirculated water. Keevil *et al.*, (1987), Rogers *et al.*, (1991,1994a, 1994b), Rogers and Keevil, (1992) used such a system - a two-stage chemostat biofilm model with controlled temperature. They suspended numerous tiles of different material into the fermenter to study the formation and development of dental plaque on acrylic surfaces, and the incorporation of *Legionella* and *Cryptosporidium* oocysts in biofilms on different material surfaces. This type of reactor allows steady-state conditions to be established, similar to those of water distribution systems where water is flowing, with the removal of samples at regular time intervals without disrupting the experiment. de Beer *et al.*, (1994) used a continuous flow reactor that incorporated 12 removable stainless-steel slides to study the direct measurement of chlorine penetration into biofilms. Stewart *et al.*, (1998) reported the analysis of biocide transport limitation in an alginate gel bead artificial biofilm system.

Biofilms may act as a reservoir for potentially pathogenic bacteria such as *Pseudomonas aeruginosa, Aeromonas hydrophila* and *L. pneumophila* (Lee and West, 1991). They may also support the growth of coliforms and this brings about another problem. Coliforms are used as indicators of drinking water quality and their presence implies that water may have become contaminated and therefore be unfit for drinking (Anon, 1994). Coliforms may get into the drinking water system by repair of the main, ingress in the distribution system, back-syphonage or breakdown in the water treatment processes. Once in the system, coliforms can survive and grow slowly in low levels of nutrient in biofilms (Block *et al.*, 1997; Packer *et al.*, 1997) and may be isolated from drinking water samples occasionally when the biofilm breaks up.

1.3 ALTERNATIVES TO BACTERIAL CULTURE

A method for the rapid assessment of microbial viability is a major requirement in many areas of public health, the water and food industries. Methods

for direct counting do not rely on the culturability of bacteria in samples and consequently give higher counts than colony counting procedures. Direct counting of bacteria on black membrane filters by epifluorescence microscopy has become the most frequently used method for total bacterial population counts and permits a rapid quantitative estimation of aquatic bacteria (Hall *et al.*, 1990). Epifluorescence is achieved by using a range of nucleic acid or protein stains that fluoresce when excited by light of a suitable wavelength. Immunofluorescence microscopy has been also widely applied to the detection and enumeration of particular microorganisms when conventional methods have proved difficult. The development of monoclonal antibodies (Kohler and Milstein, 1975; Makin and Hart, 1989; McClelland and Pinder 1994) has greatly enhanced the technique of immunofluorescence in making specific antibodies for a wide range of bacteria available.

A number of DNA specific fluorochromes are available for the detection of microorganisms. Some of these can differentiate between viable and non-viable bacteria. Additionally, monoclonal antibodies are available not only for specific bacteria , but also for protozoa (Vesey, *et al.*, 1993; Watkins, *et al.*, 1995).

1.3.1 Acridine Orange (AO)

Acridine orange (AO) has been available for over 100 years and is one of the most commonly used fluorogenic dyes in microbial ecology and environmental microbiology as a part of the acridine orange direct count (AODC) or the direct total microbiological count methods (APHA, 1989). In the UK, the AODC has also been used as a standard method for detecting all viable and non-viable bacteria, yeasts and moulds in a water sample, which is to give a rapid estimate of the total microbial biomass, living and dead (Anon, 1994).

Acridine orange binds to the charged phosphate molecules of single-stranded RNA with the RNA-AO complex fluorescing orange-red while it intercalates with double-stranded DNA and the DNA-AO complex fluoresces green. Some (McFeters *et al.*, 1995; APHA, 1989; Anon, 1994) have suggested that the reaction of AO with bacterial DNA or RNA will allow the discrimination of living or dead cells. The hypothesis is based on the fact that RNA has a

relatively short life in the cell and dead cells contain very little of it. Cells which fluoresce red contain relatively large amounts of RNA and are therefore viable whilst cells which fluoresce green contain only DNA and are therefore non-viable (Yu, *et al.*, 1995).

Acridine orange is a relatively cheap and easy stain to use and is widely used to stain microorganisms for microscopic examinations, but for flow cytometric analysis, there are only a few reports (Darzynkiewicz *et al.*, 1975, 1980, 1994; Darzynkiewicz and Chrissman, 1990; Tragnos *et al.*, 1977) most of which are for staining eukaryotic cells in cellular biology.

1.3.2 4′,6′-diamidino-2-phenylindole (DAPI)

DAPI (4′,6′-diamidino-2-phenylindole, FW; 350) as a fluorescent DNA-specific dye was first used for rapid determination of DNA contents of the eukaryotic cells in cellular biology. Dann *et al.*, (1971) first reported that DAPI passed through the nuclear envelope to bind stoichiometrically with the AT-rich regions of intact DNA molecules. DAPI was first used to stain the cell's DNA for flow cytometric analysis by Stohr *et al.*, (1977) in cellular biology.

DAPI has a very high quantum efficiency and is stable in ultra-violet (UV) light. The DNA-DAPI complex is maximally excited at 365 nm and the complex fluoresces at 465 nm with about a 20-fold increase in fluorescence as compared to DAPI alone. Recent studies have shown that the DAPI interacts not only with DNA but also with extracted and synthetic biopolymers such as double-stranded RNA (Watson, 1991), proteins (Mazzini, *et al.*, 1992) and phospholipids (Favilla *et al.*, 1993). Since the 1980s, DAPI has also been widely used to stain bacteria and environmental microorganisms mainly for direct enumeration or counting by epifluorescent microscopy. DAPI direct counting (DAPI DC) technique has been widely adapted to replace the AODC in most of the published papers (Kepner and Pratt 1994). Porter and Feig (1980) reported using DAPI for identifying and counting aquatic microflora and McCoy and Olsen (1985) adapted DAPI for determining DNA concentrations in municipal drinking water. In 1995, Zweifel and Hagstrom reported that DAPI could be also used to stain non-nucleoid-containing bacteria (ghosts).

Though DAPI was used for FCM as a DNA dye as early as in 1977 (Stohr *et al.*), the applications of DAPI by FCM have been exploited in only a limited number of environmental microbiology studies. The one limitation to the further use of the dye is that the UV units need high power laser or duel lasers which are more complex and more expensive equipment compared with the single low power laser in a flow cytometer just for visible light and red light sources, and of course includes the expensive flow cytometer itself. Robertson and Button (1989) presented a detailed discussion which included using DAPI for detecting marine bacteria by flow cytometry; Monger and Landry (1993), and Lebaron and Joux (1994) for staining the DNA content of *S. typhimurium*, and Lange *et al.*, (1997) for total sorting of airborne bacteria using flow cytometry.

The main roles of DAPI staining for environmental microbiology are for enumerating or counting the total number of cells, detecting viable cells by dual staining with other dyes, and labelling the DNA contents of biomass and demonstrating subpopulations.

1.3.3 Propidium Iodide (PI)

Propidium iodide (PI, FW 668.4, $C_{27}H_{31}N_4I_2$), like most phenanthridinium dyes, is polar, highly soluble in water and does not readily cross functionally intact external cell membranes. Unlike DNA specific stains such as DAPI, PI can bind both DNA and RNA in two binding modes. Propidium iodide fluoresces red with a maximum emission at 610nm when excited at 520nm. Propidium iodide was introduced by Hudson *et al.*, (1969) in a procedure to distinguish between the dye-dependent density of linear and closed circle DNA. Since then, PI has been widely used in the cell biology for staining non-viable cells for the flow cytometric analysis of cell viability and nucleic acid content (Watson, 1991).

As a nucleic acid specific stain with red fluorescence, PI also is used in microbiology for labelling dead cells which do not exclude PI entry in dual staining to detect viability. Donnelly and Baigent (1986) combined PI with immunofluorescence staining for the detection of *Listeria monocytogenes* in milk.

Diaper and Edwards (1994a) reported on the correlation between PI fluorescence and cellular RNA of *Staphylococcus aureus* in lakewater by flow cytometry.

1.3.4 Fluorescein Diacetate (FDA)

Fluorescein diacetate (FDA) is non-fluorescent but is broken down by esterase enzymes in the cell with the release of fluorescein which excites at 488 nm and emits at 530nm. Fluorescein accumulates inside the cell and allows viable cells to be visualised. Jorgensen *et al.*, (1992) reported that FDA had been used to determine (staining) viable biomass in water and waste water treatments and Diaper *et al.*, (1992) also reported that rapid assessment of the viability of pure cultures of bacteria stained with FDA by using flow cytometry. FDA was also used to stain living microorganisms in soil (Tsuji *et al.*, 1995).

1.3.5 Rhodamine 123 (Rh123)

Rhodamine-123 (Rh123, FW 380.8, $C_{21}H_{17}ClN_2O_3$) is a class of cationic dye which partitions into electronegative environments and has been described as a mitochondrial specific dye and the indicator of membrane potential for assessment of eukaryotic cells' viability in cell biology (Weiss and Chen, 1984). Matsuyama, (1984) first reported that living bacteria were stained with Rh123 and it could be used as a fluorescence probe for detecting the viability of microorganisms by microscopy. Resnick *et al.*, (1985) reported the flow cytometric analysis of Rh123 stained *Mycobacterium smegmatis* and Kaprelyants and Kell (1992); Diaper *et al.*, (1992); Diaper and Edwards, (1994b) also described using Rh123 and flow cytometry for the rapid assessment of the viability of pure bacterial cultures. However, for Gram-negative bacteria, the cell envelope is only slightly permeable to Rh123 and a permeation procedure such as EDTA treatment is used to obviate this problem (Matsuyama 1984). Another limitation of using Rh123 is its non-specific binding to non-cellular structures which results in extensive background fluorescence. This limits the application of Rh123 for staining environmental samples. Rh123 is a polar, water-soluble cationic fluorescent dye and has been considered the best one for staining viable bacteria (Pinder *et al.*, 1993). It is limited for staining Gram-negative bacteria

because of cell membrane permeability problems. For *Ps. aeruginosa*, the Rh123 staining percentage was only 7 % even when treated with Tris-EDTA (Diaper *et al.*, 1992).

1.3.6 Hoechst 33342 (HOE342)

Hoechst 33342 (HOE342, MW 652) dyes are benzimidazole derivatives that have a high specificity for double-helical DNA and bind preferentially to A-T base regions, but do not intercalate. They emit blue fluorescence when excited by ultraviolet light (UV) at 346nm. Like DAPI, the fluorescent dyes were also first used for staining eukaryotic cells in cellular biology since the late 1960s. Hilwig and Gropp (1973) used HOE33258 in mouse chromosome-banding studies. Latt (1973) showed that the dye was quenched when bound to bromodeoxyuridine-substituted DNA (BrdUrd) and developed a method for detecting regions of sister chromatid exchange in metaphase chromosomes labelled with BrdUrd. Arndt-Jovin and Jovin (1977) using flow cytometry, first demonstrated the use of HOE258 and HOE342 for the quantitative DNA staining and sorting of viable cells.

Hoechst dyes have been used in microbiology to stain bacteria for epifluorescence microscopy analysis since the 1980s. In the 1990s Hoechst dyes started to be used in environmental microbiology to stain bacterial DNA contents for flow cytometric analysis. Monger and Landry (1993) reported that they used HOE342 to stain marine bacteria for flow cytometric analysis (EPICS 753). Lebaron and Joux (1994) demonstrated that HOE342 was used as a DNA-specific dye for flow cytometry (ACR1400SP) to discriminate between DNA subpopulations of *Salmonella* and *Alteromonas* during starvation and recovery in seawater. HOE342 was also used for staining pure cultures of *E. coli* cell DNA by Monfort and Baleux (1996). It was clear that most of the Hoechst staining work was focused on marine cells.

1.3.7 5-cyano-2,3-ditolyltetrazolium Chloride (CTC)

5-cyano-2,3-ditolyltetrazolium chloride (CTC) is a dye and has been applied to visualise respiring aerobic and facultative bacteria in environmental

samples (Rodriguez *et al.*, 1992). CTC has also been used with flow cytometry to determine the respiratory activity and dormancy in individual *Micrococcus luteus* cells (Kaprelyants and Kell, 1993) as well as respiring autochthonous bacteria in drinking water and biofilms (Schaule *et al.*, 1993). CTC acts as an electron acceptor and is reduced to red fluorescing crystal particles in the cells from colourless. An aqueous solution of CTC is nearly colourless and non-fluorescent, while the corresponding formazan product [CTF] fluoresces in the red range at approximately 620 nm when excited at 520 nm. Soluble CTC is readily reduced to the water-insoluble fluorescent CTF product via the microbial electron transport system and indicates a mainly respiratory activity. CTF is deposited intracellularly like other formazans in a time-dependent manner and provides an indication of cumulative respiratory activity.

1.3.8 Immunofluorescence and staining *Legionella pneumophila*

It is over twenty years since Kohler and Milstein (1975) first described the production of antibodies from a single cell-line using cell-fusion techniques (Harrison and Talor, 1988). Since 1975 the technique has been widely applied and the production of monoclonal antibodies (mAbs) is now common. Although polyclonal antibodies offer some degree of specificity, the development of monoclonal antibodies gives the potential to recognise a single antigen type. The antigen-binding site may be a conformation of five or six amino acids of a protein or five or six sugar residues of a polysaccharide (Kohler and Milstein 1975). Fluorescent antibody (FA) immunofluorescence techniques have been used in environmental and ecological studies to detect a range of different bacteria including coliforms, *Salmonella* spp. (McClelland and Pinder, 1994), *Legionella* spp. (Harrison and Talor, 1988) and other species.

To detect the antibodies, and therefore the cells they recognise, they must be labelled with a fluorescent marker. Antibodies can be fluorescence-labelled for flow cytometry and epifluorescence microscopy by three essentially different methods, direct, indirect and avidin-biotin. The direct method for labelling antibodies is when the antibody is conjugated directly to the fluorophore in one of three ways including conjugation with isothiocyanates, with succinimidyl esters or

with phycobiliproteins. Although the procedures are comparatively complex, direct labelling produces covalent, and therefore, stable bonds with the antibody. Multi-colour detection of different antibodies poses no major problems, because there are no appreciable cross-reactions between labels. Fluorescent isothiocyanates such as fluorescein or rhodamine are reactive reagents for the modification of aromatic amines, such as lysine residues or free terminal amino groups on the antibody protein, and have been widely used to conjugate with antibodies e.g., FITC-mAbs. Makin and Hart, (1989) reported that they used the fluorescein-conjugated monoclonal antibody to detect *Legionella pneumophila* in environmental samples from the sites which had a history of colonisation with legionellae. The results show that direct fluorescent monoclonal antibody detection of *L. pneumophila* was more sensitive and more rapid than the indirect method and the cultural methods in detecting *L. pneumophila* in environmental water samples.

Though the most common application of flow cytometry is the measurement of surface antigens by immunofluorescence labelling using monoclonal antibodies, most of the detection of *Legionella* stained with monoclonal or polyclonal antibodies has been carried out by fluorescence microscopy (Makin and Hart, 1989; Rogers and Keevil, 1992; Lee, 1994 (unpublished data); Palmer *et al.*, 1995; Luck *et al.*, 1995; Faude and Hofle, 1997). Lee (1994, unpublished data) suggested that using flow cytometry it should be possible to detect fluorescently labelled legionellae whilst using microscopy such techniques are extremely laborious.

1.4 APPLICATIONS OF MOLECULAR PROBES IN MICROBIOLOGY

Acridine orange has always been an easy stain to use to assess microbial populations. The stain was used by Pettipher *et al.*, (1980) for the rapid enumeration of bacteria in milk. Modifications to this staining protocol have allowed Sierra *et al.*, (1997) to use AO for assessing the degree of microbial contamination of lamb carcasses. Results were available in 15 minutes meaning

that hazard assessment and critical control points (HACCP) could be applied directly in slaughter houses. The direct count was also found to correlate well with colony counts. Similar protocols have been used to monitor the quality of pork and minced beef.

The bis-benzamide derivative Hoechst 33342 (HOE342), (Monger and Landry, 1993), has been used to detect bacteria in fresh and marine waters. Rhodamine 123 (Rh123) (Morgan *et* al., 1993, Kaprelyants and Kell, 1993) and fluorescein diacetate (FDA) (Jorgensen *et al.*, 1992) have been used to determine viable biomass in water and waste water treatment. CTC, (Stellmach, 1984), has been used to detect viable bacteria in pure culture (Kaprelyants and Kell, 1993), and in secondary treated effluent (Rodriguez *et al.*, 1992). CTC has also been used to quantify planktonic and sessile respiring bacteria in drinking water (Schaule *et al.*, 1993). Bovill *et al.*, (1994) reported that CTC was used to detect metabolic activity in heat-stressed cells. Ullrich *et al.*, (1996) published the only paper up to now on the toxic effects of CTC on bacterial metabolism in environmental samples. Yamaguchi *et al.*, (1997) described the flow cytometric analysis of bacterial respiration with CTC in natural waters. In more recent years the green nucleic acid stain SYTOX was reported to stain pure cultures of bacteria for detecting viability by flow cytometry (Roth *et al.*, 1997), and now, many studies have been focused on the green fluorescent protein (GFP), which has been used to label bacteria for genetic methods for *Legionella* (Kohler *et al.*, 2000). All the above fluorescence dyes have been used in direct counting methods by epifluorescence microscopy and/or flow cytometry.

1.5 FLOW CYTOMETRY

1.5.1 History and Development of Flow Cytometry

Flow cytometry, like most scientific developments, has its roots firmly grounded in history. The Coulter blood cell counter is generally considered to be the precursor of the modern flow cytometer, in fact it was the coming together of the technologies of microscopy, ink jet technology, the Coulter counter, as well as flow technology, which provided the basis for the first flow cytometers.

In 1934, Andrew Moldayan in Montreal took a first step from static microscopy toward a flow system (Givan, 1992). He suggested the development of an apparatus to count red blood cells and neutral red stained yeast cells as they were forced through a capillary on a microscope stage. A photodetector attached to the microscope eyepiece would register each passing cell. Although it is unclear from Moldovan's paper whether he actually built this cytometer, the development of staining procedures made it obvious that the technique he suggested could be used for not only counting the number of cells but also for assessing their characteristics. In 1938, Caspersson and Schultz reported that primitive photodetectors could be used to quantify the stained images from their study of the nucleic acid metabolism in *Drosophila melanogaster* salivary gland chromosomes. It has been said that the qualitative aspects of flow cytometry take their origins from Caspersson's work in the 1930s (Givan, 1992).

In the 1950s, the Coulter technology was developed for the analysis of blood cells and soon Coulter counters became essential equipment in hospital laboratories for the rapid automated counting of white and red blood cells. These first commercial flow systems by Coulter in 1954 were used to count cells as they flowed in a stream of liquid. Analysis was based on the amount by which cells increased the electrical resistance of an orifice as they displaced isotonic saline solution while flowing through it. Cells were thereby classified more or less on the basis of their volume, since larger cells have greater electrical resistance. The Coulter counter actually incorporated many of the features of analysis that we now think of as being typical of flow cytometry; the rapid flow of single cells in file through an orifice, the detection of electrical signals from those cells and the automated analysis of those signals.

In 1953, Crossland-Taylor, working at the Middlesex Hospital in London, developed 'a device for counting small particles suspended in a fluid through a tube'. He applied the principles of laminar flow to the design of a flow system. A suspension of red blood cells was injected into the centre of a fast-flowing stream, thus allowing the cells to be aligned in a narrow central file within the core of the wider stream prior to electronic counting. This principle of hydrodynamic focusing was pivotal to the further development of flow cytometry (Givan, 1992).

In the mid-1960s, as a result of a desire to automate cervical cytology screening, Louis Kamentsky *et al.*, (1965) in New York developed a microscope-based spectrophotometer (on the pattern of one suggested by Moldavan (1934) that measured and recorded the two parameters of UV adsorption and the scatter of blue light from cells flowing 'at rates exceeding 500 cells per second' past a microscope objective. In 1965, at the same time as Kamentsky's work on cervical screening, Fulwyler (1969) at the Los Alomos laboratory developed the first cell sorter using electrostatically charged droplets, a development of Sweet's invention for ink-jet writing (1965). The technique that Fulwyler developed for sorting erythrocytes combined Coulter methodology with ink-jet technology and produced the first instrument that would charge droplets containing suspended cells, thereby allowing deflection of the cells within the droplets as dictated by signals based on the cells' measured Coulter volume (electrical resistance). Fulwyler's contribution to flow cytometry development was very important, allowing the flow cytometer a sorting ability.

Kamentsky and Melamed (1967) also elaborated this design into a sorting flow cell that provided for electronic actuation of a syringe to pull cells with high adsorption/scatter ratios out of the stream flow. Sorted cells could then be subjected to detailed microscopical analysis. In 1969, Dittrich and Gohde in Germany described a flow chamber for a microscope-based system whereby fluorescence intensity histograms could be generated based on the ethidium bromide fluorescence of alcohol-fixed cells. Also in 1969, Van Dilla and other members of the Los Alomos group reported development of the first fluorescence-detection cytometer that utilised the principle of hydrodynamic focusing (Crossland-Taylor, 1953). Unlike the microscope-based system, this had the axes of flow, illumination and detection all orthogonal to each other and also used an argon laser as the light source. Dilla's flow cytometer could support both the illumination and detection electronics of Kamentsky's device as well as the rapid flow and vibrating fluid jet of Fulwyler's sorter. Shortly afterwards, the Herzenberg group at Stanford demonstrated the use of a similar cytometer to sort fluorescent cells stained with fluorescein isothiocyanate (Givan, 1992).

During the late 1960s and early 1970s, major developments took place in fluorescence activated cell sorting at Stanford University. The sorting cytometers were able to sort lymphocytes and granulocytes into highly purified states. At the same time, these instruments began to be seen as commercially marketable objects. Up to now, the main commercial manufacturers of flow cytometers are Coulter (USA), Becton Dickinson (USA) and Ortho (USA). Modern machines are capable of sorting with five different parameters and accurately at rates of up to 10,000 cells per second. Their applications, initially based on clinical immunology, have widened into biomedical research, food and environmental microbiology.

1.5.2 Basic Principles of Flow Cytometry

Flow cytometry (FCM) combines the advantages of microscopy and biochemical analysis for the measurement of physical and chemical characteristics of individual cells as they move in a fluid stream one by one past optical or electronic sensors (Table 1.1; Figure 1.1). The technique has found widespread applications for eukaryotic cells and, more recently, the technique has been directed to the study of microorganisms. All flow cytometers work on the principle of presenting individual cells, in single file, into a focused light beam within a sensing region. This is achieved by introducing cells into the centre of a fast flowing continuous stream of liquid (usually water or buffered saline) termed the sheath fluid.

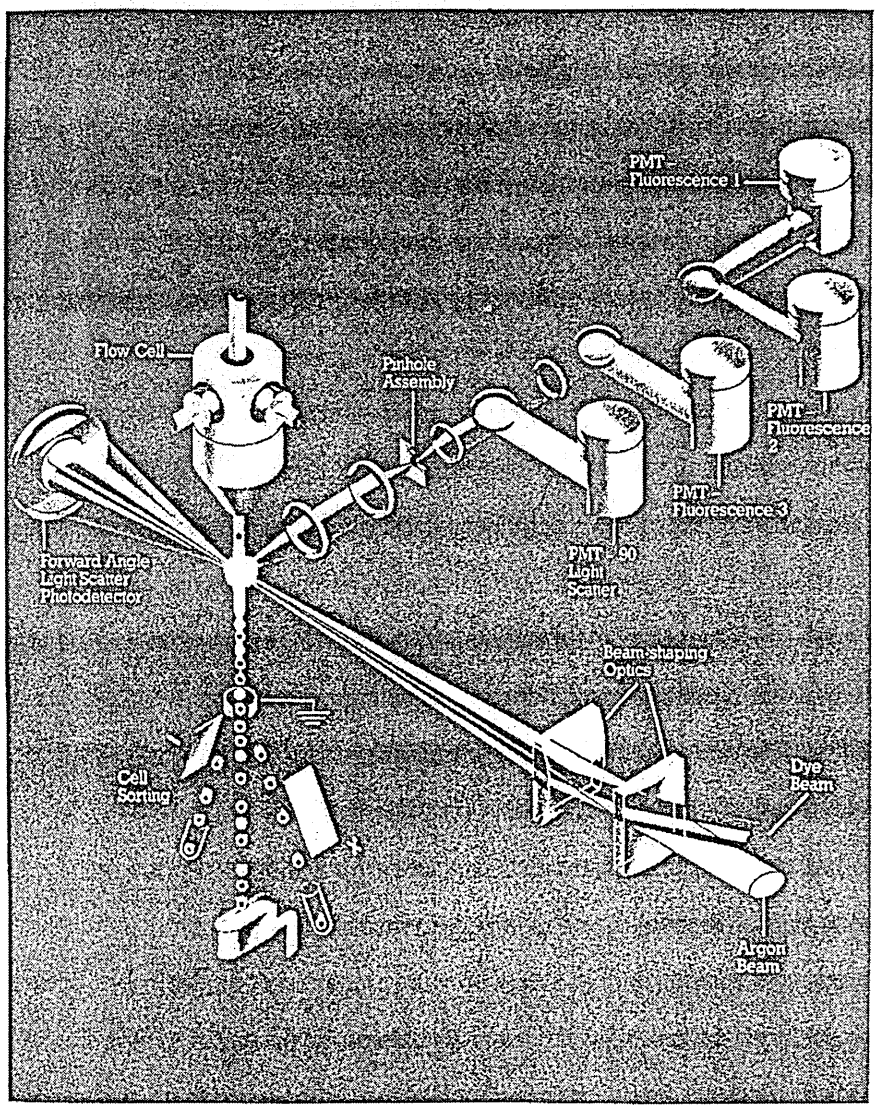
Table-1.1 A Comparison of Flow Cytometry and Solid Phase Cytometry

Specification	Flow Cytometry	Solid Phase Cytometry
	FACS Vantage (Becton Dickinson) EPICs XL (Coulter) Orthocyte (Ortho)	Epifluorescence Microscopy Image Analysis Laser scanning microscopy; Confocal Laser scanning Microscopy (CLSM) Laser Scanning Cytometry; LSC (CompuCyte) ChemScan (Chemunex)

Light source:	Lasers Arc lamp (microscope based instruments)	Lasers Arc lamp (microscope based instruments)
Detectors:	Multiple photo tubes (MPT) Electrical impedance Photo diode	Multiple photo tubes (MPT) Video camera
Parameters:	Fluorescence Light scattering Coulter volume (impedance)	Fluorescence Light Absorption Light scattering
Sample:	Suspended cells in liquid phase (Laminar flow system) Rapid flow in single file	Cells on "solid phase" (slide, filter surface) Scanning of stage and/ laser beam
Characteristics	Single cell suspension High speed (2000 cells/S) Rapid, large number of cells (Kinetics for population) Multiparameter analysis Automation Analytical + preparative: sorting	Attached cells (Solid phase e.g., biofilms without detachment) Repeated measurement of same cell possible (Kinetics for one cell) Spatial information (2- or 3-D)

(After Nebe-Von Caron, G. and Wall, G., 1998)

Figure-1.1 A flow cytometer. Illustration of the sample stream of a flow cytometer intersecting a laser beam. Also shown are portions of the beam-shaping optics, dichroi beam splitters, light scatter and fluorescence detectors, and the droplet generating and deflecting portions of the sorting apparatus (After Grogan W 1990).



The speed of flow is usually about 10 m/second, which permits the analysis of approximately 2,000 to 10,000 cells/second. The light beam can be generated either by a laser or a mercury lamp. With mercury lamps, insertion of different filters into the beam can vary the wavelength of light which serves as excitation light for fluorescent dyes used to stain the cells. In contrast, the laser excitation beam can be tuned to the desired wavelength (e.g. 300, 488 or 514nm). A cell or particle travelling at 10 m/second traverses the light beam in 0.5 to 5 μs and, as it does so, light is scattered and detected by one or more detectors in the sensing region. Most instruments have two light scatter detectors, one measures low angle scatter (LAS) the other high angle scatter (HAS). LAS comes from light scattered at the cell surface and because larger cells have greater surface area, LAS gives a measure of size. HAS arises from light bent after passage through cells and is thought to give a measure of cell refractivity and therefore internal structure. LAS and HAS measurements can be made on cells irrespective of whether they have been stained with a fluorescent dye. One or more fluorescent light detectors are also supplied and they measure fluorescence emitted from stained cells as they pass through the excitation light source. In the case of the FACS Vantage, fluorescence in region of 470, 535, 575 and >630 nm is routinely detected after light has passed through a series of dichroic mirrors and band-pass filters. There is now a wide choice of fluorescent agents (chemicals and fluorochromes attached to antibodies and nucleic acids) that are specific for different cell structures or where fluorescence is dependent on some cellular activity. The fluorescence emission, within predetermined limits, will be proportional to the amount bound to particular cell components.

The sensitivity of the scatter and fluorescence detectors can be varied to suit the cell type under observation. Sensitivity of the detectors has to be much higher for bacteria than for eukaryotic cells. The detector can collect light in either linear or logarithmic modes (high sensitivity). The light intensity (scatter or fluorescence) measured by the detectors is converted into electrical signals which are sorted in increasing order of magnitude into a number of electronic channels. The higher the channel number the greater the light intensity. All information from detectors accumulates and is stored in the computer using appropriate software. The

results can be represented by histogram distributions of range of channels or as contour maps or dotplots or as a three dimensional distribution.

1.5.3 Modern Applications of Flow Cytometry

Flow cytometry's primary use to date was in eukaryotic cell biology (Steen *et al.*, 1994; DeLeo, 1996). Recently, flow cytometry has been used successfully in the studies of the bacterial cell cycle (Monfor and Baleux; 1996) and its potential as a tool for use in microbial ecology has been recognised (Burkill, 1987; Edwards *et al.*, 1992; Diaper and Edwards 1994a; 1994b; Nebe-Von Caron *et al.*, 1998a, 1998b; Clarke and Pinder,1998). Flow cytometry also has been used to analyse the biomass in phytoplankton (Hofstraat *et al.*,1991; Jonker *et al.*, 1995; DeLeo *et al* ., 1996). Recently, flow cytometry has been shown to be capable of enumerating marine viruses stained with SYBR Green I (Marie, *et al.*, 1999) and SYBR Gold (Chen *et al.*, 2001).

Work by Kaprelyants and Kell, (1992) has demonstrated that Rh123, which is taken up in response to membrane potential can be used as a dye to detect and enumerate viable bacteria in pure culture by flow cytometry. Diaper and Edwards, (1994a) reported that he was able to detect and enumerate viable bacteria (*S. aureus)* using flow cytometry during survival studies in a lakewater microcosm. Porter *et al.*, (1993) reported using flow cytometry to enumerate and sort mixtures of *S. aureus* and *E. coli* labelled with fluorescence antibody (FITC-IgG). Flow cytometry can be used as a rapid and sensitive method for the analysis of bacterial populations and a number of fluorescence probes have been screened. No single stain is universally applicable, e.g. Rh123 works well for most Gram positive bacteria, but its staining of Gram-negative bacteria is limited because of the cells' outer membrane permeability. CTC as a new stain was reported in few cases for pure cultures of bacteria seeded in the laboratory (Kaprelyants and Kell, 1993) and bacteria in the river environment (Yamaguchi and Nasu, 1997). Flow cytometry has found applications in the food industry. Pinder *et al.*, (1990) describe a method for counting bacterial cells in pure culture by permeating cells with benzalkonium chloride and staining the DNA and RNA with ethidium bromide. Counts obtained by flow cytometry agreed well with those obtained by direct plating but were

obtained in a few minutes as opposed to several days. This was further developed to detect *Salmonella* (Pinder *et al.*, 1994; Clarke and Pinder,1998) using fluorescent antibodies and to differentially sort *S. enteritidis* and *S. typhimurium* based on a polyclonal and monoclonal antibody. One was labelled with FITC and the second with phycoerythrin giving a two-colour sort in green and red (Pinder *et al.*, 1993). Rh123 was also assessed as a viability stain and found to accumulate rapidly in cells without reducing viability. Stained cells could be sorted on the flow cytometer and grown. The sort facility gives the ability to assess the viable state of individual cells in a suspension as opposed to studying populations through cytograms. Brailsford and Gatley (1993) describe the use of flow cytometry for detecting and enumerating viable yeasts and bacteria in fruit and vegetables using a commercially available intracellular viability stain (Chemunex) based on esterase activity and intracellular accumulation of fluorochrome.

Vesey *et al.*, (1993) described a new use for flow cytometry for the detection of the parasites *Cryptosporidium* and *Giardia* in water. These two parasites have been responsible for a number of waterborne outbreaks of disease. Detection in water samples is difficult and time consuming and requires a substantial amount of microscopy. Flow cytometry was found to give specific detection, reduce the amount of microscopy and speed up the analysis time. The technique was used by Watkins *et al.*, (1995) for the examination of an upland catchment for *Cryptosporidium* during heavy rainfall. Hoffmann *et al.*, (1997) discuss the use of flow cytometry compared with direct microscopy for the detection of *Giardia* and *Cryptosporidium* in water samples. Flow cytometry was found to take less time, cost less, and could analyse a greater volume of sample. An increase in sensitivity of almost three times was observed for both parasites. Medema (1997) describes the detection of *Cryptosporidium* and *Giardia* in river and reservoir water using flow cytometry. Viability was demonstrated by DAPI/PI staining before sorting and could easily be assessed. A number of oocysts were observed to be DAPI and PI negative but internal contents could not be resolved. Deere *et al.*, (1997) discuss the use of two antibodies specific for *Cryptosporidium* labelled with different coloured fluorochromes to minimise non-specific sorting in flow cytometry together with a specific DNA probe to identify at genus or sub-

species level and demonstrate viability. Harf, *et al.*, (1997) used flow cytometry to study the endocytosis of viable *L. pneumophila* cells by the amoeba *Acanthamoeba palestinensis.* Live cells were used instead of fluorescent beads and were labelled with the lipophilic probe Cell-TrackerCM^{TM} Dil (CM-Dil) which labelled the cells but did not affect their viability nor their ability to be taken up by the amoebae. Flow cytometry could show that endocytosis increased with the size of the amoebae and that cells did not bind to the amoebae in the presence of azide. Flow cytometry allowed the quantification of large number of amoebae to generate the data.

Nebe-von Carron *et al.*, (1998a) have identified four different states of the bacterial cell. These are cells which are actively growing, that is there is cell division; cells with metabolic activity whether there is biosynthesis or enzyme activity; cells with membrane integrity in that they are able to exclude membrane permeable dyes such as propidium iodide; and cells which are membrane permeable. This type of concept differs from the viable and viable but non-culturable state of cells although the first group relates to the culturable cells, and the last group to the dead cells. These different groups can be demonstrated by different molecular probes where cell function specific reagents are used. Esterase and dehydrogenase activity can be demonstrated in metabolically active cells together with membrane potential dyes such as Rh123, but fail to stain with ethidium bromide (EB). Cells with an integral membrane but lacking metabolic activity fail to stain with propidium iodide but do take up HOE342 and EB. By the use of a combination of a number of probes, each with specific excitation and emission spectra, multi-colour sorting can be used to differentiate these groups. Nebe-von Caron *et al.*, (1998b) used this technique to establish the viability status of individual bacterial cells using flow cytometry and single cell sorting. Cells were labelled simultaneously with ethidium bromide, propidium iodide and Bis-oxonol (BOX). Cells were sorted on the basis of exclusion of ethidium bromide (metabolically active cells), uptake of ethidium bromide but exclusion of BOX (de-energised but with a polarised cell membrane), uptake of both dyes (depolarised) and permeated cells which stained with ethidium bromide. Single cells were sorted directly onto agar plates. Eighty five percent of de-energised

cells and 34% of depolarised cells could be recovered by culture. Permeabolised cells could not be cultured. This type of exercise can be used to design culture media to give the best possible recovery of environmentally damaged cells and in addition look at the media preparation and storage on the recovery of environmentally damaged cells.

An alternative stain to propidium iodide for permeabolised cells has been described by Roth *et al.*, (1997). SYTOX green, a nucleic acid stain which stains double-stranded DNA strongly and single-stranded DNA and RNA less strongly can be excited at 488nm and emits in the green at 502 – 523 nm. These effect of β-lactam antibiotics in producing permeabolised cells in *E. coli* were studied using microscopy, fluorimetry and flow cytometry.

Mason, *et al.*, (1998) describe the use of two fluorescent probes, hexidium iodide (HI) and SYTO 13 to differentiate Gram-positive and Gram-negative bacteria. Both bind to DNA and RNA but HI fluoresces red and SYTO 13 fluoresces green. Both can be excited at 488 nm and are therefore suitable for laser excitation and flow cytometry. Gram-negative organisms were found to exclude HI and therefore stain green. Gram-positive organisms stain red because the green fluorescence of SYTO 13 was quenched. Fixing Gram-negative organisms with ethanol rendered the cells permeable to HI. The authors conclude that HI could be used as an indicator of membrane integrity. The technique successfully predicted the Gram reaction of 45 strains of bacteria and was able to differentiate Gram-positive from Gram-negative bacteria in mixtures by flow cytometry. Flow cytometry was used by Xavier *et al.*, (1998) to detect rotavirus in faecal and environmental samples. The human colon carcinoma cell line CaCo-2 was used for infection and reverse transcription-PCR used to confirm infected cells. The method was found to be more sensitive than immunofluorescence and direct microscopy for detection.

1.6 *LEGIONELLA PNEUMOPHILA*

Legionella pneumophila is one member of the large family of Legionellacae. The family was initially proposed for one single genus *Legionella*

and species *L. pneumophila.* There are, at present, 41 validly described species and 62 serogroups that have been isolated from both clinical and environmental sources (Miyamoto *et al.*, 1997) and at least 14 serogroups of *L. pneumophila* and 3 sub-groups of *L. pneumophila* serogroup 1. *Legionella pneumophila* serogroup 1 is the most common cause of Legionnaires' disease in Britain and is responsible for over 95% of cases. Other serogroups of *L. pneumophila* and several other *Legionella* spp. occasionally cause pneumonia in humans.

Legionnaires' disease was first recognised in July 1976 (Dowdle, 1993) when an outbreak occurred amongst delegates attending an American Legion convention at the Bellevue Stratford Hotel in Philadelphia. The cause of the outbreak eluded scientists for several months, but in January 1997, the Centre for Disease Control (CDC) in Atlanta reported the isolation of the etiological agent which they named *L. pneumophila.* Diagnostic tests were developed and reviews of stored specimens in laboratories revealed earlier outbreaks of the disease and sporadic cases dating back to the early 1940s. This data demonstrated that the disease was not new but had managed to escape recognition because conventional media used in hospital laboratories to isolate respiratory bacteria would not grow the organism.

Legionnaires' disease is an illness characterised mainly by pneumonia. It begins quite abruptly with high fever, chills, headaches and muscle pains followed by an acute pulmonary pneumonia. Additional symptoms may include diarrhoea and involvement of the brain giving confusion and delirium. The incubation period is 2 - 7days and symptoms usually persist for a further 7 days. The mortality is usually around 10%. A second set of symptoms may appear with a rapid onset (usually within hours), is relatively short-lived and total recovery is observed. This second type of infection is called Pontiac fever. The route of infection is through inhalation of the bacterium into the lungs in the form of a fine aerosol generated in water in which the *Legionella* are growing. Legionnaires' disease is uncommon with between 100 - 200 cases being reported each year to the Communicable Disease Surveillance Centre (CDSC) and 50% of these are acquired abroad.

Legionella are Gram-negative, aerobic rods, 0.3 - 0.9 μm in width and 2 – 20 μm in length and they may be highly pleomorphic. They are motile with one,

two or occasionally more polar flagella. They do not grow on blood agar, do not reduce nitrates and have a non-fermentative metabolism. Iron salts are required for growth in vitro. Most species are defined on the basis of DNA-DNA hydridisation which has resulted in the proposal to divide the family Legionnellacae into three genera, namely *Legionella sensu stricto, Fluribacter* and *Tatlockia* (Hookey, 1995; Harrison and Taylor, 1988).

Legionella spp. are widespread in natural fresh waters, including rivers, lakes, streams and ponds and may also be found in wet soil (Atlas, 1999; Rittard et al. 2001). The first isolation of *L. pneumophila* from a natural habitat was from the mud of a stream (Morris *et al.*, 1979). Tison *et al.*, (1983) reported that after an outbreak of illness of unknown aetiology among workers exposed to lakes and streams in Mount St Helens blast zone in the USA, they examined waters inside and outside of the blast zone for *Legionella* and several species, including *L. pneumophila*, were detected by direct immunofluorescence microscopy. In lakes and rivers outside the blast zone, numbers ranged from $<10^4$ to 10^5 cells/l., whereas within the blast zone, aquatic habitats contained 10^5 to 10^7 cells/l.

In the absence of deliberate control measures for *Legionella*, the concentration of *Legionella* in water is usually less than or equal to 10^4/l and rarely exceeds 10^5/l, the level at which there may be a real risk of infections. When operating well, control measures are capable of keeping the numbers below the current limit of detection by culture i.e. about 100 cfu/l (Lee, 1994). The counts were highest in lakes receiving water from hydrothermal seeps (Lee and West, 1991). In Puerto Rico, *L. pneumophila* and other species were detected by immunofluorescence microscopy in all the samples examined, ranging from pristine fresh water to polluted estuarine and marine waters (Ortiz-Roque and Hazen, 1987). Man-made buildings have tended to create environments where the organism can grow readily, and where these environments create aerosols, outbreaks of disease can occur. There is a strong possibility of very low concentrations of the bacterium existing in all open water systems including groundwater and treated drinking water (Colbourne and Dennis, 1989). Outbreaks of Legionnaires' disease have been traced to a number of potable water sources, including contaminated water in cooling towers and air conditioning units, hot tubs, whirlpool baths, showerheads,

public fountains and even a supermarket vegetable misting machine (McEvoy 2000; Szewzyk *et al* 2000). The optimum temperature for multiplication is 37 °C, below 20 °C and above 46 °C multiplication ceases. The organism survives for a matter of hours at 50 °C, one minute at 60 °C and is killed almost instantly at 70 °C. The bacterium can become dormant at temperatures below 20 °C and return to active multiplication when higher temperatures occur. The organism appears to be insensitive to pH and has been found in cold water systems having a wide range of pH values.

Legionella pneumophila, as a primary cause of Legionnaires' disease can infect and multiply in free-living amoebae such as *Acanthamoebae*, *Naegleria* and *Hartmannella* species (Rowbotham, 1980). These species of amoebae are ubiquitous in the biofilms of the moist soil and water environments. Biofilms are a major source of *Legionella* spp. in both man-made and natural aquatic systems (Rowbotham, 1993; Marrao *et al.*,1993; Schwartz *et al.* 1998; Sessa *et al.* 2000; Murga *et al.* 2001;). *Legionella* and other environmental bacteria such as *Listeria* have evolved so that they are capable of surviving and multiplying within biofilm predators such as amoebae which offer protection against adverse conditions. Kilvington *et al.,* (1990) suggest that resistance to digestion by predator protozoa was a pre-requisite of bacterial pathogenicity and a survival mechanism for bacteria in aquatic environments. *Legionella* can multiply within the cytoplasm of *Acanthamoeba*, evading host lysosomal attack so that after 36 - 48 hours a single vesicle of motile *Legionella* fills most of the cell. The cell will eventually lyse and release many motile bacteria into the environment. *Legionella pneumophila* is known to infect five genera of amoeba (Fields *et al.*, 1993), whereas other species of *Legionella* have a more specialised host range (Fields *et al.*, 1990). Rohr *et al.*, (1998) reported that they isolated four *Hartmannella* and two *Saccamoeba* species from hot water systems at 40 – 60 °C and found that they could be cultured at 53 °C. They considered that, in the hot water system, *Legionella pneumophila* could be supported and survive in *Hartmannella* and *Saccamoeba* but not by *Acanthamoeba*, which did not colonise the central area of the hot water system investigated and which is often used as a host organism for legionellae *in vitro* (Barker *et al.*, 1992; Moffat and Tompkins, 1992; Neumeister *et al.*, 1997). Bacteria internalised by

protozoa may be given unique protection when the protozoa form cysts. Rowbotham (1986) observed that *Acanthamoeba* containing *Legionella* encyst, leading to the formation of a precyst or a mature thick-walled cyst which traps motile *Legionella*. Vogel and Isberg (1999) and Steinert *et al* (2002) outline genetic mechanisms in *Legionella*, which enable it to survive inside amoebae and alveolar macrophages.

Legionella species have been detected in sewage and concentrations were not appreciably reduced by primary or secondary treatment processes (Palmer *et al.*, 1993). This finding could be related to the protection provided by protozoa which are ubiquitous inhabitants of sewage treatment works. Not only does the amoebal cyst offer a mechanism for bacteria to evade hostile environmental conditions, but it also provides a means by which the bacteria can spread and colonise new habitats by being blown through the air (Barker and Brown, 1994). Although some bacterial species survive ingestion by protozoa, under certain environmental conditions the same organisms are eradicated. Anand *et al.*, (1983) reported that at low temperatures *Acanthamoeba* may phagocytose and digest *L. pneumophila* as food, or evict the phagosomes containing *Legionella* as faecal vesicles.

1.7 DETECTION OF *LEGIONELLA*

Routine testing for *Legionella* has now been recommended in the UK for testing cooling towers on a quarterly basis, and more frequent sampling is required when commissioning a system and establishing a treatment system. Testing is recommended for hot and cold water systems where control levels of treatment are not consistently achieved or when an outbreak of Legionnaires' Disease is suspected (HSC, 2001). The methods for the detection of *Legionella* are culture; direct immunofluorescence; rRNA-directed fluorescent oligonucleotide and polynucleotide probes; and the polymerase chain reaction (Lee, 1994; Walker *et al.* 1999).

1.7.1 Culture

The usual method of testing for *Legionella* is by culture and this requires up to 14 days for a result. The background flora normally outnumber *Legionella* considerably, therefore selective media and other pre-treatments such as acid (pH 2.2 for 5 minutes) or heat (50 °C for 30 minutes) are required to suppress the background flora.

1.7.2 Direct Immunofluorescence

Direct immunofluorescence has been used to detect *L. pneumophila* in clinical and environmental samples almost since the organism were first recognised (Cherry, *et al.*, 1978; Palmer, *et al.*, 1993, 1995). However a concentration step is still needed for water samples, quantification is relatively difficult and scanning slides can be time consuming and tiring particularly when numbers are low. Although this method has sometimes proven valuable for research and in outbreak investigations for screening a number of possible sources, it is not really practicable for routine monitoring. Although capable of providing a result within a day of collection of the sample, the technique can require more labour than culture and sensitivity depends on the experience and care of the operator. Using microscopy such techniques are extremely laborious but in conjunction with flow cytometry may offer a way forward to a semi automated system (Lee, 1994).

1.7.3 rRNA-directed Fluorescent Oligonucleotide and Polynucleotide Probes

Fluorescence-labelled rRNA-targeted oligonucleotide and polynucleotide probes have been used as a tool for the *in situ* identification of bacteria (Manz *et al.*, 1993, 1995; Amann *et al.*, 1995) and such probes have been developed for *Legionella* spp. (Manz *et al.*, 1995) and host Amoebae (Grimm *et al.* 2001). The advantages of these probes are that they can be designed to be genus specific and the degree of labelling depends on the number of ribosomes in the cell and thus can indicate viability.

1.7.4 Detection by Polymerase Chain Reaction (PCR)

The PCR method detects both culturable and non-culturable cells and the commercial PCR based kit for the detection of *Legionella* species and *L. pneumophila* in water samples is now available and has been used successfully in an outbreak investigation in a hospital (Lee, 1994). Since the result is available in hours this meant that time and costs could be saved by not having to close the ward and disinfect the water system. Some water samples contain substances inhibitory to PCR detection.

1.8 THE OBJECTIVES OF THE RESEARCH PROJECT

The aims of this project are to assess the "conventional" microbiological techniques of culture, colony counting and epifluorescence microscopy for the analysis of microorganisms in water supply systems and compare these with the use of flow cytometry. Enumerating bacteria by culture requires long incubation times. In addition these techniques will only isolate those bacteria capable of growing on the isolation media. Newer staining methods are allowing the methodology to be speeded up since specific stains can link to areas of the bacterial cell which are concerned with metabolic processes. In this way, simple staining techniques can allow the differentiation of viable from non-viable cells. The use of flow cytometry with specifically stained cells allows a rapid and perhaps more accurate method of enumerating viable cells than traditional microscopical methods. In addition, the use of monoclonal antibodies linked to fluorescent dyes can help in the rapid detection of specific microorganisms in water and related materials.

The first part of this research project is the optimisation of stain concentrations, staining times and temperatures to ensure that counting is accurate and reproducible. This information will then be used to stain bacteria in suspension for rapid flow cytometric analysis. The data obtained will allow a comparison between microscopical analysis, colony counting

and information obtained by flow cytometry. In this way we can be sure that flow cytometry will give us counts which are comparable to those obtained by with and determine the differences in counts when compared with conventional culture.

This information will be extended to the study of the formation of biofilms in the laboratory. In particular, the development of biofilms on glass slides in a bioreactor can be studied using the different counting techniques. The study will also focus on the development of *Legionella* spp. within the biofilm both from seeding laboratory cultures into the biofilm reactor and by trying to grow *Legionella* from natural water samples in the biofilm. The techniques should demonstrate whether rapid detection by epifluorescence or flow cytometry is more sensitive than conventional microbiology in the study of biofilms and the isolation of *Legionella.* If these are successful, it should be possible to use flow cytometry as a rapid and specific method for detecting *Legionella* in biofilms and water distribution system.

2.0 MATERIALS AND METHODS

2.1 MICROORGANISMS

All species of bacteria were obtained from the Yorkshire Water Bradford Laboratory and were stored at –70 °C. *Acanthamoeba polyphaga* was obtained from Dr John Barker, Sheffield Hallam University and was maintained axenically at 35 °C in PYG broth in 75 cm tissue culture flasks.

Table 2.1 Organisms

Escherichia coli	NCTC 9001
Pseudomonas aeruginosa	NCTC 10332
Staphylococcus aureus	NCTC 8532
Salmonella typhimurium	NCTC 0074
Legionella pneumophila Serogroup 1	NCTC 12821
Acanthamoeba polyphaga	

2.1.1 Growth of Bacteria and Protozoa

All bacterial species except *L. pneumophila* were maintained and grown in nutrient broth (filtered with 0.2 µm pore size syringe filter three times before using) and incubated for 18 hours at 37 °C.

Overnight broth cultures of bacteria (1ml) were added to Eppendorf tubes, centrifuged for 2 minutes (10,000 r.p.m.) at room temperature and washed twice with and resuspended in phosphate buffered saline (PBS -Sodium chloride 8.0 g/l; Potassium chloride 0.2 g/l; Disodium hydrogen phosphate 1.15 g/l; potassium dihydrogen phosphate 0.2 g/l pH 7.3). The concentration of cells was between 10^8 - 10^9 ml^{-1} by colony count on nutrient agar.

Legionella pneumophila was cultured using the Yorkshire Environmental Solutions' method (50.9.0) which was based upon the British Standards Institute method DD211:1992 [but has now been superceded by BS 6068 - Part 4.12, 1998; which is comparable to ISO 11731 1998]. This defines the isolation of *Legionella* spp., by culture on buffered charcoal yeast extract agar (BCYE supplemented with

SR110) plates with *Legionella* growth supplement at 37 °C for three days and harvested in suspension with reverse osmosis (RO) water.

Yeast extract (YE) broth was also used for the growth of *Legionella* (Baker, 1986) and the composition of the YE broth was similar to that of the BCYE agar with the important exception that neither agar nor charcoal was added to the medium. The YE broth cannot be autoclaved due to the fact that sterilisation by autoclaving could result in the release of toxic compounds which inhibit the growth of the legionellae in the absence of charcoal. The problem was overcome by filter sterilising the YE broth by passage through a syringe filter (0.22 μm) after the addition of the supplements. The medium was stored at 4 °C and was used within one month of production. All media was checked for pH, sterility and growth of target organisms before use.

Free-living amoebae were cultured using the method described by Anon (1989) by using non-nutrient agar (NNA) *E.coli* plates at 30 °C for up to 7 days.

2.1.2 Biofilms

Biofilms were prepared in the biofilm formation system set up in the Sheffield Hallam University's laboratory. These biofilms were set up for detecting *Legionella* spp. and other organisms using the flow cytometer.

2.1.2.1 Biofilm Formation System

The biofilm formation system consisted of a biofilm vessel with a recirculated water supply and two pumps (Modular Fermenter, Gallenkamp), air supply and environmental condition control unit (Plate-2.1). The biofilm vessel had a retention volume of 5 l. The flow rate from the recirculated water supply container into the biofilm vessel was 5ml min^{-1} with the same effluent flow rate from the biofilm vessel back to the supply container. The biofilm cultural conditions of the vessels were controlled using the environmental control unit.

The temperature of the biofilm vessel was maintained at 25 +/- 1 °C, or 30 +/- 1 °C for different experiments, and the temperature was measured using a metal temperature probe inserted into the aqueous phase of the vessel and the temperature was corrected using an external heating control. The dissolved oxygen (DO %) was maintained at 60% via the stirrer speed of 100 r.p.m and corrected by the air control system. pH was monitored using the glass pH probe inserted into the vessel.

2.1.2.2 Inocula

The inocula for the biofilm culture work were derived from the river Aire as as a suitable surface water supply or with *L. pneumophila* (NCTC12821) seeded in the biofilm formation system. The inoculum using Aire river water contained a range of microorganisms including bacteria algae, fungi and protozoa.

2.1.2.3 Culture media for biofilm growth

The biofilm formation system was supplied with Aire river water for growth of the biofilms. The water was taken from the River Aire at Esholt and transferred in ten litre sterile plastic containers to the Sheffield Hallam University laboratory to be used as a recirculated supply for biofilm development. The river water was found to contain *Legionella* spp., by culture on BCYE and staining with monoclonal antibody, and able to support bacterial growth in a mixed population of biofilm. The recirculated water was replaced every three days, weekly or monthly with the fresh river water depending upon the experiment.

2.1.2.4 Biofilm generation

Biofilms were generated on slides made of glass, plastic (uPVC), stainless steel or copper. Each slide had a surface area of 37 cm^2 (both sides) and slides were suspended in the biofilm vessel with wire except the glass slides which were fixed into a moulded plastic strip within the vessel. The materials were cleaned with ethanol (99%) to remove any dirt or oil before inserting into the vessel. Glass slides could be in the same vessel with the plastic or stainless steel slides, but the copper slides were separated from other kinds of slide in case any copper ions released into the vessel affected the biofilm formation on the other materials.

2.1.3 Processing Biofilm Samples

Biofilm formation was investigated at 25 °C and 30 °C after 3, 5, 7, 14, and 28 days or more growth period. The slides with biofilm were removed from the vessel and washed by complete immersion in 10 ml of the sterile RO water in 50 ml sterile centrifuge tubes with gentle movement to ensure that unwanted planktonic organisms were removed. At least two slides of each material were used; one for making the

biofilm suspension sample and other for staining, direct reading and photography. The biofilm suspension was made by scraping the whole of the slide surface with a sterile knife and resuspending in 10 ml of sterile RO water in 50ml sterile centrifuge tubes. The slide was left in the tube and then mixed with a vortex mixer for 5 minutes to disperse the organisms.

2.1.4 Environmental Samples

Treated water samples were obtained from across the Yorkshire Water distribution system and river samples from the River Aire at Esholt. Samples were either fixed and stained in suspension for total counting or filtered unfixed and stained with CTC or Rh123 for viable counting

2.1.5 Sample Fixation

Environmental and biofilm samples for AO, DAPI and PI staining were fixed, in a fume cupboard, with glutaraldehyde (0.5%) for 20 minutes at room temperature. The fixed sample could be kept for several weeks at 4 °C. Viable staining with CTC, Rh123 or HOE342 needed fresh or unfixed samples for staining. The environmental samples were directly stained or fixed and stored as above for staining.

2.1.6 Sample Staining

Samples were stained in suspension or for CTC staining, samples were filtered and stained on membranes. Suspensions were stained as described in Section 2.2. Washing was by centrifugation at 10,000 r.p.m. followed by removal of the supernatant and replacing with fresh RO water or PBS. Samples stained on membranes were washed filtering a small volume of RO water or PBS through the membrane.

2.1.7 Direct Counting by EFM with Filtration.

Direct counting by epifluorescence microscopy with membrane filtration was assessed using the method of Anon (1994). The microscope was calibrated using a certified 1mm slide micrometer on a monthly basis.

2.1.7.1 Materials

Membrane filter funnels, 25 mm and vacuum flask

Vacuum pump

Forceps

Membrane filters; 25 mm diameter 0.2 μm pore size, black, polycarbonate membranes, Poretics USA.

Universal and Eppendorf containers, sterile

Vortex mixer

Microscope with UV attachment and oil immersion lens (x100)

2.1.7.2 Fluorescence Microscopy and staining with filtration

Fluorescence microscopy is widely used in microbiology and initially it was started in the late 1930s, and now the direct counting techniques with epifluorescence microscopy play an important role in environmental microbiology.

2.1.7.3 Filtration and direct counting

Samples were stained and mixed thoroughly using a vortex mixer. 1 - 10 ml of sample was added to the membrane filter, filtered at a constant filter rate (2 ml min^{-1}) to keep an even distribution of bacteria over the surface of the membrane. 5 ml PBS was used for washing. The filter was allowed to dry for 2 min. and viewed under oil immersion for direct counting by EFM.

Determination of Effective Filtration Area (EFA) was carried out by using the formula:

EFA = πr^2 (π= 3.142, r = radius of the EFA).

EFA = 3.142 x $(10\ mm)^2$ = 314.2 mm^2

Using a x100 oil immersion lens at least 10 fields were examined to ascertain an even distribution of cells on the filter and 20 squares of a calibrated counting graticule were counted.

Final results were calculated by using the formula:

$$\text{Number bacteria ml}^{-1} = \frac{\text{EFA x number of cells per graticule}}{\text{Area of graticule x filtered volume}}$$

Direct counting (DC) of stained samples with different dyes was obtained as follows:

AODC	Green, red, orange	510nm, 590nm
CTCDC	Red, viable with respiring activity	590nm
DAPIDC	Blue	450nm
HOE342DC	Blue	450nm
PIDC	Red, non-viable	590nm
Rh123DC	Green, viable	510nm
mAb-FITC	Green, for *Legionella* spp only	510 nm

2.2 STAINING PROTOCOLS

All staining and washing solutions were filtered by 0.22 μm pore size syringe filter three times before using for staining.

2.2.1 Acridine Orange (AO) Staining

2.2.1.1 Fixative

Glutaraldehyde 5% stock solution (use in fume cupboard).

2.2.1.2 Staining solution

AO stock (Sigma) solution 1.0mg ml^{-1} (in distilled water)

AO working solution 0.1mg ml^{-1} (in distilled water)

2.2.1.3 Staining procedure

AO working solution was diluted to give a range of concentrations from 5-20 μg ml^{-1} Fixed samples were stained for 3 minutes at room temperature. The samples were washed with PBS (pH 7.2) after filtration to remove the remaining dye on the black polycarbonate membranes for direct counting using epifluorescence microscopy.

2.2.2 5-cyano-2,3-ditolyltetrazolium Chloride (CTC) Staining

2.2.2.1 Staining solution

Sodium pyruvate (80 mM) solution in distilled water

CTC stock solution 5mM in distilled water

2.2.2.2 Staining procedure

CTC staining solution was diluted in distilled water to give a range of concentrations from 0-5 mM. Samples were stained at 37 °C for 1 hour incubating in the dark. Sodium pyruvate could be added to the CTC at the start of staining to increase bacterial respiration. The stained samples were washed in PBS solution twice for direct analysis or used for further staining (dual staining) or for analysis by flow cytometry.

2.2.2.3 Dual Staining

CTC-DAPI staining

After incubation with CTC and washing twice in distilled water, DAPI working solution (final concentration 2.0 μg ml^{-1}) was added to the CTC stained sample. The samples were then incubated 20 minutes at room temperature and washed in distilled water as above. PBS was not used for washing because of precipitation of the stain.

CTC-Rh123 staining

Rh123 working stain was added to the CTC stained sample at a final concentration of 5μg ml^{-1}, incubated at 37° C for 30 minutes and washed twice in PBS.

CTC-HOE342 staining

After staining with CTC and washing, HEO342 (2.0 μg ml^{-1} final concentration) was added to the CTC stained samples with 30 minutes incubation at 37° C, then washed in distilled water. For flow cytometry, the stained sample was centrifuged at 10,000 r.p.m. for 2 minutes, washing twice in distilled water.

2.2.3 4′,6′-diamidino-2-phenylindole (DAPI) Staining

2.2.3.1 Staining solution

DAPI stock solution 1.0mg/ml in distilled water

DAPI working solution 0.1mg/ml in distilled water

2.2.3.2 Staining procedure

DAPI working solution was diluted to give a range of concentrations from 0.5-5.0 μg ml^{-1}. Samples were incubated at room temperature for 20 minutes, washed

and resuspended twice in distilled. DAPI tended to precipitate in PBS solution and any dilution made was in distilled water.

2.2.4 Hoechst 33342 (HOE342) Staining

2.2.4.1 Staining solution

HOE342 stock solution 1.0mg ml^{-1} in distilled water.

HOE342 working solution 0.1mg ml^{-1} in distilled water

The HOE342 working solution was diluted to give a range of concentrations from 0.25-4.0 μg ml^{-1} in distilled water and samples were incubated at 37 °C for 30 minutes, washed and resuspended in dH_2O. HOE342 could be used in dual staining with CTC or PI and also tended to precipitate in PBS solution like DAPI.

2.2.5 Propidium Iodide (PI) Staining

2.2.5.1 Staining solution

Propidium iodide (PI) stock solution 1.0mg ml^{-1} in distilled water

PI working solution 0.1mg ml^{-1} in distilled water

2.2.5.2 Staining procedure

PI working solution was diluted to give a range of concentrations from 0.5-10.0 μg ml^{-1}, added to the samples and incubated at room temperature for 20 minutes, centrifuged at 10,000 r.p.m., washed and resuspended in distilled water. Dual staining of PI with Rh123, HOE342 or DAPI was performed as outlined above always adding PI (final concentration 5μg ml^{-1}) in the last step for PI staining.

2.2.6 Rhodamine 123(Rh123) Staining

2.2.6.1. Staining solution

Glutaraldehyde 25% solution (use in fume cupboard).

Tris-EDTA buffer solution (Tris 10 mmol l^{-1}, EDTA 1 mmol l^{-1}, PBS pH 7.3)

Rh123 stock solution, 1.0 mg ml^{-1} in PBS

Rh123 working solution 0.1 mg ml^{-1} in PBS

2.2.6.2 Staining procedure

Staining for Gram-positive bacteria

Rh123 working solution was diluted in PBS to give a range of concentrations from 0.5-10.0 μg ml^{-1}, added to the samples, incubated for 30 minutes at 37 °C and washed twice in PBS before counting.

2.2.6.3 Staining for Gram- negative bacteria

(a) Rh123 -Tris EDTA staining: Tris-EDTA-Rh123 solution was added to the sample at a final Rh123 concentration of 5.0 μg ml^{-1}, incubated at 37 °C for 30 minutes, then washed as above for measurement.

(b) Rh123 -glutaraldehyde staining: glutaraldehyde (final concentration 0.01-0.015%) and Rh123 working solution at a final concentration of 5.0μg ml^{-1} was added to the sample, incubated at 37 °C for 30 minutes, washing twice in PBS.

2.2.6.4 Dual staining

(a) CTC-Rh123 Staining: Rh123 working solution was added to the CTC stained sample as in 2.2.2.3.

(b) Rh123- PI Staining: PI working solution was added to the Rh123 stained sample and incubated at room temperature for 20 minutes and washed twice in PBS.

(c) Rh123-DAPI Staining: DAPI working solution was added to the Rh123 stained sample, incubated at room temperature for 20 minutes and washed twice in distilled water.

2.2.7 Monoclonal Antibody Staining

Monoclonal antibody conjugated with fluorescein isothiocyanate (mAb-FITC) was used to stain *Legionella* spp. for determination by flow cytometry and epifluorescence microscopy.

For pure cultures of *Legionella* spp., colonies of *Legionella* on BCYE medium were resuspended in10 ml filtered RO water in universal containers and then fixed with glutaraldehyde (final concentration 0.5%). A 1 ml aliquot of suspension was centrifuged at 10,000 r.p.m for 2 minutes. Pellets were resuspended in 0.1 ml of PBS, 0.1 of mAb-FITC dye and incubated at 37 °C for 30 minutes. The incubation period for FCM samples was increased to 60 minutes to improve staining. Samples were washed once with distilled water.

Environmental samples from distribution systems, rivers or biofilms were concentrated by filtration with 0.2 μm pore size membranes and stained on the filtration membranes with mAb-FITC dye at 37 °C for 30 minutes in Eppendorf tubes, washed with 1 ml RO water resuspended by vortexing for further analysis.

For the direct reading of *Legionella* spp. in the biofilms the slides were drained in air for 3 – 5 minutes, fixed with formalin (2%) for 10 minutes, rinsed carefully with distilled water and then treated with mAb at 37 °C for 30 minutes in a container with a moist tissue to maintain humidity. The slides were then washed with RO water and stained with DAPI in working stock solution (2.2.3.1), washed in distilled water before mounting for microscopy.

For dual staining Dual staining of mAb with DAPI, PI or HOE33342 was performed by using mAb firstly and then with DAPI, PI or HOE33342 at working strength solutions, but for viable staining with CTC the samples were incubated with CTC initially and stained with mAb using the same protocol as described in 2.2.2.2.

The majority of the slides were attained unfixed. Suspensions of pure cultures and filtered distilled water were used as positive and negative controls in the stainings.

2.3 MEASUREMENT METHODS

2.3.1 The FACS Vantage Flow Cytometer

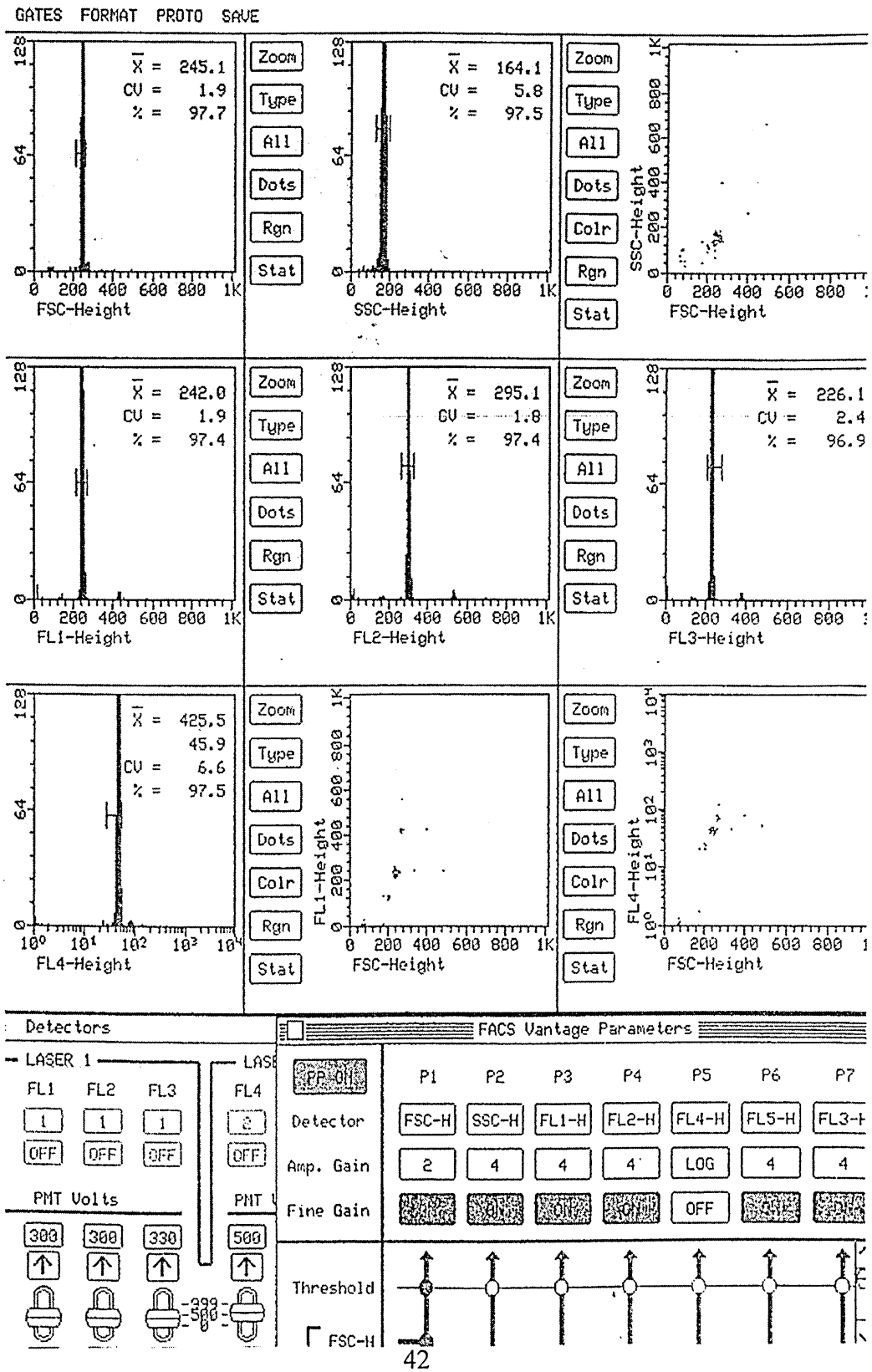
The machine used for the study was a Becton Dickinson FACS Vantage flow cytometer. This is a 'state of the art' machine capable of rapid cell detection and sorting. The soft ware operating the machine and permitting data analysis is called Lysis II. The sample is injected under pressure into a stream of liquid (the sheath fluid) and emerges from the ceramic nozzle with an opening of 70 μm. 'Hydrodynamic focusing' occurs just below the nozzle where particles in the stream of liquid are presented to the light source in single file.

The light source is an argon ion laser with a power range from 25 – 250 mW. The principal wavelength of light from the laser is blue light at 488 nm. A second wavelength at 365 nm can be used to excite dyes in the ultra-violet (UV) wavelength range. Particles passing through the light may scatter it forward (or low angle scattered light). Forward scattered light is detected by a photodiode and the detector

is designated FSC. The amount of light that is scattered will depend on the length of time that the particle stays in the light which is related to the size of the particle. Light may also be scattered to the side (side scattered or high angle scattered light). This is light which has passed through the particle and is therefore a measure of its refractivity. Side scattered light is detected by a photomultiplier tube (PMT) and this is designated SSC. The machine also has four other PMTs capable of detecting light emitted from stained cells. The wavelength of light depends upon band pass filters which permit only a certain wavelength of light through. In the FACS Vantage, the detectors are labelled as FL1 detecting green light at 530 nm, FL2 detecting light at 560 nm, FL3 detecting red light at greater than 600 nm and FL4 detecting light at 400 nm.

Each particle passing through the laser emits light and the data is gathered by the detectors. The data can be plotted graphically as a dot plot or a dot histogram. An example of a dot plot and a dot histogram is given in Figure 2.1. The machine is capable of analysing up to 20,000 events per second, but the flow of sample into the sheath fluid can be adjusted using differential pressure to optimise the rate at which events are being monitored.

Figure-2.1 Calibration of FACS VANTAGE flow cytometer
FSC-H CV; 1.9. SSC-H CV; 5.8. FL1-H CV; 1.9. FL2-H CV;1.8. FL3-H CV; 2.4
FL4-H CV; 6.6.
Laser power; 200mW. Threshold; FSC-H (56 V). 10μm DNA check bead.



Particles with similar size and fluorescence characteristics will cluster on a dot plot (see Figure 2.1). An electronic gate can be drawn around clusters and the machine asked to sort particles in these clusters out of the sheath fluid. Sorting is accomplished by vibrating the nozzle on the machine to produce individual droplets in the flow. When a particle of interest passes through the laser and the machine recognises that particle as fitting into the gated region on the dot plot, the droplet containing the particle can be charged and then deflected from the main stream of liquid by passing it past an electrically charged plate. This allows the machine operator to sort particles of interest from a complex mixture of unwanted particles. In practice, three drops are sorted, the drop containing the particle and one either side in case the particle itself is missed. Particles can be sorted into tubes, onto slides for microscopy or onto agar plates for culture.

The detectors are very sensitive to scattered light and a background noise level exists for each of the detectors. Their sensitivity can be increased by increasing the voltage supplied to each detector. Background noise can be reduced electronically by applying threshold voltages to each detector to minimise any unwanted signals. Particle characteristics may be interpreted by the computer in linear or logarithmic modes. Linear mode may give poor resolution if peaks are close together whereas logarithmic mode is better for separating peaks.

The nature of the particle and the fluorochromes used to stain the particle will determine the detectors that are used for analysing samples. For example, *Cryptosporidium parvum* can be stained with a fluorescein derived fluorochrome conjugated to a monoclonal antibody. It also has a characteristic size of 5 μm. Green fluorescence at 530 nm means that it can be sorted using the forward scattered light detector FSC and the green fluorescence detector FL1. The pulse of light produced by particles can be analysed in different ways. The height of the pulse can be measured e.g. FL1-H, the width of the pulse, FL1-W, the area under the pulse, FL1-A or the ratio of one parameter to another FL1-R. This is called pulse processing.

There are four different sort modes on the FACS Vantage, designed to give either maximum purity (but some particles may be lost) or maximum recovery but there may be of some loss in purity. The four sort modes are normal C, normal R, count and enrich. The enrich mode was used for sortings because this gives the best recovery.

2.3.2 Instrument Optimisation

In order for the machine to operate correctly, the hydrodynamic focusing has to be optimal, the laser power, focusing and position have to be optimal and the voltage levels on the detectors have to be correct. This is achieved firstly by aligning the nozzle optimally in the laser light and then using minimal voltages on the detectors (minimum sensitivity) to achieve detection of particles. The alignment was done using dot histograms which could be focused to give a maximum fluorescence channel with a minimum coefficient of variance for the histogram (Figure 2.1).

2.3.3 Setting up the Flow Cytometer

2.3.3.1 Alignment

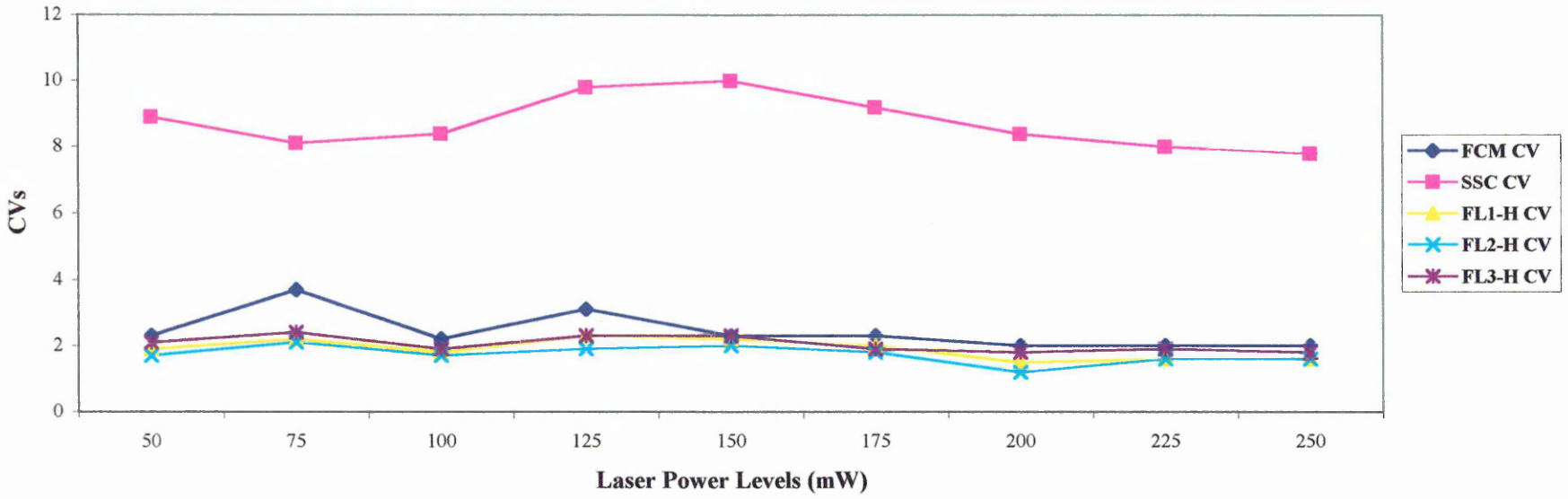
Alignment is achieved by running beads through the machine. All the beads have the same light scattering and fluorescence characteristics. If the machine is properly aligned, they will fall into a tight cluster on a dot plot or fall into a narrow dot histogram. The machine was calibrated with orange 10 μm DNA beads. This allowed optimisation of FSC, SSC, FL1-H, FL2-H and FL3-H at the same time. For FL4-H, the UV PMT, Hoechst beads were used for alignment.

Effects of the laser power levels on the calibration was investigated (Table 2.2, Figure 2.2) and suitable laser power levels with lower CVs were observed. When the laser power was 200 mW and over, the CVs of forward scatter (FSC) were maintained at 2.0% and the fluorescence detector's CVs could be below 2.0% (FL1 CV 1.5%; FL2 CV 1.2% and FL3 CV 1.8%), in order to run the flow cytometer under the good alignment conditions. A power setting of 200 mW for the laser power was selected for all flow cytometric analysis.

Table 2.2 Effects of Laser Power on the Calibration (CV) of FCM

Laser Power (mW)	50	75	100	125	150	175	200	225	250
FSC CV	2.3	3.7	2.2	3.1	2.3	2.3	2.0	2.0	2.0
SSC CV	8.9	8.1	8.4	9.8	10.0	9.2	8.4	8.0	7.8
FL1 CV	1.9	2.2	1.8	2.3	2.2	2.0	1.5	1.6	1.6
FL2 CV	1.7	2.1	1.7	1.9	2.0	1.8	1.2	1.6	1.6
FL3 CV	2.1	2.4	1.9	2.3	2.3	1.9	1.8	1.9	1.8

Figure 2.2 Effects of Laser Power Levers on the Calibration (CV) of the Flow Cytometer



2.3.4 Quality Control Following Alignment

Fluorescence beads of 2 μm and 6 μm diameter were used as standards for quality control of FACS Vantage operations. In order to check the correct alignment of the flow cytometer and optimisation of the fluidics and sorting, 2.0 μm beads (with the same size as bacteria) were counted by the machine and also sorted on the slide and checked by epifluorescence microscopy.

The daily routine checking was also carried out by adding 10 μl of 6 μm fluorescence beads (25 beads in 10 μl) into one ml of particle-free sheath solution or RO water and sorting the beads onto slides for checking by epifluorescence microscopy. For more accurate quality control, the 10 μl of beads added into the tube was directly counted first by microscopy by putting the tube on the microscope stage, counting the beads in a small spot in the tube, then diluting in sheath solution to 1 ml for running by FCM. The recovery percentage of beads was over 95% in duplication (at least two AQC bead standard material were sorted) and, if below 95%, the flow cytometer was realigned with 10 μm beads again. The AQC beads were sorted before, after and during running samples e.g. after every five samples for checking the flow cytometer operation.

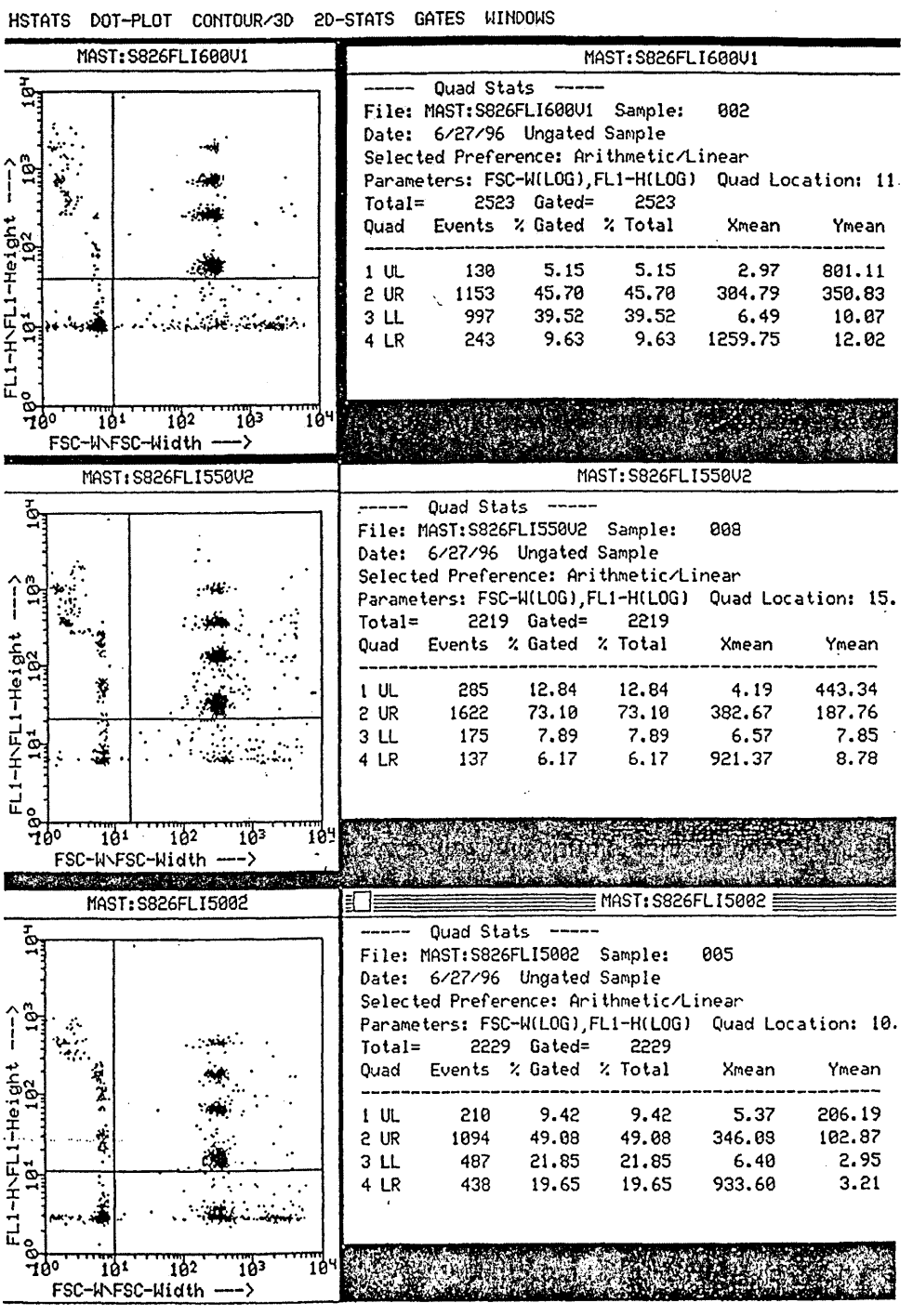
2.3.5 Measuring the Fluorescence Intensity

In order to quantify the fluorescence from the stained cells by flow cytometry, a series of fluorescence quantitation kits has been developed which were previously known as Quantitative Standards Kits, and are now called the *Quantum* ™ Series by Flow Cytometry Standards Corporation (FCSC). Each kit contains a set of calibrated fluorescence standards, with four populations of standards having different levels of fluorescence intensity (FI) and matching the emission and excitation spectra of specimens labelled with the same fluorochrome, and one reference blank population. Quantitative fluorescence standards matched for dyes used in samples allow for the direct measurement of the FI of a sample in terms of numbers of *molecules of equivalent soluble fluorochrome* (*MESF*). The correct use of the standards allows 1) quantitation of the FI of samples in terms of MESF, 2) determination of the flow cytometry fluorescence threshold, 3) determination of the flow cytometry linearity and stability, and 4) data comparison over time and between instruments.

The *Quantum*™ Series 24, 25 and 26 were routinely used as standard materials to quantitate the fluorescence intensities from stained cells by mAb-FITC and to determine the threshold levels as well as the sorting regions at the different conditions used during FCM runs.

The effects of the voltages on the *Quantum* ™ Series was investigated and Figure 2.3 shows the effects of the voltages of the detectors FL1-H on the standard beads (*Quantum*™ 26) fluorescence and the threshold levels. In dot plots of FSC-W against FL1-H, the threshold levels (blank beads' fluorescence levels) were increased from 3.2 to 12.0 on average following the voltage increasing from 500v to 600v. The region of left lower area (LL) with 10 *10 in the dot plot of FL1 against FSC-W was adopted as a background area at the 600v voltage levels. This testing was done to find the right region to be used for monoclonal antibody sorting cells stained with and is the upper left (UL) region on Figure 2.3 was used.

Figure-2.3 Quantification of the fluorescence intensity by Flow cytometry. Threshold; FSC-H (56V) with 500v, 550v and 600v FL1 PMT levels. Laser power; 200mW. Standard FITC fluorescence quantitation beads; Quantum™ 26. In dot plots; X; light Forward scatter signals, Y; Green fluorescence signals (4 different fluorescence densities' standard beads with a blank beads).



2.3.6 Optimisation of Threshold Level

Threshold is an electronic device in flow cytometry by which the flow cytometer can be made to ignore signals below a certain intensity, e.g., for ignoring background noise. The level of the threshold can affect the sorting or counting results. If the threshold is too high, the machine will ignore the signals produced by antibody stained cells and if the threshold is too low, unwanted particles may be sorted. The optimal level must be selected with the different parameters and the voltage levels. The forward scatter and FL1-H as well as FL3-H were set as thresholds and their levels were determined under the various conditions.

2.3.6.1 Effects of the threshold levels and sampling rates on the background noise

The level of threshold was determined by running filtered PBS solution as control sample at 488nm wavelength and 200 mW. Table 2.3 shows that for the green fluorescent detector (FL1); when the level rose from 50V to 210V the background noise decreased from 37744/s to 8 or 10/s with the sampling rate 6.6 μl/min (DIFF=1.0); and 250v or more high level with low background when 90.6 μl/min (DIFF=2.0). For the FSC detector, when the sample rate was 6.6 μl/min(1.0 DIFF), the threshold level over 41V, the background was reduced to a low level (2/s). When DIFF was 90.6 μl/min (DIFF 2.0), the threshold level over 53 V, the background was low, the optimal threshold level was shown in Table 2.3.

Table 2.3 Effects of the Threshold Levels and Sampling Rates on the Background

Detectors Voltages FL1-H 600 V FSC-H 600 V; Laser Power 200 mW 488 nm			
Thresholds	**Sample Rate**	**Optimal Threshold Level**	**Background***
FL1-H (green)	6.6 μl/min (DIFF;1.0)	>210V	< 8-10/s
FL1-H (green)	90.6 μl/min (DIFF;2.0)	>250V	<8-20/s
FSC-H (Forward scatter)	6.6 μl/min (DIFF;1.0)	>41V	<2/s
FSC-H (Forward scatter)	90.6 μl/min (DIFF;2.0)	>53V	<12/s
* Note; background noise level; FCM counting numbers of particles per second			

The optimal threshold levels were adapted with different detectors and sampling rates in flow cytometric analysis for reducing the background with the minimum noise levels and keeping the high sensitivities.

2.3.6.2 Effects of the PMT voltage levels on the threshold levels

The effects of the detectors' (PMT) voltages on the threshold levels were investigated for choosing the optimal threshold levels with different voltages of the detectors.

There was a strong linear correlation between the optimal FL1-H threshold levels and PMT voltage levels (r=1.0, n=5). From Table 2.4 and Figure 2.4, the strong linear relationship (Y= 0.625X+443.75; r=1.0 n=5) between the levels (X) of green fluorescence detector (FL1) as threshold and PMT voltages (Y) is shown. When FL1-H was adopted as threshold, following the increasing of PMT voltages from a range of 500V to 600V, the threshold levels which made the minimum background noise were also increased from 90V to 250V separately. For the sorting of green fluorescence labelled bacteria, the voltage of the FL1-H was often at the range of 550V to 600V and the optimal threshold levels were from 170V to 250V in flow cytometric analysis. Effects of PMT's voltage levels on the side scatter (SSC) threshold were also tested and also gave a positive correlation relationship of PMT levels with SSC levels (r= 0.92, n=5) as shown in Table 2.5 and Figure 2.5.

Table 2.4 Effects of the PMT Voltages on Threshold Levels

PMT Voltages	500	525	550	575	600	Correlation
FL1-H Threshold Levels	90	1340	170	210	250	1.0
Background No. per Sec	1-10	1-10	1-10	1-10	1-10	

Figure 2.4 FL1-H Threshold Levels

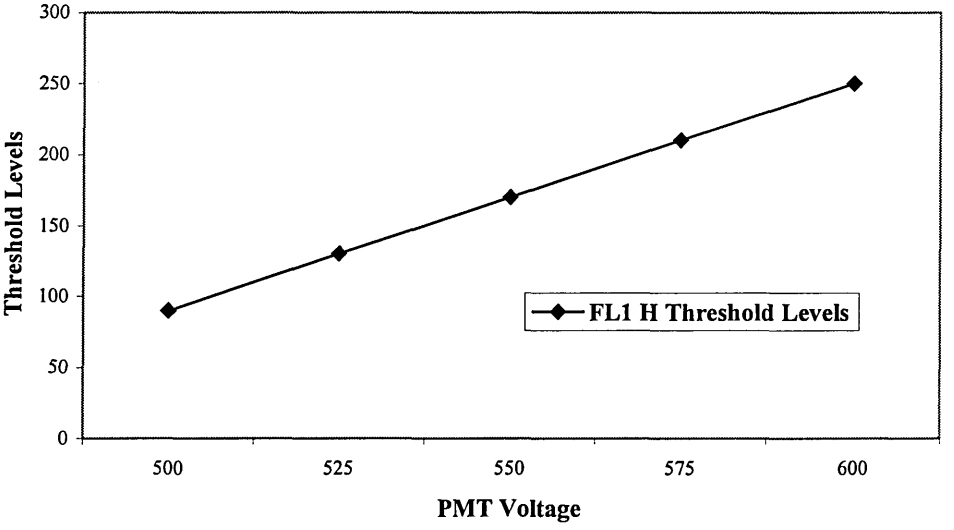
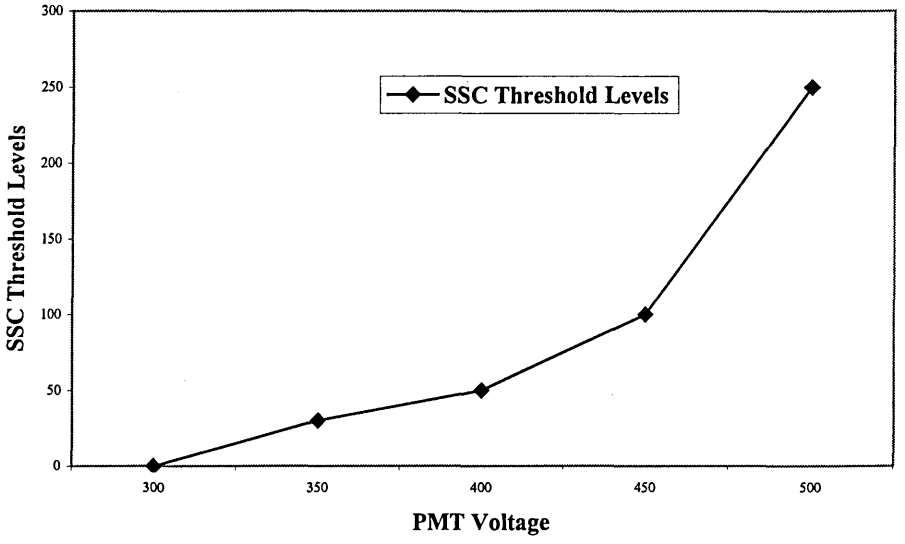


Table 2.5 Effects of PMT Voltages on Threshold Levels (Threshold;SSC-H)

PMT Voltages	300	350	400	450	500	Correlation
SSC Threshold Levels	0	30	50	100	250	0.92
Background No. per Sec	0	0	0-6	0-6	0.6	

Figure 2.5 Effects of PMT Voltages on Threshold Levels



In flow cytometric analysis, the forward scatter threshold (FSC) is most commonly used to exclude very small particles, debris, and electronic noise from acquisition. Here, the fluorescence detectors such as FL1-H and FL3-H were also used as thresholds for detecting the FITC-mAb stained and PI stained target cells separately. The selections of the thresholds depended on the targets' characteristics such as fluorescence colour, cell size, as well as background noise. In enumerating and sorting mAb-FITC stained cells, the suitable threshold was FL1-H, which could overcome the background noise and offered accurate counting results.

2.3.6.3 Effects of laser power on threshold

The effects of laser power levels on the threshold (FL1-H) were investigated and the results are given in the Table 2.6, and Figure 2.6. When the laser power was increased from 100 mW to 200 mW, the optimal threshold levels were also increased from 190V to 230V for FL1-H threshold (PMT 600v). In the current work, the laser power was fixed at 200 mW for most of flow cytometric analyses.

Red parameter FL3-H was also used as threshold for analysis of the red fluorescence cells stained by CTC or PI. The effects of the PMT voltages and the laser powers levels on the threshold levels (FL3-H) were investigated and the results are given in Table 2.7. The optimal threshold level for FL3-H was >120 for 150 mW

power and >140 for 180 mW when the voltage was 550V for FL3-H; and for the voltage 600 V, the threshold FL3-H was >140 for 150 mW laser power and > 160 for 180 mW laser power.

Table 2.6 Effects of Laser power, PMT Levels on the threshold of FL1(Green)

Laser Power	100mW	100mW	150 mW	150 mW	180 mW	180 mW	200 mW	200 mW	Laser Power
PMT Levels	550v	600v	550v	600v	550v	600v	550v	600v	PMT Levels
Threshol v	No/Sec Background		N0/Sec Background		No/Sec Background		No/Sec Background		Threshold v
0	8900	n/a	8900	8200	8780	8304	8600	8311	0
10	9100	n/a	9000	8100	9100	8400	9000	8400	10
20	7500	n/a	7700	8400	8700	8600	8900	8200	20
30	5500	n/a	5600	8600	6400	8600	7300	8200	30
40	3700	n/a	3800	8700	4500	8500	5400	8400	40
50	2300	8823	2500	8900	3100	8600	3700	8500	50
60	1350	9140	1300	9000	1800	8800	2200	8700	60
70	640	9000	700	9000	900	8900	1200	9000	70
80	300	7500	350	7580	450	8700	510	9100	80
90	120	6100	150	6100	225	7041	300	7400	90
100	50	4500	55	4300	90	5430	100	6000	100
110	25	3200	15	3124	30	3900	45	4400	110
120	**15**	2000	15	2000	15	2500	25	3100	**120**
130	**0**	1200	**15**	1200	**15**	1600	**15**	2000	**130**
140	**0**	700	**0**	700	**0**	1020	**0**	1200	140
150	**0**	350	**0**	160	**0**	480	**0**	660	150
160	**0**	150	**0**	60	**0**	240	**0**	240	160
170	**0**	75	**0**	35	**0**	110	**0**	140	170
180	**0**	30	**0**	15	**0**	35	**0**	70	180
190	**0**	**15**	**0**	**15**	**0**	15	**0**	25	**190**
200	**0**	**0**	**0**	**0**	**0**	**15**	**0**	15	200
210	**0**	**0**	**0**	**0**	**0**	**5**	**0**	**15**	**210**
220	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	220

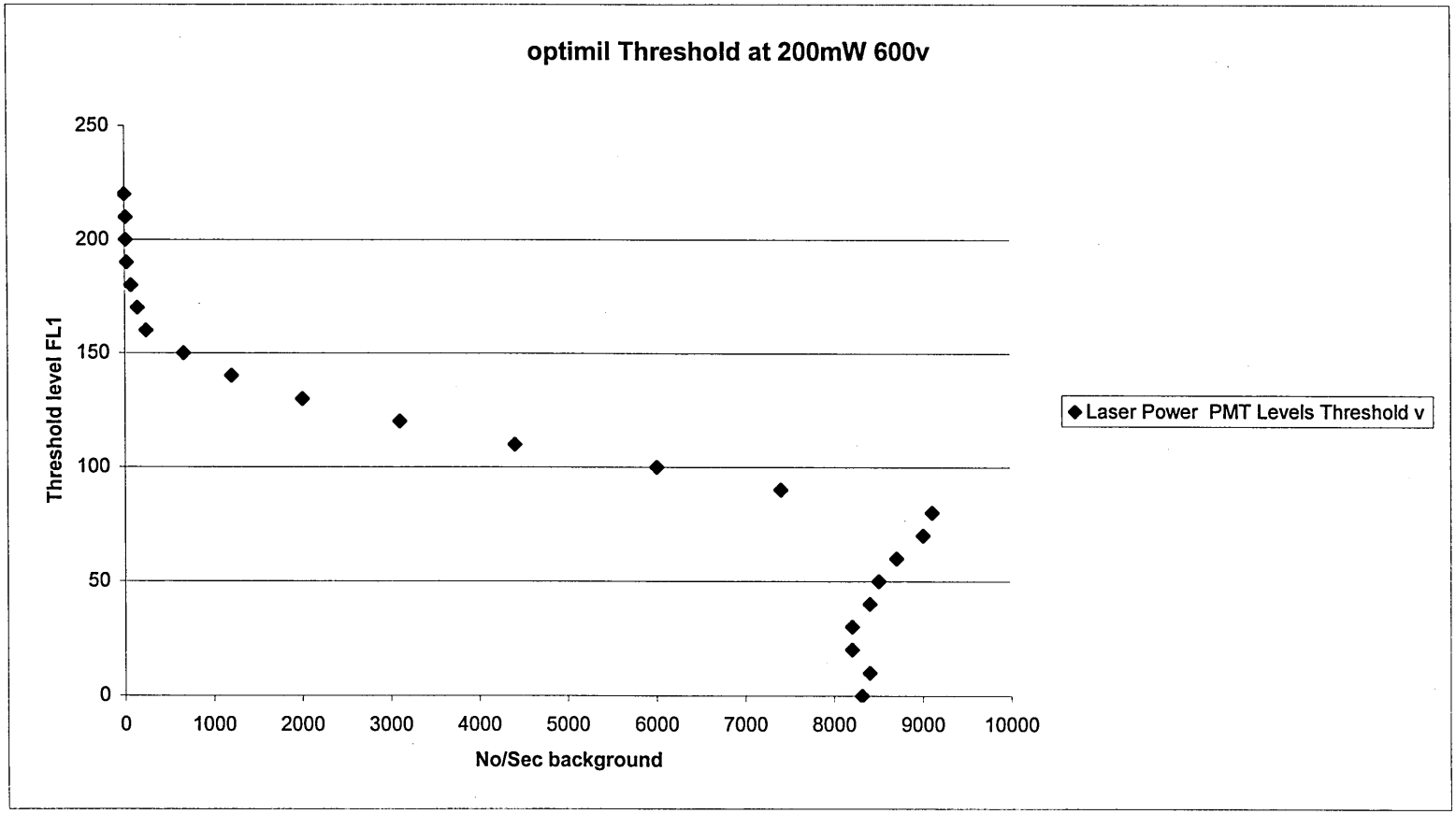


Figure-2.6 Optimil Threshold at 200 mW Laser power and 600v PMT level
Flow Cytometer; FACS VANTAGED. Threshold FL1-H (Green 530mn detector)

Table-2.7 Effects of Laser power on the Threshold levels(Threshold;FL3-H)

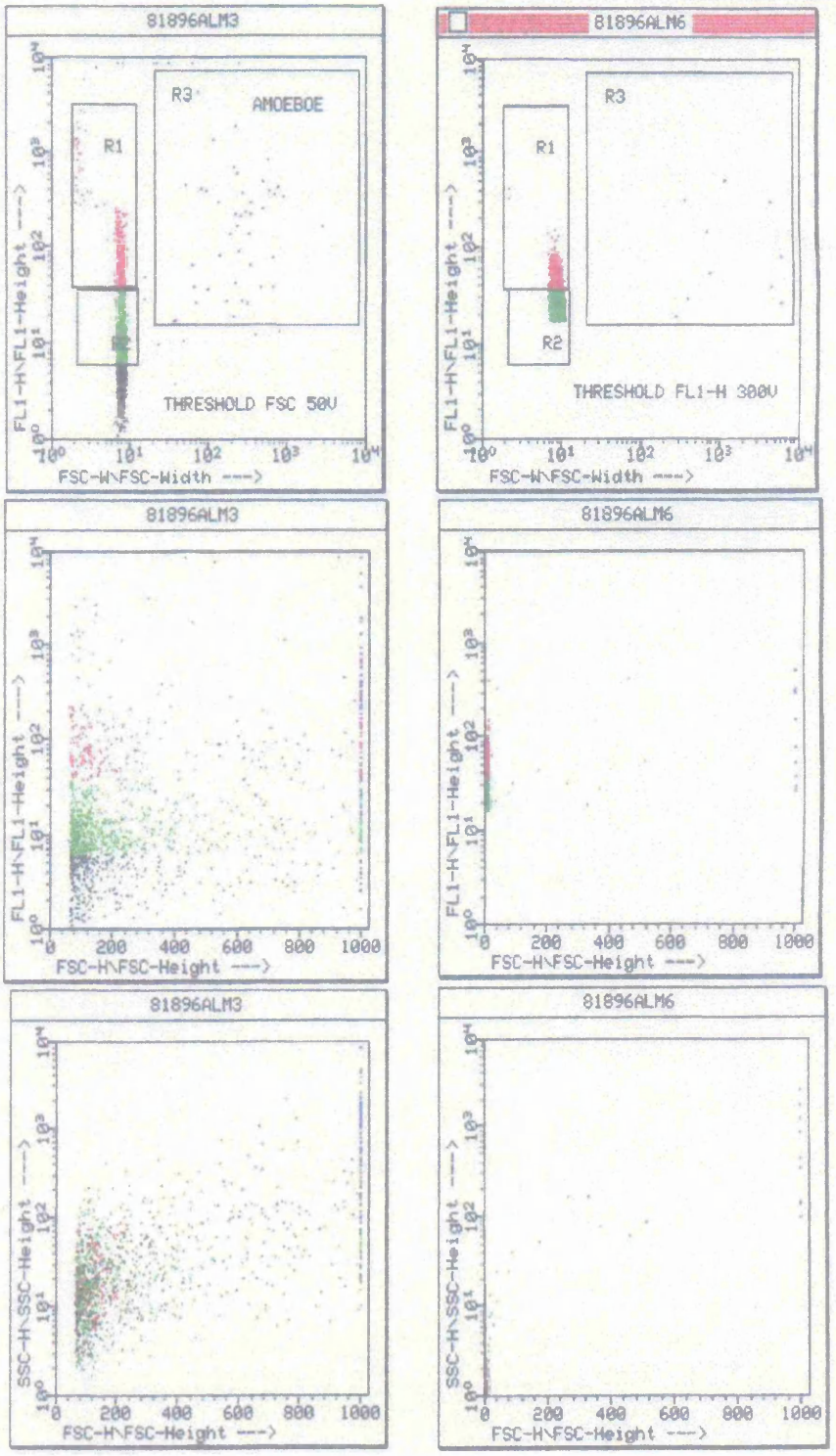
	Background No/Sec		Background No/Sec		Background No/Sec	
Laser powers mW	100mW	100mW	150 mW	150 mW	180 mW	180 mW
PMT Levels v	550v	600v	550v	600v	550v	600v
Threshold Levels FL3-H	No/Sec	No/Sec	No/Sec	No/Sec	No/Sec	No/Sec
160	4,2,2	8,4,2	4,8	2,4,2	4,2,2	**2,6,6**
150	4,2,2	2,8,2	2,4,2	2,4	2,10,4,2	8,10,16
140	2,4,2	2,8,2	2,4,2	**2,4,2**	**2,4**	4,8,12
130	2,4,2	**4,8,2**	2,4,2	2,2,4,8	4,10	8,12
120	2,4,2	10,2,4	**2,4,2**	2,4,10,14	2,6,4	22,14
110	2,4,2	2,6,14,8	**2,6,2**	12,16,16	8,4,6	34,24
100	4,2,4	14,16,20	2,8,6,4	30,52,24	4,2,6	82,74
90	**2,4,2**	36,48	4,10,4,2	80,102,118	6,8,10,6	132,174
80	2,4,10	74	2,4,6,8	170,218,206	2,14,30	n/a
70	4,6	172	4,6,10,14	376,416	6,8,10,6	n/a
60	6,10	386	10,26	820,860	24,32,34	n/a
50	26,38	792	42,48	1595	88,99,103	n/a
40	62,64	n/a	178,154	n/a	206,216,250	n/a
30		202,162.20	404,416	n/a	502,630,494	n/a
20		516,468,472	9,941,074	n/a	12,291,300	n/a
10		10,421,164	20,142,169	n/a	2602	n/a
0	2400	n/a	4588	n/a	5156	n/a

2.3.7 Selections of the Flow Cytometric Parameters (Detectors)

Modern flow cytometer such as FACS Vantage could have six main detectors and also could use all these detectors (up to eight parameters) at the same time. The information from targets could be dealt with by computers and directly viewed or saved on disks for review and reanalysis.

The selection of the detectors was dependent on the target size, shape, fluorescence colours, and other properties. The histogram (single parameter data) and the dot plot (bit map, two dimensional diagram), as well as three-dimensional diagrams were used for showing the flow cytometric analysis results. In reanalysis of the results saved in the computer, the changing parameters in dot plots were observed to be very useful, for example, when amoeba samples were sorted and the image (dot plot) of sub populations was labelled in different colours by the computer in the initial parameters, if the parameters of the dot plot were changed, amoebae sub-populations could be moved and separated depending on the parameters in dot plot. Figure 2.7 shows the FACS sorting *Acanthamoeba* samples, in the dot plot of the FSC-H/ FL1-H, the amoebae were focused on the red regular sharp sub-population on the right-hand side of the dot plot. When parameters were changed with FSC-W/ FL1-H in another dot plot, the purple colour dot amoebal subpopulation moved. They now became a separate cluster of dots around which an electronic sorting gate could be drawn.

Figure 2.7 **Flow Cytometric Imaging and Sorting of *Legionella* and Amoebae by mAb-FITC and DAPI Dual Staining and Multi-colour Gate Technique. R1;mAb-FITC Stained Region for Sorting *Legionella* (Red Dots). R2 Non-*Legionella* and Background Region (Green and Dark Dots). R3; Amoebae in Sorting Region (Blue Dots).**



2.3.8 Operations

The FACS Vantage flow cytometer was calibrated before operations by using the standard fluorescence beads.

2.3.8.1 Sorting recovery testing

10 µl of fluorescence beads containing 25 of 6 µm beads was diluted to 1 ml with RO water and sorted onto a microscope slide using the cytometer. Recovery of more than 95% was obtained and checked using epifluorescence microscopy and carried out in duplicate at least. The flow cytometer must be re-optimised if the recovery was lower less than 95% by readjusting the nozzle and the detectors.

2.3.8.2 Operation

In all analyses the laser output was set at 200 mW at an excitation wavelength of 488 nm and at 60 mW with a UV excitation wavelength of 350 nm. The flow cytometer was set up and aligned with both the 0.2 µm and 10.0 µm fluorescent beads as described in 2.3.3.1. Before each sorting, sodium hypochlorite solution (10%v/v) was passed through sample fluid tubes and nozzle to keep the system clean. The nozzle diameter for all the analyses was 70 µm. Sorting was performed in enrich mode. Samples were sorted into sterile polystyrene tubes (Flacon, Becton Dickinson) or slides or black membrane filters

Sheath and sample pressures were kept constant, and an analytical rate of approximately 1,000 events s^{-1} was maintained by sample dilution. Fluorescence at 525 nm (green) was used to detect Rh123 and mAB-FITC stained bacteria using fluorescence detector FL1 set at a photomultiplier tube voltage of 500-600 V with logarithmic gain. Fluorescence at 600 nm was used to detect CTC and PI stained bacteria using fluorescence detector FL3 set at a photomultiplier tube voltage of 550-600 V with logarithmic gain. Fluorescence at 450 nm was used to detect DAPI or HOE342 stained bacteria through fluorescence detector FL4 set at a photomultiplier tube voltage of 550 V with logarithmic gain. The threshold level for FSC was 50, and 230 for FL1 was 230 and for FL3 was 230-290. The machine was found to be difficult to align and calibrate for FL4 and when sorting with FSC and FL4, thresholds were set on FSC.

Samples were sorted by using the FACS Vantage flow cytometer, and defined parameters were set by using FACS Star LYSYS II software around the bacterial population(s) of interest, using stained and unstained, pure or environmental samples. Sort mode was enrich and sample flow rate was 100-1000 cells /second. The targeted cells were sorted onto slides or membrane filters or into sterile tubes for checking by EFM or running again by FCM. Sorted bacteria were immediately reanalysed by FCM, and the proportion of the targeted cells in each gated area and total count were enumerated and stored by the computer.

2.4 COLONY COUNTING

2.4.1 Colony count for Total Bacteria

2.4.1.1 Apparatus and Reagents

Incubator 22 °C +/-1 °C

Incubator 37 °C +/-1 °C

YEA medium (Yeast Extract 3.0g/l; Peptone 5g/l; Agar 15 g/l; pH 7.2+/- 0.2)

R2A medium (Yeast Extract 0.5g/l; Tryptone 0.25 g/l; Peptone 0.75 g/l; Dextrose 0.5 g/l; Starch 0.5 g/l; Di-potassium phosphate 0.3 g/l; Magnesium sulphate 0.024 g/l; Sodium pyruvate 0.3 g/l Agar 15 g/l; pH 7.2 +/- 0.2)

Petri dishes, sterile 90mm

2.4.1.2 Method

All colony counting for total viable colonies was performed using standard methods (Anon, 1994). YEA media and R2A media were used to enumerate the colony forming units (cfu) for detecting viable and cultured bacteria in pure cultures and environmental samples. The effect of the fluorescence dyes on the viable counts was tested using the YEA and R2A colony counting methods. The incubation time and temperature was 3 days at 22 °C and 24 hours at 37 °C for YEA colony counting and

22 °C for 7 days for R2A colony counts.

2.4.2 Buffered Charcoal Yeast Extract Agar (BCYE) Colony count for *Legionella* spp.

Legionella spp. were cultured and detected using the buffered charcoal yeast extract agar (BCYE). The bacteria were concentrated by membrane filtration or centrifugation for low density of *Legionella*. *Legionella* spp. were evaluated by counting the number of typical colonies growing on the culture medium.

Table 2. 8 Composition of BCYE Agar

Component	g/litre
ACES	10.0
KOH	2.2
α-ketoglutarate monopotassium salt	1.0
Yeast Extract	10.0
Fermtech Agar	10.0
Activated Charcoal	0.5
RO water	to 1 litre
pH 6.9 ± 0.1	
Growth Supplement SR 110	
ACES|Buffer/Potassuim Hydroxide	10.0
Ferric Pyrophosphate	0.25
L-Cysteine HCL	0.4
α-ketoglutarate	1.0

2.4.2.1 BCYE medium preparation

BCYE base (Oxoid) was added to the RO water and autoclaved at 121 °C for 15 min, cooled to 50 °C, and the growth supplement (SR110) was added at the rate of 17g BCYE base to 1000 ml distilled water to 100 ml growth supplement. The medium was dispensed into sterile Petri dishes, the plates dried and stored at 4 °C for a maximum of 4 weeks. BCYE without growth supplements medium was made using the same protocol but excluded *Legionella* growth supplement. For the isolation of

Legionella from river water selective supplements SR 111 (BMPA, Oxoid) or SR 118 (MWY, Oxoid) were added.

2.4.2.2 Sample preparation

Samples were concentrated by membrane filtration using the 142mm, 0.22μm nylon membranes for river water and cooling tower samples. The filter was cut into small pieces and organisms were removed by shaking in 20 ml of the filtered sample for 30 minutes. Samples with high concentrations of bacteria were directly inoculated without concentration treatment.

2.4.2.3 Culture for *Legionella* growth

The concentrated sample was divided into three portions. One portion was inoculated onto BCYE agar plate without treatment, one was heated and the third treated with an acid buffer at pH 2.2.

(a) Heat treatment

A minimum of 2 ml of sample was placed into a sterile universal container and heated in a water bath at 50 +/-1 $^{\circ}$C for 30 min.

(b) Acid treatment

A 10 ml sample was centrifuged at 1000g for 20 min. The supernatant was discarded and the same volume of the HCl-KCl buffer (pH 2.2) was added and resuspended by gentle shaking. The sample was left to stand for 5 min.

2.4.2.4 Inoculation of BCYE plates

Plates of BCYE were inoculated with a range of volumes from 0.05-0.5 ml of untreated sample and spread over the surface with a sterile spreader ensuring that all of the mixture was absorbed by the agar. The heat-treated samples and the acid treated samples were inoculated individually in the same way.

2.4.2.5 Incubation

All plates were incubated in a humid atmosphere at 37 $^{\circ}$C for up to ten days .

2.4.2.6 Examination of plates

Plates were examined on days 3, 5, 7 and 10 for typical colonies using a plate microscope. Colonies of *Legionella* had a grey-blue, purple, brown, lime green or red

colour. They were smooth with an entire edge and had a characteristic ground glass appearance. Each plate was carefully examined and the number of colony forming units was recorded.

2.4.2.7 Verification of *Legionella* colonies

Legionella-like colonies were selected for subculture onto BCYE with and without growth supplement. A *L. pneumophila* culture (NCTC 12821) was included as a positive control. The plates were incubated at 37 +/-1 ° C for two days and examined for growth. Colonies which had grown on BCYE without growth supplement were discarded. Those which had grown on BCYE but failed to grow on BCYE without growth supplement and showing typical colonial morphology were presumptive *Legionella.* Confirmation of isolates was by immunofluorescence.

2.5 NNA-*E.coli* TECHNIQUE FOR DETECTING AMOEBAE

2.5.1 Equipment

Filter holders for 47 mm diameter membranes, manifold unit, vacuum pump and silicone connecting tubing.

47 mm diameter 0.45 μm pore size cellulose acetate membranes.

Inverted light microscope with x 10 and x 20 objectives.

Vortex (optional).

Incubators set at 30 °C, 37 °C, 42 °C and 44 °C.

90mm polystyrene Petri dishes, glass universal containers, plastic Pasteur pipettes, flat-bottomed 96-well microtitre plates.

Sterile swabs, wax crayons or marker pens, scalpel blades.

2.5.2 Materials

Page's amoeba saline (PAS) at pH 6.8 - 6.9 was prepared (0.12 g NaCl, 0.004 g $MgSO_4.7H_2O$, 0.004 g $CaCl_2$, 0.142 g Na_2HPO_4, 0.136 g KH_2PO_4 in 1 litre of distilled water). The solution was autoclaved at 121 °C for 15 minutes and stored at room temperature.

Non-nutrient agar - *Escherichia coli* (NNA -*E. coli*) plates were prepared by seeding non-nutrient agar with a lawn of *E. coli* on which the amoebae feed. These were prepared as follows:

2.5.3 Non-nutrient agar plates (NNA).

1.5% w/v bacteriological grade agar (OXOID) in PAS was autoclaved at 121 °C for 15 minutes. The medium was distributed at 25 ml volumes into Petri dishes and dried at 37 °C for 24 hours. Plates should not be allowed to dry uncovered as cysts of free-living amoeba (FLA) may be present in the air of the laboratory. Plates were stored in sealed polyethylene bags at room temperature for up to 14 days.

Escherichia coli NCTC 10418 was grown on nutrient agar plates at 37 °C for 24 hours. A stock culture plate could be stored at 4 °C for up to 1 month. This was used to seed the entire surface area of several nutrient agar plates. Following incubation, the seeded plates were stored at room temperature in sealed polyethylene bags for up to 7 days, or at 4 °C for two weeks.

A thick portion of *E. coli* was taken from a seeded nutrient agar plate using a sterile swab and spread over the entire surface of one NNA plate. The plates could then be stored at room temperature in sealed polythene bags for up to 7 days.

2.5.4 Isolation methods

There are two methods which are recommended for the isolation of FLA. The method of choice is determined by the nature of the sample to be examined.

2.5.4.1 Filtration

The water sample was thoroughly mixed and 1-100ml (depending upon sample type) filtered through a 0.45 μm pore size cellulose acetate membrane by suction at a flow rate not exceeding 30 ml a minute. The membrane was not allowed to dry and filtration was stopped when 2 to 3 ml of sample remained above the membrane. The membrane was carefully washed *in situ* with the residual water sample, using a plastic Pasteur pipette. The whole of the residual sample used to wash the membrane was transferred into a sterile glass universal container. The membrane was placed in the same universal, and rolled so that the upper or sample surface was inward and not in contact with the wall of the universal. The universal tube was then shaken vigorously with a vortex mixer for 10 seconds.

The whole of the fluid obtained from the above technique was distributed over the surface of 2 or 3 NNA-*E. coli* plates and allow to dry at room temperature. Alternatively, the fluid was poured onto the surface of a single NNA-*E. coli* plate and left at room temperature for 2 hours. The excess fluid was then pipetted off and the plate allowed to dry. Plates should not be dried uncovered, as cysts of FLA may be present in the air of the laboratory. Uninoculated lawn plates were used as controls.
The membranes were divided into halves and each placed, face down, on the surface of a single NNA-*E. coli* plate.

2.5.4.2 Direct plating of samples

Untreated water samples may contain large numbers of FLA. This can result in failure to obtain isolates of individual amoebae because of overcrowding in the plates. To avoid this possibility, unconcentrated sample volumes should be inoculated directly onto the surface of NNA-*E. coli* plates.

1.0ml volumes of the water were pipetted onto each of 3 NNA-*E. coli* plates and left at room temperature for 2 hours. The excess fluid was pipetted off and the plates allowed to dry. The plates were covered while drying. Uninoculated plates were used as controls. Solid material was inoculated directly onto NNA-*E. coli* plates.

Swab samples were vortexed in 2ml of PAS and the liquid cultured as above.
The upper limit (ceiling) temperatures for incubation are determined by the type of amoeba to be isolated.

2.5.4.3 Incubation

Potentially pathogenic *Acanthamoeba* will grow at 37 °C. *Naegleria australiensis* will not grow above 42 °C. *Naegleria fowleri* and *N. lovaniensis* will grow at 44 °C. The following incubation temperatures are therefore recommended:

30 °C for total *Acanthamoeba* and *Naegleria.*

37 °C for pathogenic *Acanthamoeba.*

42 °C for *N. australiensis, N. lovaniensis* and *N. fowleri.*

44 °C for *N. lovaniensis* and *N. fowleri.*

Plates were incubated inverted in sealed polythene bags.

2.5.4.4 Detection of free-living amoebae

Plates were examined daily for up to 7 days with the x 10 objective of the inverted light microscope.

Free-living amoebae were observed as feeding trophozoites producing tracks and clearings in the *E. coli* lawn. Areas of amoebal growth were noted as they appeared by marking the underside of the Petri dish with a marker pen or a wax crayon. The marked areas of agar were cut out with a sterile scalpel blade and placed, seeded side down, on a fresh NNA- *E. coli* plate and incubated at the original temperature of isolation.

2.5.4.5 Provisional identification of *Acanthamoeba* and *Naegleria.*

A small area of trophozoite growth was removed using a wire loop and inoculated into one well of a microtitre plate containing 100 µl of PAS. The plate was sealed and incubated at 30 °C for 30 minutes (Anon 1989).

Trophozoite morphology was observed using the x20 objective of the inverted microscope.

2.5.4.6 Morphological characteristics of *Acanthamoeba* and *Naegleria*

Acanthamoeba trophozoites are approximately 25 to 40 µm in length and show numerous needle-like projections from the trophozoite body termed acanthopodia. A central contractile vacuole is present in the cytoplasm. Trophozoite movement is slow and polydirectional with a hyaline pseudopodium that slowly stretches out and widens.

Acanthamoeba cysts are formed on prolonged incubation on NNA-*E. coli* plates. Sizes range from approximately 15 to 28 µm depending on the species, and are double-walled. The intermittent joining of the inner wall to the outer gives rise to a polygonal arrangement. This feature enables differentiation from other FLA.

2.6 CHEMICAL ANALYSES

All chemical analyses were done at the Yorkshire Environmental Bradford Laboratory by Yorkshire Environmental's analysts as follows:

ICP (VG Plasma Quad/UK) was used for analysing the Fe, Mn, SO_4 , Al, Ca, Mg, P content of biofilm samples.

$CaCO_3$, NH_3-N, NH_2 -N, Cl, NH_4 -N. were analysed by Auto analyser Perspective®Analyzel , UK

The total organic carbon (TOC) of biofilms was analysed by TOC analyser (Model 700 TOC Analyser, College Station, Texas)

2.7 MATERIALS AND SUPPLIERS

Yeast extract agar, Merck. Merck KgaA, 64271 Darmstadt, Germany.

Yeast extract, Oxoid L21. Oxoid LTD., Basingstoke.UK.

R2A agar, Becton Dickinson DCM, USA.

Legionella CYE agar base, Oxoid CM655. Oxoid LTD., Basingstoke,UK.

BCYE growth supplement, Oxoid SR110. Oxoid LTD., Basingstoke

Legionella selective supplement, Oxoid SR118 or SR111

Nutrient broth No.2, Oxoid CM67.Oxoid LTD., Basingstoke,UK.

Nutrient Agar. Oxoid CM3. Oxoid LTD., Basingstoke, UK.

Bacteriological grade agar, Agar No.1 (Oxoid, Basingstoke, UK)

Phosphate buffered saline, Oxoid Code BR/4a. Oxoid LTD., Basingstoke, UK.

5-Cyano-2,3-di-4-tolyl-tetrazolium chloride (CTC), CAT#19292.Lot # 473951 Polysciences, Inc. Warrington, PA,USA.

Propidium Iodide (PI), Lot 57H3642. Sigma, USA.

Rhdamine 123 (RH123), Lot 51H0529. Sigma,USA.

DAPI (Sigma, USA)

Hoechst 33342 (Sigma, USA)

Acridine orange (Sigma, USA)

Bacteriological Filters (Gelman, UK)

Legionella specific Monoclonal antibody (Sanofi, France)

Hoechst alignment beads (Coulter Electronics)

Flow cytometer (Becton Dickinson, USA)

Black polycarbonate filters 25 mm, 0.2 μm(Poretics, USA))

3.0 SCREENING AND OPTIMAL STAINING FOR FLOW CYTOMETRY

3.1 CYTOMETRIC ANALYSIS

Enumeration of microorganisms and characterisation of their physical features (size and shape, etc.) are routine practices in environmental microbiology. The progenitors of some of today's enumeration techniques go back to the days when Robert Koch incorporated agar-agar into plating media (Hitchenes and Leikind, 1939). A number of enumeration protocols still popular at present were developed decades ago, such as the direct counting of bacteria using the light microscope (Baldwin 1927; Breed and Brew, 1925) and the most probable number technique (Colwell, 1979; Halvorson and Ziegler, 1933). Although these techniques are routinely used they have a number of limitations and are often extremely labour intensive. Even the comparatively simple most probable number (MPN) method requires extensive preparation. Bright-field or phase contrast direct counting methods are also in common usage but have the disadvantages of not distinguishing between bacteria and similarly shaped particles or between viable and non-viable organisms. The use of fluorescent dyes with various specificities in epifluorescence microbiology proved to be a vast improvement for enumeration (Daley 1979; Watkins and Jian 1997). Nucleic acid-specific dyes provide differentiation of cells from other microbiological materials, and dyes exist which permit one to distinguish between viable and non-viable cells (DeLeo and Baveye, 1996). Very specific immunofluorescent monoclonal antibodies allow the identification of target organisms. In spite of these recent improvements, epifluorescence direct count techniques still require a sizeable effort to obtain satisfactory results.

During the recent years there has been a rapid growth of interest in the application of fluorescent dyes to study environmental microbiology. There are two factors which have facilitated this: firstly the synthesis of a range of highly selective fluorochromes, and secondly, the availability of computer linked commercial instruments such as flow cytometry, capable of detecting and quantifying the weak signals emanating from single cells (Macey, 1994).

As stated previously, some fluorescence dyes such as AO, CTC, DAPI, Rh123, PI, FDA and HOE342 have been used for mammal cells, and antibodies have been used

in detecting bacteria, but most of the studies are based on solid staining and microscopic analysis (Watkins and Jian, 1997). As far as it is known, no more detailed studies of staining for the flow cytometric analysis of biofilms and *Legionella* and amoeba have been published.

The aim of this work was to screen the reliable dyes available commercially and optimise the staining protocols for the flow cytometric analysis of microorganisms from both pure culture and biofilms as well as environments, and include dyes for enumerating total and viable cells, and specific targets. In order to achieve this aim, it was necessary to screen and optimise the staining protocols from the current commercial dyes.

3.2 STAINING

3.2.1 Acridine Orange (AO) Staining

Acridine orange (AO) has been used in staining for a long time. The advantages are the staining speed of AO is very rapid (a few minutes at room temperature) and the dye is inexpensive. AO has also been reported to be used in the flow cytometric analysis of cancer cells with three major types of applications: (1) supravital cell staining; (2) differential staining of double-stranded versus single-stranded DNA; (3) differential staining of RNA and DNA (Darzynkiewce *et al.*, 1990, 1994). The acridine orange direct counting (AODC) is adopted as the standard method for direct counting total and viable bacteria based on the machanism that AO could stain both RNA and DNA with red and green fluorescent colours. So far as it is known no there has been no detailed study of AO for the flow cytometric analysis of bacteria.

3.2.1.1 AO staining

The overnight pure cultures of the *E. coli* and *S. aureus* and environmental samples which included raw, and treated water samples were stained by the methods in Section 2 using the acridine orange with the range of 5.0 mg/l to 30 mg/l at room temperature. The results (Table 3.1) show that the proportions of the red-orange and green bacterial cells were changed with the increasing concentrations of AO. The optimum concentration of AO staining was 10 μg/ml in three minutes using the epifluorescence microscope for direct counting of total bacteria, but for the viable

determinations, the results demonstrate that it is very difficulty to assay or enumerate the numbers of dead or living cells by red-orange or green fluorescences. This is because even if we keep all the same staining conditions, the proportion of red-orange and green fluorescence can be different for the same bacterial samples. This agrees with the results of Burton and Lanza (1985) who reported that the cells' fluorescence from AO staining (orange-red or green) is only a crude activity indicator. It agrees with the opinions of the APHA (1989), who state that 'staining cannot differentiate microbial cells on the basis of metabolic activity or viability'. In the staining performance, AO tends to remain in the fluidics system of the flow cytometer and cannot easily be washed out. For this reason, it was not used as a stain for FACS analysis.

The latest report about the AODC technique was written by Gabriel Bitton *et al.*, (1993) and they modified the direct counting method for total bacterial counts in environmental samples. They observed that the colour of the fluorescing cells (green or orange-red cells) depends on the level of the moisture on the filter and that the percentage of the green fluorescing cells decreases when the filter was dry. Moreover, an increasing number of papers published in recent years adapted the DAPI DC techniques for direct counting total microorganisms from pure and environmental samples instead of AODC.

In conclusion, AO staining could be used for total counting of bacteria by epifluorescence microscopy at a final concentration of 10 μg/ml at RT, and is not suitable for viable counting as well as for flow cytometric analysis.

Table 3.1 Effect of AO concentration on the Staining of *E.coli* and other Bacterial Cells

AO μg/ml	Green Cells (%)		Red Cells (%)
	Weak	Bright	
5.0	100	0	0
10.0	36	64	0
20.0	40	23	37

3.2.2 CTC Staining

Determination of the number of actively metabolising bacteria is an important objective of aquatic microbiologists. Several methods have been applied in this field, but none of them have been proved to be fully adequate. Many methods are available

for this analysis. Three general approaches have been used to determine bacterial numbers: (1) the number of culturable bacteria has been determined by the colony count or most-probable-number techniques; (2) direct counts have been made with stains specific to biological molecules to enumerate the total bacterial numbers and (3) direct counts have been made by using stains or microautoradiography to determine the number of active bacteria.

Previous investigations have shown that the number of metabolically active bacteria is underestimated by the colony count method. A more realistic approach is the use of nalidixic acid, a specific DNA gyrase inhibitor, which suppresses cell division in many Gram-negative bacteria. Growing cells become elongated and can be detected microscopically (King *et al.*, 1988). Viable counts detected by this method (DVC) were up to 3 orders of magnitude greater than colony count (Kogure *et al.*, 1979; Ullrich *et al.*, 1996). The problem with this technique (DVC) is that elongation may occur too slowly in the absence of exogenously supplied carbon source, or it may be insufficient for microscopic detection (Ullrich *et al.*, 1996; Rodriguez *et al.*, 1992). Another method, microautoradiography, a tracer technique based on the assimilation of radiolabeled organic solutes, combined with epifluorescence microscopy has been applied as an alternative approach for enumeration of active heterotrophic bacteria (Hoppe 1976; Tabor and Neihof, 1982; Ullrich *et al.*, 1996). There are potential problems with substrate selectivity and standardisation of the procedure (Ullrich *et al.*, 1996; Peele and Colwell, 1981). Counts comparable to those reported for the nalidixic acid and microautoradiographic technique resulted from measurements of electronic transport system (ETS) activity (Maki *et al.*, 1981; Ullrich *et al.*, 1996). The universality of ETS in living cells allows this indirect measurement of respiratory activity (Ullrich *et al.*, 1996; Packard, 1971) in a wide range of organisms, including prokaryotes and eukaryotes (reviewed by Savenkoff *et al.*, 1995). Tetrazolium salts are used as artificial electron acceptors which are reduced within the respiratory chain. This results in the intracellular formation of coloured formazans, equivalent to the respiratory activity of cells. The Tetrazolium dye 2-(p-iodophenyl)-3-p-(nitrophenyl)-5 phenyltetrazolium chloride (INT) was mainly used for this purpose in aquatic environments (Ullrich *et al.*, 1996; Aristegui *et al.*, 1995; Dufour *et al.*, 1992; King *et al.*, 1988; Maki *et al.*, 1981; Tabor and Neihof, 1982). Zimmermann *et al.*, (1978), first combined the INT assay with the acridine orange (AO) direct count method for the

simultaneous determination of total bacteria numbers and the numbers of INT-reducing (actively respiring) bacterial cells by counterstaining INT-treated samples with acridine orange. This method was later modified to improve visual detectability (Ullrich *et al.*, 1996 ; Tabor and Neihof, 1982; Dufour *et al.*, 1992). In recent publications, the tetrazolium dye 5-cyano-2,3-ditolyltetrazolium chloride (CTC) was introduced to determine the number of metabolically active bacteria. CTC had been described as an indicator of respiratory activity in tumour cells (Severin *et al.*, 1985; Stellmach, 1984) and was first applied in ecological studies by Rodriguez *et al.*, (1992) in natural and nutrient amended water samples. The advantage of CTC compared with other dyes such as INT, is simple detection due to the red fluorescence in the reduced form (Rodriguez *et al.*, 1992). The CTC method has been used extensively in studies of several aquatic environments, including municipal wastewater and groundwater (Rodriguez, 1992), seawater (Gasol *et al.*, 1995; Rodriguez *et al.*, 1992), drinking water (Schaule *et al.*, 1993; Coallier *et al.*, 1995), biofilms (Schaule *et al.*, 1993), as well as soil samples (Winding *et al.*, 1994; Yu *et al.*, 1995). The CTC assay has been applied to determine the viability of coliform bacteria exposed to Antarctic conditions (Smith *et al.*, 1994), and was also used in combination with a fluorescent antibody to detect *E. coli* in water (Pyle *et al.*, 1995). Furthermore, CTC reduction combined with Rhodamine 123 (Rh123) and [^{3}H] uridine uptake has been applied in the assessment of disinfections on metabolic activity of bacteria with biofilms (Yu *et al.*, 1994). The only CTC toxic study on bacteria was reported by Ullrich *et al.*, (1996). As far as it is known, no detailed studies on CTC stained *Legionella* and biofilms as well as drinking water and water distribution system samples for flow cytometric analysis have been published.

The aim of this work was to investigate the application of CTC for the viable staining for flow cytometric analysis of biofilms and water distribution system samples . The CTC's advantages have been documented (Rodriguez *et al.*, 1992; Schaule *et al.*, 1993; Yu *et al.*, 1995; Ullrich *et al.*, 1996; Yamaguchi *et al.*, 1997) and the one of them is that its red fluorescence could be very suitable for the dual staining combined with the fluorescent antibody which is usually linked with FITC (green fluorescence) and with other DNA dyes such as DAPI for flow cytometric analysis.

3.2.2.1 CTC Staining

(i) Effect of CTC concentration on staining

A range of CTC concentrations were tested for optimising the CTC concentration for staining microorganisms from pure cultures and environmental samples using the methods outlined in Section 2. The results show that the CTC concentration was adequate between 2.0 and 4.0 mM final concentration. The maximum fluorescence density and CTC-staining percentage (80%) appeared at the range of 2.0mM to 4.0mM in CTC stained *S. aureus* and *Ps. aeruginosa* populations separately by flow cytometry with the incubation time from one and a half to three hours at the incubation temperature 37 °C (Table 3.2, Table 3.3, Figure 3.1).

Table 3.2 Effect of Stain Concentration on CTC and DAPI Staining Using *S. aureus* and FCM

CTC (mM)	Red (%) (1.5 hours)	Blue (%) (1.5 hours)	Red (%) (3.5 hours)	Blue (%) (3.5 hours)
0.0	0.1	99.8	0.1	99.8
0.25	6.6	93.4	4.1	95.9
0.50	11.9	88.1	8.1	91.2
1.0	18.2	81.7	15.1	84.8
2.0	37.1	62.8	31.6	68.3
3.0	56.7	43.2	51.1	48.8
4.0	73.2	24.4	80.0	20.0

Table 3.3 **Effect of Stain Concentration on CTC Staining Using *Ps. aeruginosa* and FCM**

CTC (mM)	CTC Stained (Red Cells %)	Fluorescence Density FL3 CHANNEL
0.0	0	102
0.25	44	335
0.50	37	315
1.0	47	334
2.0	66	440
3.0	62	448
4.0	66	412
5.0	64	367
7.0	58	355

(ii) Effect of the incubation time on the CTC staining

The effect of the incubation time on the CTC staining was investigated using the methods outlined in the Section 2. The CTC assay was performed with incubation times ranging from 0.5 to 4 hours using flow cytometry and epifluorescence microscopy (Table 3.2, Table 3.4; Figure 3.2).

Figure 3.1 **Effect of CTC Concentrations on Staining**

CTC concentration (mM); 0.0, 0.25, 0.5, 2.0, 3.0, 4.0.

Staining Time 0-1.5 h and 0-3.5 h. Bacteria *S. aureus*

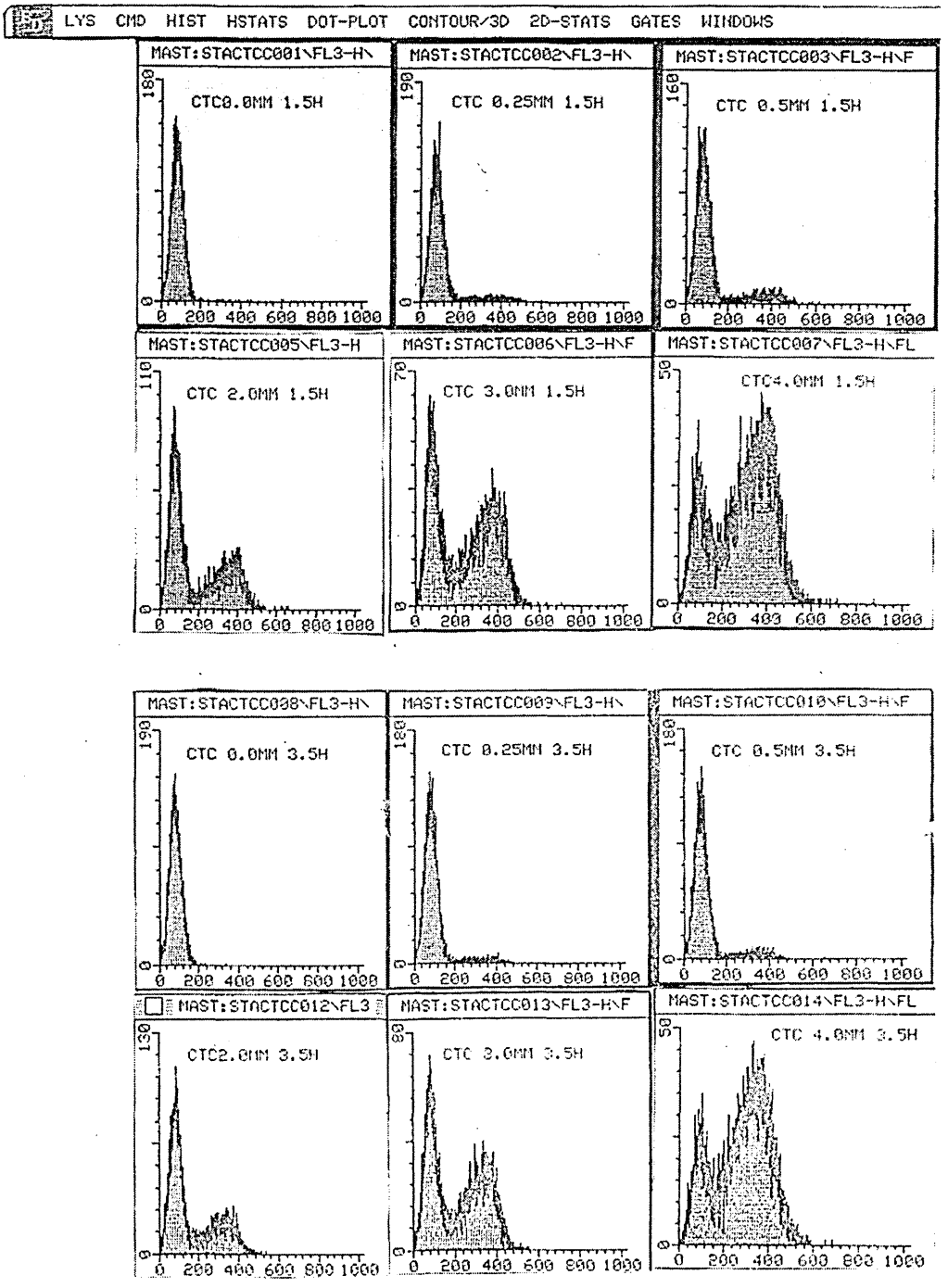


Table 3.4 Effect of Incubation Times on CTC Staining Using *S. aureus* and FCM

Time (Hours)	Peak Channel	Fluorescence Density (Red)
0.5	50	491
1.0	86	512
1.5	93	525
2.5	92	520
3.5	105	545
4.0	116	544
Control	21	134

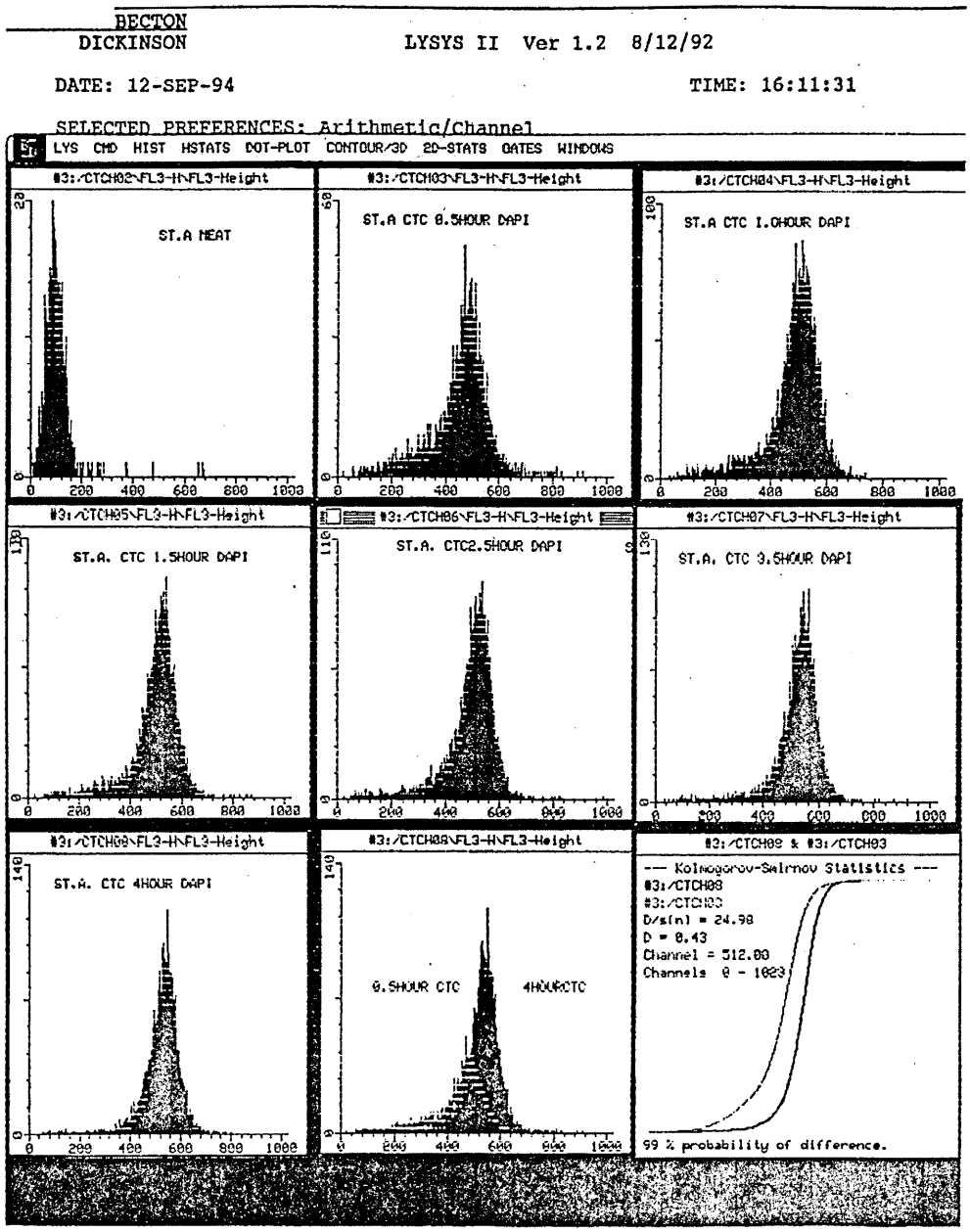


Figure-3.2 Effects of incubation time on the CTC staining

Incubation time; 0.0h, 0.5h, 1.0h, 1.5h, 2.5h, 3.5h, 4.0h at 37°C in dark .
CTC concentrations; 4mM. Bacteria; *S. aureus.*
Flow cytometry; 200mW laser power, threshold FSC.

The flow cytometric analysis results show that the stained *S. aureus* cells with 4mM CTC concentration fluoresced the maximum red colour after 2 hours and up to 4 hours incubation time at 37 °C, which agreed with the epifluorescence microscopic checking. At least one and half hours incubation time was suitable and chosen for both flow cytometry and epifluorescence microscopy and to ensure that diffusion and uptake of the CTC were not time limited.

(iii) Effect of the incubation temperature on the CTC staining

Further investigation was carried on testing the effect of incubation temperature on the CTC staining. The CTC staining was performed at the room temperature, 37 °C and 44 °C with the same CTC concentration (4 mM), same incubation time (2hours). The results show that there were no differences either from the stained *Ps. aeruginosa* or the stained *S. aureus* as well as raw water samples and treatment water samples from the water distribution system (Table 3.5).

Table 3.5 Effects of the Incubation Temperature on CTC Staining

Temperature (°C)	*S. aureus* (% red)	*Ps. aeruginosa* (% red)	Raw Water (% red)	Treated Water (% red)
22	73	65	0.8 - 4.0	0.2
37	73	66	0.9 - 4.0	0.2
44	70	62	0.9 – 3.9	02.

Note: The pure culture bacteria were stained in suspension. The raw and treated water samples were stained on membranes after filtration. All final CTC concentrations were 4 mM with 2 to 4 hours incubation times. DAPI was used for total cell staining.

(iv) Effect of the sodium pyruvate and glucose supplements on the CTC staining

It was reported that some reagents such as sodium pyruvate or glucose as well as R2A liquid medium (Schaule *et al.*, 1993) may play an activation role to the electron

acceptors in the electron chain reactions in bacteria. In the commercial R2A solid medium, there is 0.03% of sodium pyruvate (0.3 g/litre total weight).

The effect of the sodium pyruvate on CTC staining was investigated using flow cytometry and epifluorescence microscopy. The result (Table 3.6) show that for the fresh pure cultures there were no differences with or without added sodium pyruvate; but for environmental samples, such as raw water, sodium pyruvate increased the cells' fluorescent density. Large size (0.5 μm diameter) red CTC-formazan crystal particles were found in the bacterial cells stained with CTC plus sodium pyruvate, and smaller size (0.1-0.2 μm diameter) particles only with CTC without sodium pyruvate.

Table 3.6 Effects of Sodium Pyruvate on CTC staining

Samples Size	Na pyruvate (mM)	CTC (mM)	Red Cells* (%)	CTC Formazan Particles
E. coli	4.0	3	80	0.5 μm
E. coli	0	3	80	0.4 – 0.5 μm
S. aureus	4.0	3	73	Whole cell red
S. aureus	0	3	72	Whole cell red
Raw water	4.0	3	1.9 – 16	0.2 – 0.4 μm
Raw water	0	3	0.8 - 4	0.1 – 0.2 μm
Final water	4.0	3	0.7	0.2 – 0.4 μm
Final water	0	3	0.2	0.1 – 0.2 μm

***Pure cultures gave stable counts; raw waters were found to give a wider range of counts**

Samples were stained at 37 °C for 3 hours.

The effect of glucose on the CTC staining was also investigated using pure culture and raw water samples. The microscopical studies have shown that there is not much difference between with and without using glucose in the CTC staining from enumeration results and morphological characteristics. This result does not agree with the report from Schaule *et al.*, (1993) who found that glucose enhanced CTC staining. Yamaguchi *et al.*, (1997) reported that in the CTC staining, supplementation with R2A medium or Meldola's blue resulted in an increase of background noise signals for pure cultures of bacteria. This was also seen in distribution samples containing significant amounts of iron. This non-specificity could be a problem with certain samples.

(v) Staining *Legionella* by CTC

As far as it is known there is are no detailed reports for the staining of *Legionella* by using CTC for the viable determinations by both flow cytometer and epifluorescence microscopy. The aim of this work was to investigate the application of CTC for staining the viable *Legionella* for the flow cytometric analysis.

The staining of *Legionella* spp. with CTC was investigated using different incubation temperatures (room temperature, 37 °C and 44 °C) and times, as well as a range of CTC concentrations with or without supplements.

Table 3.7 Effects of Temperature on the CTC Staining of *Legionella*

Temperature (°C)	Glucose (mM)	Sodium Pyruvate (mM)	Growth Supplements (%)	Staining Result
4	0	0	0	-
4	1	0	0	-
4	0	4.0	0	-
4	0	0	1	-
20	0	0	0	-
20	1	0	0	-
20	0	4.0	0	-
20	0	0	1	-
37	0	0	0	-
37	1	0	0	-
37	0	4.0	0	-
37	0	0	1	-
42	0	0	0	-
42	1	0	0	-
42	0	4.0	0	-
42	0	0	1	-

CTC concentration 0.5 – 4 mM, staining times 3-24 h.

The results show (Table 3.7) that pure cultures of *Legionella* cells from three-day colonies or up to ten days using BCYE colony counting methods did not stain with CTC staining under experimental conditions. The *L. pneumophila* cultured by YE broth were stained by CTC but the positive proportion was only 20% by flow cytometric analysis. By reading both FACS sorting and manually made slides, the CTC stained *Legionella* cells were mostly of fresh and smaller size cells and longer cells and filaments of *Legionella* were negative for CTC staining. So, CTC staining for the *Legionella* would be not suitable for the determination of its viability. The original hope was that if CTC was suitable for the staining of *Legionella* cells, the dual staining by CTC with mAb-FITC could be very easy for determining the total and viable cells by flow cytometry. In reality, only low number cultural *Legionella* cells could be stained under the current conditions, so other dyes would be considered for adoption in the dual staining such as mAb-FITC with PI, and the details will be given later. No explanation has been found in the literature for this phenomenon.

In conclusion, CTC staining can be used for staining most pure cultures of bacteria and real samples e.g. raw water, at the range of 2.0 mM to 4.0 mM final concentration and at least 2 hours incubation times at room temperature or 37^{o}C for both epifluorescence microscopy and flow cytometric analysis. Sodium pyruvate could be used to increase the CTC staining speed for the water distribution system samples and environmental samples. For the *Legionella* species, CTC staining is not suitable due to the fact that the *Legionella* cells from the BCYE cultural medium cannot been stained and secondly that only a low proportion of CTC stained *Legionella* cells were achieved using the yeast extract (YE) broth.

3.2.3 DAPI Staining

DAPI staining was investigated using the method outlined in Chapter 2 and the results are given in Table 3.8.

Table 3.8 Effect of the Concentration and Incubation Times on DAPI Staining

Sample	DAPI (μg/ml)	Incubation Time at 37 °C		
		15 min	30 min	60 min
E. coli 10^8/ml	0.5	-	-	-
	1.0	+	+	+
	2.0	++	++	++
	3.0	++	++	+++
	5.0	+++	+++	+++
Ps. aeruginosa 10^8/ml	0.5	-	-	-
	1.0	-	-	-
	2.0	++	++	++
	3.0	++	++	++
	5.0	+++	+++	+++
S. aureus 10^8/ml	0.5	-	-	-
	1.0	+	+	+
	2.0	++	++	++
	3.0	++	++	++
	5.0	+++	+++	+++
Salmonella spp. 10^8/ml	0.5	-	-	-
	1.0	+	+	+
	2.0	++	++	++
	3.0	++	++	+++
	5.0	+++	+++	+++
Raw waters and treated waters $10^5 - 10^6$/ml	0.5	-	-	-
	1.0	+	+	+
	2.0	++	++	++
	3.0	++	++	+++
	5.0	+++	+++	+++

Comments - poor staining ++ good staining - easy reading
+ weak staining +++ good staining - bright background staining

3.2.3.1 Effects of DAPI concentrations on the staining

To determine the optimal concentrations for various samples, a range of 0.5,1.0, 2.0, 3.0, and 5.0 μg/ml DAPI (final concentrations) were tested by the methods outlined in Chapter 2. The blue fluorescence of DAPI-stained bacteria increased with an increase in the dye's concentration from 2.0 μg/ml to 5.0 μg/ml for both pure cultures and environmental samples. The optimal concentration was observed at 3.0 μg/ml and concentrations over 5.0 μg/ml caused stronger background (Table 3.8) using epifluorescence microscopy. Lebaron *et al.*, (1994) adopted 2.5 μg/ml as the optimal concentration.

3.1.3.2 Effects of the incubation time and temperature on the DAPI staining

The incubation time conditions were chosen from 15 minutes and 30 minutes up to one hour at 37 °C. The optimal incubation factor was observed by epifluorescence microscopy (EFM) from fifteen to thirty minutes (Table 3.8) for both pure cultures and environmental samples and the fifteen minute incubation time was essential for DAPI staining.

Further staining was carried out at 37 °C and at room temperature (data not presented and there was no difference in staining. This agrees with most other published literature (Kepner *et al.*, 1994; Zweifel *et al.*, 1995; Lang *et al.*, 1997). The optimal staining conditions for DAPI were chosen at room temperature (in the dark) and for at least fifteen minutes with the final concentrations between 2.0-5.0 μg/ml for both epifluorescence microscopy and flow cytometric analysis for pure cultures and environmental samples. DAPI direct counting (DAPI DC) has more suitable advantages than AODC and this will be discussed in a later chapter. Such staining conditions were also suitable for labelling the walls of the amoeba cysts and *Cryptosporidium* oocysts which agrees with the fact that DAPI is not only specific for the DNA, but also for bacteria without nucleoids (Zweifel *et al.*, 1995).

Lower DAPI concentrations could be adopted but these need a longer incubation time e.g. at least two hours with 0.5 μg/ml for marine bacteria (Monge *et al.*, 1992).

DAPI is generally believed to bind to DNA preferentially at the AT-rich region within the minor groove of B-DNA in solution, but the specificity of DAPI toward

nucleic acids has been questioned by the finding that the dye interacts not only with DNA but also with extracted or synthetic biopolymers such as double-stranded RNA, proteins and phospholipids as well as staining the bacteria which are without nucleoids. It is also questioned that DAPI staining intensity, especially from the environmental samples such as chlorinated water samples, was variable and shows poorly and highly fluorescence bacteria with different subpopulations using flow cytometry (Saby *et al.*, 1997). The reason is that the DAPI staining of bacterial cells varied with the DNA-DAPI complex and the cell envelope (Matsunaga, *et al.*, 1995; Saby *et al.*, 1997). Fortunately, all the samples were fixed first with 2% formalin to increase cell membrane permeability prior to DAPI staining in our testing.

3.2.4 HOE342 Staining

3.2.4.1 Effect of the concentrations and staining time

To determine the optimal concentration for both pure cultures of bacteria and environmental samples, a range of 0.25, 0.50, 1.0, 2.0, 4.0 μg/ml final concentrations of HO342 were tested in the methods outlined in Section 2. The blue fluorescence of HOE342 stained bacteria increased slightly with dye concentrations from 0.25 to 1.0 μg/ml, but it was unclear for reading or counting because of the poor and weak blue fluorescence. A range of HOE342 concentrations from 2.0 to 4.0 μg/ml were chosen for staining most bacteria and environmental samples because it gave a bright blue fluorescence from the stained cells which were easily read and counted. A dye concentration of 2.0 μg/ml was chosen for HOE342 staining (Table 3.9).

The incubation times were investigated at 37 °C. From 15 minute to 30 minutes, the blue fluorescence was stable and increased when incubated up to one hour. The optimal incubation time was chosen at 30 minutes at 37 °C with the 2.0 μg/ml of final concentration of HOE342 (Table 3.9).

Table 3.9 **Effects of the Concentrations and Incubation Times on HOE342 Staining**

Sample	HOE342 (μg/ml)	Incubation Time at 37 °C		
		15 min	30 min	60 min
E. coli 10^8/ml	0.25	-	-	-
	0.5	-	-	-
	1.0	+	+	++
	2.0	++	++	+++
	4.0	++	+++	+++
Ps. aeruginosa 10^8/ml	0.25	-	-	-
	0.5	-	-	-
	1.0	+	+	++
	2.0	++	++	+++
	4.0	++	+++	+++
S. aureus 10^8/ml	0.25	-	-	-
	0.5	-	-	
	1.0	+	+	++
	2.0	++	++	+++
	4.0	++	+++	+++
Salmonella spp. 10^8/ml	0.25	-	-	-
	0.5	-	-	-
	1.0	+	+	++
	2.0	++	++	+++
	4.0	++	+++	+++
Raw waters and treated waters $10^5 - 10^6$/ml	0.25	-	-	-
	0.5	-	-	-
	1.0	-	+	++
	2.0	++	++	++
	4.0	++	+++	+++

Comments - poor staining ++ good staining - easy reading
+ weak staining +++ good staining - bright background staining

The lower concentrations of HOE342 (0.5 μg/ml) have been used for staining marine bacteria with at least 2 hours incubation time for flow cytometric analysis (Monger *et al.*, 1993; Lebaron *et al.*, 1994). Unfortunately, for the fresh water samples and pure cultures, the 0.5 μg/ml concentration was too low with weak blue fluorescence to be seen in our conditions. It was necessary not only for epifluorescence microscopy but also for flow cytometry to adopt 2 μg /ml of final concentration of HOE342 for staining for our purpose. So the choice of optimal conditions must be considered with both analysis methods, and the microscopic analysis is the only way for checking or viewing the sorted target samples.

In conclusion, the Hoechst 33342 could be used to stain the pure bacterial cultures and environmental samples with 2.0 μg/ml at 37 °C in 30 minutes incubation.

3.2.5 PI Staining

A ranging of PI concentrations were tested for staining pure cultures and environmental samples using epifluorescence microscopy and flow cytometry by the methods outlined in Section 2. The results show that the optimum PI concentrations were found to be from 5.0 μg to 10.0 μg /ml (final concentration) for staining both heat treated *E. coli* and *S. aureus* (Table 3.10) by epifluorescence microscopy. The flow cytometric analysis of the PI staining showed the same result (Table 3.11) that when the PI concentrations ranged from 5.0 to 15.0 μg/ml, the positive percentages of stained *Legionella* remained at 92-93%. The optimum concentration of PI was observed at 5 μg/ml (Table 3.11).

Table 3.10 Effect of stain concentration on PI staining using heat-treated bacteria (10 minutes at 100 °C)

PI (μg/ml	*E. coli*	*S. aureus*
0.0	-	-
0.5	-	-
1.0	±	±
2.0	+	+
3.0	+/++	+/++
4.0	+/++	+/++
5.0	++	++
7.5	++	++
10.0	+++	+++

- \- Poor staining
- \+ Weak staining
- ++ Good staining - easy to read
- +++ Good staining - bright background

Table 3.11 Effects of PI Concentration on the Staining of *Legionella* by FCM

PI (μg/ml)	Total count (0.5 ml cells)	RI Background	R2 +ve Area	Mean Fluorescence	Peak Channel *
0.0	2685	1473 54%	1136 42.3%	237	168
1.0	6630	1247 18%	5407 81%	359	171
5.0	10000	932 8.0%	9269 92%	414	251
10.0	10000	754 7.54%	9313 93%	413	222
15.0	10000	795 7.95%	9222 92%	412	251

FACS Vantage Threshold FL3-H 140V, FL3-H Detector 600V.* Peak channel level

Legionella suspension 0.5 ml for each testing.

Propidium iodide has been used to stain eukaryotic cells to determine the viability of cells for a long time. In order to see if PI could be used for viable staining for bacteria, the effects of the PI on the dual staining were investigated.

The effect of the PI on the RH132 staining was shown in Table 3.12 and the numbers of green labelled cells (Rh123 positive) were reduced from 100% to 50% or 0% for *Ps. aeruginosa* and *E. coli* when the concentrations of PI was increased from 3.0 to 4.0 μg/ml, This means that dual staining of Rh123 with PI was not suitable for detecting the viability of bacteria.

Propidium iodide has been used for the direct staining of non-viable cells and also for viable staining with HOE342 (dual staining). It was suggested that dual staining with the Rh123 could also be used for viable staining (Grogan *et al.*, 1990). The effect of PI on viable staining in conjunction with Rh123 was investigated and the results are given in Table 3.12.

Table 3.12 Effect of Stain Concentration (PI) on Viability Staining of a Culture of *Ps. aeruginosa* and *E. coli*

Rh 123 (μg/ml)	PI (μg/ml)	*Ps. aeruginosa* (10^8/ml)	*E. coli* (10^8/ml)
5.0	1.0	100% green	100% green
5.0	2.0	100% green	25% red/75% green
5.0	3.0	25% red/75% green	50% red/50% green
5.0	4.0	50% red/50% green	100% red

In conclusion, PI could be used for the staining of non-viable bacterial cells by the flow cytometry. The optimal staining concentration is 5 μg /ml in final concentration at room temperature (Table 3.11). In viable staining via dual staining, the DAPI-PI could be used to indicate the viable cells (Blue with DAPI only), while the Rh123-PI due staining could not be used for the viable staining due to the unstable results. For the *Legionella* cells, the mAb-FITC with PI could be used for viable staining by flow cytometry.

3.2.6 Rh123 Staining

Rh123 is a polar, water-soluble cationic fluorescent dye and can be concentrated rapidly in living cells as a membrane potential indicator. Rh123 has been considered the best one for staining viable bacteria (Pinder *et al.*, 1994). It is limited for staining Gram-negative bacteria because of cell membrane permeability. For *Ps. aeruginosa*, the Rh123 staining percentage was only 7% even when treated with Tris-EDTA (Diaper *et al.*, 1992).

To try to overcome this problem, glutaraldehyde was first used for staining Gram- negative bacteria with Rh123. The effect of glutaraldehyde on the Rh123 staining was investigated by flow cytometry and EFM. The optimum concentration was determined as 0.01 to 0.015% for *Ps. aeruginosa* (Table 3.13 and 3.14), and also for *E. coli* (Table 3.15). At the above range, the staining percentage was from 90 to100% and with higher fluorescence density. Since the first use of Rh123 for staining bacteria (Matsuyama, 1984), the application of Rh123 has been limited to pure cultural species only (Diaper *et al.*, 1992; Kroll *et al.*, 1993; Pinder *et al.*, 1994), one of the main reasons is that Rh123 is only suitable for staining the Gram-positive bacteria, this can be overcome using gluteradehyde which will largely extend the application of Rh123 in the determination of the bacterial viability for both Gram- negative and positive species.

Effects of Rh123 concentrations, incubation time and temperature were also investigated and the optimum concentration was 5 μg/ml (Table 3.14) in final concentration which agreed with other reports (Kroll *et al.*, 1993) and for 30 minutes incubation at RT or 37 °C.

Table 3.13 Effect of Rh 123 Concentration on Staining on *Ps. aeruginosa* using FCM

Rh 123 (μg/ml)	Gluteraldehyde (%)	Fluorescence Density (Green)
0	00.01	149
0	0.01	159
0.5	0.01	233
1.0	0.01	272
2.0	0.01	307
3.0	0.01	326
4.0	0.01	343
5.0	0.01	360
7.5	0.01	347
10.0	0.01	372

Table 3.14. Effect of Gluteraldehyde Concentration on the Staining of *Ps. aeruginosa*

Rh 123 (μg/ml)	Glutaraldehyde (%)	Staining (%)	Direct Count (No per ml EFM)
5	0.0025	6	1.26×10^8
5	0.005	10	1.96×10^8
5	0.01	100	1.96×10^9
a			1.70×10^9

Table 3.15 **Effect of Glutaraldehyde on RH 123 Staining (5.0 μg/ml) (FCM) Using *E. coli***

Gluteraldehyde (%)	Fluorescence Density (Green)
0	204
0.005	251
0.0075	292
0.01	410
0.15	458
0.02	446
0.04	450
0.05	427

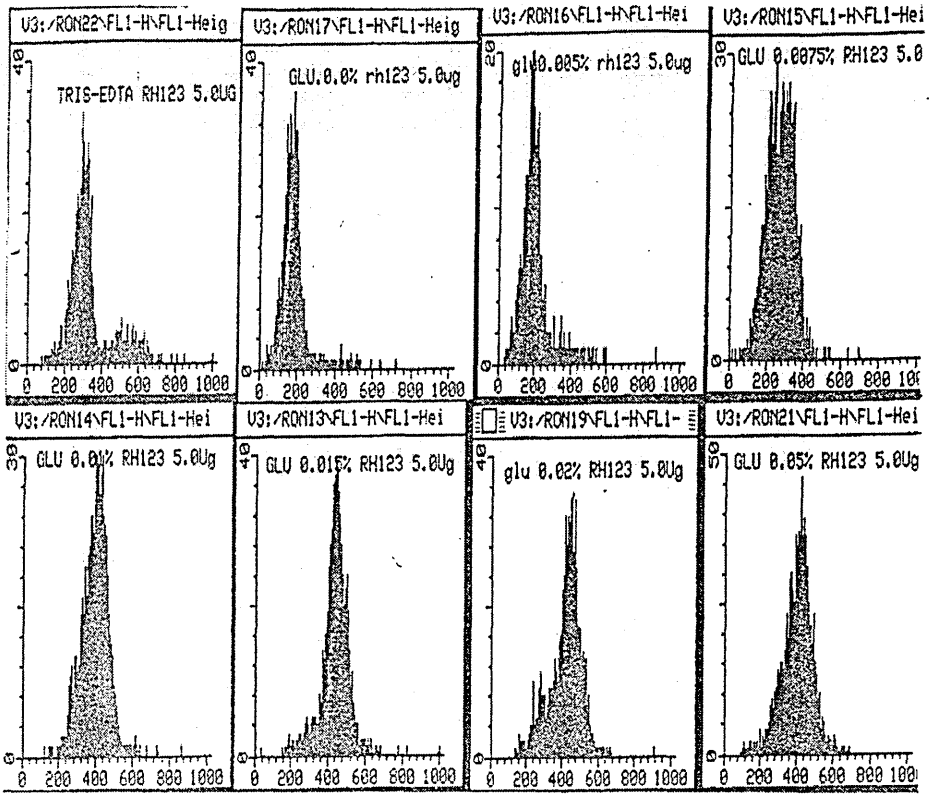


Figure-3.3 Effect of glutaraldehyde on the Rh123 staining of G(-) bacteria.
Rh123 concentration; 5μg/ml. Glutaraldehyde % (v:v); 0.0%, 0.005%, 0.0075%0.01%, 0.015%, 0.02%, 0.05%. Bacteria; *E. coli*. Flow cytometer; laser power 200mW, threshold; FSC.

In conclusion, Rh123 staining can be used for detecting and enumerating bacterial viability at a final concentration 5 μg/ml for 30 minutes at RT, and for Gram-negative species, the glutaraldehyde could be used (0.01 – 0.015%) to improve the membrane permeability and extend Rh123 viable staining to the Gram-negative bacterial species.

3.2.7 Staining *Legionella* Using mAb-FITC Immunofluorescence Probe

The commercial monoclonal antibody kit (Sanofi, France) was chosen as the special dye for staining *L. pneumophila* from water samples after screening three kinds of antibodies which were used in the routine testing of water samples at Yorkshire Environmental Solutions' pathogen laboratory (Bradford).

3.2.7.1 Effects of the concentration on the staining

The effect of the monoclonal antibody's concentration (%) was investigated by the methods outlined in the Chapter 2. Table 3.16 shows the flow cytometry of a range of mAb concentrations from 3.1 to 50% for staining the suspension of a pure culture of *L. pneumophila* (NCTC 12821) and the optimal concentration of mAb was between 12.5% and 25% for both 30 minutes and 60 minutes incubation time at 37 °C. Flow cytometry demonstrated that when using 50% of monoclonal antibody (1:1,V/V, final %), the background noise was very high and this concentration would not be suitable for the flow cytometric analysis of *Legionella* spp.

Table 3.16 Effect of mAb Concentrations (%) on Staining *L. pneumophila* by FCM Counting

MAb (%)	3.1	6.25	12.5	25	50	Incubation Time (min)
T-Count	1711	6459	16485	23720	33041	30
+ve Count	1343	5340	14584	19005	14506	30
+ve/T-C	71%	77%	80.6%	76.6%	38%	30
T-Count	4062	11511	25280	28840	53066	60
+ve Count	3166	9641	22735	23650	26463	60
+ve/T-C	70.4%	78%	86.6%	82%	49%	60

Further investigations by comparison of the flow cytometry of mAB staining with epifluorescence microscope analysis using DAPI direct counting (Table 3.17) indicated that when *L. pneumophila* was stained with the monoclonal antibody kit using 12.5 - 25% concentrations at 37 °C for 60 minutes incubation time, the enumeration results were very close (Table 3.16, 3.17); DAPI DC: 2.18×10^4 ; mAb-FITC by FCM: $2.1\text{-}2.3 \times 10^4$ and $2.06\text{-}2.2 \times 10^4$ by EFM for the same pure culture of *L. pneumophila.*

Table 3.17 Effect of mAb (%) on Staining for *L. pneumophila* by Direct Counting

mAb (%)	Incubation Time/Direct Counting			
	30 min	No. x 10^3	60 min	No. x 10^3
0	-	0	-	0
3.1	++	2.4	++	4.4
6.25	++	6.24	++	12.4
12.5	++/+++	13.2	++/+++ Good	20.64
25	++/+++	20.6	+++ Good	22.3
50	+++	17.8	+++ Good	20.6

DAPI total counting 2.18 x 10^4

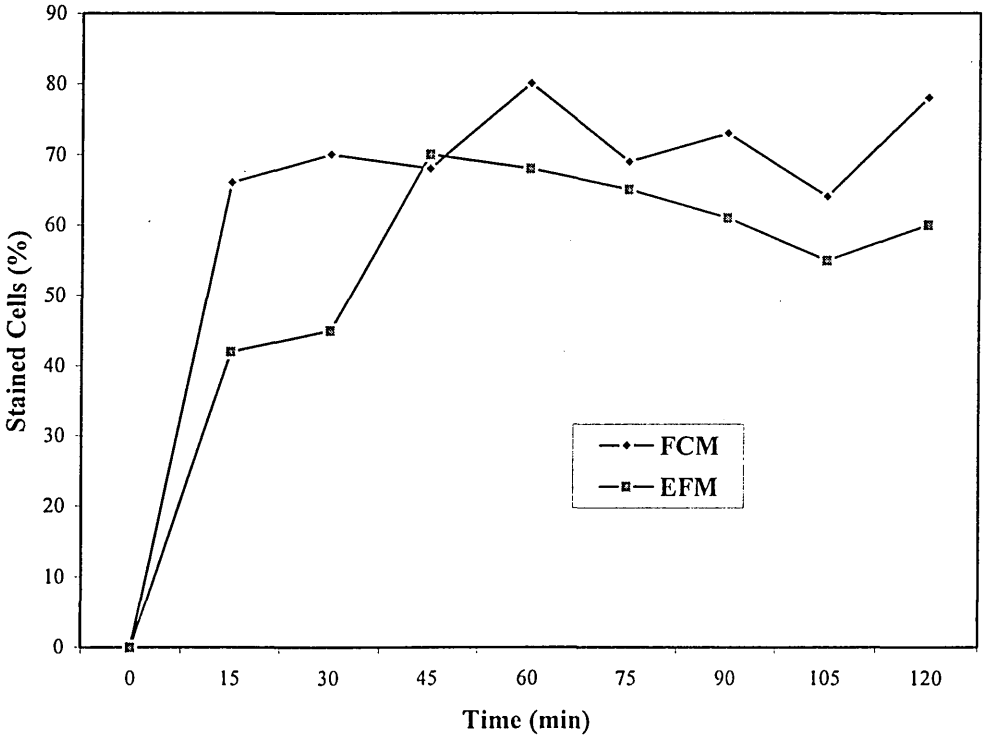
3.2.7.2 Effects of the incubation time on the staining

The effects of the incubation time on the mAb staining for *Legionella* spp. were investigated by flow cytometry (Table 3.18, Figure 3.4). The results show that from 15 to 120 minutes of incubation times, the highest positive percentage (80%) of mAb labelled *Legionella* cells is from the 60 minutes incubation time, which agreed with results obtained by comparison with epifluorescence microscopic analysis (Table 3.18). Flow cytometry of the mAb staining for *Legionella* show that the 30 minutes incubation time suggested by the manufacturers of the mAb Kit should be extended to 60 minutes.

Table 3.18 Effect of Incubation Time on the mAbs-FITC Staining

Voltage/Time	15'	30'	45'	60'	75'	90'	105'	120'	-VE
600V No. +VE	66%	70%	68%	80%	69%	73%	64%	78%	14% FCM
550V No. +VE	41%	39%	37%	40%	51%	51%	41%	46%	5.5% FCM
500V No. +VE	12%	25%	21%	31%	32%	33%	28%	32%	1.3% FCM
EFM BG/Tot.	42%	45%	70%	68%	65%	61%	55%	60%	0% EFM

Figure 3.4 Effect of Incubation Time on the mAb FITC Staining for *Legionella* X; incubation time (min). Y; percentage of mAb FITC stained cells



3.2.7.3 Dual staining of *Legionella* for detecting total and viable cells

The original hope was that CTC-mAB-FITC could be ideal for the viable analysis but in fact the CTC could not be used due to the difficulty in staining *Legionella* species. So the PI-mAb FITC duel staining was investigated for the viable staining of *Legionella* by flow cytometry. Figure 3.5 shows that heat killed *Legionella* cells were stained by the PI (with strong red fluorescence) and mAb FITC (with green fluorescence also) and the viable cells were only stained by mAb-FITC with green colour. The current limited work only show the viable analysis, and in the staining, full washing must be carried out to wash out the remaining PI on the cells after the PI staining. For accurate viable enumerating, further work would be needed.

Figure-3.5 Dual staining by PI and mAb-FITC for heated *Legionella*
MAb-FITC; 12.5% (V:V). PI; 5.0µg/ml.
Flow cytometer; laser power; 200mW, threshold; FSC, PMT levels; 550v, 600v, 650v. Bacteria; *L. pneumophilla.*

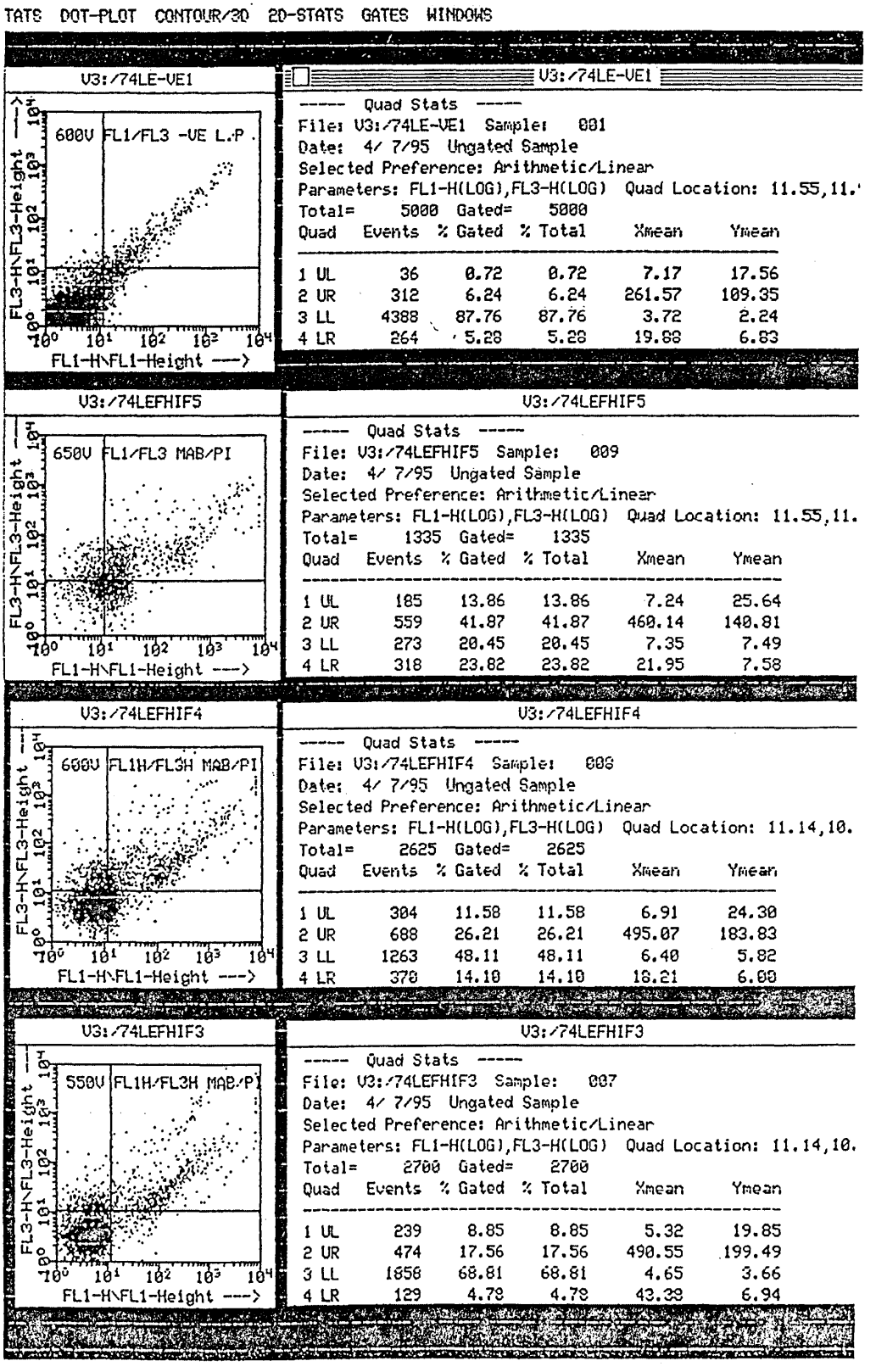
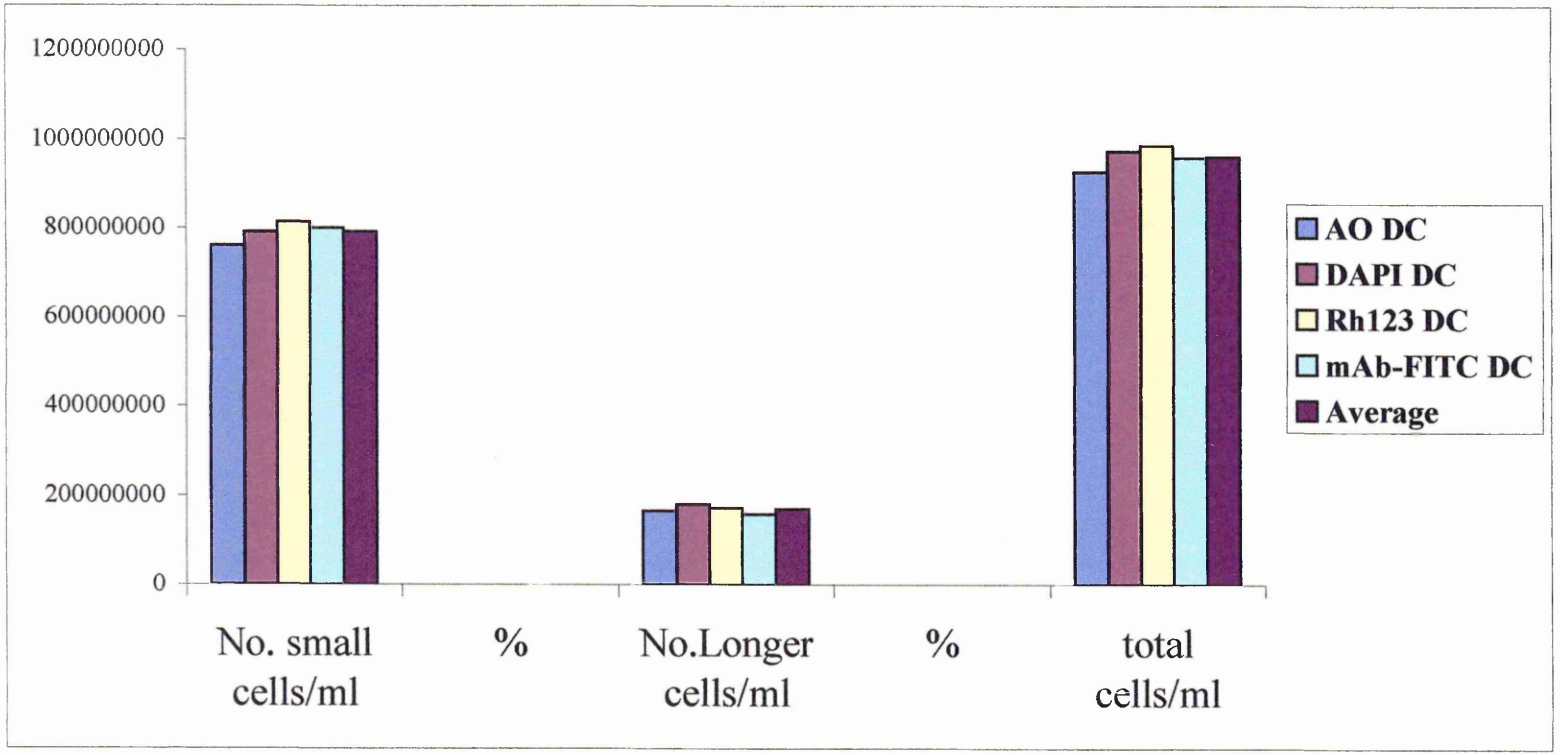


Table 3.19 Comparison of Different Dyes with Colony count for Enumerating Pure Cultures of *Legionella*

Dyes	No. Small Cells/ml	%	No. Longer Cells/ml	%	Total Cells/ml	DC/BCYE PC %
AO DC	7.6×10^8	81.9	1.66×10^8	18.0	9.28×10^8	92.8
DAPI DC	7.92×10^8	80.0	1.82×10^8	20.0	9.74×10^8	97.4
Rh123 DC	8.14×10^8	82.5	1.73×10^8	18.0	9.87×10^8	98.7
MAb-FITC DC	8.0×10^8	83.3	1.60×10^8	17.0	9.60×10^8	96.0
Average	7.92×10^8	81.9	1.70×10^8	18.0	9.62×10^8	96.23
SD	22,883,764		9,464,874		25,355,801	
CV(%)	3		6		3	
PI DC	8.16×10^7	59.2	8.45×10^7	51.0	1.66×10^8	16.7
CTC DC				n/a		
BCYE PC (cfu/,ml)					1.0×10^9	100

Figure 3.6 **Comparison of Direct Count with Colony count for Enumerating *Legionella***



The comparisons of different dyes for staining of *Legionella* cells were carried out by using AO, DAPI, Rh123, PI, and CTC, mAb-FITC and the results are given in the Table 3.19 and Figure 3.6. The average percentage of the stained *L. pneumophila* cells by the four dyes (AO, Rh123, DAPI, mAb-FITC) is 96.23% compared with BCYE colony counts (CFU), the Rh123 direct count and DAPI count shown the higher counting results (98.7% Rh123, 97.4% DAPI), mAb-FITC direct counting shows 96.23%. CTC staining was negative for the *Legionella* suspension from the three-day CFU by BCYE media. PI direct counting showed only 16.7% of *Legionella* cells had been stained with PI.

The current work indicated that the flow cytometry of monoclonal antibody staining for *L. pneumophila* demonstrated suitable mAb staining conditions, and the 12.5-25% of mAb concentrations (final %) at 37° C with 60 minutes incubation time showed the best fluorescence intensity with less background noise for both flow cytometry and epifluorescence microscopy and were subsequently used in further studies for staining legionellae from water samples.

3.3 DISCUSSION

AO and DAPI worked well for rapidly staining bacteria for direct counting of the total population by EFM and flow cytometry (for DAPI only). CTC, Rh123 and HOE342 were used to directly count viable bacteria in pure cultures, raw waters and final treated waters from the water works by EFM and flow cytometry.

The CTC stained bacteria were very easy to count because the CTC-formazan fluoresces primarily in the red region of the visible spectrum. It was readily distinguishable from most background fluorescence and autofluorescing abiotic particles, which typically emit in the blue-green region of the visible spectrum in most natural water samples. The CTC-DAPI dual stained samples in the same preparation could be viewed simultaneously with a 365nm excitation filter and a 450 nm emission filter, both viable (with respiring activity) bacteria with red crystal CTC-formazan particles and the total population (with blue colour) were clearly read by epi-fluorescence microscopy.

In the raw water samples, the CTC microscopic counts exceeded the YEA and R2A colony counts by nearly 3 orders of magnitude and 1 order of magnitude respectively. These data suggest that the CTC staining method might provide a more sensitive indicator of viable (i.e. actively respiring) bacteria in environmental samples. Sodium pyruvate was

first used to improve the CTC staining for the environmental samples and it largely increases the CTC staining speed.

Fluorescein diacetate (FDA) had been tested for the determination of living cells in the current work and the main disavantage was that the dye must be dissolved by using the organic solution acetone and the FDA stock and working solution could not be filtered by using the membranes or syringe filter (0.22 μm size) for keeping the particles free before staining the bacteria. The fluorescence density from the stained bacterial cells was weak and so FDA was not suitable for staining the bacteria in environmental samples under the current limited work (data not presented). Tsuji *et al.* (1995) also reported that the FDA method has the disadvantage that many species of soil microorganisms fail to stain and FDA does not efficiently penetrate some types of membranes, and the fluorescein produced from FDA tends to leak from cells. The new fluorescence probe 5-(and 6-) sulfofluorescein diacetate (SFDA) has been adopted to replace the FDA (Tsuji *et al.*, 1995).

Rh123 and HOE324 were also good stains for the direct counting of viable bacteria with a greater number being counted than with colony counts and CTC counts, which might contain total viable bacterial populations (VC+VNC). The DAPI direct count was for total bacterial population and gave the highest number of cells which included all viable and non-viable bacteria (VC+VNC+NV). Rh123-CTC dual stained samples also could be counted in the same preparation (black filter membrane filtration) simultaneously with a 510 nm emission filter for detecting and enumerating viable bacteria.

The direct counting by EFM was a very useful and fast method for the rapid detection and enumeration of bacteria stained with fluorescent dyes, and the CTC-DAPI dual staining was very suitable for viable detection and enumeration of both pure cultures and environmental samples. The dual stain CTC-DAPI will be used in this study. It might be possible that different viable stains will reflect different metabolic states in environmental bacteria and this will be considered in future work.

In conclusions work has been undertaken to screen and determine the dyes and their staining conditions for the flow cytometric analysis of bacteria from pure culture and environmental samples (Table 3.20). The following dyes and conditions have been adopted for the purposes of this thesis:

DAPI will be adopted for the total direct counting of bacteria as well as protozoa by flow cytometry and epifluorescence microscopy. DAPI will also be used in the determination of viability by the dual staining with CTC (CTC-DAPI), and Rh123 (Rh123-DAPI), as well as PI (DAPI-PI). The CTC-DAPI dual staining can be the best way for the viable staining.

CTC will be adopted for the determination of the bacterial viability (respiring viability) by both flow cytometry and epifluorescence microscopy. The 2-4mM final concentration with as least 2 hours incubation time in the dark can make most viable bacterial species show their respiring activity. The CTC (reduced CTC) with red fluorescence can be easy used with blue (DAPI, HOE342), green (Rh123, mAb-FITC) in dual fluorescence staining. Sodium pyruvate can be used in the CTC staining for increasing the CTC staining speed for the environmental bacteria. CTC will not be used in the staining of *Legionella* under the current conditions.

Rh123 will be adopted in staining both Gram-positive and Gram-negative bacteria for the viable staining by flow cytometry and microscopy. The pre-treatment of Gram-negative bacterial cell walls by using the glutaraldehyde will extend the application of the Rh123 for staining viable Gram-negative bacteria.

PI will be used for staining the dead cells and also can be adopted in the total counting of the bacteria after the pre-treatment (heat treatment, or fixing). In the dual staining PI can be used with DAPI (DAPI-PI) for viable staining, or with mAb-FITC for determining special targets such as *Legionella*. PI can be used in both flow cytometry and microscopy. The Rh123- PI dual staining will not be adopted in the project due to the unstable staining.

In conclusion of the flow cytometry of monoclonal antibody staining for *L. pneumophila* in this study demonstrated suitable mAb staining conditions, and the 12.5-25% mAb concentrations at 37 °C with 60 minutes incubation time showed the best fluorescence intensity with less background noise for both flow cytometry and epifluorescence microscopy and were subsequently used in further studies for staining legionellae from water samples.

The current work to screen the dyes and staining of microorganisms for flow cytometric analysis of bacteria was demonstrated that the AO, CTC, DAPI, PI, Rh123 and mAb can be used in the determination of bacteria and the conclusion is in Table 3.20.

Table 3.20 Outlook for the Dyes for Staining Bacteria for Flow Cytometry

Name of Dye	Total Sorting	Viable Sorting	Special Target Sorting
AO	EFM	N/A	N/A
DAPI	FCM/EFM	N/A	N/A
CTC	N/A	FCM/EFM	N/A
CTC -DAPI	FCM/EFM	FCM/EFM	N/A
CTC-Rh 123	N/A	FCM/EFM	N/A
PI	FCM/EFM	N/A	N/A
DAPI/PI	FCM/EFM	FCM/EFM	N/A
Rh 123	N/A	FCM/ECM	N/A
Rh 123-PI	N/A	N/A	N/A
mAb-FITC	N/A	FCM/EFM	FCM/EFM
mAb-FITC-CTC	N/A	FCM/EFM	FCM/EFM
DAPI-mAb-FITC	FCM/EFM	N/A	FCM/EFM
PI-mAb-FITC	FCM/EFM	EFM/EFM	FCM/EFM
DAPI-CTC-mAB-FITC	FCM/EFM	FCM/EFM	FCM/EFM

4.0 DETERMINATION OF BACTERIA IN WATER DISTRIBUTION SYSTEM AND TAP WATER BIOFILMS

4.1 Introduction

The microbiological quality of drinking water is an issue of global concern. Among the possible sources of microbial contamination are surface-associated biofilms, which are common in drinking water systems. The methods required for evaluation of the bacteriological quality of potable water are often based on cultivation of planktonic bacteria in a sample, such as heterotrophic colony counts (HPC), such as YEA and R2A. However, subculture techniques often require lengthy incubation times of several days or more, thus, there is a need for more rapid and convenient monitoring methods for quantitative assessment of the viability of microorganisms and total biomass.

The evolution of the bacterial quality of drinking water in the water distribution systems (Networks) is a major interest. Many studies dealt with this problem and attempted to correlate bacterial regrowth with turbidity, free disinfectant residual and temperature (Goshko *et al.*, 1983; McCoy and Olson, 1986). However, the correlation coefficients were low. In order to better understand the development of bacteria in the system, more recent investigations into the formation of biofilms Manz *et al.*, 1993; Rogers *et al.*, 1994a, b) and of biodegradable dissolved organic carbon (BDOC) in distribution systems (Servais *et al.*, 1992; Norton and LeChevallier, 2000) have taken into account the residence time of water in the system on the water biological quality (Kerneïs *et al.*, 1995).

Recently, some novel methods have been reported to be used in determination of bacteria in aquatic environments (Watkins and Jian, 1997) which include direct counting methods by using novel DNA probes with EFM, and flow cytometry. But for the water distribution system, there are a few papers have been reported. Schaule *et al.*, (1993) reported that CTC was used to quantify the planktonic and sessile respiring bacteria in drinking water and biofilms. Watkins and Jian (1997) reported the use of the flow cytometer with PI staining for enumerating bacteria in water distribution systems.

The aim of this work is to assess conventional microbiological methods for analysis of microorganisms in water distribution system and to compare these with the

applications of the flow cytometer. Real water distribution system samples and tap water biofilms will be have been investigated and are reported here.

4.2 DETERMINATION OF BACTERIA IN WATER DISTRIBUTION SYSTEMS

4.2.1 Colony counting bacteria in water distribution system

The water distribution system samples were analysed by conventional microbiological methods i.e. colony counts. The results are given in Table 4.1 and shown that all 90 samples from the nine different water distribution systems, replicated 10 times,did not contain coliform bacteria in 100ml and met the drinking water standard (coliform <1cfu/100ml), but with high cfu numbers of total bacteria. For the YEA 1 day testing, the average density of cfu/ml was 2 cfu/ml in 90 samples, and 5 cfu/ml by YEA 3 day colony counts; 256 cfu/ml by the R2A colony counts. The ratios of three colony counting methods (n=90) were 0.78% (YEA1d/R2A), 2% (YEA3d/R2A). The statistical analysis shows no correlation between YEA1d PC with YEA3d PC (r=-0.0832 n=90), but good positive correlation between YEA 3d PC with R2APC (r=0.6090, n=90). The average CVs of these colony counting results for total bacterial cfu in three methods was 106.3%.

4.2.2 Direct Counting of Bacteria in Water Distribution Systems

The samples from water distributions systems were analysed by the direct counting methods outlined in the Chapter 2 and the results were shown in the Table 4.1 and Table 4.2.

The direct counting of the DAPI stained bacteria gave the total densities of the bacterial population in the water distributions systems. The average bacterial numbers were high, up to 174525 cells /ml in a total of 90 samples from ten different water distribution systems, with 10.4% CV in average. The DAPI DC results were 680 times higher than the R2A colony counting results and the ratio of the R2A with DAPI DC was 0.15%. The similar results was reported by Schaule, *et al.*, (1993) and their total counting numbers by DAPI direct counting were from 10^5 to 10^6 /ml. The relationship between R2A PC (total cfu) and DAPI DC (total cells) was not very significant and the r

value ranged from 0.2995 (sample 3, n = 10) to 0.8744 (sample 9, n = 3) with an average of 0.5734 in a total of 9 samples.

Table 4.1 Determinations of Bacteria in Water Distribution Systems by Direct Counting and Colony counting Methods

Sample	Mean Value (n = 10)	Correlation Ratio	
Sample 1			
YEA 1D cfu/ml	0		
YEA 3D cfu/ml	7.3	0.5583	YEA 3D/DAPI
R2A 7D cfu/ml	212.7	0.9570	R2A 7D/YEA 3d
DAPI DC No/ml	195,586	0.4884	R2A 7D/DAPI
Sample 2			
YEA 1D cfu/ml	0.4		
YEA 3D cfu/ml	0.7	-0.2492	YEA 3D/DAPI
R2A 7D cfu/ml	46.6	-0.2297	R2A 7D/YEA 3d
DAPI DC No/ml	191,300	0.7984	R2A 7D/DAPI
Sample 3			
YEA 1D cfu/ml	0		
YEA 3D cfu/ml	0.7	0.3131	YEA 3D/DAPI
R2A 7D cfu/ml	104.1	0.6614	R2A 7D/YEA 3d
DAPI DC No/ml	168,044	0.2995	R2A 7D/DAPI
Sample 4			
YEA 1D cfu/ml	0.3		
YEA 3D cfu/ml	0.7	0.4320	YEA 3D/DAPI
R2A 7D cfu/ml	86.7	0.6386	R2A 7D/YEA 3d

Table 4.1 continued

Sample	Mean Value (n=10)	Correlation	Ratio
DAPI DC No. ml	160,148	0.4318	R2A/DAPI
Sample 5			
YEA 1D cfu/ml	0		
YEA 3D cfu/ml	1.5	-0.4285	YEA 3D/DAPI
R2A 7D cfu/ml	248	-0.1122	R2A 7D/YEA 3d
DAPI DC No/ml	176,890	0.6481	R2A 7D/DAPI
Sample 6			
YEA 1D cfu/ml	0.4		
YEA 3D cfu/ml	3.9	0.3351	YEA 3D/DAPI
R2A 7D cfu/ml	304	0.5709	R2A 7D/YEA 3d
DAPI DC No/ml	164,170	0.3126	R2A 7D/DAPI
Sample 7			
YEA 1D cfu/ml	0.8		
YEA 3D cfu/ml	9.1	-0.3136	YEA 3D/DAPI
R2A 7D cfu/ml	803	-0.1021	R2A 7D/YEA 3d
DAPI DC No/ml	171,570	0.4757	R2A 7D/DAPI
Sample 8			
YEA 1D cfu/ml	0		
YEA 3D cfu/ml	18	0.3786	YEA 3D/DAPI
R2A 7D cfu/ml	393	0.6059	R2A 7D/YEA 3d
DAPI DC No/ml	198,710	0.8639	R2A 7D/DAPI
Sample 9			
YEA 1D cfu/ml	0.4		
YEA 3D cfu/ml	2.1	0.592	YEA 3D/DAPI
R2A 7D cfu/ml	102	0.3346	R2A 7D/YEA 3d
DAPI DC No/ml	143,940	0.8744	R2A 7D/DAPI

The individual data points for this Table can be found in Appendix A

Table 4.2 Analysis of the Bacteria in Water Distribution System by Direct Counting and Colony counting, Total Samples 9; n=10 for Each Overall Mean Result

Test	Total Mean Value	S. D	CV (%)	Correlation Ratio	
YEA 1D cfu/ml	0.26	0.278887	109	-080832	YEA 1D/YEA 3D
YEA 3D cfu/ml	4.9	5.791469	118	0.6090	YEA 3D/R2A
R2A 7D No. ml	256	234.9409	92	0.1423	R2A/DAPI DC
DAPI/DC No/ml	174,525	18067.32	10.40	0.4990	YEA 1D/R2A

The determination of the bacterial viability in water distribution systems was carried out by CTC viable staining and direct counting by EFM. The results are given in Table 4.3 and show that, for the raw water samples, the ratio of CTC positive cells against DAPI stained cells was 0.5 – 72.9% and the ratio of R2A PC with CTC DC was 1.5% - 33%. For the final samples, the ratios of CTC with DAPI DC was 0.4% - 9.2% (ratio R2A PC/CTC DC). The calculated CTC positive cells removal rate between raw and treated water was 94%, DAPI DC cells removal rate was 86%, and cfu (by R2A PC) removal rate was 99.6% high.

Table 4.3 Determination of Bacterial Viability in Water Distribution Systems

Name/Sample Number	Raw/82	Final/81	Raw/84	Final/83	Raw/65	Final/68	Raw/21	Final/20
CTC DC No/ml	960	320	240	160	42,105	33,684	498,240	0
DAPI DC No/ml	28,160	47,360	50,560	39,360	2,760,000	364,000	683,520	8,160
YEA 1D cfu/ml	2	0	0	0	264	8	1340	5
YEA 3D cfu/ml	2	2	0	1	928	4	N/A	N/A
R2A 7D cfu/ml	320	14	6	6	2,560	15	7,440	8
CTC/DAPI (%)	3.40	0.70	0.50	0.40	1.50	9.20	72.90	N/A
R2A/CTC (%)	33	4.30	2.50	3.70	6	0.04	1.50	N/A

Raw = Untreated Water Final = Treated water

N/A = Not applicable

4.2.3 Flow cytometric analysis of the bacteria in water distribution system

The water distribution systems samples were analysed by flow cytometry and the results are given in Table 4.4, Figure 4.1, Table 4.5 and Figure 4.2. The fresh samples were fixed by using 2% formalin (final concentration), stained with PI by the methods outlined in Chapter 2, and then sorted using the FCM. Tables 4.4 and 4.5 show that most of the FCM counting results agree well with the EFM results apart from samples 160806 and 160807. The higher counting could be from tiny particles stained by PI, which may be smaller in size than the cells.

Table 4.4 **Flow Cytometric Analysis of Water Distribution System Samples Stained with PI at 10 μg/ml**

Test Method	Counts (Numbers per ml for direct counts, cfu/ml for colony counts)							
Sample No	160805	160806	160807	160808	160809	160810	160811	160813
FCM	50,500	40,000	24,000	8,100	8,250	14,000	25,000	40,000
EFM	48,000	18,000	8,600	7,800	9,600	13,000	24,000	38,000
YEA1D	0	2	0	0	0	1	1	0
YEA3D	1	3	0	0	2	4	4	0
R2A	19	0	18	8	46	173	145	99
FCM/EFM	104%	220%	300%	103%	85%	107%	104%	105%

A control sample using RO water and FCM had a count of 700 per ml.

Figure 4.1 **A Comparison Between FCM and EFM Analyses of Bacteria in Water Samples**

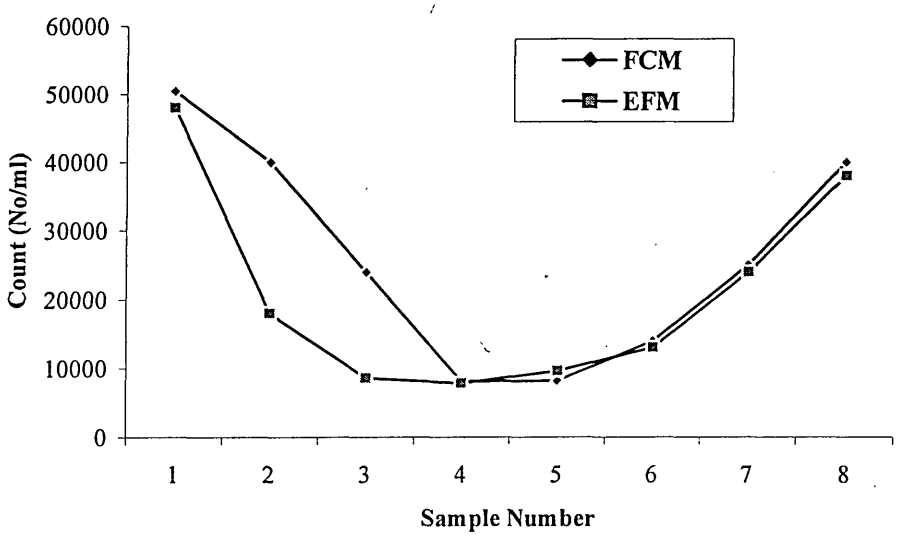


Table 4.5 **Flow Cytometry Analysis of the Bacteria in the Water Distribution System**

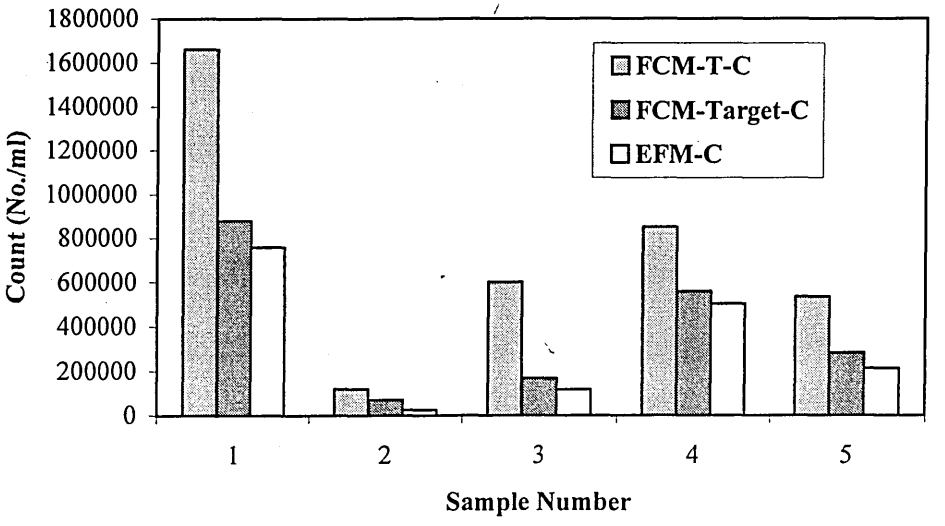
Sample Number	Counts (No/ml)			EFM/FCM
	FCM -TC	FCM-Target-C	EFM	
16113433	1,660,000	880,000	760,000	86.3%
16113434	120,000	70,000	24,000	34%
16113435	600,000	168,000	117,000	70%
16113436	850,000	560,000	504,000	90%
16113444	536,000	284,000	212,000	74%

FCM - TC = Flow cytometry, total counting

FCM - Target - C = Flow cytometry, bacterial counts in the sort region

EFM = Epifluorescent microscopy total bacterial counting

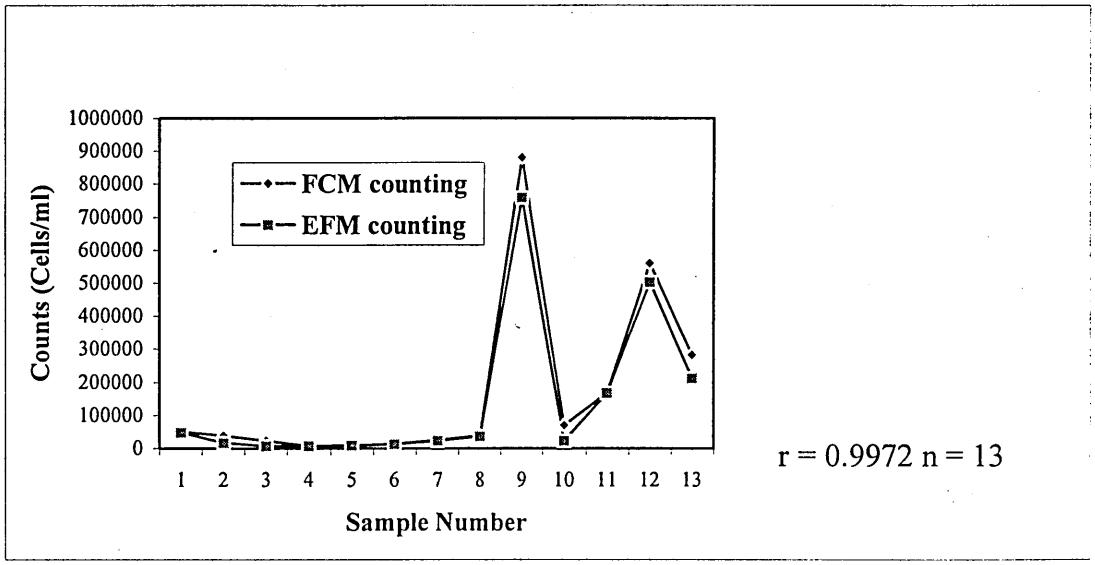
Figure 4.2 Flow Cytometric Analysis of Bacteria in Water Distribution Systems



Note: PI staining 10 μg/ml, DAPI staining 5 μg/ml. Samples were fixed with 2% formalin. Laser power 160mW, 488 nm, FL3-H 600V, threshold 140 V.

The statistics results show that there is a strong positive correlation relationship ($r = 0.9972$, $n = 13$) between flow cytometric analysis and epifluorescence microscopic direct counting for counting bacteria in the water distribution systems using samples stained with PI and DAPI.(Figure 4.3).

Figure 4.3 Comparison of the FCM Analysis with EFM Direct Counting



4.3 THE FLOW CYTOMETRIC ANALYSIS OF *LEGIONELLA* AND AMOEBAE IN TAP WATER BIOFILMS

The biofilms in a tap water supply were formed with seeded *L. pneumophila* and maintained for 4 weeks at 30 °C, and aerated with filtered air and the pH, DO and temperature were monitored on-line. Both planktonic and biofilm samples were collected for colony counting, microscopic and flow cytometric analysis.

The tap water biofilms with seeded *Legionella* (NCTC12821) were analysed by using the FACS flow cytometer and BCYE colony counting methods as well as epifluorescence microscopy.

Table 4.6 Recoveries of Flow Cytometric Sorting of *L. pneumophila* on Slides from Tap Water Biofilms

Days 30° C in water	0	3	7	14	21	28	Mean	Corr.
Biofilm Sample No.	1f	2f	3f	5f	6f	7f		
No. cells FCM	0	836	122	231	171	122	247	0.9929
No. cells EFM	0	769	112	231	120	36	211	
Recovery (%)	N/A	92	92	100	68	29	76	
Days 30 °C	0	3	7	14	21	28		
Planktonic Sample No.	1p	2p	3p	5p	6p	7p		
No. cells FCM	0	778	573	884	193	39	411	0.9980
No. cells EFM	0	784	523	908	180	39	406	
Recovery (%)	N/A	100.8	91.5	102	93	100	98.3	

N/A = Not applicable

Figure 4.4a Recoveries of FCM Sorting of *L. pneumophila* Cells From Tap Water Biofilms

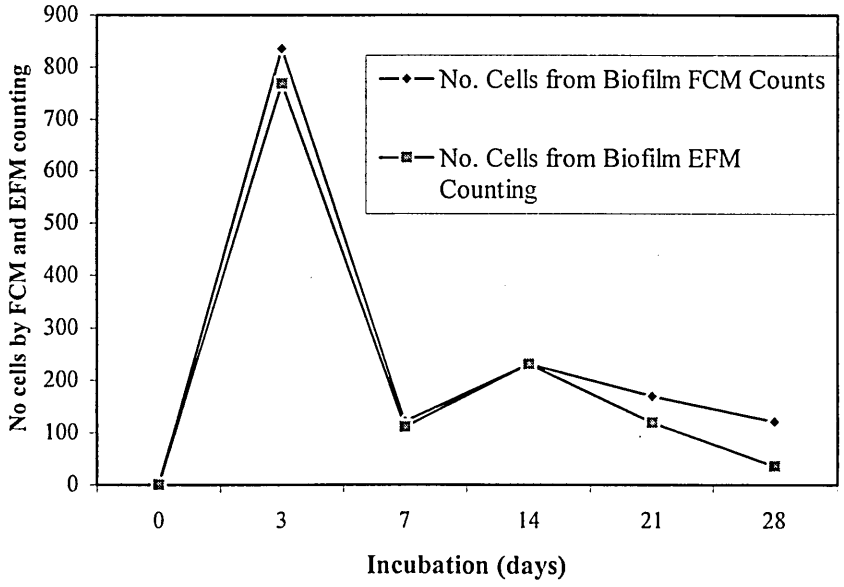
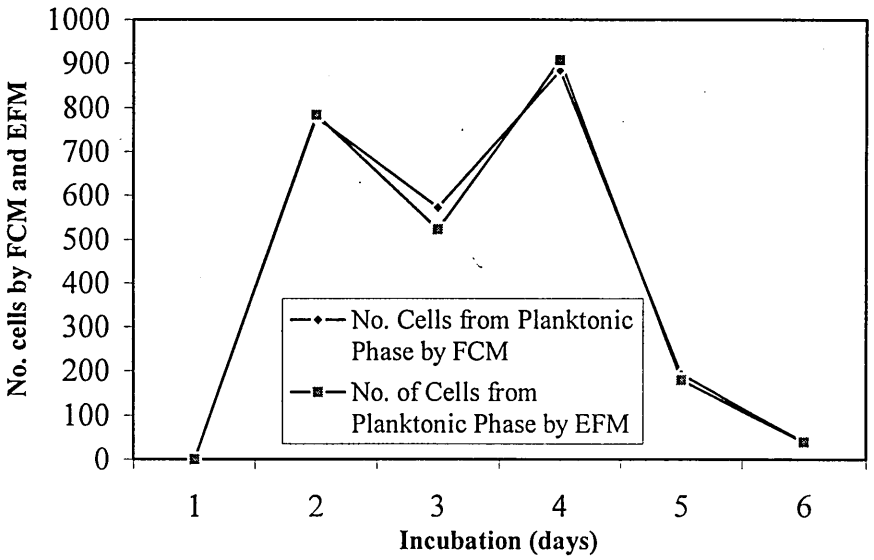


Figure 4.4b Recoveries of FCM Sorting of *L. pneumophila* Cells from Tap Water Planktonic Phase



Note: Both biofilms and planktonic samples were stained with mAb (1:3 V:V) and DAPI at 5 μg/ml.

Table 4.6, Figures 4.4a, 4.4b show the results of the *Legionella* bacteria in both biofilm samples and planktonic samples taken from a range of 0 to 28 days biofilms. The recovery of the FCM sorting against rechecking by EFM for counting *Legionella* cells sorted on the slides was 68 - 100% (EFM/FCM x 100) for most of the biofilm samples ($r = 0.9929$, $n = 6$), and at 91.5 - 102% ($r = 0.9980$, $n = 6$) for all planktonic samples. The recovery of the 28 day biofilm sample was lowest at 29%, implying that the flow cytometer could be easier to use to sort planktonic samples with good recovery rates (92-102%) because of the uniform suspension of cells in it. For sorting biofilm samples, the recovery rate had a bigger range due to the non-uniform nature of the cellular contents.

Table 4.7a Flow Cytometric Analysis of Tap Water – Planktonic Phase of Biofilm Generator

Days	0	3	7	14	21	28
Total Count for Planktonic Bacteria	71,240	40,260	38,049	7,538	2,702	8,108
	74,473	38,150	35,228	7,689	2,699	5,010
	72,416	34,358	31,681	6,713	3,019	6,163
Average	72,710	37,589	34,986	7,313	2,807	6,727
SD	1,636	2,991	3,191	525	184	1,203
CV (%)	2.2	8	9	7	7	18
Mab-FITC for*Legionella* in Planktonic	34,142	6,149	7,585	3,591	205	45
	35,500	6,441	7,204	3,295	214	44
	34,685	6,175	7,040	3,024	200	50
Average	34,776	6,345	7,276	3,309	206	46
SD	684	148	280	275	7	3.2
CV (%)	2	2	4	8	3	7

Table 4.7b Flow Cytometric Analysis of Tap Water – Biofilm Phase of Biofilm Generator

Days	0	3	7	14	21	28
Total Count for Biofilm Bacteria	0	27,121	1,066	15,572	17,354	4,062
	0	33,767	1,000	17,987	19,978	3,809
	0	31,990	1,082	15,960	20,400	3,993
Average	0	30,959	1,049	16,506	19,244	3,955
SD	0	3,441	43	1,297	1,650	131
CV (%)	0	11	4	8	9	3
Mab-FITC for*Legionella* in Biofilm	0	1,149	127	319	154	120
	0	1,074	122	313	137	123
	0	956	124	306	136	121
Average	0	1,060	124	313	142	121
SD		97	2.5	6.5	10	1.5
CV (%)		9	2	2	7	1

The precision of flow cytometric analysis of *Legionella* cells in the planktonic and the biofilm phases was conducted by sorting at least three times for each biofilm sample and the results are given in Tables 4.7a and 4.7b). For the planktonic samples, the average CV is 6% for total counting and 3% for the *Legionella* cells in the target sorting region, while for biofilms samples the average CV is 7% for total counting and 4.2% for *Legionella* cells in the target sorting region.

Table 4.8 Determinations of *Legionella* From the Tap Water Biofilms by Flow Cytometer

Days	0	3	7	14	21	28	Average	Correlations		Biofilms
EFM DC(No/cm^2)	0	14622	1784	4297	2243	1541	4897			*Legionella*
FCM Counts (No/cm^2)	0	12927	1679	4143	1846	1630	4445			*Legionella*
BCYE PC (cfu/cm^2)	0	738	32	154	305	259	248			*Legionella*
BCYE PC/FCM (%)		5.70	1.90	3.70	16.5	15.9	8.74	0.9026	n=6	*Legionella*
FCM/EFM (%)		88.40	94.10	96.40	82	106	93.38	0.9993	n=6	*Legionella*
Days	0	3	7	14	21	28	Average	Correlations		
EFM DC (No/cm^2)	0	514	486	2946	1081	1892	1384			Amoeba
FCM counts (No/cm^2)	0	1003	797	2311	1662	2581	1671			Amoeba
EFM Recheck No/cm^2	0	662	378	2351	1054	2162	1321			Amoeba
FCM /EFM DC (%)		195	163	78	153	136	145	0.8927	n=6	Amoeba
Recovery % by EFM		129	78	80	98	114	100	0.9656	n=6	Amoeba
Days	0	3	7	14	21	28	Average	Correlations		Planktonic
EFM DC (No/ml)	1920000	640000	512000	192000	20000	5130	548188			*Legionella*
FCM Counts (No/ml)	1780000	617500	350000	152000	20500	5000	487500			*Legionella*
BCYE PC (cfu/ml)	13000	1290	2000	1500	950	80	3137			*Legionella*
FCM/EFM (%)	93	97	69	79	103	98%	90	0.9969	n=6	*Legionella*

Figure 4.5a ***Legionella*** **in Tap Water Biofilms by Flow Cytometer**

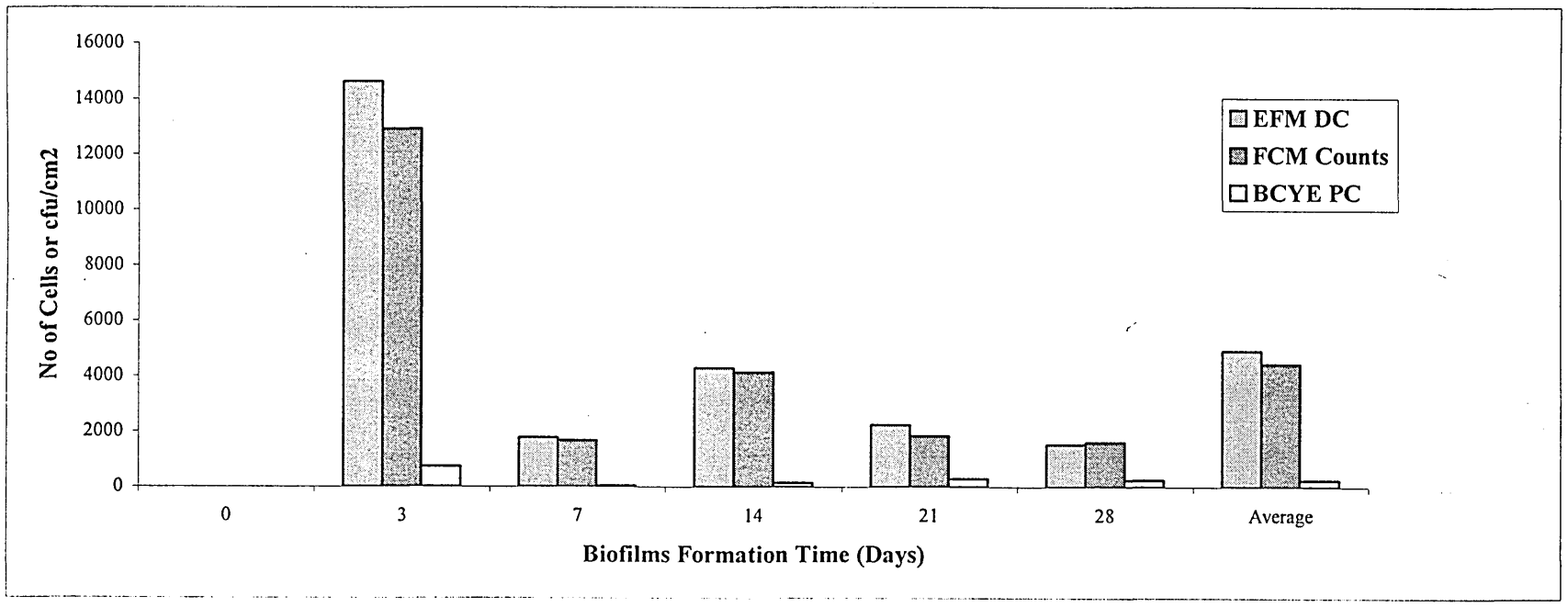


Figure 4.5b Amoebae in Tap Water Biofilms by FCM

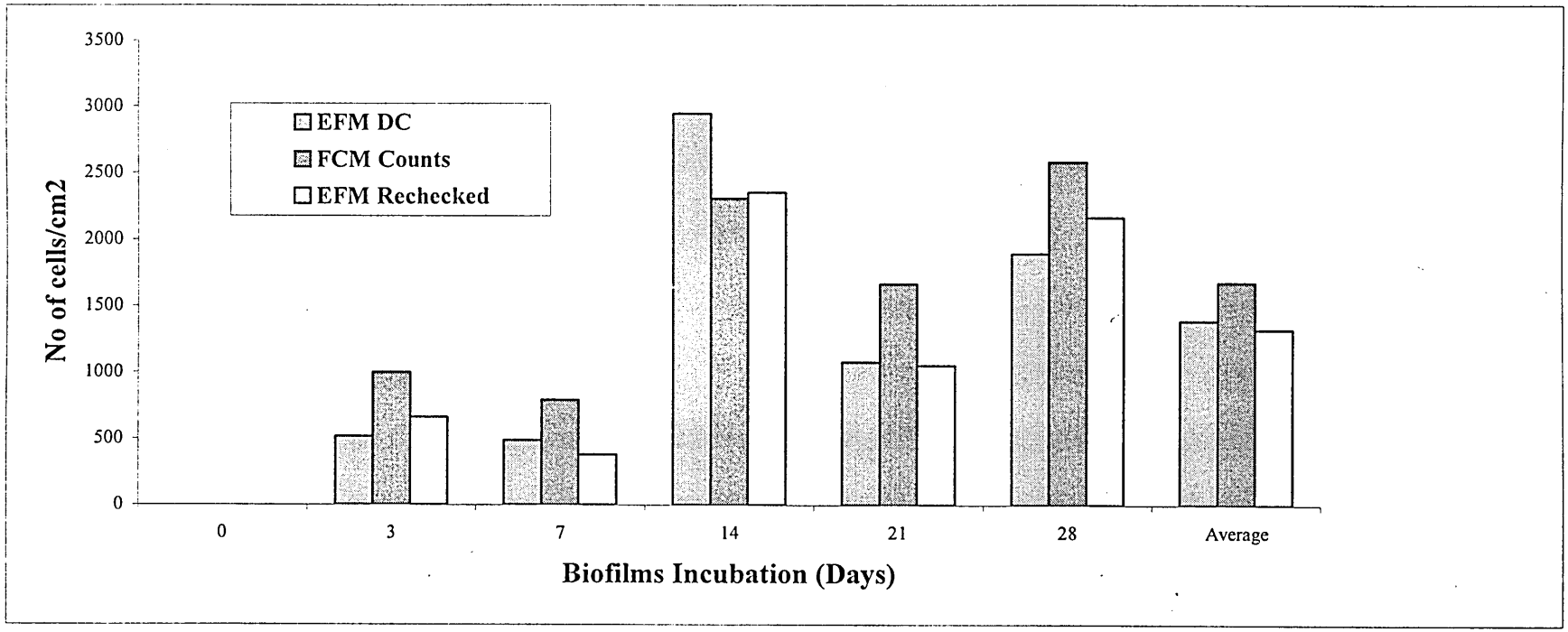
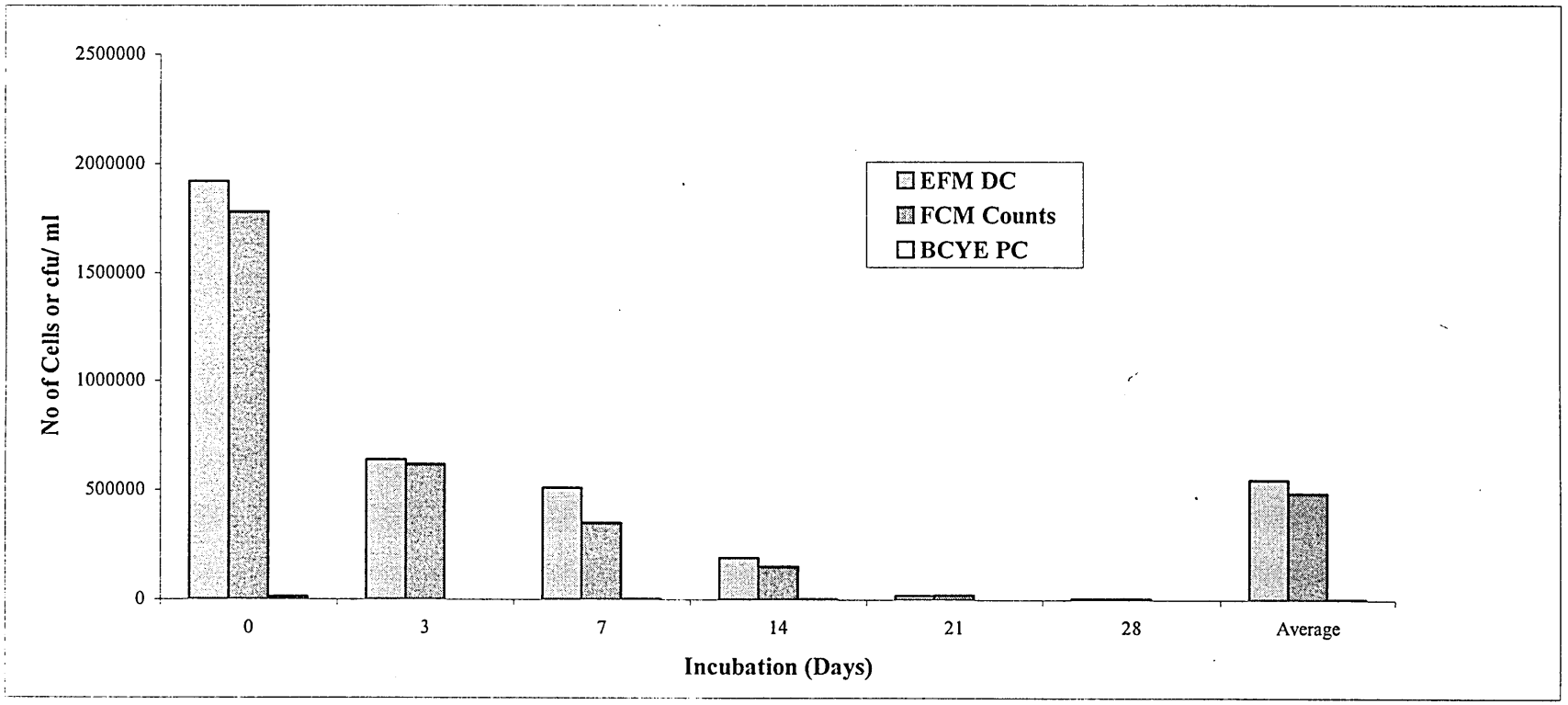


Figure 4.5c ***Legionella*** **in Planktonic Phase**



The *Legionella* cells and amoebae in biofilms and planktonic samples from 0 to 28 days were analysed by using FCM and compared with EFM counting and BCYE colony counts. The results are given in Table 4.8 and Figures 4.5a, 4.5b and 4.5c. The *Legionella* density in tap water biofilms peaked at the third day (737.8 cfu/cm^2) and then decreased down to 32.4 cfu/cm^2 by the seventh day, then increased again to 305.4 cfu/cm^2 at the 21st day. Up to 28days the density fell to 258.5 cfu/cm^2. The average of the density of *Legionella* in biofilms over 28 days was 273 cfu/cm^2. The average culturable *Legionella* (BCYE PC) in the total *Legionella* population (mAb-FITC FCM or EFM) was 8.76% and the highest level which was obtained was on the 21st day (16.6%). For the flow cytometric analysis of *Legionella* in biofilms, in comparison with EFM direct reading, the average of EFM DC/FCM counting was 108% in the range from 95% to 122%. The amoebae in biofilms were also analysed by both FCM and EFM and the results are given in Table 4.8. The recovery for comparison of FCM with EFM for detecting amoebae in biofilms was 71.4% (EFM DC/ FCM sorting) on average, and the results of rechecking the sorted amoebae on the slides is close to the direct reading by EFM methods (EFM DC/ rechecking amoebae sorted by FCM on the slides: 99.7%). The flow cytometer, therefore, could recover 99% of amoebae from the tap water biofilms samples.

4.4 DISCUSSION

The water in the water distribution system can be thought of as a perishable product, which has a shelf life, packaging, and a preservative. For potable water, the shelf life is the time the water spends in the distribution system, including storage, on its way to the customer's tap. The packaging is the piping and storage facilities used to convey the water, and the preservative could be the disinfectant, either free chlorine or chloramines. To preclude water quality problems, the following aspects need to be addressed: microbiological, chemical, physical, and aesthetic, and all these categories of problems can occur in distribution systems.

From a microbiological standpoint, bacteria can grow within the water distribution system, creating a potential regulatory compliance problem. Coliform and heterotrophic colony count organisms would be of greatest concern to system operators. Nitrification of ammonia in chloraminated water can result in increases

in heterotrophic bacteria and depletion of the residual chloramine. Both the re-growth of bacteria and nitrification are partially caused by longer retention times in piping and storage facilities in the distribution system (Kirmeyer 2000).

From a physical and chemical standpoint, the loss of residual chlorine is perhaps the most serious and prevalent problem. In the United States, maintenance of a secondary disinfectant residual throughout the distribution system is crucial to meeting regulations and maintaining low bacterial counts. In addition, with time and in the presence of free chlorine in the distribution system, disinfection by-products can increase due to the interaction of naturally occurring organic matter with the chlorine. Leaching of inappropriate or inadequately cured linings and coatings from pipes and storage facilities can result in volatile organic chemicals entering the water, with both health and aesthetic consequences. In addition, pH can fluctuate in the water distribution system, resulting in increased uptake of metals by the water. These pH variations are often attributed to low buffering intensity (capacity) of the treated water as well as the corrosion reactions themselves. Sediment and suspended particles, such as iron and manganese can settle in the piping system and in storage facilities, where they can harbour micro-organisms or be re-suspended and enter the piping on the customer's premises.

From an aesthetic standpoint, distribution systems can cause yellow, red, or rusty water as the water picks up iron from ferrous materials such as unlined cast iron, steel or galvanised pipe. In addition, the taste and odour of the water can be affected as the water travels through the piping and storage system.

Data obtained in the current study has shown that flow cytometry is a far more rapid and sensitive method than the colony counting methods as well as direct counting by microscopy. As far as it is known, no detailed study has been undertaken on the flow cytometric analysis of bacteria, particularly *Legionella* and amoeba in the water distribution systems and drinking water biofilms.

4.4.1 Colony counting Bacteria

The colony count methods are a conventional approach for quantification of viable heterotrophic bacteria in drinking water and aquatic environmental samples. A disadvantage of the colony count method is, however, the long incubation time required for the colony growth. The direct counting methods with

fluorescent dyes, as described here may provide a much more sensitive and rapid approach for enumerating total and viable cells in drinking water. The results presented show that for the water distribution system samples, up to 7 days incubation, the total bacterial cfu densities could increase from 2 (2 cfu/ml in 1day) to 5 (5 cfu/ml in3 days) and up to 256(256 cfu/ml in 7 days) in average (n = 90) and the ratio of the YEA PC 1d/YEA PC 3d/ R2A PC 7D was 0.8/2/100. Although the coliform bacteria and *Legionella* spp. were not detected from all these water distribution systems, the total viable and cultural bacterial densities ranged from 0 to1054 cfus /ml. Similar results were reported by Schaule *et al.*, (1993) and the R2A PC's numbers ranged from about 10 to 10^3 cfu/ml. Though HPC bacterial density has no direct impact on human health, it has sometimes been reported to promote the development of coliform bacteria, protozoa and of macro-invertebrates such as *Asellus* and *Nais* (Kerneïs *et al.*, 1995).

4.4.2 Direct Counting of Bacteria

Direct counting of bacteria by staining and with epi-fluorescence microscopy from water distribution systems has provided a very useful way for studying the bacteria in water distribution systems. Many authors (Manz *et al.*, 1993; Schaule *et al.*, 1993; Saby *et al.*, 1997; Norton and LeChevallier, 2000) have reported their work on biological regrowth and biological qualities, in water distribution systems, and the vast majority of these have concentrated on pilot scale experiments and laboratory devices. The work described in this thesis was based on the real water distribution systems via 9 different sampling sites in the whole drinking water network systems. The DAPI direct counting of the total bacteria has been investigated and the results show that the total bacterial density was high, up to 10^5 cells/ml on average (n = 90, CV% = 10%) and a similar result of 10^5 to 10^6 cells /ml was reported by Schaule *et al.*, (1993).

The CTC staining has been successfully used to detect the viable bacteria in the water distribution systems from the current work. The CTC direct counting showed that in the treated water there were 0.7% (density;10 cells/ml) of total cells (by DAPI DC) with respiratory activity. And in total of active respiratory cells (CTC positive cells), only 0.2% could be detected by the colony counting method.

The direct counting method has provided a more reliable and sensitive way for studying the water distribution system and also allowed the total assessment of the biomass and the density changes in the microbiological populations if combined with colony count methods. The current work has shown that the analysis of both raw and treated samples in the water distribution system by direct counting and colony count methods together have given a clear view of total biomass and viable species before and after process treatments. In the present data, the density of DAPI DC expressed the total bacteria (both dead and alive species) in the system and it remained a constant density (CV was about 10%) and the CTC DC and colony counts indicated the changes in viable species.

4.4.3 Flow Cytometry Analysis of Bacteria in Water Distribution Systems

Water distribution system samples, which include raw and treated water samples, were analysed by flow cytometry in the current work. The results showed that flow cytometry could be used for enumerating and sorting the water distribution system samples at fast analysis speed (1ml sample /min and with the events rate 1000 events/s). A count can be obtained in 30 seconds to a minute for each sample. In comparison with the epifluorescence microscopic analysis, the average percentage of the FCM sorting against EFM direct counting for the PI stained samples was 148%. There is a strong correlation between flow cytometry counting with epi-fluorescence microscopic direct counting ($r = 0.9972$, $n = 13$). PI and DAPI could be used for staining dead and total cells by single or dual staining to detect the total and viable cells in samples. CTC staining was also used successfully to label the living cells in water distribution system samples. The CTC positive cells in the total population were 1.9-16% in raw samples and 0.7% in treated samples (CTC DC/ DAPI DC) by both flow cytometric and epifluorescence microscopic analysis. The ratio of CTC positive cells between treated and raw samples was 6% which means that the removal rate for respiring bacteria could be 94% by current water treatment processes. The ratio of total viable cells (TVC) by R2A colony counts was 0.4% between treated and raw samples, while the removal rate of TVC could be 99.6%. The above results show that even after the treatment process, there are still high densities of viable bacteria

(6833 cells for CTC +ve/ml) in final treated samples from water distribution system.

The CTC staining method was modified by using filtration membranes (0.2μm pore size) for concentrating the cells, and filtering any reducing substances which could reduce the CTC to CTF and which could form bacteria-like particles in staining. Staining the concentrated samples on the filtration membranes was carried out in the dark in moist conditions in cabinets at 37 °C for at least 2 hours. These methods could also reduce the applied volume of CTC which is very expensive.

Detecting the total and viable cells, which includes viable and culturable cells (VC) and viable but not culturable cells (VNC), and biomass would be very useful in water microbiology for drinking water quality. Direct counts can be obtained very quickly and because there is a good correlation between direct count and colony count, the colony count could be predicted. The colony count only gives a small proportion of the total count and does not reflect what is happening in water in the distribution system.

The current preliminary work using flow cytometric analysis combined with the CTC, PI and DAPI staining offered a fast and reliable method. Further work will need to be done, especially screening the novel dyes for viable labelling which will greatly improve both FCM and EFM analysis.

4.4.4 Conclusion

The current work presented here has indicated that flow cytometry could be used as a rapid and sensitive technique to detect bacteria in water distribution systems. The comparison of the flow cytometric analysis with direct counting methods as well as colony counts has shown that the flow cytometric analysis combined with novel fluorescence dyes such as CTC, and DAPI or PI could elucidate both total and viable enumeration and provide sorting or selection of the microorganisms in the water distribution systems. A strong statistical correlation of flow cytometric analysis with direct counting was observed ($r = 0.999$, $n = 13$, $p<0.01$).

The study of bacteria in water distribution systems has shown that traditional microbiological methods could only demonstrate 0.15% of the total bacteria in water distribution systems (R2A PC/DAPI DC) up to 7 days and there was a good correlation relationship between R2A PC and DAPI direct counting. The CTC direct counting indicated that even in the final effluent samples, there are still high numbers (10^3) of viable bacteria per ml on average, and cfu densities about 10/ml by R2A PC and 0 cfu /ml by YEA 1d and 3d colony counts. Coliform cfu also was 0/100 ml. The current limited study shows that flow cytometry and direct counting methods are the best methods to study the microorganisms in water distribution systems.

Flow cytometry can be used to quickly and accurately detect the bacteria and other microorganisms in water samples from water distribution systems, and can include total counting and sorting and viable counting by combining staining with DNA probes and monoclonal antibodies. A further application of flow cytometry could be on-line monitoring and analysing of biomass in water distribution systems.

4.4.5 Flow Cytometric Analysis of Tap Water Biofilms

The aim of this work was to see if flow cytometry could be used for analysis of tap water biofilms and provide a practical method for determining the content of biofilms in water distribution systems.

4.4.5.1 Flow cytometric analysis of tap water biofilms

Data obtained in the current study has shown that flow cytometric analysis is suitable for detecting and counting bacteria in tap water biofilms. The recovery of *Legionella* cells using flow cytometry counting and sorting on to slides for rechecking by epifluorescence microscopy was at the range of 68-100% ($r = 0.9929, n = 6$) for most of the biofilm samples, and at 92-101% ($r = 0.9980, n = 6$) for planktonic samples. For the biofilm samples, the flow cytometric counts were all higher than the microscope counts which were used for rechecking the sorting results. This meant that flow cytometry could recover nearly all of the target cells from tap water biofilm samples using the sorting function such as enrich sort mode, which can give high recovery but with high background noise.

The recovery of amoebae from tap water biofilm samples was 99% by sorting in comparison with EFM results.

For the comparison of flow cytometric analysis with epifluorescence microscopic direct counting, the average ratio of FCM counting with epifluorescent microscopic counting was 93.45% ($r = 0.9993, n = 6$) for *Legionella* from tap water biofilms samples. For the amoebae, the comparison of FCM sorting with EFM direct counting showed the average percentage was 145% (FCM/EFM, $r = 0.8927$, $n = 6$). The recovery rate for amoebae by flow cytometry was 99% on average ($r = 0.9656, n = 6$).

In the 28 days of biofilms formation, the average number of *Legionella* cfu was 297.6 /cm^2 and the highest number of *Legionella* cfu was obtained at 21 days on glass surfaces at 30 °C. Rogers *et al.*, (1994a) reported 1700 cfu /cm^2 at 30 °C with glass surfaces and the highest numbers of *Legionella* were also achieved at 21 days. The highest ratios of viable *Legionella* cells (cfu) against total *Legionella* populations were achieved on the 21st day with 16.5% for biofilms and 4.63% for planktonic phase, and averages were 8.76% (biofilms; $r = 0.9026, n = 6$) and 1.46% (planktonic phase; $r = 0.9560, n = 6$) by flow cytometry and R2A colony counts. The highest amoebal density (3251/cm^2) was achieved on day 14 (average density 1321 amoebae/cm ^{2}) from the biofilms.

4.4.5.2 Conclusion

Work has been undertaken to determine the *Legionella* and amoebae as well as other biomass in tap water biofilms and has compared the flow cytometer with other microbiological methods. The results have been shown that:

1. The flow cytometer was suitable for analysing tap water biofilms. The recovery rates of the flow cytometer were 86% ($r = 0.9929$, $n = 6$) at the range of 68 to 100% for sorting *Legionella* cells and 99% ($r = 0.9980$, $n = 6$) for sorting amoebae in biofilms. The flow cytometric analysis of *Legionella* in biofilms in 28 days strongly correlated with the epifluorescence microscopic analyses and colony counts by BCYE (FCM-EFM, $r = 0.9993, n = 6$; for BCYE-FCM, $r = 0.9026$, $n = 6$). For *Legionella* cells, the average ratio was 93.4% for FCM-FEFM and 100-145% for amoebae by FCM/EFM.

2. Viable *Legionella* cells in biofilms were higher than in the planktonic phase in the biofilm systems. The ratios of the cfu against total cells for *Legionella* were 8.76% in biofilms and 1.46% in the planktonic phase.

3. In seeded tap water biofilm systems at 30 °C, *Legionella* cells achieved their highest density after 3 days, then fell, then gradually increased over three weeks. The current work for tap water biofilms provided an indication that flow cytometry could be used to analyse biofilm bacteria and protozoa in water distribution systems

4. Monoclonal antibody fluorescence dye (mAb-FITC) has been first reported to stain tap water biofilms for the flow cytometry analysis of *Legionella* spp. The optimal staining conditions may be extended for use in the study of the environmental biofilms.

5. The strong statistical correlation relationship between numbers of *Legionella* cfu by BCYE PC and by mAB FCM and EFM could provide a method for predicating the *Legionella* cfu number by flow cytometric analysis with mAb staining.

5.0 FLOW CYTOMETRIC ANALYSIS OF MICROORGANISMS IN BIOFILMS

5.1 INTRODUCTION

5.1.1 History of Biofilms

Most bacteria or other microorganisms in natural habitats can exist in two distinct physical environments; the planktonic state, whereby they function as individuals, and the sessile state, whereby they attach to surfaces to form biofilms and function as an integrated community. Biofilms can be defined as microorganisms and their extracellular products associated with a substratum (McFeters, 1984). In natural environments, the bacteria in the biofilms immobilised at a substratum surface are typically embedded in an organic-polymer matrix of bacterial origin. The matrix of exopolymers has been defined as " those materials which can be removed from microorganisms without disrupting the cells and without which the microorganisms are still viable" (Gehr and Henry, 1983). Such biofilms are ubiquitous in flowing aqueous environments, are not necessarily uniform in time and space, and may trap inorganic and organic substances within the polymer matrix. Biofilms develop on virtually all surfaces immersed in natural aqueous environments irrespective of whether the surface is biological (aquatic plants and animals) or abiological (stones, particles, metal, concrete, etc.).

Biofilms form particularly rapidly in flowing systems where a regular nutrient supply is provided to the bacteria and other microorganisms. Extensive bacterial growth, accompanied by excretion of copious amounts of extracellular polymers, leads to the formation of visible slimy layers (biofilms) on the solid surfaces.

Biofilms play a crucial role in a variety of disciplines, including medicine, immunology, biotechnology, biocorrosion, biofouling, biodeterioration and process engineering. Biofilms have been successfully used in water treatment for over a century (Atkinson, 1975). It was not until the early 1980s, however, that the

advantages of the biofilm reactors became a focus of interest for a considerable number of researchers, not only in the field of water and waste water treatment, but also in many other areas of biotechnology (Adler, 1987; Bryers, 1993). Biofilms or immobilised cell systems have also been successfully used in the production of substances such as acetic acid, polysaccharides, ethanol, cellulose production, biosensor applications and recombinant gene expression (Bryers, 1993).

Biofilms play a very important role in the maintenance and survival of microorganisms in both the general water environment and man-made water systems (Costerton *et.al.*, 1987; Characklis *et al.*, 1990). Biofilms not only serve to allow for the growth of micro-organisms in water systems but also protect them from antimicrobial substances (Keevil *et al.*, 1987; 1995; Brown and Gilbert., 1993). Biofilms can be a major source of *Legionella* species and other pathogenic microorganisms such as free-living amoeba in both man-made water systems (Rowbotham, 1993) and natural aquatic environments (Marrao *et al.*, 1993). The concentration of bacteria within the biofilms provides excellent opportunities for attack by predators such as protozoa, and parasites such as bacteriophages and *Bdellovibrio* species (Characklis *et al.*, 1990). The biofilm/water interface also attracts ciliates, flagellates and amoebae which graze the surface, seeking food. The bacterial pathogens capable of survival and/or multiplication in the protozoa in biofilms include *Legionella*, *Listeria*, *Mycobacterium* and *Vibrio* (Barker and Brown, 1994) which cause human diseases. Colonisation of water systems by pathogenic bacteria such as *L. pneumophila* have been implicated as a cause of water-born diseases and these microorganisms are known to be widely distributed in the biofilms of water distribution systems , cooling towers and hot water systems as well as being present in untreated waters and groundwaters (Colbourne and Dennis, 1989). They can also grow on fittings within buildings.

Most sections of human and animal gastrointestinal tracts are colonised by specific groups of bacteria (the normal microbiota) giving rise to natural biofilms that provide a degree of protection from pathogenic species. Insertion of prosthetic devices into the human body often leads to the formation of biofilms on the surface of the devices by *Staphylococcus epidermidis*, other coagulase-negative staphylococci, and Gram-negative bacteria (Marshall, 1992). These normal skin inhabitants possess

a high degree of adhesiveness to the inanimate prosthetic device surfaces. Within a biofilm they are protected from the effects of antibiotics, and hence the biofilm continues to provide a source of infection to other parts of the body by bacterial detachment and biofilm sloughing. A well publicised example of this problem was the deaths following massive infections of patients receiving total artificial hearts (Jarvik hearts). Similarly, in cystic fibrosis patients, the production of large amounts of alginate exopolymer by strains of *Ps. aeruginosa* limits diffusion and thus prevents effective control by antibiotics (Marshall, 1992). Other examples of biofilms of medical importance include water reticulation systems, wherein potentially pathogenic bacteria may be protected from the effects of chlorination in biofilms; teeth, where the dental plaque represents a biofilm that can lead to tooth decay; *Legionella* contamination of dental unit waters (Atlas, *et al.*, 1995) and contact lenses, where the bacterial biofilm formed on the contact lenses and storage cases may induce severe eye irritation and inflammation (Mclaughlin-Borlace, *et al.*, 1998).

5.1.2 Current Methods for Detection of Biofilms

The methods used to study biofilms have been reviewed by Characklis (1973, 1981, 1990); Guezennec and Fera, (1987) and Lazarova and Manem, (1995) who describe various methods for direct and indirect biofilm estimation. Biomass quantity is one of the most important parameters in characterising biofilms in drinking water distribution systems and environmental water quality. On the other hand, the key parameter from the viewpoint of water and waste water treatments, public health and drinking water quality is the *active biomass* or *biofilm activity.* Total biofilm amount can be estimated either by physical or biochemical parameters such as biomass, density, thickness and TOC, COD and BOD. Physical and biochemical properties mainly determine the mass transfer properties of the biofilm and include nutrient diffusion and frictional resistance measurements. Biofilm composition also can be described in more detail by measuring different specific biofilm constituents (exopolysaccharides, proteins), total cell count or various cellular components such as peptidoglycan, lipopolysaccharides and lipids.

5.1.2.1 Parameters for studying biofilms

The total biofilm amount is often measured either in terms of dry weight (Bratbak and Dundas, 1984; Bratbak, 1985; Nouvion *et al.*, 1987) or of volatile solids (Oga *et al.*, 1991). The main disadvantage of using these parameters for biofilm characterisation is that their estimation includes not only active microorganisms, but also inert mass, exopolymers and adsorbed organic matter. Biofilm density is usually calculated from experimental values of biolume (biofilm thickness) and biofilm mass. Several reports have pointed out the correlation between the biofilm thickness and density. Biofilm density could increase up to 105 mg/cm^3 during the initial steps of the growth and after a ''critical '' thickness of 100 μm to a relatively stable value of 25-30 mg/cm^3 (Bhamidimarri *et al.*, 1987, Hoehn and Ray, 1973).

Biofilm thickness depends on the volume of fixed biomass and varies during the different steps of biofilm growth. Biofilm thickness has been determined using various methods such as direct measuring by using light microscopy and indirect methods such as image analysis (Senthilnathan *et al.*, 1989), or by determining the thermal resistance (Vieira *et al.*, 1993), or electrical conductance (Hoehn and Ray, 1973) of biofilms. Santegodes and Ferdleman, (1998) reported that the biofilm thickness could be determined by positioning a thin glass needle mounted on a micromanipulator on the surface of the biofilm, moving the needle down until it touched the substratum, which was detected by the bending of the needle when viewed through a dissection microscope. The biofilm thickness was inferred from micromanipulator readings.

The biochemical parameters for biofilms include TOC, COD and BOD. Total organic carbon (TOC) represents approx. 50% of cell biomass (Harris and Kell, 1985) and can also be used for indirect quantification of total biofilm amount. The sensitivity and precision of the TOC method are high (2.0 + 0.45 μg/cm^3) respectively (Characklis *et al.*, 1982). Over the last few years there has been an increasing interest in the measurement of biodegradable dissolved organic carbon (BDOC) in the water distribution systems or in drinking water (Frias, 1994). Bacterial growth in the biofilms of distribution network may occur even at low amounts of BDOC, so the drinking water may harbour a large variety of bacteria. The measurement of the oxidisable matter in biofilms, expressed by the chemical

oxygen demand (COD), is another physico-chemical method used for the indirect estimation of the fixed biomass (Bryers and Characklis, 1981; Murgel *et al.*, 1991). The method offers the same advantages as the TOC, that is to say high precision, + 0.1 μg O_2/cm^2 and a low detection limit, 6 μg O_2/cm^2 (Characklis *et al.*, 1982). BOD and BDOC for measuring the biochemical properties of biofilms have a drawback in that the time taken between the time of sampling and the availability of the results is at least 5 days, which is not compatible with appropriate monitoring of the biofilms.

Three specific cellular components are present in bacterial envelopes: peptidoglycan (PG), lipopolysaccharides (LPS in Gram-negative cells) and lipids and have been used for estimation of the bacterial mass in biofilms (Lazarova and Manem, 1995). PG is a part of the cell wall and is composed of chains of N-acetyl-glucosamine and of N-acetyl-muramic acid (NAM), linked by short chains of specific amino acids, such as D-alanine and diaminopimelic acid. NAM and D-alanine are universal components of all types of bacteria and with considerable variations in specific quantities depending on the type of bacteria (Geesey and White, 1990). The main disadvantages of the analytical procedures are their complexity and long duration (hydrolysis, followed by chromatographic separation). LPS are situated in the upper part of the outer membrane and are only present in Gram-negative bacteria. An important advantage of measuring LPS is that they are found in remarkably constant quantities in the various strains and under various physiological conditions (Lazarova and Manem, 1995). The LPS in biofilms can be determined by the use of the limulus amebocyte lysate (LAL) test or by GC/MS analysis (Geesey and White, 1990). Lipids are cell membrane components and the main advantage of measuring them is the high specific content which is relatively constant Up to 90-98% of bacterial membrane lipids are present in the form of phospholipids and their determination can be done by colorimetric methods which are simple, reproducible and sensitive (Geesey and White, 1990).

The main components of biofilms are bacterial cells as well as other microorganisms (total cells) and their activities (viable cells). The increasing control and monitoring as well as use of biofilms requires the development of new analytical approaches for detecting and enumerating microorganisms in biofilms.

Biofilm cellular mass can be selectively estimated by the total cell count consisting of direct or indirect cell enumeration after a preliminary step of fixed biomass removal and aggregate disintegration. Several approaches have been used for the cell counting, which include colony counts and microscopy techniques such as light microscopy, epifluorescence microscopy, electron microscopy, image analysis, atomic force microscopy, confocal laser scanning microscopy, and the novel technique flow cytometry.

Biofilm cellular mass can be estimated by the standard colony count of colony forming units (CFU), one of the best known and oldest microbiological techniques (Wood *et al.*, 1996). The main disadvantage of the colony count method is analytical procedure length (ten days for *Legionella* spp), high cost and the underestimation of the total number of microorganisms by viable but non- culturable (VNC) species. Biofilm cellular mass also can be directly or indirectly estimated by the total cell count after staining then using microscopy. Fluorescence staining is far superior to traditional stains for direct counting of cells in biofilms by microscopy. The other staining techniques such as monoclonal antibody labelled with fluorescent dyes, 16s rRNA probe, and FISH, have also been successfully used in labelling microorganisms in biofilms for more sensitively and more accurately detecting the cellular mass by microscopy and flow cytometry (Manz *et al.*, 1993; Christensen *et al.*, 1998).

A key parameter in monitoring and control, as well as use of biofilms, is the biomass activity. Common techniques for estimation of the biomass activity consist of biochemical tests to measure either certain specific enzymes or specific products of the bacterial metabolism such as ATP, DNA and RNA, or measurement of the electron transport system activity. The measurement of substrate removal rate is a conventional technique for determining biofilm activity. In recent years, many research works have focused on directly targeting and viewing the viable cells in biofilms.

For bacterial activity characterisation, Strange *et al.*, (1963) and Holm-Hanson and Booth (1966) have developed an ATP determination test and it was over fifty years ago McElroy in 1947 (Lappalainen, 2001) firstly made the finding that insect luciferase enzyme reaction requires adenosine triphosphate (ATP). ATP is a

fundamental component if living matter which is involved in metabolic activities and disappears immediately after the death of the cells (White *et al.*, 1979; Atlas, 1982; Lappalainen, 2001). Jorgensen *et al.*, (1992) reported that the estimation of viable biomass in waste water and activated sludge by determination of ATP and oxygen utilization rate and FDA hydrolysis. Kooij *et al.*, (1995) adapted the ATP and AOC (assimilable organic carbon) to study on the biofilm formation on the surfaces of glass and teflon in water treatments. The principle of the test is based on the measurement of the quantity of light produced when luciferin is oxidised in the presence of ATP by the enzyme luciferase.

$$\text{D-Luciferin} + \text{ATP} + O_2 \xrightarrow[\text{Luciferase}]{Mg^{2+}} CO_2 + \text{Oxyluciferin} + \text{AMP} + \text{Ppi} + \text{Light (560 nm)}$$

The method is not standardised (Stevenson *et al.*, 1979; Lazarova and Manem, 1995; Lappalainen, 2001) and the major disadvantages are its extreme sensitivity to the extraction procedure and in environmental monitoring the low toxin concentrations may cause a general increase in metabolism resulting in depleted ATP pool (Lappalainen, 2001). Additional difficulties are experienced in extracting ATP from biofilms. On the other hand, ATP is still an indirect parameter for biofilm activity which does not meet the demand of water standards in saying how many viable pathogenic species are in it.

DNA and RNA are other fundamental components of bacterial cells whose synthesis is proportional to the growth rate. One approach used to measure DNA is spectrophotometrically, after extraction and purification (Lazarova and Manem, 1995). Another technique is the incorporation of radioisotopes. The radioisotope methods are highly sensitive, enabling the detection of the slightest changes in bacterial activity (Karl, 1981). Nevertheless, the requirements of assay safety and versatility have fostered the development of other detection systems like nucleic acid probes which can be labelled by fluorescent dyes, enzymes, monoclonal antibodies, and other novel probes. In recent years, flow cytometry has been used to detect bacterial DNA. Lebaron and Joux, (1994) reported that the cellular DNA content of *S. typhimurium* and *A. haloplanktis* in artificial seawater was analysed by using an ACR 1400 flow cytometer with DAPI and HEO342 staining. They considered that

flow cytometry may provide a new approach to understanding dynamic and physiological changes in bacteria by detecting cellular heterogeneity in response to different growth conditions.

The direct viable count (DVC) method has been used for detecting the viable microorganisms in marine environments and in food such as milk (Harris and Kell, 1985; Buchrieser and Kaspar, 1993; Joux and Lebaron, 1997). Kogure *et al.*, (1979) first reported using the DVC method for determining living marine bacteria by microscopy, and recently Joux and Lebaron, (1997) improved this method by using an antibiotic cocktail for marine bacteria.. The original DVC method is based on the incubation of samples with a single antibiotic (nalidixic acid or ciprofloxacin) as a specific inhibitor of DNA synthesis which prevents cell division without affecting other cellular metabolic activities (Goss *et al.*, 1964; Buchrieser and Kaspar, 1993). The resulting cells can continue to metabolise and become elongated after incubation, then the elongated viable cells can be directly read by microscopy. The main disadvantage of the DVC method is the long time for incubation and it cannot be used to detect the slow-growing bacterial dormant cells (Joux and Lebaron, 1997). In fact, there could be a potential use if the DVC method was combined with flow cytometry.

Compared with other biochemical parameters used to estimate bacterial activity, the measurement of the electron transport system activity (ETS) offers a specific advantage in that it enables the use of microscopy techniques to directly read the viable cells in biofilms. All active cells have a dehydrogenase activity resulting from a set of respiratory chain enzyme activities (Haddock and Jones, 1997). The principle of dehydrogenase activity determination consists of deviating the ETS electron flow for the reduction of a chemical indicator under determined conditions. Various chemical compounds are used as indicators; triphenyl tetrazolium chloride (TTC), 2-(p-iodophenyl)-3-(p-nitrophenyl-5-phenyltetrazolium chloride (INT) or 5 cyano-2,3 diotolyl-tetrazolium chloride (CTC) . These substances are colourless and after reduction, are transformed into monoformazans of a strange and stable red colour which can easily be quantified by both spectrophotometry and microscopy. Rodriguez *et al.*, (1992) reported a new technique for the direct counting of respiring cells after staining with the fluorochrome CTC. de Beer *et al.*, (1994) and Huang *et al.*, (1995) detected the bacterial respiratory activity within biofilms during

disinfection process by using CTC and the fluorescence microscope. The main advantages of this CTC staining method in comparison with conventional microbial techniques are its relative simplicity and rapidity.

5.1.2.2 Instrumentation for determining biofilms

The techniques of light (including fluorescence) and electron microscopy have been and continue to be the basic methods of investigation for biofilm structure and formation, composition, as well as biofilm activity estimation. The new microscopic techniques include transmission electron microscopy (TEM), scanning electron microscopy (SEM), environmental scanning microscopy (ESEM), episcopy differential interference contrast microscopy (DIC) with and without florescence, Hoffman modulation contrast microscopy (HMC), atomic force microscopy (AFM), and scanning confocal laser microscopy (SCLM). Surman *et al.*, (1996) compared different microscope techniques for the examination of biofilms and they found that TEM analysis gave useful information about the spatial relationships of microorganisms within the biofilm matrix, whilst SEM enabled the surface topology of the biofilms to be examined at high magnification. But the preparation required for TEM and SEM may, however, result in the inclusion of artefacts. ESEM and AFM allow direct visualisation of intact hydrated specimens at high magnifications. AFM images may be rotated and manipulated to provide accurate measurements of individual microorganisms with relative ease. SCLM can be used to investigate not only the presence and the viability of the biofilm's consortium but also biofilm/substrata interactions (Surman *et al.*, 1996). HMC allows the *in situ* examination of biofilms and a clear image is produced in the examination of intact biofilms. DIC may be used to examine biofilms on opaque surfaces and if used in conjunction with fluorescent vital stains can be used to assess the viability of the microbial population. Light or fluorescence microscopy can be useful as preliminary step in biofilm studies supplying information on the general appearance of the fixed biomass (Kristensen and Christensen, 1982; Robinson *et al.*, 1984; Lazarova *et al.*, 1992, 1994). Rogers and Keevil, (1992) reported that the *L. pneumophila* in biofilms could be directly observed by episcopic differential interference contrast microscopy. Santegoeds and Ferdleman, (1998) observed the sulphate reducing

bacteria in biofilms by epifluorescence microscopy. Following the developments of the applications of the new dyes or stains (DAPI, INT, CTC), monoclonal antibodies, or rRNA targeting probes, light microscopy, especially epifluorescence microscopy has been used as an essential tool in the study of biofilms in most laboratories.

The main advantages of light microscopy are simplicity, rapidity and the possibility of directly or indirectly observing the biomass immediately without preliminary treatment. Resolution, however, is relatively low and close to the limit of bacterial cell dimensions i.e. 0.345 μm/pixel (Sieracki *et al.*, 1985).

The development of confocal scanning laser microscopy (CSLM) has recently extended the possibilities of in-depth visual observation of biofilm structure by means of 3-D images which provide a bridge between light microscopy and electron microscopy (Caldwell *et al.*, 1992; Caldwell *et al.*, 1993). Peshwa *et al.*, (1993) reported that using the confocal microscopy with 3-D image technique, they could clearly view the difference in the structure of bacterial aggregates as a function of the concentration of calcium and biomass age and with a non-homogeneous spatial distribution of active and dead cells stained by fluorescence dyes. CSLM was also used by de Beer *et al.*, (1994) to demonstrate the complex structure of aerobic biofilms formed by discrete aggregates of densely packed cells and interstitial voids. Moller *et al.*, (1997) reported using CSLM and 16s rRNA targeting probe (in situ hybridisation) and AO dye to detect *P. putida* in biofilms.

The use of scanning electron microscopy (SEM) for studying biofilms avoids the drawbacks of the previous technique by providing very high image resolution (McLaughlin-Borlace *et al.*, 1998). Furthermore, this technique could be coupled with the X-ray microanalysis to determine the elemental cell and biofilm composition. Krambeck *et al.*, (1981) developed a computer system enhancing cell size measurements from scanning electron micrographs, and the hydraulic resistance of immobilised cells was studied by Fowler and Robertson, (1991) using a similar technique. TEM can also be used to study internal biofilm structures such as the gram-negative cell wall structure and the types of exopolymer matrix in biofilms (Eighmy *et al.*, 1983; Surman *et al.*, 1996).

The major disadvantage of SEM or TEM is the slow, complex and expensive sample preparation procedure which may induce specimen damage,

distortion or biofilm loss (Chang and Rittmann, 1986). A better micrograph quality could be obtained without gold coating by means of low voltage observation using a field emission gun (Lazarova *et al.*, 1992).

Image analysis (IA) can be defined as the acquisition of an image followed by the quantification and classification of the components within it (Magennis, 1997) and the automated image analysers usually include microscopy, CCD camera, personal computer and monitor. The computer-enhanced technology is a powerful tool for the analysis of light and electron microscopic images which facilitates and improves cell count and biomass estimations. Early absorbency systems used ultraviolet (UV) light, but modern analysers work in the visible range. Sieracki *et al.*, (1985) and Pernthaler *et al.*, (1997) applied image cytometry analysis to epifluorescence microscopy for counting and cell measurements of bacteria. The greatest advantage mentioned is the rapidity of this method. By using the same approach, Siebel and Characklis, (1991) were able to determine bacterial cell dimensions in various biofilms. The potential of image analysis is the continuous *in situ* characterisation of the initial surface colonisation (Lawrence *et al.*, 1987; Escher and Characklis, 1988; Caldwell *et al.*, 1993; Pernthaler *et al.*, 1997). By observing the biofilm development over time, they determined that different types of bacteria utilise different attachment manoeuvres resulting in colony growth as monolayers or perpendicularly to the surface. The major drawback of IA coupled with bright-field and phase-contract microscopy is its limited application to transparent surfaces.

Over recent years, flow cytometry has been successfully used for enumerating and characterising bacteria and other microorganisms from both pure cultures and environmental samples in laboratories and man-made or natural environments, e.g. for water samples: *Cryptosporidium* (Vesey *et al.*, 1993), activated sludge with rRNA probe (Wallner *et al.*, 1995), bacterial respiratory and enzymatic activity in the river (Yamaguchi and Nasu, 1997), bacteria in mineral water and river water (Lebaron *et al.*, 1998); airborne bacteria in the atmosphere (Lange *et al.*, 1997); Gram-negative aerobic bacteria in soil samples (Thomas *et al.*, 1997). For biofilm studies, however, most of the methods are based on the traditional microbiological and microscope techniques. There is an increasing use of enumeration and characterisation of the microorganisms and their physical features

(size and shape, etc.) from water, food and medical fields by using novel automated techniques. Flow cytometry will offer more contributions which include fast, accurate counting and sorting total biomass and viable cells, and characterising special targets in biofilms, determining DNA contents, and also for determining components in biopolymers for studying biofilms combined with other methods. It is true that the modern staining, targeting and labelling methods for microscopy used to study biofilms have provided proof and possibility for adapting flow cytometry for determining biofilms.

5.2 FLOW CYTOMETRIC ANALYSIS OF THE MICROORGANISMS IN WATER ENVIRONMENTS AND BIOFILMS

5.2.1 Introduction

Flow cytometric analysis performed in environmental microbiology laboratories is often more stringent than that required for the analysis of mammalian cells and can push sensitivities close to limits of operation. This is because the volume, nucleic acid and protein content of bacteria are approximately 1000x less than in mammalian cells. Since detection involves identification of light scatter, the signals produced by bacteria are generally several orders of magnitude lower than those from eukaryotic cells. For example, the DNA content of the *E. coli* chromosome is some 1400 times less than that of diploid human cells (Steen *et al.*, 1994) This means that measurement of the bacterial DNA content with sufficient precision for applications like determination of cell-cycle distribution, i.e., with coefficients of variation (CV) of the order of a few percent, requires a combination of highly fluorescent staining and a sensitive instrument.

On the other hand, bacteria in some situations have relatively much higher RNA content than typical mammalian cells, notably when they grow under optimal conditions. This means that dyes with some affinity for RNA, like ethidium bromide and propidium iodide, are not suitable, except if RNA has been removed, for example, by treating the cells with RNase.

Bacteria differ from eukaryotic cells in that the chromosome does not contain histones and other proteins which inhibit the binding of many DNA-specific

dyes and thereby destroy the stoichiometry of the staining. Presumably the bacterial DNA is more loosely packed so that " chromatin structure " may not be expected to affect the staining of such cells.

Many bacterial species, such as *E. coli*, are rods rather than spheres and may therefore create orientation artefacts in some flow cytometers, especially laser-based instruments with near-parallel excitation light. In contrast to mammalian cells which, with few exceptions, are spherical in suspension and with a nucleus which is roughly concentric with the cell membrane and with a size roughly constant relative to that of the cell, bacteria may vary greatly with respect to the intracellular distribution of the their DNA, depending on growth conditions and other factors. Thus, while under certain conditions the DNA appears to be evenly distributed in all of the cytoplasm, it may be concentrated into a minor portion of the cell volume in other cases (Steen, *et al.*, 1994).

The volume of bacteria is typically three orders of magnitude smaller than that of mammalian cells. In some instruments this creates problems with the light scattering measurement; the large angle (90°) detection especially does not have sufficient sensitivity. The cell wall of Gram-negative bacteria is quite different from that of mammalian cell membrane. In addition to the cytoplasmic membrane which is similar to that of mammalian cells, bacteria exhibit a complex cell wall consisting primarily of peptidoglycans, lipoproteins, and lipopolysaccharides. The permeability of this envelope is significantly different from that of the plasma membrane; hence, the knowledge one may apply to the staining of mammalian cells, and especially living cells, is not necessarily applicable to bacteria.

Vital staining of bacteria is further complicated by the fact that some bacteria have the ability to excrete some dyes very efficiently. Thus, as demonstrated by Steen *et al.*, (1994), even some DNA binding dyes which permeate the cell wall are pumped out so efficiently that hardly any staining occurs.

It may appear that the main reason that flow cytometry has been limited to applied bacterial studies can be found in some of the above problems from the viewpoint of the bacteria. On the other hand there are some problem from the viewpoint of flow cytometry: traditionally they are amongst the more expensive of laboratory instruments and require highly skilled personnel to operate them; there is

poor overlap between the emission lines of the argon laser, and the absorption spectra of the most DNA- specific dyes may cause weak fluorescence yields.

Recently flow cytometers have been used to great effect for microbiological diagnosis and even more recently (DeLeo and Baveye, 1996; Walner *et al.*, 1997; Wilkins *et al.*, 1999; Gunasekera *et al.*, 2000) they have been applied in environmental microbiology. However, applications of the flow cytometric analysis of *Legionella* and amoebae are very few and some reports were only limited to analysis of pure cultures in laboratories (Ingram *et al.*, 1982; Harf *et al.*, 1997; Borazjani *et al.*, 2000). As far as it is known there are no details reported on the flow cytometric analysis of the *Legionella* from biofilms, as well on amoebae.

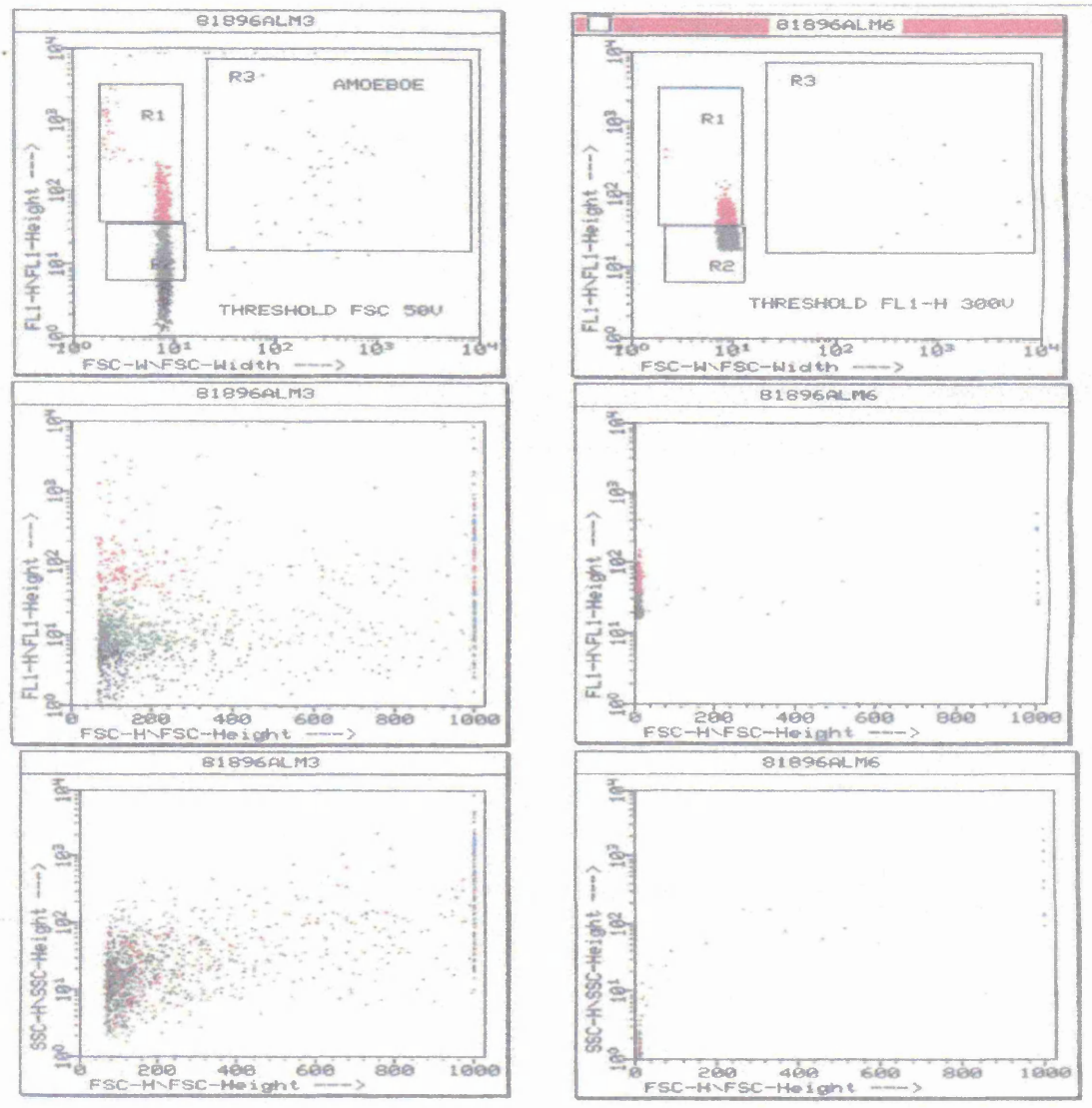
The objective of this work was to apply flow cytometry to detect *Legionella,* amoebae as well as other microorganisms in biofilms and compare the flow cytometric results with conventional microbiological methods.

5.2.2 Analysis of Flow Cytometer for Detecting Microorganisms in Biofilms and Water Environments

5.2.2.1 Images of the pure cultures of bacteria and *Amoeba* by flow cytometry

The image of the pure culture of amoebae and seeded *L. pneumophila* was obtained by FACS and is given in Figure 5.1 In the dot plot of FSC-W with FL1-H, the green dots in the sort region R1 is the *Legionella* sub-population which has the lower forward scatter light due to the smaller size and the purple dots in the sort region R3 are the amoeba subpopulation with higher forward scatter light because of their bigger size. In the dot plot of FSC-H with FL1-H, following the parameters forward scatter change from FSC-Width to FSC-Height, the dots in the R1 and R3 as well as the R2 regions are separated by their forward scatter-High signal levels. The dot plots of the FL1-H / FSC-W were used for sorting *Legionella* and *Amoeba* samples in the current work.

Figure 5.1 Flow Cytometry of *Legionella* and *Amoeba*



5.2.2.2 Flow cytometric analysis of pure cultures of bacteria and amoeba

Pure cultures of *S. aureus* cells stained with CTC and DAPI were counted and sorted onto slides by FCM and rechecked by EFM (see Table 5.1). The results

show that the FCM counting is very accurate (r= 0.9999 n=5, for sorting CTC stained cells) and can also sort the target cells from the mixture populations.

Table 5.1 Flow Cytometry Sorting of CTC-DAPI Stained Bacteria

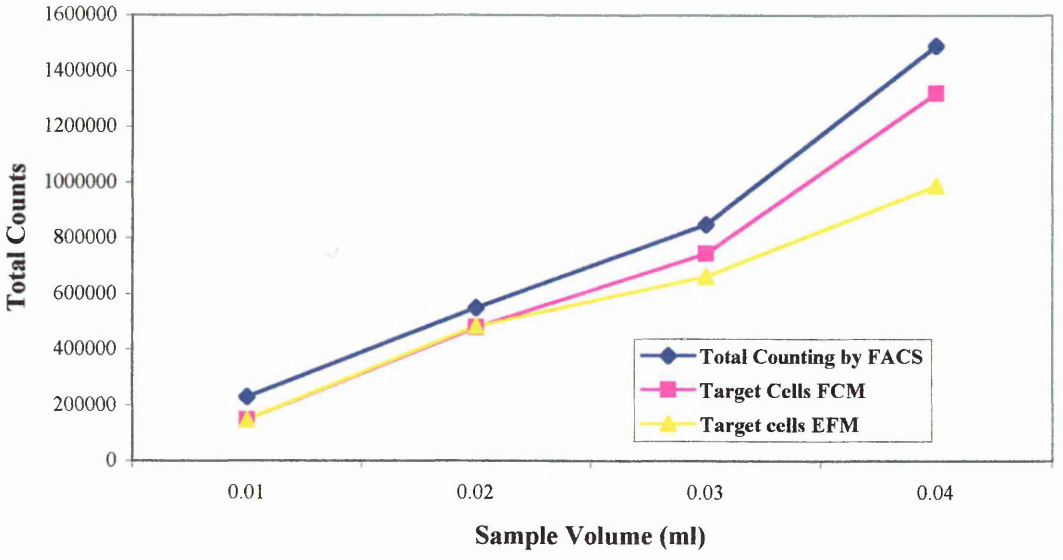
Left Sort for CTC Stained Cells		Right Sort for DAPI Stained Cells	
68	68	8	6
46	44	7	6
41	39	12	12
65	62	15	10
256	257	33	31
Correlation 0.9999		Correlation 0.9803	

In order to see if flow cytometry could be used for detecting *Legionella* spp, the mAb stained pure culture *L . pneumophila* suspension in a range of densities was sorted using flow cytometry and the results (correlation; r= 0.9887 n=4) are given in Table 5.2 and Figure 5.2 . Following the *Legionella* cells density (volume) increasing from 10 µl up to 40 µl of *L. pneumophila* cells suspended in the same 0.5ml of RO water, the total counting of FCM kept in the linear ranges, and comparison with EFM (1x10µl =1.48 x10^5 and BCYE colony count (1x10 µl=1.49 x 10^5) results, FCM total counting results including background was 20% higher than the both EFM and PC results. The counting numbers in the target region (R2) of *L. pneumophila* is very close to the epifluorescence microscope results (99—100% FACS: EFM Ratio) at ranges of 10 µl to 20 µl. Over 30 µl, the FCM counting result were higher than the EFM counts, the reason being that the optimal counting speed for FCM should be below 2000 particles a second. With 10 to 20 µl of *Legionella* suspension in 0.5 ml of RO water, the counting speeds were below 2000/S (600-1200/S), for the 30 to 40 µl, the counting speeds were over the optimal range (>2200/S), so the results were not in the linear range. The sorting speed has been kept to1000/S in the following FCM analyses.

Table 5.2 Flow Cytometer Counting of *L. pneumophila* Cells

Sample Volumes	Total Counting (Particles)	Target Cells (FCM)	Target Cells (EFM)	FCM/EFM (%)
1 x 0.01 ml	230,000	148,000	148,000	100
2 x 0.01 ml	550,000	478,000	482,000	99
3 x 0.01 ml	850,000	744,000	744,000	112
4 x 0.01 ml	1,490,000	1,320,000	988,000	133
Confirmations	Total Counting No.	*Legionella* Cells	*Legionella* Cells	Correlation 0.9887

Figure-5.2. Flow Cytometer Counting of *Legionella*



The pure culture of *L. pneumophila* suspension was also analysed by FCM to sort a range of dilutions from 10^{-10} to 10^{-4}. Table 5.3 and Figure 5.3 show that between the concentrations of 10^{-7} to 10^{-4}, the target counting for *Legionella* cells was increased ten times.

Table 5.3 Flow Cytometer Counting of mAb Stained *L. pneumophila* Cells

Dilution	Total Count	Target Region (R1) Count	Region 2 (R2) Count	R1/Total (%)	Abort	Dilution	Log Total Count	Log Target R2 Region	Log Dilution Times
10^{-10}	193	74	107	38	24	10^{-10}	2.29	1.87	-10
10^{-9}	165	60	102	36	20	10^{-9}	2.22	1.78	-9
10^{-8}	149	49	97	33	4	10^{-8}	2.17	1.69	-8
10^{-7}	436	258	164	59	3	10^{-7}	2.64	2.41	-7
10^{-6}	3,383	2,056	306	61	39	10^{-6}	3.53	3.31	-6
10^{-5}	23,697	21,123	2,366	89	416	10^{-5}	4.37	4.32	-5
10^{-4}	247,386	208,285	38,974	84	10,484	10^{-4}	5.39	5.32	-4
Control	534	295	219	55	3				

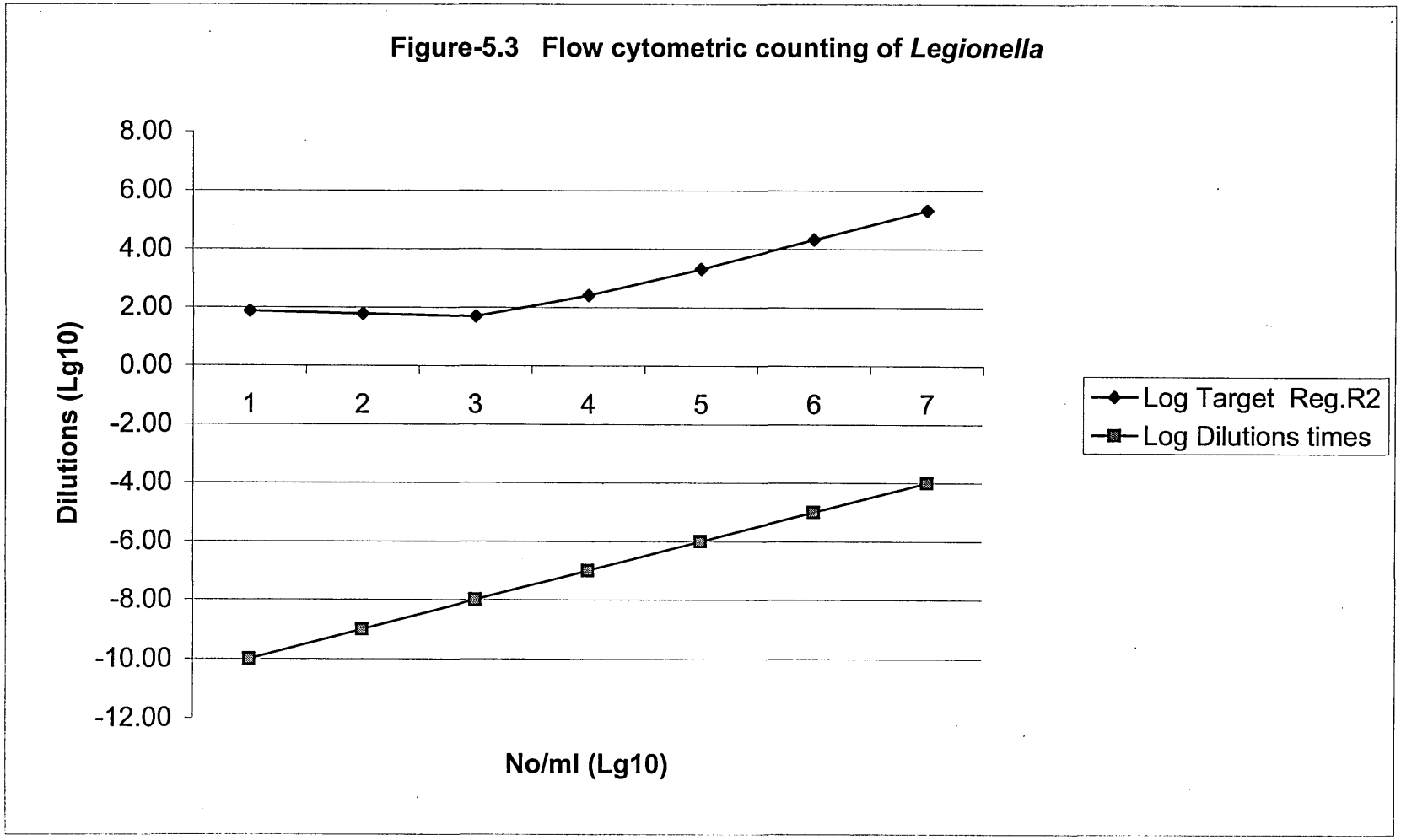


Figure-5.3 Flow cytometric counting of *Legionella*
8.00
6.00
4.00
2.00
0.00
-2.00
-4.00
-6.00
-8.00
-10.00
-12.00
Dilutions (Lg10)
1
2
3
4
5
6
7
No/ml (Lg10)
Log Target Reg.R2
Log Dilutions times

5.2.2.3 Flow cytometric analysis of *Legionella* and amoeba in biofilms

The Aire river water was used to maintain biofilm formation with or without seeding *L. pneumophila*. pH, DO, and temperature were monitored on line. Both biofilms and planktonic samples were collected every three to five days for analysis of biomass by colony count methods (YEA, R2A, BCYE) and stained with mAb-FITC, DAPI, PI., and CTC for FCM and EFM analysis.

(i) Aire river water biobackground

Aire River water was used as a water supply for biofilms and the river water biobackground was investigated. The results are given in the Table 5.4. The river water pH was in the range of 7.56 to 7.73 and the temperature was 11 to 14° C. The BCYE colony count results were positive with 4 cfu/ml or less during the sampling period in November and December 1995.

Table 5.4 Aire River Water Background

Date	pH	Temperature °C	BCYE (cfu/ml)	YEA 3D (cfu/ml)	R2A 7D (cfu/ml)
17/10/95	7.50	11.9	2.5	4×10^4	5×10^4
23/11/95	7.75	11.1	4		
27/11/95	7.73	12.0	<4		
30/11/95	7.69	14.0	<4	2×10^5	2×10^5
05/12/95	6.61	13.0	<4	6×10^5	
12/12/95	7.72	11.8	<4	1.2×10^4	7×10^4

(ii) Flow cytometric analysis of the biofilms (25 °C) with Aire river water recirculated supply.

The aim of this work after analysis of the tap water biofilms by FCM was to attempt to see if the real *Legionella*-positive river water could be used for producing biofilms with bacteria and other microorganisms including *Legionella*

species, and then analyse this biofilm by FCM and other microbiological methods as well as comparing them with each other.

The Aire river water (total 13.5 litres, 3.5 litres in reactor, 10 litres in supply tank) was used as a recirculated supply (flow rate in/out 300 ml/h) for biofilms growing up to 34 days at 25 °C , with dilution rate 0.37/h. The DO % was monitoring on line and controlled to keep the DO >60 % (Table 5.5).

Table 5.5 The Maintenance Conditions of the Biofilm System Supplies with River Aire Water

Sample Number	Days	pH	DO (%)	Temperature (^{o} C)	Stirring Speed (rpm)
1	0	7.07	80	18	0
2	1	7.87	68	25	150
3	2	7.82	68	25	125
4	3	7.80	58	25	100
5	4	8.0	70	25	100
6	5	8.12	62	25	100
7	6	8.14	66	25	130
8	7	8.12	67	25	140
9	8	8.13	54	25	130
10	9	8.04	60	25	100
11	10	8.12	58	25	140
12	11	8.13	64	25	130
13	12	8.13	66	25	140
14	13	8.09	64	25	110
15	14	7.97	61	25	110
16	15	7.93	62	25	140
17	16	7.92	68	25	120
18	17	7.92	62	25	120
19	18	7.93	64	25	140
20	19	7.93	64	25	150
21	20	7.95	65	25	125

Both planktonic phase samples and biofilms were analysed by colony counting on YEA (3 days), R2A (7 days) and BCYE for detecting the total bacteria and *Legionella* species. The results are given in the Tables 5.6a and b and Figures 5.4a, b and c. In the planktonic phase, *Legionella* numbers increased from an initial 2.5 cfu/ml to 356 cfu/ml after 16 days at 25 °C, then decreased to 36 cfu/ml at 19 days. Total bacterial numbers decreased for the first three days for both YEA (41,000 down to 28,000 cfu/ml) and R2A (52,000 down to 34,000 cfu/ml), and then increased for R2A at six days up to 19 days (250,000 cfu/ml) - a four fold increase. For the river water supply tank, the *Legionella* numbers slowly increased from 2.5 cfu/ml initial density to 20 cfu/ml at the ninth day then decreased to 8 cfu/ml at the 19th day. The main reason could have been that the river water supply tank was at a lower temperature (room temperature 18 to 20 °C).

Table 5.6a Detection of Total Bacteria and *Legionella* in Planktonic Phase of River Water Supply

Days	BCYE (cfu/ml)	R2A 7D (cfu/ml)	YEA 3D (cfu/ml)	BCYE/R2A (%)
0	2.5	52,000	41,300	0.005
3	10	600,000	95,400	0.002
6	16	160,000	42,100	0.010
9	20	278,000	48,000	0.007
13	15	250,000	20,000	0.006
16	10	171,000	8,570	0.009
19	8	180,000	553,000	0.004

Table 5.6b Detection of Total Bacteria and *Legionella* in Planktonic Phase of Biofilm Tank

Days	BCYE (cfu/ml)	R2A 7D (cfu/ml)	YEA 3D (cfu/ml)	BCYE/R2A (%)
0	2.5	52,000	41,300	0.005
3	20	54,000	28,000	0.059
6	32	122,000	18,000	0.026
9	48	244,000	71,000	0.020
13	200	176,000	19,500	0.114
16	356	250,000	65,000	0.142
19	36	245,000	497,000	0.02

Figure 5.4a *Legionella* in Planktonic Phase of Biofilm Tank

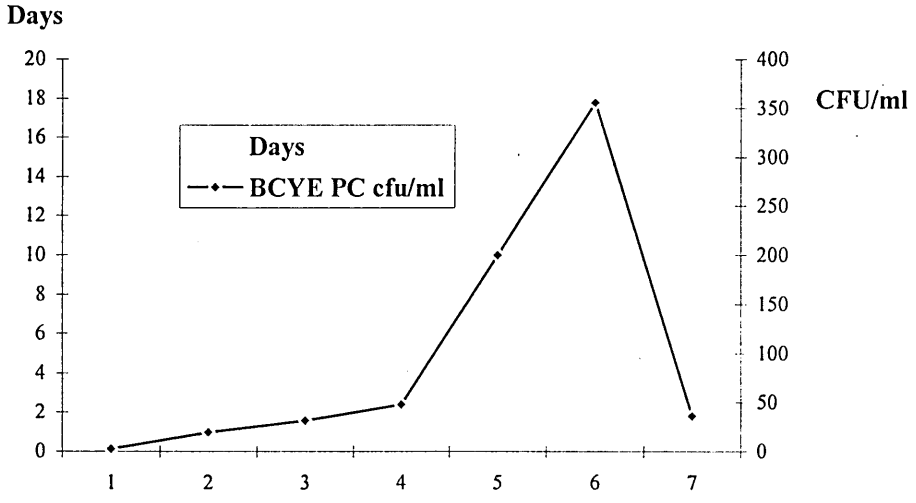


Figure 5.4b ***Legionella*** **(%) in Total Bacterial Population in Planktonic Phase**

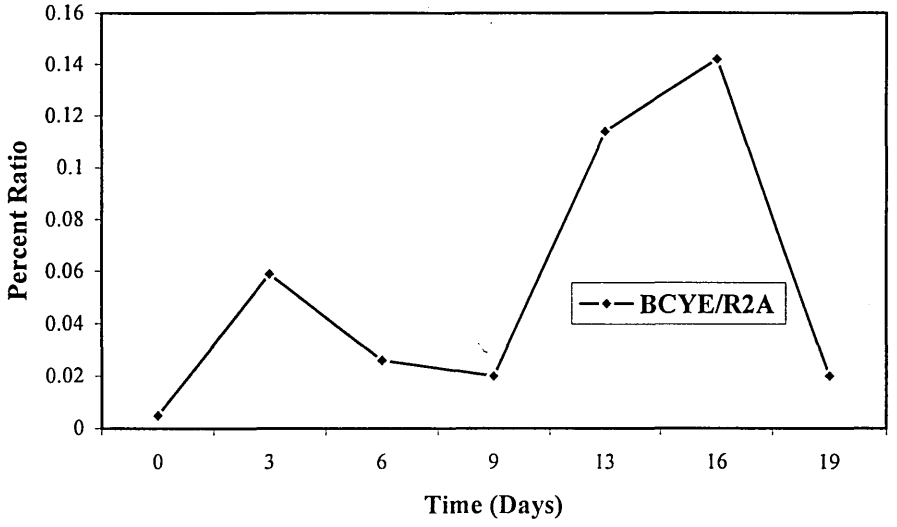


Figure 5.4c **Total Bacteria in Planktonic Phase of Biofilm Tank**

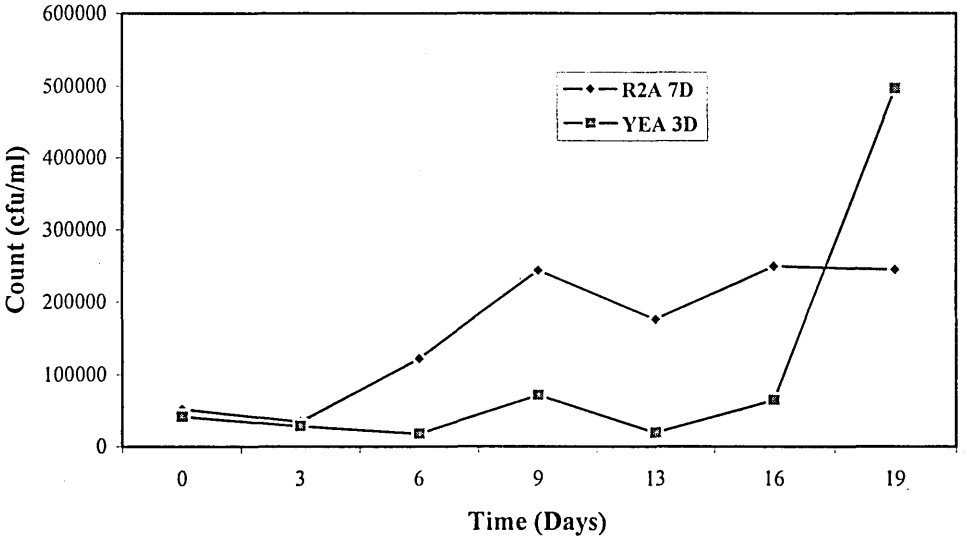


Table 5.7a Counts of *Legionella* in Biofilm

Days	BCYE (cfu/cm$^{2)}$	EFM (No./cm^2)	FCM (No./cm^2)
0	0	0	0
3	5	4,000	5,400
6	15	4,900	6,480
9	49	8,000	7,300
13	75	13,400	13,800
16	100	11,600	12,000
19	32	9,000	7,700
34	23	na	na

Fig.5.5a *Legionella* in Biofilms

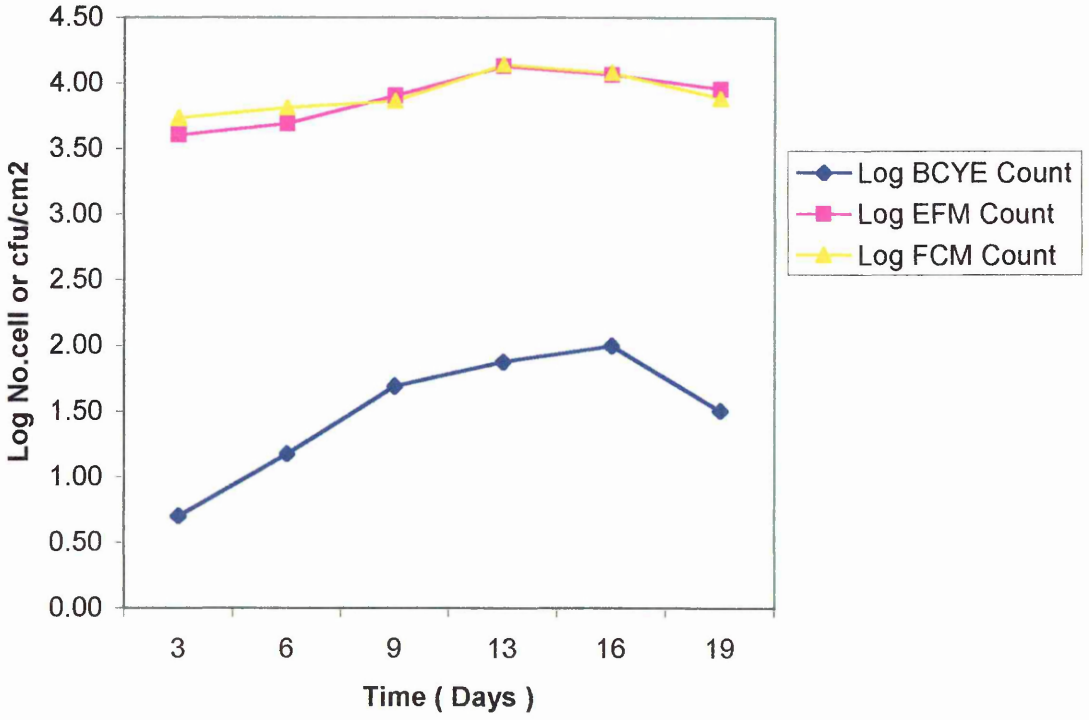
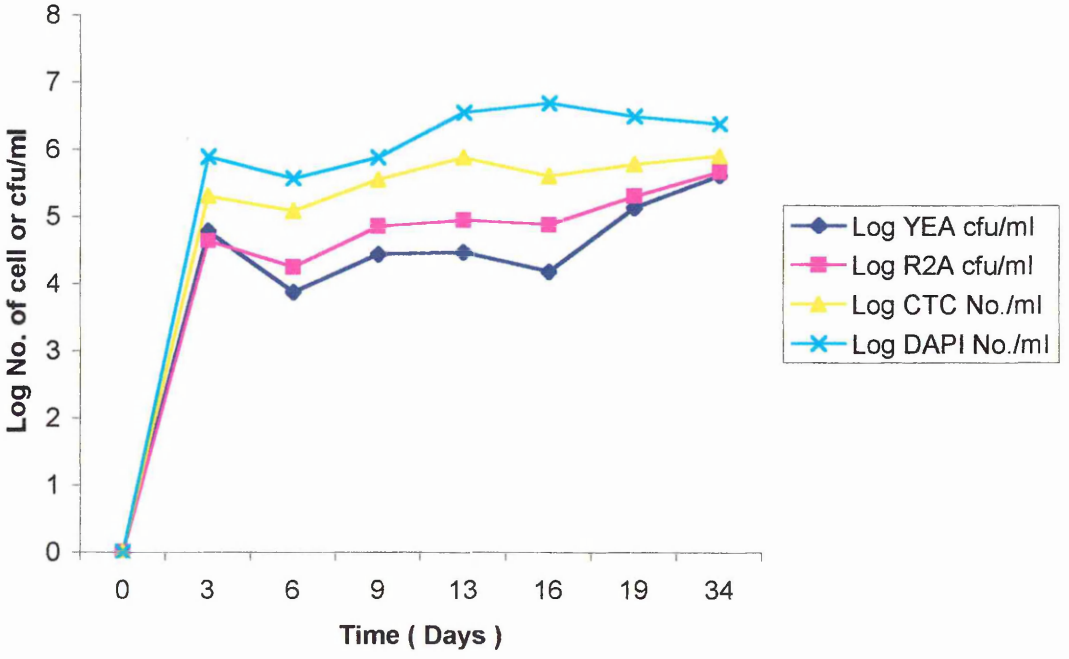


Table 5.7b Detection of Total Bacteria in Biofilms

Days	YEA 3D (cfu/cm^2)	R2A 7D (cfu/cm^2)	DAPI DC ($No./cm^2$)	CTC DC (No. /cm2)
0	0	0	0	0
3	60,000	43,200	777,000	200,000
6	7,400	17,300	364,000	120,000
9	27,000	71,000	754,000	350,000
13	29,000	87,000	3,510,000	750,000
16	15,000	75,500	4,860,000	400,000
19	135,000	200,000	3,100,000	600,000
34	410,000	460,000	2,400,000	800,000

Fig.5.5b Comparison of Counts Obtained from Biofilms Using Direct Counting, CTC DAPI



The biofilm was analysed and the results are given in Tables 5.7a and b and Figures 5.5 a, b. The flow cytometric analysis results for *Legionella* in

biofilms strongly correlated with the direct counting results by epifluorescent microscope ($r = 0.9746$, $n = 8$) and the ratio of EFM DC with FCM sorting was 96%. The density of *Legionella* cells detected by using BCYE colony count increased from the initial zero cfu/cm^2 to 100 cfu/cm^2 at sixteen days which was in agreement with the planktonic phase results where the highest density of *Legionella* also appeared at sixteen days, and then decreased to 23 cfu/cm^2 at 34 days. The total bacterial densities detected by both YEA and R2A PCs kept increasing during the 34 days. The ratio of *Legionella* against total bacteria in biofilms was 0.058% (BCYE PC: R2A PC; $r = -0.0177$, $n = 8$) on average, and the highest rate (0.132%) was obtained at day sixteen. The direct reading of *Legionella* and total bacteria in biofilms (biofilms slides) stained with CTC, mAb as well as DAPI were carried out by using EFM and the results are presented in Table 5.7a and b and Figure 5.5 a and b showing that the percentage of respiring bacteria against total bacteria (CTC/DAPI DC) was 27 % ($r = 0.6863$, $n = 8$) on average and the total *Legionella* cells (mAb DC) was 0.4 % of the total bacterial population (DAPI DC) of biofilms. The viable and culturable *Legionella* cells (CFU) was low (0.47% in total *Legionella* populations by mAb DC) and the BCYE DC strongly correlated with the total *Legionella* cells by flow cytometry (ratio; 0.47% BCYE PC: mAb FCM sorting, $r = 0.8757$, $n = 8$) and epifluorescent microscopic counting (ratio; 0.471%, BCYE PC: mAB EFM DC, $r = 0.8940$,$n = 8$). The strong correlation relationship of *Legionella* cfu and total *Legionella* cells stained by mAb FITC implied that flow cytometry of total *Legionella* cells could be used to predict the culturable *Legionella* density in biofilms as well as in the planktonic phase.

In the comparison of the FCM with EFM counting of total *Legionella* cells stained with mAb-FITC, the average percentages of BCYE PC against FCM counting (0.465%) was close to BCYE PC against EFM counting (0.47%). The total culturable bacteria (R2A PC) was 25% (Ratio; R2A/CTC; $r = 0.7605$, $n = 8$) on average of total viable with respiring cells (CTC DC).

(iii) Flow cytometric analysis of *Legionella* in biofilms incubated at 30 °C with Aire water supply

The aims of this work were to analyse biofilms at the moderate temperature condition (30° C) by using flow cytometry and other microbiological methods to focus on *Legionella* and amoeba. *L. pneumophila* (NCTC 12821) was seeded into the biofilm system in order to achieve a high density of the cells for taking more samples for analysis. Biofilms were formed in the same systems outlined in Chapter 2 and the incubation temperature was changed to 30 °C. The Aire river water was still recirculated into the system and the *Legionella* (NCTC12821) was seeded into the system. The biofilms were incubated up to 28 days and the maintenance conditions and biomass in both planktonic and biofilms were analysed by taking samples on different days.

1. Analysis of the maintenance conditions in biofilm systems

The maintenance conditions in biofilm systems were monitoring and the results are shown in Table 5.8. The maintenance conditions were monitored by analysing TOC, NH_4-N, NO_3, NO_2, $CaCO_3$, conductivity, Fe, Al, Mn, $CaSO_4$, P, pH, temperature, DO from the planktonic phase.

Table 5.8 shows the changes in the conditions. Changes of ammonia, nitrite and nitrate were as followings; in the 28 days, ammonia decreased from the initial 2.42 mg/l down to the 0.03 mg/l at the 7th day, and then remained level (0.03 mg/l) to day 28. The average of the ammonia concentration was 0.54 mg/l. Nitrite and nitrate were increased to their highest levels at the 7th day and then fell slowly to the 28 day. The changes of the nitrogenous substances in the system show that the nitrification process was taking place and this is usual under the aerobic conditions of the fermenter.

The total organic carbon (TOC) was not changed much and was marginally down from the initial 6.88 mg/l to 5.3 mg/l at the 28th day and the average of the TOC concentration was 5.7 mg/l. The lowest level of TOC appeared on the 7th day with the 5.2 mg/l concentration. The pH changed from an initial 7.82 to 8.36 on the 7th day and rose to 8.4 by the 28th day. The average conductivity was 514 $\mu s/cm^2$, and the level decreased from an initial 544 to 516

$\mu s/cm^2$ by the 28th day. The ICP analysis of the inorganic ions is also given in Table 5.8. Fe concentration decreased from an initial 0.46 mg/l down to <0.01 mg/l at the 7th day and was similar on the 28th day. P also decreased from an initial 0.408 mg/l down to <0.1 mg/l at the 28th day with 0.23 mg/l average concentration.

Table 5.8 The Maintenance Conditions of the Biofilm System (30 °C) with River Aire Water

No	1	2	3	4	5	6	7	8	9
Days	0	1	4	7	11	15	20	24	28
Cl mg/l	50.6	53.6	55.3	55.2	57.6	54.8	55.5	54.6	56.2
NO_3 (mg/l)	5.73	5.99	7.77	8.0	7.15	7.82	7.08	6.55	6.38
NO_2 (mg/l)	0.073	0.126	1.76	1.60	0.136	0.008	0.02	0.025	0.013
NH_4-H (mg/l)	2.42	1.95	0.32	0.03	0.03	0.03	0.03	0.03	0.03
$CaCO_3$ (mg/l)	122	116	74.3	104	109	107	109	115	117
pH	6.88	6.96	5.8	5.2	5.4	5.9	4.7	5.2	5.3
TOC (mg/l)	6.88	6.96	5.8	5.2	5.4	5.9	4.7	5.2	5.3
Cond. $\mu s/cm^2$	544	520	481	534	498	520	497	523	516
Fe (mg/l)	0.46	0.027	0.024	<0.01	0.019	<0.01	0.02	<0.01	<0.01
Al (mg/l)	0.059	<0.01	0.032	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Mn (mg/l)	0.149	<0.002	0.008	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002
Ca (mg/l)	59.5	68.3	68.4	68.7	67.7	62.3	67.6	66.5	66.6
Mg (mg/l)	6.37	7.55	6.92	7.61	7.53	6.66	7.33	7.30	7.23
SO_4 (mg/l)	54.8	64.8	60.1	65.2	63.9	58.4	62.0	62.5	63.9
P (mg/l)	0.41	0.37	0.16	0.35	0.41	0.27	0.12	<0.1	<0.1

From the above results, it appeared that the seventh day was a key period when some conditions such as ammonia, nitrite and nitrate, Fe, pH, changed up or down in the planktonic phase of biofilms. It is clear that the nitrification process was taking place in the biofilm system which can be seen from the decreasing levels of ammonia and with the increasing levels of nitrite and nitrate. Further analysis of the relationships of these changes with those of the biomass will be discussed later.

2. Flow cytometric analysis of the biofilms

The biofilms (slides in the system) were taken every three days and analysed immediately by flow cytometry and colony counting, and epifluorescence microscopy with or without pretreatments such as fixing and staining.

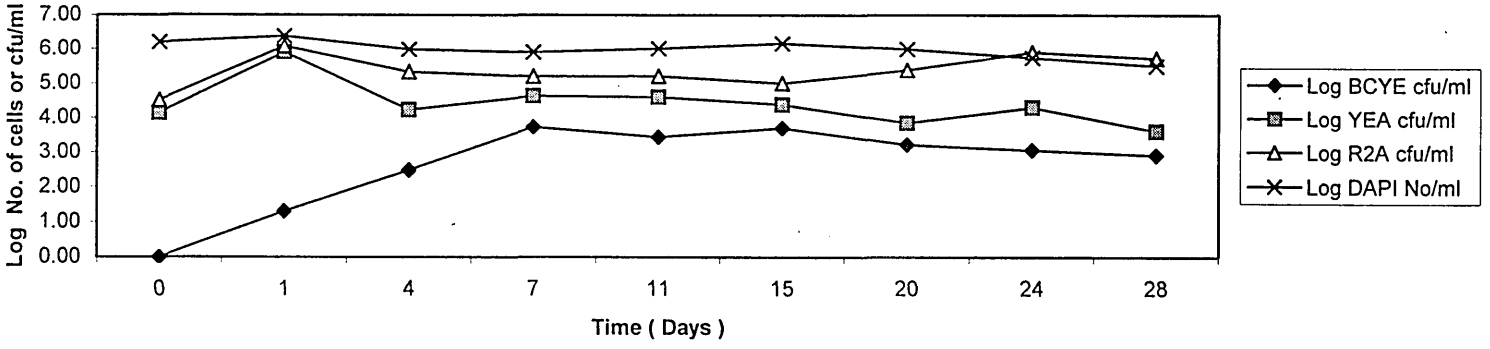
(1) Analysis of planktonic phase.

The planktonic phase was analysed by direct counting methods outlined in Chapter 2 and the results are given in Table 5.9 and Figure 5.6.

Table 5.9 Determination of Bacteria in Planktonic Phase of the Biofilm System (30 °C, River Aire Water Supply)

Planktonic samples	1	2	3	4	5	6	7	8	9	Averages	Ratio%
DAYS	0	1	4	7	11	15	20	24	28		
BCYE CFU/ml	0	20	300	5300	2700	4960	1630	1140	800	1,872	0.5%BCYE/R2A
YEA CFU/ml	14,000	800,000	17,000	42,000	40,000	24,000	7,000	20,000	4,000	107,556	28% YEA/R2A
R2A CFU/ml	33,000	1,200,000	210,000	156,000	160,000	100,000	240,000	800,000	530,000	381,000	
DAPI DC No/ml	1,600,000	2,400,000	960,000	790,000	1,040,000	1,460,000	993,000	550,000	316,000	1,123,222	34%R2A/DAPI
Correlation	R2A/DAPI	YEA/R2A	BCYE/YEA		BCYE/R2A		BCYE/DAPI DC				
r (n=9)	0.2899	0.7770	-0.3081535		-0.4489958		-0.182724				
r n=5 (11 days)	0.82313688										
r n=4 (7 days)	0.81272359										

Figure 5.6 Determination of Bacteria in Planktonic Phase



The viable culturable *Legionella* cells in initial river water were unable to be detected and 20 cfu/ml were found in the planktonic phase after seeding the *Legionella* cells (NCTC12821) on the first day. The highest density of *Legionella* cfu in planktonic phase also appeared on the 7th day with 5300 cfu/ml, falling to 800 cfu/ml at 28 days at 30 °C. The average percentages of *Legionella* (cfu/ml by BCYE PC) in all the viable culturable bacterial population (CFU by YEAPC or R2APC)) was 1.7% for BCYE/YEA and 0.5% for BCYE/R2A. To compare with total direct counting by DAPI DC method, *Legionella* (cfu) was only 0.17% (BCYEPC/DAPI DC), total viable culturable bacteria by YEA PC was 9.6% (YEAPC/DAPI DC) and 33.4% by R2A (R2APC/DAPI PC). The YEA colony counting showed a lower recovery for detecting the culturable bacteria with a 28% ratio of YEA/R2A counting (YEAPC/R2APC).

Statistical analysis showed that there was a good linear correlation between R2A colony counts with DAPI direct counting over the first 11 days ($r = 0.8231$, $n = 5$), but for the total 28 days there was not a linear correlation for the R2A PC with DAPI DC ($r = 0.2899$, $n = 9$).

(2) Analysis of the Biofilms

The biofilms formed at 30 °C with seeded *Legionella* and Aire water supply were also analysed by flow cytometry, colony counting methods and direct counting by using epifluorescence microscopy for detecting total viable bacteria, *Legionella* and amoeba. Results are given in Table 5.10 and Figures 5.7a,b,c and d.

Table-5.10 Determinations of bacteria and *Amoebe* in Biofilms (30 °C ,Aire River Water supply)

Planktonic samples	1	2	3	4	5	6	7	8	9	Averages	Correlation
DAYS	0	1	4	7	11	15	20	24	28		r; n=9
BCYE CFU/ml	0	20	300	5300	2700	4960	1630	1140	800	1,872	
YEA CFU/ml	14,000	800,000	17,000	42,000	40,000	24,000	7,000	20,000	4,000	107,556	
R2A CFU/ml	33,000	1,200,000	210,000	156,000	160,000	100,000	240,000	800,000	530,000	381,000	
DAPI DC No/ml	1,600,000	2,400,000	960,000	790,000	1,040,000	1,460,000	993,000	550,000	316,000	1,123,222	
BCYEPC/R2APC %	na	0.002%	0.143%	3.397%	1.688%	4.960%	0.679%	0.143%	0.151%	1.40%	-0.4490
YEAPC/R2APC %	42%	66.7%	8.1%	26.9%	25.0%	24.0%	2.9%	2.5%	0.8%	22.1%	0.7770
R2APC/DAPI DC %	2%	50%	22%	20%	15%	7%	24%	145%	168%	50%	0.2899
BCYEPC/DAPI DC %	na	0.001%	0.031%	0.671%	0.260%	0.340%	0.164%	0.207%	0.253%	0.241%	-0.1827
Biofilms											
BCYE CFU/cm^2	0	43	459	1,526	1,890	2,106	10,260	2,241	1,223	2,469	
YEA CFU/cm^2	0	16,200	54,000	118,800	32,400	18,900	18,900	12,150	4,050	34,425	
R2A CFU/cm^2	0	32400	145,800	324,000	43,200	23,760	70,200	37,800	19,980	87,143	
DAPI DC No/cm^2	0	432000	764,100	977,400	405,000	418,500	405,000	345,600	429,300	522,113	
DAPI DC No/cm^2	0	148500	383,400	1,587,600	1,347,300	1,991,600	1,482,300	926,100	882,900	1,093,713	on biofilms
CTC DC No/cm^2	0	999	2,700	7,128	34,290	29,700	32,400	59,400	13,500	22,515	on biofilms
mAb DC No/cm^2	0	2700	15,300	14,256	16,389	29,700	15,120	6,210	9,180	13,607	on biofilms
Amoeba No/cm^2	0	4590	12,825	14,256	12,096	15,660	34,830	14,958	11,394	15,076	on biofilms
BCYE PC/R2A PC	na	0.13%	0.31%	0.47%	4.38%	8.86%	14.62%	5.93%	6.12%	5.1%	-0.0023
BCYE PC/mAb DC	na	1.59%	3.0%	10.7%	11.5%	7.1%	67.9%	36.1%	13.3%	18.9%	0.2861
BCYE PC/ *Amoebe*	na	1%	4%	11%	16%	13%	29%	15%	11%	12.5%	0.9316
Legionella /*Amoebe*	na	59%	119%	100%	135%	190%	43%	42%	81%	96%	0.5018
BCYE PC/ DAPI DC	na	0.01%	0.06%	0.16%	0.47%	0.50%	2.53%	0.65%	0.28%	0.58%	-0.0296
CTC /DAPI on biofilm	na	0.7%	0.7%	0.4%	2.5%	1.5%	2.2%	6.4%	1.5%	2.0%	0.5174
R2A PC/*Amoeba* %	na	706%	1137%	2273%	357%	152%	202%	253%	175%	657%	-0.1277

Figure 5.7a Determination of *Legionella* and Amoebae in Biofilms

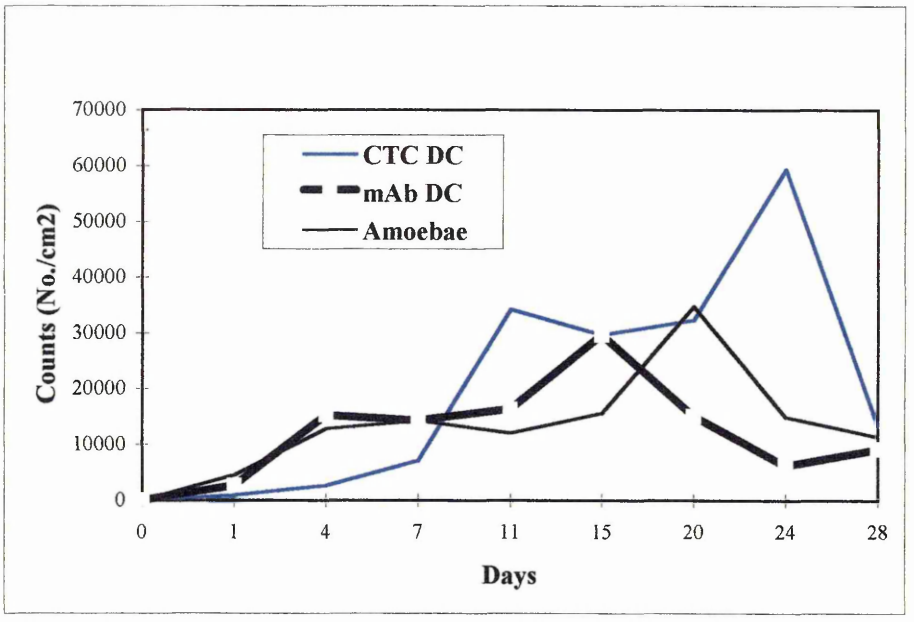


Figure 5.7b Determination of *Legionella* and Total Bacteria in Biofilms

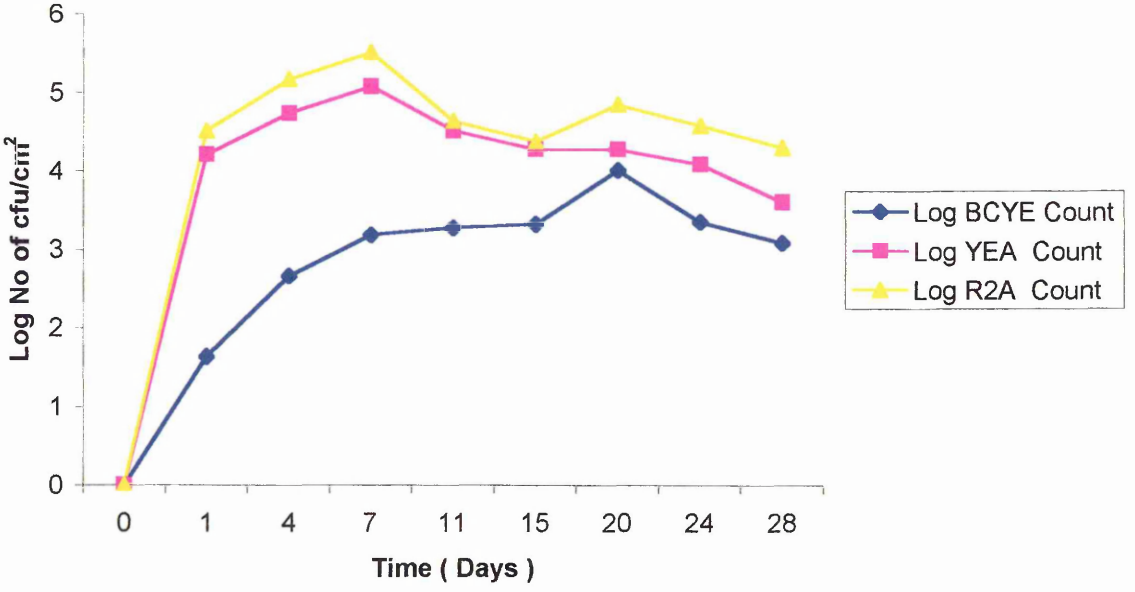


Figure 5.7c Direct Counting of Total Bacteria by DAPI Staining

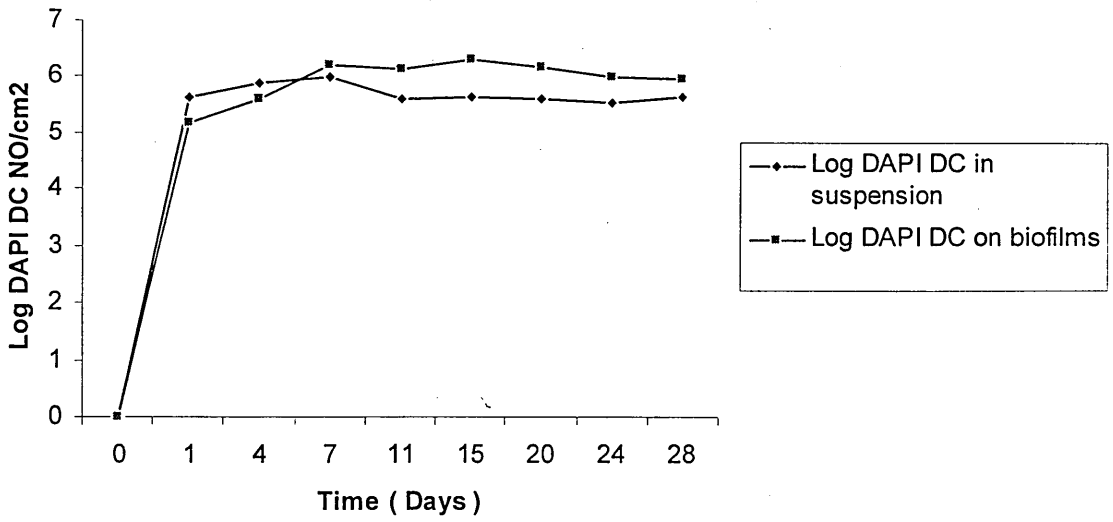
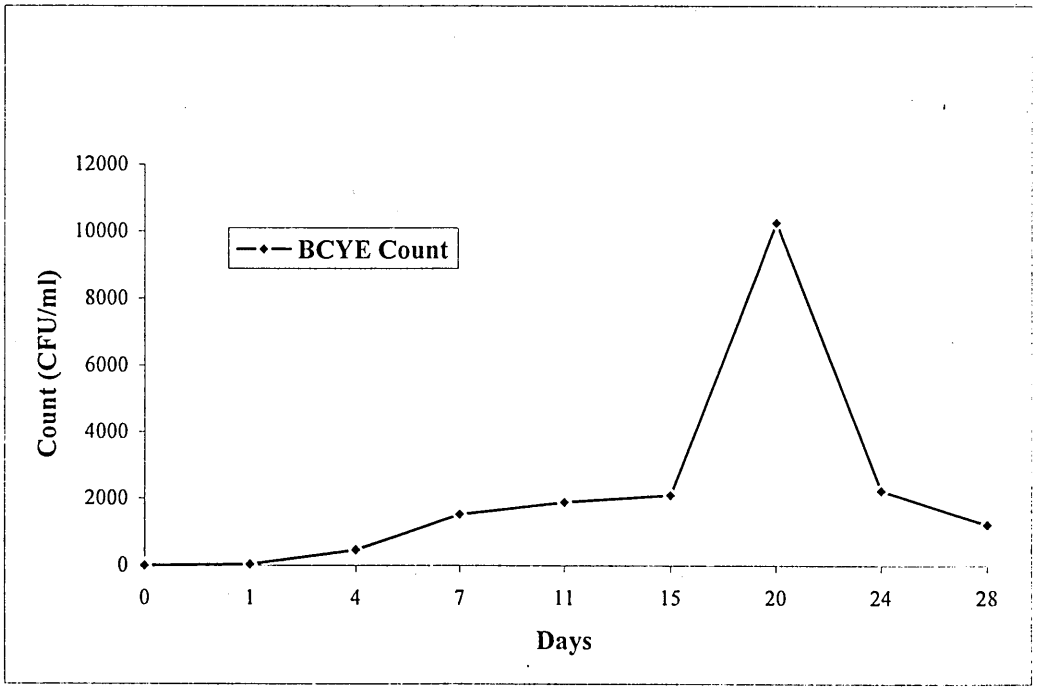


Figure 5.7d Colony counting of *Legionella* in Biofilms



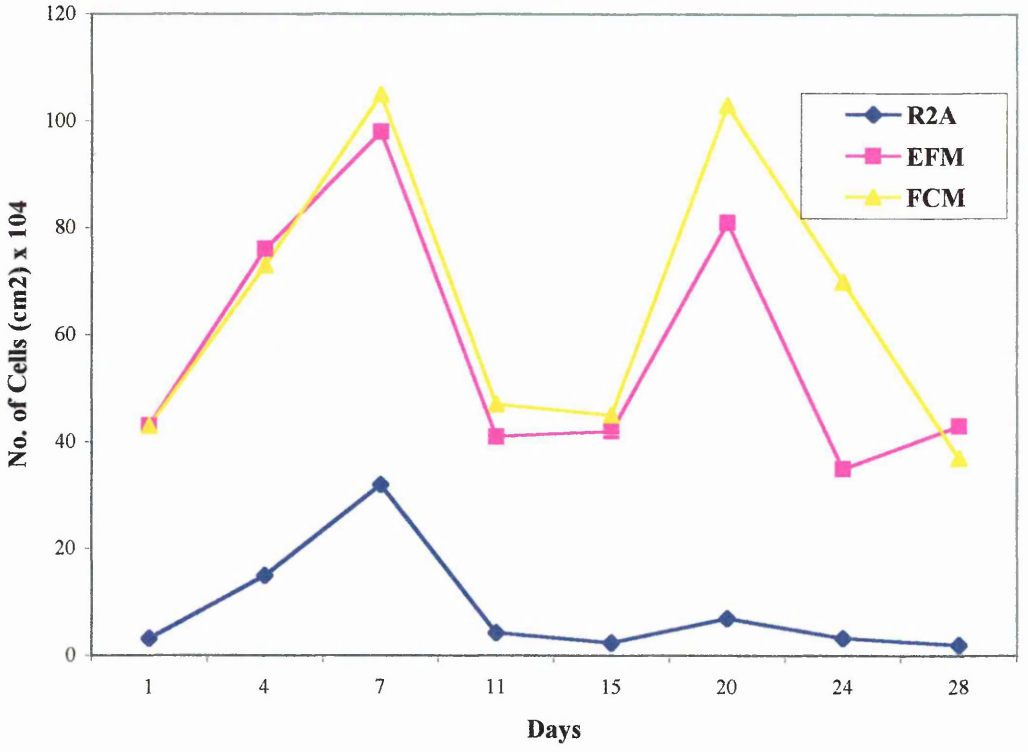
Total direct counting by DAPI DC demonstrated that total cell numbers at 7 days (977,400 cells/cm^2) is 1.87 times more than average one (522,113 cells/cm^2 in 28 days) and the initial total bacterial number was 432,000/cm^2 and then down to 405,000 cells /cm^2 at day 11, remaining to day 28 at a constant level. Viable culturable bacteria detected by the R2A colony counting also at 7 days were 324,000 cfu/cm^2 which is 3.7 times and 10 times more than average (87,143 cfu/cm^2) and the initial density respectively, then decreased quickly to 19,980 cfu/cm^2 which is only 22.9% of average density after 28 days. *Legionella* (cfu) peaked in biofilms at day 20 (10260 cfu/cm^2) which is 4.16 times more than average one (2469 cfu/cm^2) from initial 43 cfu/cm^2 after a day. The maximum percentage ratio of BCYE PC/R2APC (ratio of the *Legionella* against total bacteria) was 14.62% at day 20 and the initial only 0.13%, and the average ratio was 5.1%. Direct counting of biofilms on slides was carried out for total counting of all bacteria by DAPI DC and by CTC DC methods for total active bacteria (respiring) and for total *Legionella* cells by mAB-FITC stained and counting. Amoebae were also counted directly on biofilms. Direct readings of total *Legionella* in biofilms stained by mAb FITC were carried out and the highest density (29,700 cell/cm^2), which is 2.18 times and 11 times more than average one and initial ones was achieved at 15 day. The highest level, 67.9% (cfu/total cells per cm^2) of viable culturable *Legionella* in the total *Legionella* population also appeared at day 20 in biofilms at 30 °C with Aire river water supply. Amoebal species were directly counted on the biofilms (slides) and the highest density also appeared at day 20 (34,800 /cm^2) which is 2.31 times more than the average amoebal count at 28 days and is 7.58 times more than the initial 4590/cm^2. Total average active bacteria in biofilms by CTC DC methods was 22,515 cells/cm^2 which is only 2% (CTC DC/DAPI DC) of total average bacteria by DAPI DC (1,093,713cells/cm^2 in 28 days). Total average *Legionella* cells (13,607cells/cm^2) was 1.2% of the total average bacteria (mAb DC/DAPI DC).

Flow cytometric analysis of biofilms which were formed at 30 °C results are given in Table 5.11 and Figure 5.8. There was strong correlation between the flow cytometric analysis of total bacteria in biofilms and those by EFM ($r = 0.8600$, $n = 8$) and as well as by R2A colony counts ($r=0.7100$, $n=8$).

Table 5.11 FCM Analysis of Biofilms (30 °C)

Days	R2A (CFU/cm^2)	EFM ($N0/cm^2$)	FCM No/cm^2	R2A/EFM	R2A/FCM	FCM/EFM
1	3.2×10^4	4.3×10^5	4.3×10^5	7.50%	7.50%	100%
4	1.5×10^5	7.6×10^5	7.3×10^5	19.74%	21.0%	96%
7	3.2×10^5	9.8×10^5	1.05×10^6	32.65%	30.84%	107%
11	4.3×10^4	4.1×10^5	4.7×10^5	10.94%	9.15%	115%
15	2.4×10^4	4.2×10^5	4.5×10^5	5.71%	5.0%	107%
20	7.0×10^4	8.1×10^5	1.03×10^6	8.64%	6.8%	127%
24	3.3×10^4	3.5×10^5	7.0×10^5	9.0%	5.0%	200%
28	2.0×10^4	4.3×10^5	3.7×10^5	5.0%	5.41%	86%
Correlation ® (n=8)				0.8631	0.7172	0.8600

Figure 5.8 Analysis of Bacteria in Biofilms



To compare with colony counting methods and epifluorescence microscopic direct counting, the average percentages of viable culturable bacteria in total bacterial populations was 15% (R2A PC/EFMDC) and 13.2% (R2A PC/FCM sorting), and for total counts, the average percentage of FCM/ EFM DC in 28 days with 8 times sampling was 117%. The FCM result is 17% higher than EFM DC results which basically agreed with each other in tendency (Figure 5.8), but flow cytometric analysis is faster and more accurate. Flow cytometric analysis of *Legionella* in biofilm samples (10 µl for each sample only) stained by mAb-FITC was investigated and the results (Table 5.12 and Figure 5.9) show that the average percentage of the viable and culturable *Legionella* cells by BCYE colony counting in 28 days is 11% in total *Legionella* population. The ratio (Table 5.12) of flow cytometric analysis of *Legionella* against those by EFM was 108% on average for the 30° C biofilms (r=0.9811, n=9). The amoebae in biofilms were analysed by both flow cytometer (FACS) and direct reading (EFM) on biofilms (slides) and the results in Table 5.12 show that the flow cytometric analysis results

of amoebae by EFM is only 53% of the flow cytometric result. *Legionella* density against amoebae was 188% on average (Ratio: *Legionella* by FCM/amoeba by FCM).

Table 5.12 FCM Analysis of *Legionella* and Amoebae in Biofilms

Days	1	4	7	11	15	20	24	28	Correlatn.
FCM T-C (No/cm^2)	$2.22x10^6$	$2.52x10^6$	$3.6x10^6$	$1.74x10^6$	$1.5x10^6$	$5.25x10^6$	$1.86x10^6$	$1.82x10^6$	
FCM (No/cm^2)	$1.18x10^4$	$1.53x10^4$	$1.66x10^4$	$2.84x10^4$	$2.53x10^4$	$4.86x10^4$	$2.02x10^4$	$2.84x10^4$	
EFM DC (No/cm^2)	$1.1x10^4$	$1.7x10^4$	$1.2x10^4$	$2.3x10^4$	$2.3x10^4$	$4.3x10^4$	$2.1x10^4$	$2.9x10^4$	
Biofilms									
EFM DC (No/cm^2) *Legionella*	$2.7x10^3$	$1.53x10^4$	$1.43x10^4$	$1.64x10^4$	$2.97x10^4$	$1.51x10^5$	6.21x103	$9.81x10^3$	
BCYE (CFU/cm^2) *Legionella*	43	459	$1.52x10^3$	$1.89x10^3$	$2.11x10^3$	$1.03x10^4$	$2.24x10^3$	$1.22x10^3$	
Amoebae DC (No/cm^2)	$4.59x10^3$	$1.28x10^4$	$1.43x10^4$	$1.21x10^4$	$1.57x10^4$	$3.48x10^4$	$1.5x10^4$	$1.14x10^4$	
Amoebae FCM (No/cm^2)	$4.85x10^3$	$4.15x10^4$	$2.93x10^4$	$3.21x10^4$	$1.15x10^4$	$3.51x10^4$	$4.94x10^4$	$2.16x10^4$	
BCYE/Amoebae EFM	1%	4%	11%	16%	14%	29%	15%	11%	0.9316
BCYE/Amoebae FCM	1%	1%	5%	6%	18%	29%	5%	6%	0.3589
Amoebae/Amoebae (EFM/FCM %)	95	31	48	38	136	97	31	52	0.5664
FCM/EFM (%)	104	88	133	122	109	112	95	97	0.9811
BCYE PC/EFM DC	0.40	2.70	12.70	8.20	9.20	23.08	10.60	4.20	0.8163
BCYE/FCM R2 (%)	0.36	3.00	9.17	6.7	8.3	21.1	11.1	4.3	0.8752

Figure 5.9a Enumeration of *Legionella* in Biofilms

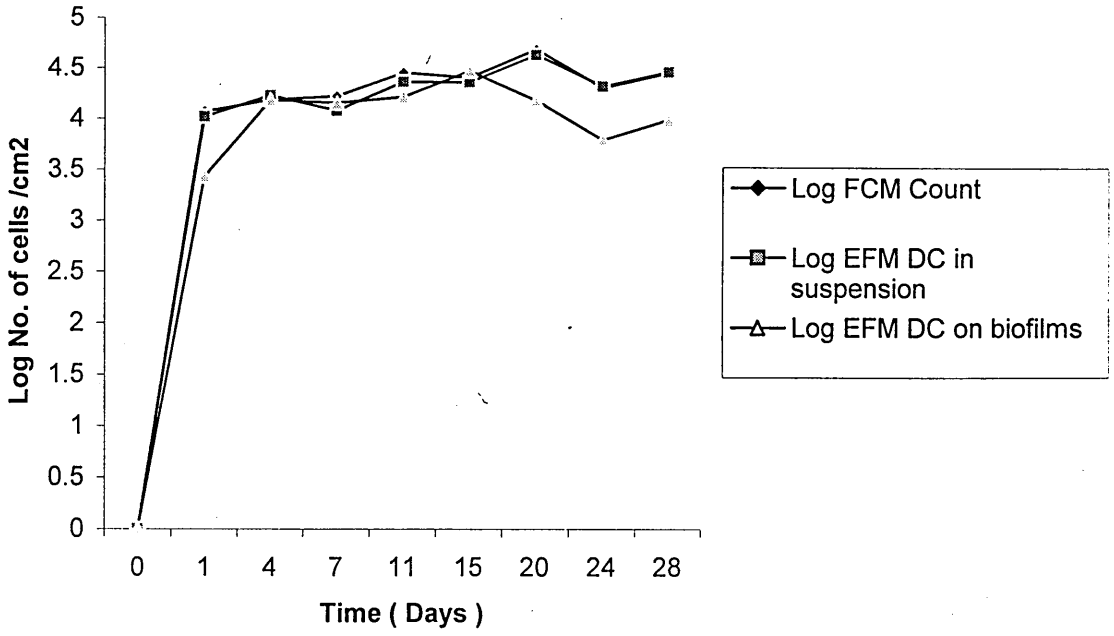
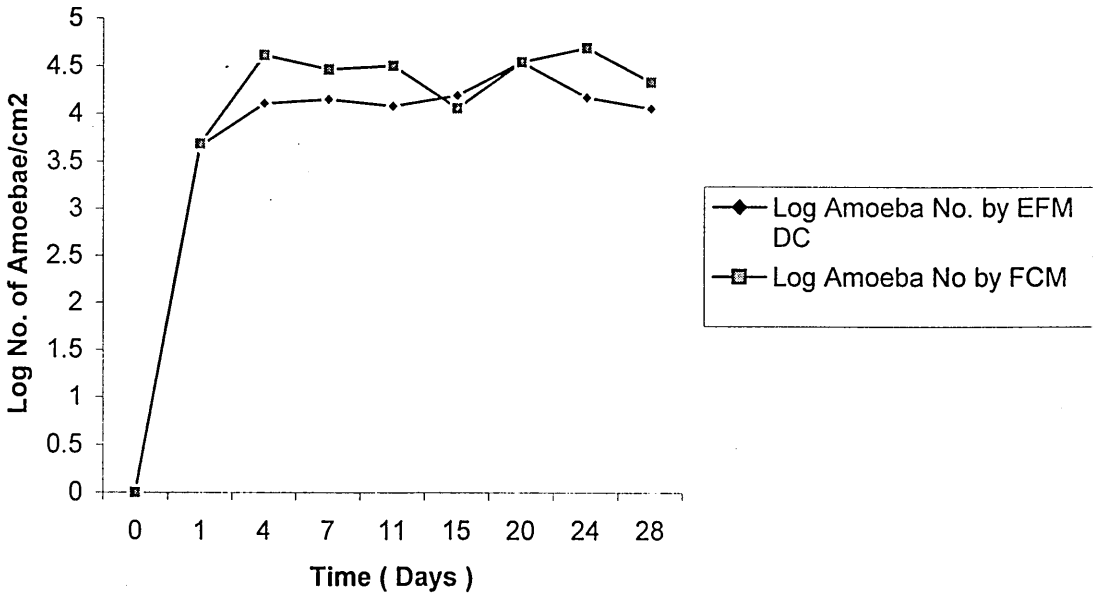


Figure 5.9b Amoebae in Biofilms



5.3 DISCUSSION OF FLOW CYTOMETRIC ANALYSIS OF BIOFILMS

The primary objective of this project was to study the application of flow cytometry to determine micro-organisms in biofilms, to focus on *Legionella* and other species, and to compare flow cytometry with conventional microbiological methods such as colony counts. For such a comparison to be meaningful, it was necessary to establish the operational conditions of flow cytometric analysis, of the flow cytometer and produce biofilms under different conditions.

5.3.1 Imaging of Bacteria and Other Microorganisms by flow Cytometry

The flow cytometer as an advanced instrument cannot only be used for enumerating and sorting, but also for imaging bacteria and other biomass and their sub-populations in whole samples. In previous work, the flow cytometric images were often used to view the targets by using light scatter detectors such as side scatter (SSC) and forward scatter (FSC). Allman *et al.*, (1992) reported that a mixture of pure culture bacterial species which included *E. coli* and *Legionella*, could be imaged by flow cytometry to recognise each subpopulation by light scatter characteristic and autofluorescence.

This work for flow cytometric images has shown that the best way to view the targets is to label or stain them initially using mAb to stain the *L. pneumophila*. The population in the mixture was then very easily viewed by fluorescent detectors and light scatter detectors and by setting up the sort region. It proved very difficult to set up the target region by using light scatter detectors only for the same mixture without staining.

Multicolour gates are one of very useful function for FCM images. Normally all dots which present the events are white, multicolour gates show events (dots) in colours representing the different gates (built-up regions). The colours show the distributions of sub-populations in data plots. In a certain image picture, such as a 2-D plot, with the multicolour gate which makes up to 8 regions

with different colours, each individual region has the same colour dots (events). When the parameters of the plot are changed by other ones, the dots' colour remains but the dots' distributions could be rebuilt by their characteristics. In this way, it is very easy to view and trace the sub-populations and their changes following parameter changes and to find which parameters could be suitable for imaging and further analysis.

Following computer technique developments, the imaging technique, which includes the data analysis, would be more useful for FCM analysis and make the flow cytometric analysis more perfect (Jonker *et al.*, 1995). An Artificial Neural Network (ANN) is a computational method inspired by the remarkable pattern recognition abilities of the biological brain. The ANN is able to learn to recognise and classify such data through the use of a set of training examples and an appropriate training procedure. Once an ANN has trained it is a very computationally efficient procedure for classifying novel data. This makes it a very suitable method for application to the rapid analysis of flow cytometry data. Artificial neural networks can be of great help in discrimination of single species (Balfoort *et al.*, 1992; Wilkins *et al.*, 1994). Wilkins *et al.*, (1999) reported that they identified phytoplankton from flow cytometric data by using Radial Basis function neural networks. The optimised Gaussian Radial Basis Function (RBF) network could recognise 34 species of marine and freshwater phytoplankton with a 91.5% success overall.

5.3.2. Flow Cytometric Analysis of Pure Cultures of Bacteria and Other Microorganisms

Many workers have analysed pure cultures of bacteria (Ingram *et al.*, 1982; Diaper *et al.*, 1992; Jepras *et al.*, 1995; DeLeo and Baveye, 1996) as well as fungi (Brailsford and Gatley, 1993) and algae (Premazz *et al.*, 1989; Jonker *et al.*, 1995) using flow cytometry, but for *Legionella* species (Ingram *et al.*, 1982; Harf *et al.*, 1997) and for protozoa such as amoebae (Flores *et al.*, 1990; Harf *et al.*, 1997; Avery *et al.*, 1995; Borazjani *et al.*, 2000) few papers have been published.

The work of Ingram *et al.*, (1982) was to detect pure cultures of *L. pneumophila* stained with polyclonal antibody by flow cytometry, and the work

mainly focussed on viewing the histogram of pure cultures of *L. pneumophila* cells only. Harf *et al.*, (1997) detected the endocytosis of viable *Legionella* cells by *Acanthamoeba palestinensis*. Borazajani *et al.*, (2000) reported the detection of cultures of *Acanthamoeba* spp. in contact lens disinfecting solutions by using flow cytometry and duel staining with PI and FDA.

In the current work, pure cultures of mAb labelled *L. pneumophila* were fully analysed using flow cytometry under different conditions. The results have shown that the flow cytometer could be successfully used to accurately enumerate and recognise pure cultures of *Legionella* species. By comparison with epifluorescence microscopy and BCYE colony counting, the FCM results correlated well (Table 5.13).

Table 5.13 Comparison of FCM Analysis with EFM and BCYE Colony counting

Test Method	Counts/ml
FCM	**1.48×10^9**
EFM	**1.48×10^9**
BCYE	**1.44×10^9**

Note n = 3. MAb-FITC staining for both FCM and EFM. BCYE PC; 3 day cfu.

The monoclonal antibody (mAb) is superior to polyclonal antibodies and other dyes for labelling the *Legionella* species for detection with flow cytometry. The mAb is suitable for staining *Legionella* cells in suspension though it is normally used for the solid stage staining (staining the fixed cells on the slides) within the range 12.5% to 25% final concentrations (V:V). Higher concentrations (50% or higher) of stain would cause high background noise. Further tests showed that there were no cross-reactions when using the mAb to discriminate *L. pneumophila* in a mixed population from *E. coli* or *Ps. aeruginosa.*

Current work for flow cytometric analysis of pure cultures of *E. coli*, *Ps. aeruginosa*, and *S. aureus* have shown that flow cytometry also can be used to enumerate and sort the other bacterial cells which were stained by Rh123; and that after treatment by glutaraldahyde, it could be extended to staining of Gram-negative bacteria by DAPI, as well as PI and CTC dyes. Direct total counting of single or mixed pure cultures of bacteria could be easily done by flow cytometry due to the similar size and low background noise.

Pure cultures of amoeba cysts were analysed by flow cytometry and the work showed that the amoeba cysts could be quickly and accurately sorted by flow cytometry after staining with DAPI dye. For the mixed samples of amoeba with bacteria and environmental samples, the flow cytometer can also clearly image, sort, and enumerate the bacterial and amoeba sub-populations by light scatter and fluorescent detectors.

Flow cytometry has been used for analysis of bacterial DNA content, as well as cell membrane potentials (Lebaron and Joux, 1994; Robertson *et al.*, 1998) for flow cytometry of marine bacterial DNA content by DAPI staining. In this work, the different growth time *Legionella* cfus were analysed by flow cytometry after staining with mAb-FITC, and DAPI in suspension. Unfortunately, the preliminary work showed that only the numbers of the larger size sub-populations were increasing with incubation time, which was due to the increasing number of filaments of *Legionella* cells, as confirmed by epifluorescent microscopy after sorting these sub-populations onto slides by flow cytometry.

5.3.3 Flow Cytometric Analysis of Biofilms

River water is far more complex than the tap water in microbiological species, nutrients and general background. The current work was to produce the biofilms in river water, with or without seeding with *Legionella* cells, and sorting the biomass by flow cytometry and other microbiological methods. The aim of the work was to see if the flow cytometry could be successfully used for analysis of biofilms from the natural environment. The work focused on *Legionella* and amoebae, as well as total biomass population.

5.3.3.1 Flow cytometric analysis of the biofilms incubated in river water at 25 °C

The biofilms were incubated in the river water which contained the *Legionella* for up to 34 days. In the planktonic phase of the biofilm system (biofilm tank 25 °C, supply tank 20 °C), the *Legionella* densities increased at different rates. In the biofilm tank the average density was 99.2 cfu/ml and the highest level (356 cfu/cm) at day 16 was 141 times that of the initial density (2.5cfu/ml); and in the supply tank, the average density was 11.6 cfu/ml and the highest level was only 7 times higher than the initial density (2.5cfu/ml). The temperature conditions could be the main reason affecting the *Legionella* growth rate. It has been widely reported that the optimum temperature for multiplication of *Legionella* in the laboratory is around 37 °C and below 20 °C multiplication can be considered insignificant. (Anand *et al.*, 1983; Mauchline *et al.*, 1994). It is possible that temperature may have affected the *Legionella* gene expression which products determine virulence, as *Acanthamoeba* can kill and digest *L. pneumophila* at 20° C (Anand *et al.*, 1983). The current work was in very close agreement with the above ones. At 20 °C, *Legionella* grew very slowly and in 19 days the density was only increased 3.5 times on average from the initial level. For the *Legionella* density in aquatic environments, Lee and West, (1991) reported that the numbers ranged from $<10^4$ to 10^5/ l. In the current work the density in Aire river water was 2.5 - 4 x10^3/l.

For the biofilms at 25 °C the current work showed that flow cytometric analysis of *Legionella* in biofilms was in very close agreement with the microscopic direct counts (average percentage of EFM/FCM was 96%; the correlation; r = 0.9744, n = 8). The highest number of *Legionella* was also obtained at day 16 (100 cfu/cm^2) and the average density was 43 cfu/cm^2 after 34 days. The numbers of

Legionella (cfu) in biofilms was 0.058% of the total bacterial population (total counts were determined by R2A PC) on average, and the ratio was close to the one in the planktonic phase (0.061%). For the CTC direct counts, the average ratio of

the total viable and culturable cells (cfu from R2A PC) against cells with respiratory activity (CTC +ve) was 17% (R2APC/CTC DC). The ratio of CTC positive cells was 27% of the total bacterial population by DAPI direct count.

Conclusion

1. The flow cytometer can be used for analysis of river water biofilms. The comparison of flow cytometric analysis with epifluorescence microscopic counting showed the average percentage of EFM DC against FCM sorting for mAb stained *Legionella* cells was 96% (Ratio of EFM/FCM) for the biofilms at 25 °C with river water. There is a statistic correlation between flow cytometric analysis with epifluorescent microscopic analysis for enumeration of *Legionella* cells in biofilms (r =.0.9744, n = 8; density $8x10^3$ cells/cm^2).

2. The viable *Legionella* cfu was 0.058% of the total bacterial population (cfu by R2A PC) in biofilms and 0.054% in planktonic phase on average. The viable *Legionella* cfu in total *Legionella* cells (counting by flow cytometer and epifluorescence microscopy) was 0.47% in biofilms on average. Total respiring bacteria (CTC +ve) in the total bacterial population (by DAPI DC)) was 27% and the viable, culturable bacteria (cfu by R2A PC) was 17% compared with the respiring bacteria.

3. The *Legionella* numbers of cfu in both biofilms and planktonic phase needed 16 days to achieve the highest densities at 25 °C from the initial 2.5 cfu /ml density in river water at 11 °C.

4. The biofilm formation system in the current work could be used to recover *Legionella* from the river water at 25 °C. However, for flow cytometry analysis the
amounts of the samples that could be used from these conditions were low and limited. The higher temperature, and seeding the *Legionella* cells in such biofilms system would be needed and this work was carried out in next stage.

5.3.3.2 Flow cytometric analysis of the river water biofilms with seeding *Legionella* cells at 30 °C

The aim of this work was to provide more target *Legionella* cells as well as other microorganisms for using the flow cytometer to analyse the biofilms by (i) increasing the cultural temperature from 25 to 30 °C and the latter would be considered the best condition for both *Legionella* and amoebal growth; (ii) seeding the *Legionella* cells (*L. pneumophila* NCTC 12821) into the biofilms systems with the Aire river water. The planktonic phase, chemical and physical characteristic in the biofilm system was monitored in all cultural process.

(i) Maintenance conditions

The current investigation has shown that in the planktonic phase of biofilms system, the seventh day was a key day and that some conditions such as ammonia, nitrite and nitrate, Fe and pH changed up or down during the first week. The nitrification process was taking placed in the biofilm system which was shown by decreasing ammonia concentration and increasing nitrate concentration. The TOC concentration, with 5.7 mg/l on average, was at a high level, which means the river water or planktonic phase was in a pollution situation (the Aire river flowed into the effluents of a waste water treatment works upstream of the sampling point). Yamaguchi (1997) reported that TOC levels of polluted river waters were at 6.1 mg/l with numbers of cfu 10^4 to 10^6 /ml; and in unpolluted river waters the TOC values were from 1.0 to 1.8 mg/l with the 10^3 cfu/ml of bacteria. In our planktonic phase, the total bacterial count was $1.4x10^4$ for YEA colony counting (average 1.1×10^5 cfu/ml) and 3.3×10^4 cfu /ml for R2A colony counting (average 3.8×10^5 cfu/ml) initially.

The above results show that the planktonic phase in the curent biofilm systems had a high oganic substance level and aerobic condition (nitrification process must be in aerobic phase) for supporting the biofilm formation.

(ii) Flow cytometric analysis of biomass in 30 °C biofilms

1. Planktonic phase

The number of *Legionella* cfu was negative for the initial river water and after seeding with the *L. pnuemophila* (NCTC12821) the numbers of the *Legionella* cfu was achieved at 20 cfu/ml at the start of the biofilms. The highest level (5300cfu/ml) of *Legionella* cfu which was more than 264 times as high as the initial density and was also achieved at day 7 and, in comparison to 25 °C biofilm system, the latter's highest level appeared at day 16 with 141 times higher than the initial level. The fast growth rate of *Legionella* in the planktonic phase at 30 °C indicated that the temperature altered the *Legionella* growth which is in general agreement with other workers (Rogers and Keevil, 1992; Rogers *et al.*, 1994). The ratio of the *Legionella* cfu against total bacterial cfu was 1.7% (BCYE PC/YEA PC) and 0.5% (BCYE PC/R2A PC) in the planktonic phase of biofilms system at 30 °C.

2. Analysis of the bacteria and amoeba in biofilms

Flow cytometric analysis.

In the 28 days incubation period, the average percentage of flow cytometric analysis of total bacteria against direct counting by epifluorecsence microscopy was 117% (ratio FCM/EFM; r = 0.8600; n = 8) for biofilms at 30 °C with the high bacterial density (10^5cells/cm^2). For the *Legionella*, flow cytometric analysis showed that the average density of the cells in biofilms was 21636 cells/cm^2 (n=8) while the density of *Legionella* in tap water biofilms was 4445 cells /cm^2. The average numbers of amoebae in biofilms by flow cytometer was 25028 cells /cm^2 and the ratio of EFM DC /FCM for amoebae was 53%. In the tap water biofilms (at 30 °C), the amoebal density was 1811 cells /cm^2 and the ratio was 71.4% (EFM DC/FCM). The ratio of cfu (total bacteria by R2A PC) against flow cytometric analysis (total bacterial cells numbers by FCM) was 13.2% which was 1.8% less than the ratio of R2A PC/EFM DC (15%). The ratio of *Legionella* against amoeba was 203% in this biofilm by flow cytometric analysis while in tap water biofilms the ratio was 245% at 30 °C. To compare the results

by direct reading of *Legionella* cells in biofilms with those in suspensions by EFM and flow cytometry, the direct reading on biofilms was lower and was only 68% of EFM and 63% of FCM results.

Direct counting was carried out in analysing the biofilms with DAPI, CTC, mAb-FITC by directly counting the cells in the biofilms on the slides. The average numbers of active bacteria (CTC positive cells) was 2% of total by DAPI direct counting. The total *Legionella* cells by mAb-FITC direct counting was 1.2% of total numbers of bacteria by DAPI DC.

To compare the counting results of *Legionella* cells by the BCYE PC against flow cytometric analysis of the numbers of FITC-mAb stained cells, at 30 °C, the ratios range from 5.4% (Aire river water biofilms) to 8.8% (tap water biofilms), while for the 25 °C biofilms (without seeding with *Legionella*), the ratio was very low percentage (0.47%), which means that the temperature of 30 °C largely supported increasing numbers of the active *Legionella* in whole populations more than at 25 °C.

The culurable *Legionella* numbers achieved highest density at day 20 (BCYE PC; 2600 cfu/cm^2) which was close to the tap water biofilms (at day 21). The total activated bacterial count reached its highest level at 7 days (R2A cfu; $3.24x10^5$ /cm^2).

(iii) Conclusion for the FCM analysis of the 30 °C biofilms

1. The data obtained from the current work have further indicated that flow cytometry can be succesfully used in the study of organisms on the biofilms. By analysis of 30 °C biofilms with seeded high numbers of *Legionella* (density; 10^4/cm^2) show that flow cytometric analysis of both *Legionella* as well as total bacteria have a good statistical correlation with direct counting by epifluorescent microscopy. The ratio of FCM with EFM is 117% (r=0.8600, n=8) for the enumeration of total bacteria in biofilms at 30 °C. The ratio of FCM with EFM for *Legionella* (density; 2 x 10^4 cells/cm^2) is 110% (r=0.9811, n=9) for the 30° C biofilms.

Table 5.14 Comparison of Flow Cytometric Analysis of Biofilms with EFM Direct Counting

Biofilms	Ratio% FCM/EFM for *Legionella*	Ratio % for total Bacteria
30 °C seeding	110% r = 0.9811, n = 9 ($2x10^4/cm^2$)	117%, r = 0.8600, n = 8, ($6x10^5/cm^2$)
25 °C No seeding	104%, r = 0.9745, n = 7 ($8x10^3/cm^2$)	N/A
30 °C Tap seeding	93% r = 0.9993, n = 6 ($4x10^3/cm^2$)	N/A
Pure Culture	99-133% r = 0.9887 n = 4 (1-$9x10^5$/ml)	

N/A = Not applicable

2. The current data (Table 5.15) show that in different biofilms, the ratios of the bacteria are different, the 25 °C biofilms contain lower densities of viable *Legionella* (cfu) which is close to natural environmental biofilms. The density (9.9×10^4cfu/l) in the planktonic phase is in the normal range $<10^4$ to 10^5cfu /l (Lee and West 1991), the culturable *Legionella* cfu is at the low level ratio (0.475% cfu/total cells) in the total *Legionella* population. The 30 °C biofilms contain higher densities of *Legionella* while the planktonic phase density reached the highest level ($1.87x10^6$cfu /l), which is in the risk range (10^5 to 10^7cfu /l). The ratio of cfu/ total *Legionella* cells is at a high level (6.7% cfu/ total *Legionella* cells). The tap water biofilms are also in the high level of *Legionella* density ($3.1x10^6$cfu /cm^2) and its ratio of *Legionella* cfu/total *Legionella* cells is also at the same high level (6.6%) within the 30 °C biofilms.

Table 5.15 Comparison of Bacterial Densities in the Biofilm

Biofilms	30° C (river water) seeded	25° C (river water) unseeded	30° C (tap water) seeded
Legionella (FCM cells/cm^2)	21600	8000	4000
Legionella BCYE (cells/cm^2)	2470	43	297
BCYE/FCM (%)	11	0.47	6.6
BCYE /R2A (%)	5.1	0.06	na
Legionella BCYE (cells/ml)	1870000	99000	3100000

6.0 GENERAL DISCUSSION

6.1 GENERAL DISCUSSION

A comprehensive study on the application of flow cytometry for the analysis of biofilms has been undertaken and the results presented in this thesis. It has been shown that flow cytometry can been successfully used to enumerate, sort and image the bacteria and amoebae in biofilms and water distribution systems as a rapid and sensitive semi-automated technique compared with the conventional microbiology. The novel fluorescent dyes and (fluorescein-labelled monoclonal antibodies) from the most current commercial dyes also have been screened and the staining protocols have been optimised and adopted for flow cytometric analysis and direct counting by EFM. Optimisation was done using pure cultures and samples from drinking water distribution systems. Biofilms were generated using both treated drinking water and river water with and without seeding with *L. pneumophila.* The tap water biofilms and river water biofilms were analysed by the flow cytometer and direct counting methods as well as by conventional microbiological methods (colony counting). The bacterial populations in real water distribution systems have been fully investigated and the total, viable bacteria were determined by the above methods.

It has been shown that the results of flow cytometric analysis of total *L. pneumophila* cells have a strong statistical correlation with the numbers of *Legionella* by BCYE colony counting methods for biofilms and planktonic phases. There are also strong statistical correlations between flow cytometric analysis and epifluorescent microscopic analysis (direct counting) for determination of bacteria, including *Legionella*, *E. coli, Salmonella*, *Pseudomonas* and amoeba, and total and viable cells in pure cultures. A similar statistical correlation was seen with bacteria in water distribution systems and biofilms.

6.2 OPTIMISATION OF THE FLOW CYTOMETER

Threshold and its levels of selection are very important for flow cytometric analysis. Optimal threshold and its levels optimise the results with lower background noise and give accurate counting and high recovery sorting. For the same samples,

for example mAb-FITC stained *L. pneumophila* suspension, results using fluorescent detector FL1-H at 530 nm as threshold could give 100% of real cells in the samples, but only 10% of cells if using forward scatter detector FSC-H as threshold. Current work for threshold parameters shows that for counting or sorting bacteria stained with certain dyes, it is better to choose the same fluorescence colour parameter with the dye used to stain the target cells as the threshold for the flow cytometric analysis (Table 6.1).

Table 6.1 Selections of Parameters as Threshold for Different Staining

Stain	Threshold Detector
CTC	FL3 Red detector (600 nm)
DAPI	FL1-H Green detector (530 nm)
HOE33342	FL1-H Green detector (530 nm)
PI	FL3 Red detector (600 nm)
Rh123	FL1-H Green detector (530 nm)
Mab-FITC	FL1-H Green detector (530 nm)

Note: DAPI is often used in dual staining with green or red fluorescent dyes so the threshold detector should be FL1-H or better still FL2-H.

Threshold levels could be affected by laser power levels, PMT voltage levels as well as sample flow rates. For the green fluorescence threshold (FL1), the relationship between PMT voltage(Y) and the threshold levels (X) could be described as the strong linear correlation ($Y = 0.625X + 443.75$, $r = 1.0$, $n = 5$). For the sample flow rate, the optimal is at the rate of 1000 events /second for both accurate enumerating and sorting under our experimental conditions. The laser power was fixed at 200 mW and PMT level was at 600 V in this study.

6.3 OPTIMISATION OF MOLECULAR STAINS FOR FLOW CYTOMETRY

The thesis set out to optimise a variety of stains for the detection and enumeration of bacteria. Some stains will provide total counting and other stains

which can be transformed by bacteria can give an indication of viability. A list of the stains together with their excitation and emission spectra are given in Table 6.2 together with data on optimum times and staining concentrations for EFM and FCM analysis.

Table 6.2 **List of Stains used in the Study Together with their Excitation and Emission Spectra and Optimal Time/temperature for Staining.**

Stain	Excitation (nm)	Emission (nm)	Concentration	Time (min)	Tempertature (^{o}C)
AO	503	530 (DNA) 640 (RNA)	10 μg/ml	5	RT
CTC	530	600	2-4mM	120	RT/37
DAPI	372	455	2-5 μg/ml	15	RT
FDA	505	530	Unsuitable for environmental samples		
HOE342	395	450	2 μg/ml	30	37
mAb	490	530	12.5-25%	60	37
PI	500	630	5 μg/ml	20	RT
Rh123	485	546	5	30	RT

The best stain for counting total bacteria was found to be DAPI. The best stain for counting viable bacteria was CTC although *L. pneumophila* stained very poorly with CTC. CTC staining was shown to improve with the addition of sodium pyruvate (4 mM). As far as it is known there are no details of CTC staining of *Legionella* which have been published, but the CTC's toxic effects on the bacteria has been reported by Ullrich (1996). The data from the current staining work have shown that even for pure cultures of bacteria, the highest CTC positive cell numbers never reached 90% or more of the total count and ratios ranged from 60% to 80% (Tables 3.2, 3.5 and 3.6). The reasons for this could be due to the CTC toxicity, or, the fact that in pure bacterial populations some bacteria could be dead or lose their

metabolic activity. The traditional colony count methods can only show the viable and culturable cells (VC) and direct counting by flow cytometry could detect all the cells including VC cells, viable non-culturable cells as well as dead cells in the whole population. One explanation of the failure of staining *L. pneumophila* by using CTC could be that *Legionella* expels the CTC by the efflux pumps on its cell membrane. The further investigation using the lower temperature 4 °C to reduce the membrane pump's ability, has shown that in normal staining time (4 h) up to three days, CTC was still negative for staining *L. pneumophila*. By incubating for a few weeks and up to two months at 4 °C (keeping the slides in the fridge), microscopic examination could find very low numbers of CTC positive *Legionella* cells in view fields which included the normal small size cells and the *Legionella* filaments. This would tend to indicate that there is not a pumping mechanism involved in the failure of CTC to stain the cells and it might be a subject for future research.

AO could be used for total cell counting by EFM but is not suitable for viability staining or flow cytometry. The proportion of red (viable cells) was found to increase with stain concentration. Fluorescein diacetate stained bacteria in environmental samples poorly and was therefore not used for studying biofilms using EFM or FCM. Rh123 and HOE342 were also found to be good for counting viable bacteria and Rh123 could be used as a dual stain with CTC. Propidium iodide was found to be useful for staining non-viable cells for both EFM and FCM and it could be used in combination with DAPI to give total and viable counts. Combining PI with Rh123 gave unstable staining. CTC and DAPI combined well for counting total and viable cell counts and it was decided to use these two stains for the study of the biofilms. Monoclonal antibody (mAb) is suitable for detecting *L. pneumophila* in biofilms and water samples by flow cytometry and the data from the current work have demonstrated there were no cross reactions by using the mAb for labelling the *L. pneumophila*. The mAb FITC labelling of *L. pneumophila* could be used to predict the viable cultural cells (CFU) due to the strong correlation between the two methods. The literature for the applications of flow cytomety to determine *Legionella* and amoebae is mainly limited to pure culture studies on these organisms. Flores *et al.* (1990) reported the use of mAb and flow cytometry for the differentiation of *Naegleria fowleri* from *Acanthamoeba* species. Muldrow (1982) described the use of flow cytometry for the study of free-living amoebae and,

recently, Harf *et al.*, (1997) reported the use of flow cytometry to study the endocytosis of pure culture, viable *L. pneumophila* by *Acanthamoeba palestinensis* and his work provided a valuable method for the recovery of viable *Legionella*. Avery *et al.*, (1995) reported the application of flow cytometry to sort soil amoebae. As far as it is known there are no details of flow cytometric analysis of amoeba and *Legionella* from tap water biofilms and environmental water biofilms.

6.4 COMPARISON OF CULTURAL, MICROSCOPIC AND FLOW CYTOMETRY METHODS FOR THE DETECTION OF BACTERIA

Counting using the standard colony counting media is the basic method for assessing the hygienic quality of treated waters and the numbers of bacteria in environmental waters. It can also be used to assess the quality of water in buildings and installations such as cooling towers. One disadvantage of this technique is the long incubation required for growth and EFM or FCM can be used to reduce this significantly.

6.4.1 In Pure Cultures

Flow cytometry of pure cultures was shown to give very good correlation with EFM. Cells of *S. aureus* counted using FCM and at the same time sorted onto microscope slides for EFM gave very accurate results (Table 5.1). Similar results were obtained for counting stained cells of *L. pneumophila* (Table 5.2).

6.4.2 In Environmental Samples

The current work has indicated that for colony counting methods the R2A colony count method is more sensitive than the YEA colony count methods using 1 day and 3 days incubation. The ratio is 2:5:256 (0.8:2:100 = YEA 1d cfu: YEA 3d cfu: R2A 7d cfu) for the determination of bacteria in water distribution systems. The coliform and *Legionella* spp were negative in the water distribution system samples in the current work, but the total viable and culturable bacteria (VC) was 256 cfu /ml on average and the highest density was 1054 cfu /ml. Though HPC bacterial

density has no direct impact on human health, it has sometimes been reported to promote the development of coliform bacteria and of macro-invertebrates such as *Asellus* and *Nais* (Kerneïs, *et al* 1995).

This study of bacteria in water distribution systems has shown that the traditional microbiological methods could only show 0.15% of total bacteria in water distribution systems when comparing R2A colony counts with DAPI counts using EFM (Tables 4.1 and 4.2) within up to 7 day testing and there was a good correlation between R2A PC and DAPI direct counting. The CTC direct counting indicated that even in treated water samples, there are still high numbers (10^3) viable bacteria per ml on average while the cfu densities were 10.7/ml by R2A PC and 3.25 cfu /ml by YEA 1d and 2.3 by 3d colony counts (Table 4.3). The coliform cfu = 0/100 ml. The current study shows that flow cytometry and direct counting methods can be used to quantify microorganisms in water distribution systems combined with the traditional microbiology in future. The big advantage here is that direct counting using EFM or FCM can provide data in as little as 60 minutes. One disadvantage is the cost of buying and running the flow cytometer compared with conventional culture methods.

6.4.3 In Biofilms

Colony counting of bacteria in biofilms was compared with direct counting for the river Aire generated biofilms. Like the water distribution samples, direct counting with DAPI and CTC gave a much higher recovery than conventional colony counts. In addition, R2A gave higher counts than YEA (Table 5.9, Figure 5.7b). The difference in counts is clearly demonstrated in Figure 5.5b where development of the biofilm can be seen much more clearly by direct counting using DAPI. Similar data can be seen in Table 5.9 and Figure 5.6 although bacteria decrease rather than increase.

6.5 COMPARISON OF CULTURAL METHODS AND FLOW CYTOMETRY FOR THE DETECTION OF *LEGIONELLA*

6.5.1 Tap Water Biofilms

One biofilm experiment was done using tap water seeded with *Legionella* at 30 °C. Recoveries from tap water biofilm when analysed by FCM and EFM gave

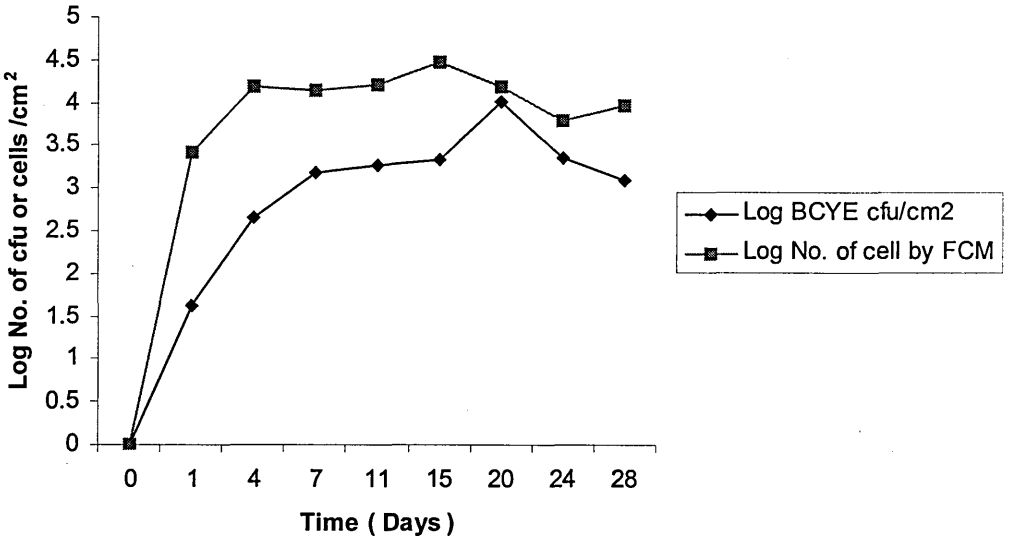
good correlation (Table 4.6). The analysis was repeated on three occasions (Table 4.7a and 4.7b) for both the planktonic and biofilm phases and again there was good correlation. *Legionella* peaked in the tap water biofilms after 3 days and then numbers fell until a peak at 14 days (Table 4.8). Numbers in the planktonic phase declined. Significantly lower results are obtained by culture in both planktonic and biofilm phases compared with direct counting.

6.5.2 In River Aire Water Biofilms

Two biofilms were generated using river Aire Water. The first was not seeded and incubated at 25 $^{\circ}$ C because low numbers of *L. pneumophila* were detected in the source water (Table 5.4). The second was seeded with *L. pneumophila* and the temperature increased to 30 $^{\circ}$ C. In the first experiment, the numbers of *Legionella* in the recirculated water remained low (probably because the recirculated water was only at 18 $^{\circ}$ C, the numbers in the planktonic phase increased from 2.5 cfu/ml to 365 cfu/ml in 16 days (Table 5.6a) and to 100 in the biofilm (Table 5.7a). However, numbers detected in the biofilm by both EFM and FCM were three orders of magnitude higher (Table 5.7a, Figure 5.5a) than those obtained by direct counting. There is also good agreement between counting by EFM and FCM.

In the seeded biofilm at 30 $^{\circ}$ C the numbers of *Legionella* peaked at day 7 (5,300 cfu/ml) with a second peak at day 15 (4,960 cfu/ml) before declining to 800 cfu/ml in 28 days (Table 5.10). The numbers in the biofilm peak on day 20 by using culture (10,260 cfu/cm^2) whereas they peak at day 15 by mAb direct counting (29,700 cfu/cm^2). Direct counting always gives higher results but at the higher temperature, the difference between the two is much smaller, particularly beyond the initial stages of the experiment.

Figure 6.1 Comparison of *Legionella* Counting in the Biofilm at 30 ° C Using BCYE Culture and mAb Direct Counting



This would suggest that at the higher temperature of 30 ° C the *Legionella* are more active in their growth and that because of this better recoveries have been obtained on BCYE.

Rogers (1994 a and b) reported the study of *Legionella* biofilms by using colony counting and microscopy. She showed that in the biofilm system, the planktonic flora contained a total microbial flora of 10^4 to 10^6 cfu/ml by using R2A PC, with numbers of *Legionella* CFU by BCYE PC between 10^3 to 10^4/ml. In her biofilms (on glass material), the total flora was $1.9x10^6$ cfu/cm^2 and *Legionella* cfu was $1.7x10^3$/cm^2 on average in 28 days in tap water at 30 °C. The ratio of *Legionella* with total flora was 0.9%.

The current work by flow cytometry shows that for *Legionella* in tap water biofilms, the number of cfu was $2.9x10^2$/cm^2 which is lower than those of Rogers and in the 30 °C river water biofilms, *Legionella* cfu was $2.4x10^3$/cm^2 (Table 6.3). The ratios of numbers of *Legionella* cfu in biofilms and the planktonic phase were different; in the tap water biofilms system, the current result was 9.6% compared with Rogers results of 101%. In the 30° C biofilms, the ratio from the current work was 132%, and in the 25° C river water biofilms, was 43%. Rogers only adopted the

colony counting methods (BCYE PC and R2A PC) to enumerate the cfu numbers of total bacteria and *Legionella* and there have been no details about the relationship between direct counting by flow cytometry or epifluorescent microscopy and colony counting for *Legionella* published up to now.

For the relationship between total bacteria and *Legionella* in a biofilm system, Rogers (1994 a and b) concluded that no direct relationship existed between total biofouling and the number of *L. pneumophila* cfu. The current work shows (Table 6.3) that in the river water biofilms seeded with *Legionella* (30 °C), the ratio of *Legionella* cfu to total bacteria (BCYE PC/R2A PC%) was 0.13% to 14.62% with 5.1% average, and the correlation between *Legionella* cfu and total bacteria cfu (R2A PC) was not good ($r = 0.2107$, $n = 9$); and 0.06% ($r = -0.0177$, $n = 8$) in 25 °C biofilms. The *Legionella* cfu could not be predicted accurately from the total bacterial cfu present in the biofilms. But the current work (Table 5.12; Table 6.3) has shown that there were strong relationships between *Legionella* cfu and total *Legionella* cells in biofilms.

In 30 °C river biofilms with seeded *Legionella*, the ratio of BCYE PC/ FCM count (total count with mAb) ranged from 0.36% to 21% with 6.68% average with a strong statistic correlation ($r = 0.8572$, $n = 8$) (Table 5.12). The further work by direct reading of *Legionella* cells stained on the biofilms directly with mAb-FITC in the biofilms show very poor correlation between *Legionella* cfu and total cells in biofilms ($r = 0.2861$, $n = 8$) and the ratio was ranged from 1.6% to 68% (19% average) for BCYE DC/mAb DC (Table 5.10).

Table 6.3 Comparison of Ratios of BCYE PC and mAb FCM (EFM) in Biofilms

Biofilm System	Ratio of BCYE PC mAb FCM Counting			
	%	r	n	
30 °C river water seeded FCM	8	0.8752	8	
30 °C river water seeded EFM	9	0.8163	8	
25 °C river water unseed FCM	0.47	0.8757	8	
25 °C river water unseed EFM	0.47	0.8940	8	
30 °C tap water seeding FCM	8.74	0.9026	6	
30 °C tap water seeding EFM	8	0.9084	6	
Average		0.8788		
	Ratio of BCYE PC/R2A PC			
30 °C river water seeded FCM	5.1	-0.0023	9	Biofilm
30 °C river water seeded FCM	0.5	-0.4490	9	Planktonic
25 °C river water unseed FCM	0.06	-0.0177	8	Biofilm
25 °C river water unseed FCM	0.05	0.5219	7	Planktonic

In the 25 °C river water biofilms without seeding with *Legionella* cells, the ratio of BCYE/ mAb FCM strongly correlated ($r = 0.8757$, $n = 8$) with an average of 0.47% (BCYE PC/ mAb FCM). The ratio of BCYE PC/ mAb EFM DC was 0.47% on average ($r = 0.8940$, $n = 8$). Again, in tap water biofilms (30°C with seeded *Legionella* cells), the ratio (BCYE PC/ mAb FCM) was 8.74% on average ($r = 0.9026$, $n = 6$). So, it is clear that the current work indicates that there is a direct, strong statistical correlation between the number of *Legionella* CFU and total number of *Legionella* cells in the *Legionella* population both in biofilms and planktonic phases at different temperatures and it may provide a method to predict the culturable *Legionella* (CFU) in tap water biofilms and river water biofilms by using flow cytometry or microscopy with mAb labelling.

6.6 COMPARISON OF CULTURAL METHODS AND FLOW CYTOMETRY FOR THE DETECTION OF AMOEBAE

The literature for the applications of flow cytomety to determine *Legionella* and amoebae is mainly limited to pure culture studies on these organisms. Flores *et al.*, 1990 reported the use of mAb and flow cytometry for the differentiation of *Naegleria fowleri* from *Acanthamoeba* species. Muldrow (1982) described the use of flow cytometry for the study of free-living amoebae and, recently, Harf *et al.*, (1997) reported the use of flow cytometry to study the endocytosis of pure culture, viable *L. pneumophila* by *Acanthamoeba palestinensis* and his work provided a valuable way for the recovery of viable *Legionella* by growing the *Legionella* in amoebae. Avery *et al.*, (1995) reported the application of flow cytometry to sort soil amoebae. As far as it is known there are no details of flow cytometric analysis of amoeba and *Legionella* from tap water biofilms and environmental water biofilms.

6.6.1 In the Tap Water Biofilms

Amoebae could be counted in biofilms by both EFM and FCM. Numbers were seen to increase to a maximum of 2,946 cells per cm^2 in the biofilm phase by day 14 and 2,581 in 28 days by FCM (table 4.8). This corresponds to a decrease in the numbers of *Legionella.*

6.6.2 In River Aire Water Biofilms

Amoebae were counted in the Aire river biofilms at 30 °C. Counts were maximum at day 20 at 34,830 cells per cm^2 (Table 5.10).

6.6.3 Determination of Biofilm Development on Surfaces Other than Glass

Attempts were made to generate biofilms on other surfaces (uPVC, copper and stainless steel. It was found to be difficult to read biofilm development on the other materials because uPVC in particular was white and reflected too much light. Also, it was difficult to remove biofilms from the other materials. uPVC in particular was very soft and scraping removed the base material. Only low numbers developed on copper.

6.7 FURTHER WORK

Referring back to the objective of this project, results have been obtained which clearly indicate that flow cytometric analysis was successful for the enumerating, sorting and imaging of bacteria and protozoa in pure culture, water distribution systems, and biofilms as a more rapid and accurate method than the conventional microbiology. Further work should be carried out as follows:

6.7.1 Flow Cytometry

In the preliminary flow cytometric analysis reported in the thesis only the green detector (FL1-H, 530nm), red detector (FL3-H, 590 nm) and forward scatter detector (FSC-H) were chosen as thresholds for the analysis. The UV detector should also adopt 390 nm as a threshold, which may offer the two and three multiple colour detectors together to image the target samples stained with two or three different dyes, and give more accurate 2-D and 3-D total population, viable subpopulation images for enumerating and separate sorting. In the current work, the UV fluorescence has not been accurately detected by the flow cytometer due to the fact that the UV detector could not be accurately calibrated, and the UV fluorescence was mainly viewed by the epifluorescent microscope. Better detection of UV could be achieved by using a specific UV laser as opposed to a dual laser with UV as the second line. This would allow a more accurate calibration of the UV fluorescence detector.

The flow cytometric auto-recognising target technique will be very useful and reliable for determination of environmental microorganisms. In this study, the preliminary work was only carried out using the multiple colour gating technique to view, trace and image the target cells and population changes, distributions and recombinations following changing parameters in two D or three D plots.

6.7.2 Staining and Screening New Dyes for FCM Application

In the current work, commercial dyes, which are usually used for solid staining have been screened for flow cytometric analysis. For total enumeration, DAPI and PI have been recognised as the best dyes for flow cytometric analysis, and CTC as a best probe for detecting the viability. Rh123 has been successfully used for Gram-negative bacteria staining by using glutaraldehyde pre-treatment. CTC for

staining *Legionella* was not successful in the current study and further work would be needed to find the reasons which could identify the mechanisms of *Legionella* metabolism and understand why the cells can live in the low nutrient and high temperature environments. Novel dyes for detecting the viability of *Legionella* could also be screened.

The current work has shown that there is a strong correlation between BCYE PC (CFU) with mAb-FITC-stained total *L. pneumophila* cells by flow cytometry in biofilms and in the planktonic phase. Similar studies on the relationship between BCYE PC and flow cytometry with mAb FITC in environmental samples should be carried out.

6.7.3 Applicability of these Findings to the Future Analysis of Water and Environmental Samples

Flow cytometry can be used to obtain a more accurate and faster count than colony counting. In addition, it can detect cells which cannot be grown by conventional cultural techniques. Its application would be in the study of the development of the organisms in biofilms and the rapid screening of samples which might contain sufficient organisms to be a public health problem.

7.0 REFERENCES

Alder, I. (1987)

Proceedings of the International European Congress on Biotechnology, **1**:1. Amsterdam, The Netherlands.

Allison, D. G. and Gilbert, P. (1992)
Bacterial biofilms. Scientific Progress, Oxford, **76**: 305-321.

Allman, R., Hann, A. C., Phillips, A. P., Martin, K. L. and Lloyd, D. (1990)
Growth of *Azotobacter vinelandii* with correlation of Coulter cell size, flow cytometric parameters, and ultrastructure.
Cytometry, **11**: 822-831.

Allman, R., A. Hann, A. C., Manchee, R. and Lloyd, D. (1992)
Characterization of bacteria by multiparameter flow cytometry.
Journal of Applied Bacteriology, **73**: 438-444.

Amann, R. I., Ludwig, W. and Schleifer, K. H. (1995)
Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiological Reviews, **59**: 153-169.

Alvarez, A., Ibáñez, M. and Rotger, R. (1993)
ß-Galactosidase activity in bacteria measured by flow cytometry.
Biotechniques, **15**: 974-976.

American Public Health Association (1989)
Standard methods for the examination of water and wastewater, 17th edition.
American Public Health Association, Washington, D.C.

Anand, C. M., Skinner, A. R., Malic, A. and Kurtz, J. B. (1983)
Interaction of *Legionella pneumophila* and free-living amoebae (*Acanthamoebae palestinesis*). Journal of Hygiene (Cambridge), **91**: 167 - 178.

Anon, (1982)
The bacteriological examination of drinking water supplies.
HMSO London. Report **71**: 54-55.

Anon, (1989)
Isolation and identification of *Giardia* cysts, *Cryptosporidium* oocysts and free-living amoebae in water etc. HMSO, London.

Anon, (1994)
The microbiology of water 1994. Part 1 - drinking water.
Reports on Public Health and Medical Subjects No. **71**: HMSO, London.

Aristegui, J. and Montero, F. M. (1995)
The relationship between community respiration and ETS activity in the ocean. Journal of Plankton Research, **17**: 1563-1571.

Arndt-Jovin, D. J. and Jovin, T. M. (1977)
Analysis and sorting of living cells according to deoxyribonucleic acid content. Journal of Histochemistry and Cytochemistry, **25**: 585-589.

Atkinson, B. (1975)
Biochemical Reactors. Point Press, London.

Atlas, R. M. (1982)
Enumeration and estimation of microbial biomass. In Experimental Microbiological Ecology, edited by Burns, R. G. and Slater, J. H., 84-101. Blackwell Scientific, Oxford.

Atlas R., Williams, J. F. and Huntington, M. K. (1995)
Legionella contamination of dental-unit waters. Applied and Environmental Microbiology, **61**:1208-1213.

Atlas R.M. (1999)
Legionella: from environmental habitats to disease pathology, detection and control. Environmental Microbiology, **1**: 283 - 293

Avery, S., Harwood, J. and Lloyd, D. (1995)
Quantification and characterization of phagocytosis in the soil amoeba *Acanthamoeba castellanii* by flow cytometry. Applied and Environmental Microbiology, **61**: 1124-1132.

Baldwin, E. R. (1927)
Tuberculosis: bacteriology, pathology and laboratory diagnosis, 59-61. Lea and Febiger, Philadephia.

Balfoort, H. W., Snoek, J., Smits, J.R.M., Breedveld, L.W., Hofstraat, J. W., and Ringelberg, J.(1992)
Automatic identification of algae: neural network analysis of flow cytometric data. Journal of Plankton research, **14**: 575-589.

Barer, M. R., Gribbon, L. T., Harwood, C. R. and Nwoguh C. E. (1993)
The viable but non-culturable hypothesis and medical bacteriology. Reviews in Medical Microbiology, **4:** 183-191.

Barer, M. R. (1997)
Viable but non-culturable and dormant bacteria: time to resolve an oxymoron and a misnomer?
Journal of Medical Microbiology, **46**: 629-631.

Barker, J., Farrell, D., and Hutchisonet, J. G. P. (1986)
Factors affecting growth of *Legionella pneumophila* in liquid media.
Journal of Medical Microbiology, **22**: 97-100.

Barker, J., Michael R., Brown, W., Collier, P. J., Farrell, I. and Gilbert, P. (1992)
Relationship between *Legionella pneumophila* and *Acanthamoeba polyphaga*: physiological status and susceptibility to chemical inactivation.
Applied and Environmental Microbiology, **58**: 2420-2425.

Barker, J. and Brown, M. R. (1994)
Trojan Horses of the microbial world: protozoa and the survival of bacterial pathogens in the environment.
Microbiology, **140**: 1253-1259.

Barker J., Scaife, H. and Brown, M. R. W. (1995)
Intraphagocytic growth induces an antibiotic-resistant phenotype of *Legionella pneumophila.*
Antimicrobial Agents and Chemotherapy, **39**: 2684-2688.

Bhamidimarri, S. M, R., Greenfield, P. F. and Bell, P. R. F. (1987)
Biofilms characteristics in a fluidized – bed bioreactor.
Proceedings of the 42nd Industrial Waste Conference, 103-111. Purdue University, West Lafayette, Ind.

Bitton, G., Koopman, B., Jung, K., Voiland, G. and Kotob, M. (1993)
Modification of the standard epifluorescence microscopic method for total bacterial counts in environmental samples.
Water Research, **27**:(7) 1109-1112.

Bjornsen, P. K. (1986)
Automatic determination of bacterioplankton biomass by image analysis.
Applied and Environmental Microbiology, **51**: 1199-1204.

Block, J. C., Muteauk, L., Gatel, D., and Reasoner, D. J. (1997)
Survival and growth of *E. coli* in drinking water distribution systems.
In Coliforms and *E. coli* Problems or Solution? Edited by Kay, D. and Fricker, C., 157 - 167.

Bogosian, G., Patricia, J., Morris, L. and O'Neil, J. (1998)
A mixed culture recovery method indicates that enteric bacteria do not enter the viable but non-culturable state.
Applied and Environmental Microbiology, **64**:(5) 1737-1742.

Borazjani, R. N., May, L., Noble, J. A., Avery, S. V. and Ahesrn, D. G. (2000)
Flow cytometry for determination of the efficacy of contact lens disinfecting solutions against *Acanthamoeba* spp..
Applied and Environmental Microbiology, **66**: 1057-1061.

Boulanger, C. and Edelstein, P. (1995)
Precision and accuracy of recovery of *Legionella pneumophila* from seeded tap water by filtration and centrifugation.
Applied and Environmental Microbiology, **61**: 1805-1809.

Bovill, R. A., Shallcross, J. A. and Mackey, B. M. (1994)
Comparison of the fluorescent redox dye 5-cyano-2,3-ditolyltetrazolium chloride with p-iodonitrotetrazolium violet to detect metabolic activity in heat-stressed *Listeria monocytogenes* cells.
Journal of Applied Bacteriology, **77**: 353-358.

Bratbak, G. (1985)
Bacterial biovolume and biomass estimations.
Applied and Environmental Microbiology, **49**: 1488-1493.

Bratbak, G. and Dundas, I. (1984)
Bacterial dry matter content and biomass estimations.
Applied and Environmental Microbiology, **48**: 755-757.

Brailsford, M. A. and Gatley, S. (1993)
Industrial application of flow cytometry for the rapid detection of microorganisms.
In New Techniques in Food and Beverage Microbiology. Edited by Kroll, R. G., Gilmour, A. and Sussman, M. Society for Applied Bacteriology, Technical Series 31, Blackwell Scientific Publications, 67 - 84.

Breed, R. S. and Brew, J. D. (1925)
Counting bacteria by means of the microscope.
Technical Bulletin, 49. N.Y. Agricultural Experiment Station. Geneva. N.Y.

British Standard (1992a)
Methods for Sampling for *Legionella* Organisms in Water and Related Materials, BS7592:1992.
British Standard Institution, London.

British Standard (1992b)
Methods for Detection and Emumeration of *Legionella* Organisms in Water and Related Materials, DD 211:1992.
British Standard Institution, London.

British Standard (1998)
Detection and Enumeration of *Legionella*. BS 6068 Part 4.12. British Standard Institution, London.

Brown, M. R. W. and Gilbert, P. (1993)
Sensitivity of biofilms to antimicrobial agents.
Journal of Applied Bacteriology Symposium Supplement, **74**: 87S-97S.

Bruetschy, A., Laurent, M. and Jacquet, R (1994)
Use of flow cytometry in oenology to analyse yeasts.
Letters in Applied Microbiology, **18**: 343-347.

Bryers, J. D. and Characklis, W. (1981)
Early fouling biofilm formation in a turbulent flow system: overall kinetics. Water Research, **15**: 483-491.

Bryers, J. D. (1993)
The biotechnology of interfaces.
Journal of Applied Bacteriology Symposium Supplement, **74**: 98S-109S.

Buchrieser, C. and Kaspar, C. B. (1993)
An improved direct viable count for the enumeration of bacteria in milk.
International Journal of Food Microbiology, **20:** 227-236.

Burkhill, P. H. (1987)
Analytical flow cytometry and its application to marine microbial ecology.
In Microbes in the Sea, 139-166. Edited by Sleigh, M. A., Chichester : Ellis Horwood.

Burton, G. A. and Lanza, G. R. (1985)
Sediment microbiological activity tests for the detection of toxicant impacts.
In Aquatic Toxicology and Hazard Assessment. Edited by Caldwell, R. D. and Purdy, R. C., 7th Symposium. 214-228. ASTM. Philadeiphia, Pa.

Calwell, D. E., Korber, D. R. and Lawrence, J. R. (1992)
Confocal laser microscopy and digital image analysis in microbial ecology. Advances in Microbiological Ecology, **12**: 1-67.

Calwell, D. E., Korber, D. R. and Lawrence, J. R. (1993)
Analysis of biofilm formation using 2D vs 3D digital imaging.
Society for Applied Bacteriology Symposium Supplement, **74**:(22) 52S-66S.

Caspersson, T. O. and Schultz, J. (1938)
Nucleic acid metabolism of the chromosomes in relation to gene reproduction. Nature, **142**: 294-295.

Chang, H. T. and Rittmann, B. E. (1986)
Biofilm loss during sample preparation for scanning electron microscopy.
Water Research, **11**: 1451-1456.

Characklis, W. G. (1973)
Attached microbial growths - I. Attachment and growth.
Water Research, **7**: 1113-1127.

Characklis, W. G. (1981)
Fouling biofilm development: a process analysis.
Biotechnology and Bioengineering, **23**: 1923-1960.

Characklis, W. G., Trulear, M. G., Bryers, J. D. and Zelver, N. (1982)
Dynamics of biofilm processs: methods.
Water Research, **16**: 1207-1216.

Characklis, W. G. (1990)
Laboratory biofilm reactor.
In Biofilms. Edited by Characklis, W. G. and Marshall, K. C., 55-89.
Wiley, New York.

Chen, H. C. and Wu, S. D. (1992)
Agar medium for enumeration of fecal coliforms.
Journal of Food Science, **57**:(6) 1454-1457.

Chen, J. and Koopman, B. (1997)
Effect of fluorochromes on bacterial surface properties and interaction with granular media.
Applied and Environmental Microbiology, **63**:(10) 3941-3945.

Chen F., Lu, J. R., Binder, B., Liu, Y. C. and Hodson, R. (2001)
Application of digital image analysis and flow cytometry to enumerate marine viruses stained with SYBR Gold.
Applied and Environmental Microbiology, **67**: 539-545.

Christensen, B., Sternberg, C., Andersen, J., Eberl, L., Möller, S., Givskov, M. and Molin, S. (1998)
Establishment of new genetic traits in a microbial biofilm community.
Applied and Environmental Microbiology, **64**: 2247-2255.

Cherry, W.B., Pittman, P. P., Harris, G. A., Herbert, B. M., Thomason, B. L., Thacker, L. and Weaver, R. E. (1978)
Detection of Legionaires'disease bacteria by direct immunofluorescent staining. Journal of Clinical Microbiology, **8**: 329-338.

Clarke, R. G. and Pinder, A. C. (1998)
Improved detection of bacteria by flow cytometry using a combination of antibody and viability markers.
Journal of Applied Microbiology, **84**: 577-584.

Coallier, J., Prvost, M. and Rompre, A. (1994)
The optimization and application of two direct count methods for bacteria in distributed drinking water.
Canadian Journal of Microbiology, **40**: 830-836.

Colbourne, J. and Dennis, P. (1989)
The ecology and survival of *Legionella pneumophila.*
Journal of the Institution of Water Engineers and Scientists, **3**: 345-350.

Colwell, R. R. (1979)
Enumeration of specific populations by the most-probable-number (MPN) method. 56-61.
In, Native Aquatic Bacteria: Enumeration, Activity and Ecology. Edited by Costerton, J. W. and Colwell, R. R.. American Society for Testing and Materials, Philadelphia.

Costerton, J. W., Cheng, K. J., Geesey, K. G., Ladd, P. I., Nickel, J. C., Dasgupta, M. and Marrie, T. J. (1987)
Bacterial biofilms in nature and disease.
Annual Reviews in Microbiology, **41**: 435-464.

Crosland -Taylor, P. (1953)
A device for counting small particles suspended in fluid through a tube.
Nature, **171**: 37-38.

Coulter, W. H. (1956)
High speed automatic blood cell counter and cell size analyser.
Proceedings of a National Electrophoresis Conference, **12**: 1034-1042.

Daley, R. J. (1979)
Direct epifluorescence enumeration of native aquatic bacteria: uses, limitations and comparative accuracy. 29-45.
In, Native Aquatic Bacteria: Enumeration, Activity and Ecology. Edited by Costerton, J. W. and Colwell, R. R.. American Society for Testing and Materials, Philadelphia.

Dann, O., G. Bergen, G., Demant, E. and Vogl, G. (1971)
Trypanocide diamine des 2-phenyl-benzoforan, 2-phenylindoles.
Liebigs Annalen Der Chemie, **749**: 68-79.

Darzynkiewicz Z., Traganos, F., Sharpless, T. and Melamed, M. R. (1975)
Conformation of RNA *in situ* as studied by acridine orange staining and automated cytophotometry.
Experimental Cell Research, **95**: 143-153.

Darzynkiewicz Z., Traganos, F. and Melamed, M. (1980)
New cell cycle compartments identified by multiparameter flow cytometry. Cytometry, **1**: 98-108.

Darzynkiewicz, Z., Crissman, H (Eds) (1990)
Flow cytometry.
Methods in Cell Biology,Volume **33**: ISBN 0-12-564133-8. U.S.A.

Darzynkiewicz, Z., Robinson, H., and Crissman, H.(eds) (1994)
Flow cytometry, Seond Edition, Part A. Vol.**41**:
Methods in Cell Biology.

Darzynkiewicz, Z., Robinson, H., Crissman, H(eds) (1994)
Flow cytometry, Seond Edition, Part B. Vol. **42**:
Methods in Cell Biology.

de Beer, D., Stoodley, P., Roe, F. and Lewandowski, Z. (1994)
Effects of biofilms structures on oxygen distribution and mass transport.
Biotechnology and Bioengineering, **43**: 1131-1138.

Deere, D., Vesey, G., Dorsch, M., Ashbolt, N., Williams, K. and Veal, D. (1997)
Optimisation of *in-situ* ribosomal RNA labelling of *Cryptosporidium parvum* in suspension.
Proceedings of the second symposium on health related water microbiology, University of Warwick. Eds. Morris, R. and Gammie, A.

DeLeo, P. and Baveye, P. (1996)
Enumeration and biomass of bacteria in aquifer microcosm studies by flow cytometry.
Applied and Environmetal Microbiology, **62**: 4580-4586.

Diaper, J. P., Tither, K. and Edwards, C. (1992)
Rapid assessment of bacterial viability by flow cytometry.
Applied Microbiology and Biotechnology, **38**: 268-272.

Diaper, J. P. and Edwards, C. (1994a)
Survival of *Staphylococcus aureus* in lake water monitored by flow cytometry. Microbiology, **140**: 35-42.

Diaper, J. P. and Edwards, C. (1994b)
The use of fluorogenic esters to detect viable bacteria by flow cytometry.
Journal of Applied Bacteriology, **77**: 221-228.

Dittrich, W. and Gohde, W. (1969)
Impulsfluorometrie dei einzelzellen in suspensionen.
Zeitschrift Fur Naturforsch, 24b: 360-361.

Doggett, M. S. (2000)
Characterization of fungal biofilms within a municipal water distribution system.
Applied and Environmental Microbiology, **66**:(3) 1249-1251.

Donnelly, C.W. and Baignet, J. G. (1986)
Methods for flow cytometric detection of *Listeria monocytogenes* in milk. Applied and Environmental Microbiology, **52**: 689-695.

Dowdle, W. (1993)
In *Legionella*, Current status and emerging perspectives; Keynote Address 1976: Lessons learned, 1 - 4. Edited by Barbaree, J. *et al.*, American Society for Microbiology.

Dufour, P. and Colon, M. (1992)
The tetrazolium reduction method for assessing the viability of individual bacterial cells in aquatic environmets: improvements, performance and applications. Hydrobiologa, **232**: 211-218.

Duncan, S., Glover, L., Killham, K. and Prosser, J. (1994)
Luminescence-based detection of activity of starved and viable but non-culturable bacteria.
Applied and Environmental Microbiology, **60**: 1308-1316.

Edberg, S. C. and Allen, M. J. (1988)
National field evaluation of a defined substrate method for the simultaneous enumeration of coliforms and *Escherichia coli* from drinking water: comparison with the standard multiple tube fermentation method.
Applied and Environmental Microbiology, **54**: 1595-1601.

Edwards, C., Porter, J., Saunders, J. R., Diaper, J., Morgen, J. A. W. and Pickup, R. W. (1992)
Flow cytometry and microbiology. In: Flow Cytometry, Society for General Microbiology, **19**: 105-108.

Eighmy, T. T., Maratea, D. and Bishop, P. L. (1983)
Electron microscopic examination of wastewater biofilm formation and structural components.
Applied and Environmental Microbiology, **45**: 1921-1931.

Escher, A. R. and Characklis, W. G. (1988)
Microbial colonization of a smooth substratum: a kinetic analysis using image analysis.
Science and Technology, **20**: (11/12) 277-283.

Faude, U. and Hofle, M. (1997)
Development and application of monoclonal antibodies for *in situ* detection of indigenous bacterial strains in aquatic ecosystems.
Applied and Environmental Microbiology, **63**: 4534-4542.

Favilla, R., Stecconi, G., Cavatorta, P., Startor, G. and Mazzini, A. (1993)
The intereaction of DAPI with phospholipid vesicles and micells.
Biophysics and Chemistry, **46**: 217-226.

Fields, B. S., Barbaree, J. M., Sanden, G. N. and Morrill, W. E. (1990)
Virulence of a *Legionella anisa* strain associated with Pontiac fever: an evaluation using protozoan cell culture and guinea pig models.
Infection and Immunology, **58**: 3139-3142.

Fields, B. S., Uteley-Fields, S. R., Chin Loy, J. N., White, E. H., Steffens, W. L. and Shotts, E. B. (1993)
Attachment and entry of *Legionella pneumophila veriformis*.
Journal of Infectious Diseases, **167**: 1146-1150.

Flores, B. M., Garcia, C. A., Stamm, W.E. and Torian, B. E. (1990)
Differentiation of *Naegleria fowleri* from *Acanthamoeba* species by using monoclonal antibodies and flow cytometry.
Journal of Clinical Microbiology, **28**: 1999-2005.

Foster, J. W. and Hall, H. K. (1991)
Inducible pH homeostasis and the acid tolerance response of *Salmonella typhimurium*.
Journal of Bacteriology, **173**: 5129 - 5135.

Fouchet, P., Jan, S., Courtois, J., Courtois, B., Frelat, G. and Barbotin, J. N. (1995) Quantitative single-cell detection of poly (ß-hydroxybutyrate) accumulation in *Rhizobium meliloti* by flow cytometry.
FEMS Microbiology Letters, **126**: 31-36.

Fowler, J. D. and Robertson, C. R. (1991)
Hydraulic permeability of immobilized bacterial cell aggregates.
Applied and Environmental Microbiology, **57**: 102-113.

Frais,J., Ribas, F. and Lucena, F. (1994)
Substrate affinity from bacterial strains and distribution water biofilms.
Journal of Applied Bacteriology, **76**:182-189.

Fry, J. C. and Zia, T. (1982)
A method for estimating viability of aquatic bacteria by slide culture.
Journal of Applied Bacteriology, **53**: 189-98.

Fry, J. C. (1988)
Determination of biomass.
In Methods in Aquatic Bacteriology. Edited by Austin, B.J., Wiley, New York.

Fulwyler, M. J. (1969)
Electronic separation of biological cells by volume.
Science, **150**: 910-911.

Gasol, J. M., Zweifei, U. L., Peters, F., Fuhrman, J. A. and Hagström, Å. (1999)
Significance of size and nucleic acid content heterogeneity as measured by flow cytometry in natural planktonic bacteria.
Applied and Environmental Microbiology, **65**: 4475-4483.

Geesey, G. G. and White, D. C. (1990)
Determination of bacterial growth and activity at solid-liquid interfaces.
Annual reviews in Microbiology, **44**: 579-602.

Gehr, R. and Henry, J. G. (1983)
Removal of extracellar material. Techniques and pitfalls.
Water Research, **12**: 1743-1748.

Gilbert, P., Evans D. and Brown, M. (1993)
Formation and dispersal of bacterial biofilms *in vivo* and *in situ.*
Journal of Applied Bacteriology, Symposium Supplement, **74**: 67S-78S.

Givan, A. (1992)
Flow cytometry; first principles.
Wiley-Liss. John Wiley and Sons, Inc.

Gorman, S. P. and Scott, E. M. (1980)
A review; antimicrobial activity, uses and mechanism of action of glutaraldehyde.
Journal of Applied Bacteriology, **48**: 161-190.

Goshko, M. A., Minnigh, H. A., Pipes, W. O., and Christain, R. R. (1983)
Relationships between standard plate counts and other parameters in water distribution systems.
Journal of the American Water Works Association, **75**: 568-571.

Goss, W. A., Deitz, W. H. and Cook, T. M. (1964)
Mechanism of action of nalidixic acid on *E. coli.*
Journal of Bacteriology, **88**: 1112-1118.

Goulder, R. (1991)
Metabolic activity of freshwater bacteria.
Scientific Progress, Oxford (1991), **75**: 73-91.

Grimm, D., Ludwig, WF., Brandt, BC., Schleifer, KH., Hacker, J and Steinert, M (2001) Development of 18S rRNA-targeted oligonucleotide probes for specific detection of *Hartmanella* and *Neigleria* in *Legionella*-positive environmental samples. Syst. Appl. Microbiology **24** (1) 76 - 82

Guezennec, J. and Fera, P. (1987)
Utilisation detechniques bacteriologiques et biochimiques pour l'etude du biofilm bacterien.
Revue De L Institut Francals Du Petrole ET Annales Des Combustibles, **42**: 599-615.

Gunasekera, T. S., Attfield, P. V. and Veal, D. A. (2000)
A flow cytometry method for rapid detection and enumeration of total bacteria in milk.
Applied and Environmental Microbiology, **66**: 1228-1232.

Haddock,B.A; Jones, C.W (1997)
Bacterial respiration. Bacteriology Review **41,** 47-99

Hall, G. H., Jones, J. G., Pickup, R. W. and Simon, B. M. (1990)
Methods to study the bacterial ecology of fresh water environments.
Methods in Microbiology, **23**: 181-210.

Halvorson, H. O. and Ziegler, N. R. (1933)
Application of statistics to problems in bacteriology.
Journal of Bacteriology, **25**: 101-121.

Harf, C., Monteil, H. and Vetter, M. T. (1987)
Relations amoebes libres-*Legionella* dans l'environment.
In Collection Fondation Faulte M. Merieux, 139 - 143. Edited by Fleurette, J. N., Bornstein, D., Surgot, M.. Lyon: Colloque *Legionella* 6-7 Mai 1987.

Harf, C., Goffnet, S., Meunier, O., Monteil, H. and Colin, D. A. (1997)
Flow cytometric determination of endocytosis of viable labelled *Legionella pneumophila* by *Acanthamoeba palestinensis.*
Cytometry, **27**: 269 - 274.

Harris, C. M. and Kell, D. B. (1985)
The estimation of microbial biomass.
Biosensors, **1**: 17-84.

Harrison, T. and Talor, A. (eds) (1988)
A laboratory manual for *Legionella.*
Edited by Harrison, T. and Talor, A. ISBN 0 471 91861 X.

Harrison, T. G. and Saunders, N. A. (1994)
Taxonomy and typing of *Legionellae.*
Reviews in Medical Microbiology, **5**: 79-90.

Hickey, E. W. and Hirshfield, I. N. (1990)
Low pH induced effects of patterns of proteins synthesis and on internal pH in *Escherichia coli* and *Salmonella typhimurium.*
Applied and Environmental Microbiology, **56**: 1038 - 1045.

Hilwig, I and Gropp, A (1973).
Experimental Cell Research, **81**: 474-477.

Hitchens, A. P. and Leikind, M. C. (1939)
The introduction of agar-agar into bacteriology.
Journal of Bacteriology, **37**: 485-493.

Hoehn, R. C. and Ray, A. D. (1973)
Effects of thickness on bacterial film.
Journal of the Water Pollution Control Federation, **45**: 2302-2320.

Hoffman, R. M., Standridge, J. H., Prieve, A. F., Cucunato, J. C. and Bernhardt, M. (1997)
Using flow cytometry to detect protozoa.
Applied and Environmental Microbiology, **89**:(9) 104-111.

Hofstraat, J. W., de Vreeze, M. E. J., van Zeijl, W. J. M., Peperzak, J. C. H. and Balfoort, H. W. (1991)
Flow cytometric discrimination of phytoplankton classes by fluorescence emission and excitation properties.
Journal of Fluorescence, **1**: 249-265.

Holm-Hanson, O. and Booth, C. R. (1966)
The measurement of adenosine triphosphate in the ocean and its ecological significance.
Limnology and Oceanography, **11**: 510-519.

Hookey, J., (1995)
Typing and taxonomy of the *Legionellae*: an update.
PHLS Microbiology Digest, **12**:(4), 223 - 226.

Hoppe, H. G. (1976)
Determination and properties of actively metabolizing heterotrophic bacteria in the sea, investigated by means of micro-autoradiography.
Marine Biology., **36**: 291-302..

HSC (2001)
Legionnaires' Disease: The control of Legionella bacteria in water systems.
Approved Code of Practice and Guidance. ISBN 07176 177726

Huang, C. H., Yu, F. P., Mcfeters, G. A. and Stewart, P. (1995)
Non-uniform spatial patterns of respiratory activity within biofilms during disinfection.
Applied and Environmental Microbiology, **61**: 2252-2256.

Hudson, B. W., Upholt, B., Devinny, J. and Vinograd, J. (1969)
The use of an ethidium analogue in dye-buoyant density procedure for the isolation of closed circular DNA: the variation of the superhelix density of mitochondrial DNA.
Proceedings of the National Acadamy of Science, USA., **62**: 813-820.

Huq, A., Colwell, R. R., Rahman, R., Ali, A., Chowdhury, M. A. R., Parveen, S., Sack, D. A. and Russek-Cohen, E. (1990)
Detection of *Vibrio cholerae* in the aquatic environment by fluorescent monoclonal antibody and cultural methods.
Applied and Environmental Microbiology, **56**:(8) 2370 - 2373.

Hutter, K. J. and Eipel, H. E. (1978)
Flow cytometric determinations of cellular substances in algae, bacteria, moulds and yeasts.
Antonie van Leeuwenhoek, **44**: 269-282.

Ingram, M., Cleary, T.J., Price, B. J., Price,R. L., and Castro, A. (1982)
Rapid detection of Legionella pneumophila by flow cytomerty.
Cytometry,**3:**134-137.

Jepras, K. L., Carter, J.,Pearson, S. C.,Panl, F.E., and Wilkinson, M.J., (1995)
Development of a robust flow cytometric assay for determining numbers of viable bacteria.
Applied and Environmetal Microbiology, **61**: 2696-2701.

Jones, J. G. and Simon, B. M. (1975)
An investigation of errors in direct counts of aquatic bacteria by epifluorescence microscopy, with reference to a new method for dyeing membrane filters. Journal of Applied Bacteriology, **39**: 317-329.

Jonker R. R., Meulemans, J. T., Dubelaar, G. B. J., Wilkins, M: F. and Ringelberg, J. (1995)
Flow cytometry: A powerful tool in analysis of biomass distributions in phytoplankton.
Water Science and Technology, **32**:(4) 177-182.

Jorgensen, P. E., Eriksen, T. and Jensen, B. K. (1992)
Estimation of viable biomass in wastewater and activated sludge by determination of ATP, oxygen utilisation rate and FDA hydrolysis.
Water Research, **26**:(11) 1495-1501.

Joux, F. and Lebaron, P. (1997)
Ecological implications of an improved direct viable count mehtod for aquatic bacteria.
Applied and Environmental Microbiology, **63**;(9) 3643-3647.

Kalmbach, S., Manz, W. and Szewzyk, U. (1997)
Isolation of new bacterial species from drinking water biofilms and proof of their *in situ* dominance with highly specific 16S rRNA probes.
Applied and Environmental Microbiology, **63**: 4164-4170.

Kamentsky, L. A., Melamed, M. R. (1967)
Spectrophotometric cell sorter.
Science, **156:**1364-1365.

Kaprelyants, A. S. and Kell, D. B. (1992)
Rapid assessment of bacterial viability and vitality by rhodamine 123 and flow cytometry.
Journal of Applied Bacteriology, **72**: 410-422.

Kaprelyants, A. S. and Kell, D. B. (1993)
Dormancy in stationary-phase cultures of *Micrococcus luteus*; flow cytometry analysis of starvation and resuscitation.
Applied and Environmental Microbiology, **59**:(10) 3187-3196.

Kapuscinski, R. L. and Skoczylas, B. (1977)
Analytical Biochemistry., **102**: 344-352.

Karl, D. M. (1981)
Simultananeous states of ribonucleic acid and deoxyribonucleic acid syntheses for estimating growth and cell division of aquatic microbial communities.
Applied and Environmental Microbiology, **42:** 802-810.

Keevil, C., Bradshaw, D. J., Dowsett, A. B., and Feary, T. W. (1987)
Microbial film formation: dental plaque deposition on acrylic tiles using continuous culture techniques.
Journal of Applied Bacteriology, **62**: 129 - 138.

Keevil, C., Rogers, J. and Walker, J. (1995)
Potable water biofilms.
Microbiology Europe, **3**:(6) 10-14.

Kepner, R. L. and Pratt, J. (1994)
Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present.
Microbiological Reviews, **58**: 603-615.

Kerneïs, A., F. Nakache, F., Deguin, A. and Feinberg, M. (1995).
The effects of water residence time on the biological quality in a distribution network.
Water Research, **29**: 1719-1727.

Kilvington, S. and Price, J. (1990)
Survival of *Legionella pneumophila* within cysts of *Acamthamoeba polyphaga* following chlorine exposure.
Journal of Bacteriology, **68**: 519-525.

King, L. K. and Parker, C. B. (1988)
A simple, rapid method for enumerating total viable and metabolically active bacteria in groundwater.
Applied and Environmental Microbiology, **54**: 1030-1031.

Kirmeyer, G (2000)
AWWARF manual helps utilities maintain water quality in distribution systems. Drinking Water Research. Pub: AWWA Research Foundation pp2 - 5

Kogure, K., Simidu, U. and Taga, N. (1979)
A tentative direct microscopic method for counting living marine bacteria. Canadian Journal of Microbiology, **33**: 415-420.

Kohler, G. and Milstein, C. (1975)
Continuous cultures of fused cells secreting antibody of pre-defined specificity. Nature, **256**: 495-496.

Kohler, R., Bubert, A., Goebel, W., Steinert, M., Hacker, J. and Bubert, B. (2000)
Expression and use of the green fluorescent protein as a reporter system in *Legionella pneumophila*.
Molecular and General Genetics, **262**: 1060-1069.

Kooij, D., Veenendaal, H., Baars-Lorist, C., Klift, D. and Drost, Y. (1995)
Biofilm formation on surfaces of glass and teflon exposed to treated water. Water Research, **29**: 1655-1662.

Kopp, V., Tanghe, N. and Faivre, M. (1993)
Tertiary refining by nanofiltration of surface water in the Paris region.
Water Supply, **11**: 271-280.

Krambeck, C., Krambeck, H. J. and Overbeck, J. (1981)
Microcomputer-assisted biomass determination of plankton bacteria on scanning electron micrographs.
Applied and Environmental Microbiology, **42**: 142-149.

Kristensen, G. H. and Christensen, F. R. (1982)
Application of cryo-cut method for measurements of biofilm thickness.
Water Research, **16**: 1619-1621.

Kroll, R. G., Gilmour, A. and Sussman, M. (1993)
New techniques in food and beverage microbiology. 67-86.
Society for Applied Bacteriology, Technical Series No. 31, Blackwell Scientific Publications, London.

Lange, J., Thorne, P. and Lynch, N. (1997)
Application of flow cytometry and fluorescent *in situ* hybridization for assessment of exposures to airborne bacteria.
Applied and Environmental Microbiology, **63**:(4) 1557-1563.

Lappalainen, J. (2001)
Improved use and new applications of luminescent bacteria.
ISBN 951-29-1880-3. ISSN 0082-7002.Gillot Oy- Turku, Finland, 2001.

Latt, S. A. (1973)
Proceedings of the National Academy of Science, U.S.A, **70**: 3395.

Lawrence, J. R., Delaquis, P. J., Korber, D. R. and Caldwell, D. E. (1987)
Behaviour of *Ps. fluorescence* within the hydrodynamic boundary layers of surface microenvironments.
Microbiological Ecology, **14**: 1-14.

Lazarova, V., Capdeville, B. and Nikolov, L. (1992)
Biofilm performance of a fluidized bed biofilm reactor for drinking water denitrification.
Water Science and Technology, **26**:(3/4) 555-566.

Lazarova,V., Pierzo, V., Fontvielle, D., and Manem, J. (1994)
Integrated approach for biofilm cheracterization and biomass activity control.
Warwe Science and Technology, 29:(7) 345-354.

Lazarova, V. and Manem, J. (1995)
Biofilm characterisation and activity analysis in water and wastewater treatment.
Water Research, **29**:(10) 2227-2245.

Lebaron, P. and Joux, F. (1994)
Flow cytometric analysis of the cellular DNA content of *Salmonella typhimurium* and *Alteromonas haloplanktis* during starvation and recovery in seawater.
Applied and Environmental Microbiology, **60**:(12) 4345-4350.

Lebaron, P., Parthuisor, N. and Catala, P. (1998)
Comparison of blue nucleic acid dyes for flow cytometric enumeration of bacteria in aquatic systems.
Applied and Environmental Microbiology, **64**:(5) 225-230.

Lechevallier, M., Babcock, T. and Lee, R. G. (1987)
Examination and characterization of distribution system biofilms.
Applied and Environmental Microbiology, **53**: 2714-2724.

Lee, J. V. and West, A. A. (1991)
Survival and growth of *Legionella* species in the environment.
Journal of Applied Bacteriology, Symposium Supplement, Pathogens in the Envrionment, **70**: 121S - 129S.

Lee, J. V. (1994)
The control of *Legionella*. (unpublished data)
PHLS, Nottingham, UK.

Liu, D., Lau, L., Chau, Y. K. and Pacepavicius, G. J. (1993)
Charcterisation of biofilm development on artificial substratum in natural water. Water Research, **27**:(3) 361-367.

LÜck, P. C. H., Helbig, J. H., Ehret, W. and Ott, M. (1995)
Isolation of a *Legionella pneumophila* strain serogically distinguishable from known serogroups.
Zentralblatt Fur Bakteriologie-International Journal of Medical Microbiology Virology and infectious Diseases. Zbl. Bakt. **282**: 35-39.

Macey G. M. (ed) (1994).
Flow Cytometry; Clinical Applications.
Edited by Macey, G. M., Blackwell Scientific Publications. ISBN 0-632-03673-7.

Magennis, D. P. (1997)
Nuclear DNA in histological and cytological specimens: measurement and prognostic significance.
British Journal of Biomedical Science, **54**: 140-148.

Maki, J. S. and Remsen, C. C. (1981)
Comparison of two direct-count methods for determing metabolizing bacteria in freshwater.
Applied and Environmental Microbiology, **41**: 1132-1138.

Makin, T. and Hart, C. A. (1989)
Detection of *Legionella pneumophila* in environmental water samples using a fluorescein conjugate monoclonal antibody.
Epidemiology and Infection, **103**: 105-112.

Manz, W., Szewzyk, U., Ericsson, P., Amann, R., Schleifer, K. H. and Thor-Axel, Stenstrõm (1993)
In situ identification of bacteria in drinking water and adjoining biofilms by hybridization with 16S and 23S rRNA directed fluorescent oligonucleotide probes.
Applied and Environmental Microbiology, **59**:(6) 2293-2298.

Manz, W., Amann, R., Szewzyk, R., Szewzyk, U., Thor-Axel Stenstrõm, Hutzler, P. and Schleifer, K. H. (1995)
In situ identification of Legionellaceae using 16S rRNA-targeted oligonucleotide probes and confocal laser scanning microscopy.
Microbiology, **141**: 29-39.

Marie, D., Brussaard, C. P. D., Thyrhaug, R., Bratbak, G. and Vaulot, D. (1999)
Enumeration of marine viruses in culture and natural samples by flow cytometry.
Applied and Environmental Microbiology, **65**: 539-545.

Marie, D., Vaulot, D. and Partenskyet, F. (1996)
Application of the novel nucleic acid dyes YOYO-1, YO-PRO-, and picogreen for flow cytometric analysis of marine prokaryotes.
Applied and Environmental Microbiology, **62**:(5) 1649-1655.

Marrao, G., Verissimo, A., Bowker, R. G. and da Costa, M. S. (1993)
Biofilms as major sources of *Legionella* spp. in hydrothermal areas and their disperson into streams.
FEMS Microbiological Ecology, **12**: 25-33.

Marshall, K. (1992)
Biofilms: An overview of bacterial adhesion, activity and control at surfaces. Features ASM News, **58**:(4) 202-207.

Mason, D. J., Shanmuganathan, S., Mortimer, F. C. and Gant, V. A. (1998)
A fluorescent Gram stain for flow cytometry and epifluorescence microscopy. Applied and Environmental Microbiology, **64**:(7) 2681-2685.

Matsunaga, T. M., Okochi, M. and Nakasono, S. (1995)
Direct count of bacteria using fluorescent dyes: application to assessment of electrochemical disinfection.
Annnals of Chemistry, **67**: 4487-4490.

Matsuyama, M. T. (1984)
Staining of living bacteria with rhodamine 123.
FEMS Microbiology Letters, **21**: 153-157.

Mauchline, W. S., James, B.W., Fitzgeorge,R. B., Deninis, P.J., and Keevil, C.W (1994)
Growth temperature reversibly modulates the virulence of *Legionella pneumophila.*
Infection and Immunity, **62**: 2995-2997.

Mavridon, A. (1992)
A study of the bacterial flora of a non-carbonated natural mineral water.
Journal of Applied Bacteriology, **73**: 355-361.

Mazzini, A., Cavatorta, P., Iori, M., Favilla, R. and Sartor, G. (1992)
The binding of 4′,6′-diamidino-2-phenylindole to bovine serum albumin.
Biophysics and Chemistry, **46**: 217-226.

McClelland, R G. and Pinder, A. C. (1994)
Detection of low levels of specific *Salmonella* species by fluorescent antibodies and flow cytometry.
Journal of Applied Bacteriology, **77**: 440-447.

McCoy, W. F. and Olson, B. H. (1985)
Fluorimetric determination of the DNA concentration in municipal drinking water.
Applied and Environmental Microbiology, **49**: 811-817.

McCoy, W. F. and Olson, B. H. (1986)
Relationship among turbidity, particle counts and bacteriological quality within water distribution line.
Water Research, **20**: 1023-1029.

McElroy, W. D. (1947)
The energy source for bioluminescence in an isolated system.
Proceedings of the National Acadamy of Science, USA, **33**: 342-345.

McEvoy, M., Batchelor, N., Hamilton, G., MacDonald, A., Faiers, M., Sills, A., Lee, J and Harrison, T (2000)
A cluster of cases of Legionnaires' Disease associated with exposure to a spa pool on display., Communicable Disease in Public Health/PHLS **3**: 43 - 45

McFeters, G. A. (1984)
Biofilm development and its consequences.
In Microbial Adhesion and Aggregation (Edited by Marshall, K. C.), 109-124, Springer, Berlin.

McFeters, G.A., Singh, A., Byun, S., Callis, P. R. and Williams, S. (1991)
Acridine orange staining reaction as an index of physiological activity in *E. coli.* Journal of Microbiological Methods, **13**: 87-97.

McFeters, G. A., . Yu, F..P., Pyle, B..H., and Stewart, P. S. (1995)
Physiological assessment of bacteria using fluorochromes.
Journal of Microbiological Methods, **21**: 1-13.

Mckay, A. M. (1992).
A Review. Viable but non-culturable forms of potentially pathogenic bacteria in water.
Letters in Applied Microbiology, **14**: 129-135.

Mclem, G. and Collins, J. (eds) (1990)
Guide to flow cytometry methods, U.S.A. ISBI:0-8247-8330-1

Mclaughlin-Borlace, L., Stapleton, F., Matheson, M. and Dart, J. K. G. (1998)
Bacterial biofilm on contact lenses and lens storage cases in wearers with microbial keratitis.
Journal of Applied Microbiology, **84**: 827-838.

Medema, G. J. (1997)
Detection of viable *Cryptosporidium* and *Giardia* in water by flow cytometry and propidium iodide staining.
Abstract for the International Symposium on *Cryptosporidium*, Long Beach, California.

Meyer-Reil, L. A. (1978)
Autoradiography and epifluorescence microscopy combined for the determination of number and spectrum of actively metabolizing bacteria in natural waters.
Applied and Environmental Microbiology, **36**: 506-512.

Miyamoto, H. H., Yamamoto, K., Arima, J., Fujii, K., Maruta, K., Izu, T., Shiomori, T., Yoshida, S-I (1997).
Development of a new semi-nested PCR method for detection of legionella species and its application to surveillance of Legionellae in hospital cooling tower water.
Applied and Environmental Microbiology **63**: (7) 2489 - 2494

Moffat, J. F. and Tompkins, L. S. (1992)
A quantitative model of intracellular growth of *Legionella pneumophila* in *Acanthamoeba castellanii.*
Infection and Immunity, **60**: 296-301.

Moldavan, A. (1934)
Photo-electric technique for the counting of microscopical cells.
Science, **80**: 188-189.

Møller, S., Korber, D. R., Wolfaardt, G. M., Molin, S. and Caldwell, D. E. (1997)
Impact of nutrient composition on a degradative biofilms community.
Applied and Environmental Microbiology, **63**: 2432-2438.

Monfort, P. and Baleux, B. (1996)
Cell cycle characteristics and changes in membrane potential during growth of *Escherichia coli* as determined by acyanine fluorescent dye and flow cytometry.
Journal of Microbiological Methods, **25**: 79-86.

Monger, B. C. and Landry, M. R. (1993)
Flow cytometric analysis of marine bacteria with Hoechst 33342.
Applied and Environmental Microbiology, **59**:(3) 905-911.

Morgan, J. A. W., Rhodes, G. and Pickup, R. W. (1993)
Survival of non-culturable *Aeromonas salmonicida* in lake water. Applied and Environmental Microbiology, **59**:(3) 874-880.

Morris, G. K., Patton, C. M., Feeley, J. C., Johnson, S. E., Gorman, G., Martin, W. T., Skaliy, P., Malliinson, G. F., Politi, B. D. and Mackel, D. C. (1979)
Isolation of the Legionaires' disease bacterium from environmental samples. Annals of Internal Medicine, **90**: 664-666.

Muldrow, L. L., Tyndall, R. L. and Fliermans, C. B. (1982)
Applications of flow cytometry to studies of pathogenic free-living amoebae. Applied and Environmental Microbiology, **44**: 1258-1269.

Murga, R., Forster,TS., Brown, E., Pruckler, JM., Fields, BS and Donlan, RM (2001).
Role of Biofilms in the survival of *Legionella pneumophila* in a model potable water system. Microbiology **147**: 3121 - 3126

Murgel, G. M., Lion, L. W., Acheson, C., Shuler, M. L., Emerson, D. and Ghiorse, W. (1991)
Experimental apparatus for selection of adherent microorganisms under stringent growth conditions.
Applied and Environmental Microbiology, **57**: 1987-1996.

Nebe-von Caron, G., Stephens, P. and Muller, S. (1998a)
Bugs in the beam. A manual for flow cytometry.
Prepared for the ISAC XIX congress February 1998, Colorado Springs.

Nebe-von Caron, G., Stephens, P. and Bradley, R. A. (1998b)
Assessment of bacterial viability status by flow cytometry and single cell sorting.
Journal of Applied Microbiology, **84**: 988-998.

Nebe-Von Caron, G. and Wallner, G. (1998c)
The use of fluorescent probes for the detection, differentiation and viability assessment of bacteria based on direct cytometric single cell analysis.
Worshop at the summer conference '98 of the Society for Applied Microbiology, in collaboration with the cytometry group of the Royal Microscopical Society.

Newsome, A., Scott, T. M., Benson, R. F. and Fields, B. S. (1998)
Isolation of an amoeba naturally harboring a distinctive *Legionella* species. Applied and Environmental Microbiology, **64**: 1998.

Neumeister, B., Schoniger, S., Faigle, M., Eichner, M. and Dietz, D. (1997)
Multiplication of different *Legionella* species in Mono Mac 6 cells and in *Acanthamoeba castellanii.*
Applied and Environmental Microbiology, **63**: 1219 - 1244.

Neumeister, B., Reiff, G., Faigle, M., Deitz, K., Northoff, H. and Lang, F. (2000)
Influence *of Acathamoeba castellanii* on intracellular growth of different *Legionella* species in human monocytes.
Applied and Environmental Microbiology, **66**: 914-919.

Norton, C. and LeChevallier, M. (2000)
A pilot study of bacteriological population changes through potable water treatment and distribution. Applied and Environmetal Microbiology, **66**:(1) 268-276.

Nouvion, N., Block, J. C. and Faup, G. M. (1987)
Effects of biomass quantity and activity on TOC removal in a fixed–bed reactor. Water Research, **21**: 35-40.

Nuraida, L., Grigolava, I., Owens, J. D. and Campbell-Platt, G. (1992)
Oxygen and pyruvate as external electron acceptor for *Leuconostoc* spp..
Journal of Applied Bacteriology, **72**: 517-522.

Oga, T., Sutherson, S. and Ganczarczyk, J. J. (1991)
Some properties of aerobic biofilms.
Environmental Technology, **12**: 431-440.

Oritz-Roque, C. M. and Hazen, T. C. (1987)
Abundance and distribution of *Legionellaceae* in Puerto Rican waters.
Applied and Environmental Microbiology, **53**: 2231-2236.

Packard, T. T. (1971)
The measurement of respiratory electron transport activity in marine plankton. Journal of Marine Research, **29**: 235-244.

Packer, P. J., Holt, D. M., Colbourne, J. S., and Keevil, C. W. (1997)
Does *Klebsiella oxytoca* grow in the biofilm of water distribution systems? The effect of different water sources on coliform growth in a chemostat model.
In Coliforms and *E. coli* problems or solution? Edited by Kay, D. and Fricker, C. 157-167.

Palmer, C., Tsai, Y. L., Paszko-Kova, C. and Sangermano, L. R. (1993)
Detection of *Legionella* species in sewage and ocean water by polymerase chain reaction, direct fluorescence antibody and plate culture methods.
Applied and Environmental Microbiology, **59**: 3618-3624.

Palmer, C., Bonilia, G. F., Roll, B., Paszko-Kolva, C., Sangermano, L. R. and Fujioka, R. S. (1995).
Detection of *Legionella* species in reclaimed water and air with the Environamp *Legionella* PCR kit and direct fluorescent antibody staining.
Applied and Environmental Microbiology, **61**: 407-412.

Peele, E. R. and Colwell, R. R. (1981)
Application of a direct microscopic method for enumeration of substrate-responsive marine bacteria.
Canadian Journal of Microbiology, **27**: 1071-1075.

Pernthaler, J., Alfreider, A., Posch, T., Andreatta, S. and Psenner, R. (1997)
In situ classification and image cytometry of pelagic bacteria from a high mountain lake (GossenkÖllesee, Austria).
Applied and Environmental Microbiology, **63**: 4778-4783.

Peshwa, M. V., Kyung, Y. S., McClure, D. B. and Hu, W. S. (1993)
Cultivation of mammalian cells as aggregates in bioreactors: effects of calcium concentration on spatial distribution of viability.
Biotechnolgy Bioengineering, **41**: 179-187.

Pettipher, G. L., Mansell, R., Mckinnon, C. H. and Cousins, C. M. (1980)
Rapid membrane filtration epifluorescence microscopy technique for direct enumeration of bacteria in raw milk.
Applied and Environmental Microbiology, **39**: 423-429.

Pickup, R. W. and Sauders, J. R. (1990)
Detection of genetically engineered traits among bacteria in the environment. Trends in Biotechnology, **8**:(11) 329-335.

Pickup, R.W. (1991)
Development of molecular methods for the detection of specific bacteria in the environment.
Journal of General Microbiology, **137**: 1009-1019.

Pickup, R. W. and Rhodes, G. (1997)
Detection of specific bacteria in water: implications of survival strategy.
In The Microbiological Quality of Water, edited by Sutcliffe, D. W., Freshwater Biological Association.

Pinder, A. C., Purdy, P. W., Poulter, A. G. and Clark, D. C. (1990)
Validation of flow cytometry for rapid enumeration of bacterial concentrations in pure culture.
Journal of Applied Bacteriology, **69**: 92-100.

Pinder, A. C., Edwards, C., Clarke, R. G., Diaper, J. P. and Poulter, A. G. (1993)
Detection and enumeration of viable bacteria by flow cytometry.
In New Techniques in Food and Beverage Microbiology, edited by Kroll, R. G., Gilmour, A. and Sussman, M. Society for Applied Bacteriology, Technical Series 31, Blackwell Scientific Publications, 67-84.

Pinder, A. C. and McClelland, R. G. (1994)
Rapid assay for pathogenic salmonella organisms by immunofluorescent flow cytometry.
Journal of Microscopy, **176**: 17-22.

Porter, K. G. and Feig, Y. (1980)
The use of DAPI for identifying and counting aquatic microflora.
Limnology and Oceanography, **25**: 943-948.

Porter, J., Edwards, C., Alun, J., Morgon, W. and Pickup, R. W. (1993)
Rapid, automated separation of specific bacteria from lake water and sewage by flow cytometry and cell sorting.
Applied and Environmental Microbiology, **59**:(10) 3327-3333.

Premazz, G., Buonaccorsi, G. and Zilio, P. (1989)
Flow cytometry for algal studies.
Water Research, **23**:(4) 431-442.

Pyle, B. H., Broadaway, S. C. and McFeters, G. A. (1995)
A rapid, direct method for enumerating respiring enterohemorrhagic *Escherichia coli* O157:H7 in water.
Applied and Enviornmental Microbiology, 61: 2614-2619.

Ravdin, P., Clark, G. M., Hough, J. J., Owens, M. A. and McGuire, W. L. (1993)
Neural Network Analysis of DNA flow cytometry histograms.
Cytometry, **14**: 74-80.

Reasoner, D. J. and Geldreich, E. E. (1985)
A new medium for the enumeration and sub-culture of bacteria from potable water.
Applied and Environmental Microbiology, **49**: 1-7.

Resnick, M., Schuldiner, S. and Bercovier, H. (1985)
Bacterial membrance potential analyzed by spectrofluorometry.
Current Microbiology, **12**: 183-186.

Riggs, J. L., Seiwald, R.J., Buckerhalter, J.H (1958)
Isothiocyanate compounds as fluorescent labelling agents for immune serum. American Journal of Pathology. **34**: 1081-1086.

Rittard,S., Douglass, S., Brooks, T., Springthorpe, L., Filion, LG and Sattar, SA (2001). Occurrence of *Legionella* in groundwater: an ecological study.
Water Science and Technology **12**: 99 - 102

Robertson, B. R. and Button, D. (1989)
Characterizing aquatic bacteria according to population, cell size and apparent DNA content by flow cytometry.
Cytometry, **10**: 70-76.

Robertson, B., Button, D. and Koch, A. (1998)
Determination of the biomasses of small bacteria at low concentrations in a mixture of species with forward light scatter measurements by flow cytometry. Applied and Environmental Microbiology, **64**: 3900-3909.

Robinson, R. W., Akin, D. E., Nordstedt, R. A., Thomas, M.V. and Aldrich, H. C. (1984) Light and electron microscopic examinations of methane-producing biofilms from anaerobic fixed–bed reactors.
Applied and Environmental Microbiology, **48**: 127-136.

Rodriguez, G. G., Phipps, D., Ishiguro, K. and Ridgway, H. F. (1992)
Use of a fluorescent redox probe for direct visualization of actively respiring bacteria.
Applied and Environmental Microbiology, **59**: 1801-1808.

Rogers, J., Dowsett, A. B., Lee, J. V. and Keevil, C. W. (1991)
Chemostat studies of biofilm development on plumbing materials and the incorporation of *Legionella pneumophila*, 458-460.
In Biodeterioration and Biodegradation, edited by Rossmore, H. W., Vol. 8. Elsevier Science Publishers Ltd., Barking, UK.

Rogers, J. and Keevil, C. W. (1992)
Immunogold and fluorescein immunolabelling of *Legionella pneumophila* within an aquatic biofilm visualized by using episcopic differential interference contrast microscopy.
Applied and Environmental Microbiology, **59**:(6) 2326-2330.

Rogers, J., Dowsett, A. B., Dennis, P. J., Lee, J. V. and Keevil, C. W. (1994a)
Influence of temperature and plumbing material selection on biofilm formation and growth *of Legionella pneumophila* in a model potable water system containing complex microbial flora.
Applied and Environmental Microbiology, **60**:(5) 1585-1592.

Rogers, J., Dowsett, A. B., Dennis, P. J., Lee, J. V. and Keevil, C. W. (1994b)
Influence of plumbing materials on biofilm formation and growth of *Legionella pneumophila* in potable water systems.
Applied and Environmental Microbiology, **60**:(6) 1842-1851.

Rohr, U., Weber, S., Michel, R., Selenka, F. and Wilhelm, M. (1998)
Comparison of free-living amoebae in hot water systems of hospitals with isolates from moist sanitary areas by identifying genera and determining temperature tolerance.
Applied and Environmental Microbiology, **64**: 1820-1824.

Roszak, D. and Colwell, R. R. (1987)
Survival strategies of bacteria in the natural environment.
Microbiological Reviews, **51**: 365-379.

Roszak, D. B., Grimes, D. J. and Colwell, R. R. (1984)
The viable but non-recoverable stage of *Salmonella enteritidis* in aquatic systems. Canadian Journal of Microbiology, **30**: 334-338.

Roth, B., Poot, M., Yue, S. and Mallard, P. (1997)
Bacterial viability and antibiotic susceptibility testing with SYTOX green nucleic acid stain.
Applied and Environmental Microbiology, **63**: 2421-2431.

Rowbotham, J. (1980)
Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae.
Journal of Clinical Pathology, **33**: 1179 - 1183.

Rowbotham, J. (1986)
Current views on the relationships between amoeba, *Legionellae* and man. Israel Journal of Medical Science, **22**: 678-689.

Rowbotham, J. (1993)
Legionella-like amoebal pathogens.
In Legionella; current status and emerging perspectives, 137-140.
Edited by Barbaree, J. M. *et al.*, Washington DC: American Society for Microbiology.

Saby, S., Sibille, I., Mathieu, L., Paquin, J. L. and Block, J. C. (1997)
Influence of water chlorination on the counting of bacteria with DAPI (4′,6′-diamidino-2-phenylindole).
Applied and Environmental Microbiology, **63**: 1564-1569.

Sanden, G., Morrill, W., Fields, B., Breiman, R. and Barbaree, J. (1992)
Incubation of water samples containing amoebae improves detection of legionellae by the culture method.
Applied and Environmental Microbiology, **58**:(6) 2001-2004.

Santegoeds C. M. and Ferdelman, T. M. (1998)
Structural and functional dynamics of sulfate-reducing populations in bacterial biofilms.
Applied and Environmental Microbiology, **64**: 3731-3739.

Sartory, D. P. and Howard, L. (1992)
A medium detecting β-glucuronidase for the simultaneous membrane filtration enumeration of *Escherichia coli* and coliforms in drinking water.
Letters in Applied Microbiology, **15**: 273-276.

Sartory, D. P. (1995)
Improved recovery of chlorine-stressed organisms with pyruvate supplemented media.
Water Science and Technology, **31**: 255-258.

Savenkoff, C., Packard, T. T., Rodier, M., Gérino, M., Lefèfre, D. and Denis, M. (1995). Relative contribution of dehydrogenases to overall respiratory ETS activity in some marine organisms.
Journal of Plankton Research, **17**: 1593-1604.

Schaule, G., Flemming, H. C. and Ridgway, H. F. (1993)
Use of 5-cyano-2, 3-ditolyl tetrazolium chloride for quantifying planktonic and sessile respiring bacteria in drinking water.
Applied and Environmental Microbiology, **59**:(11) 3850-3857.

Schotts, E. B. and Wolley, R. E. (1990)
Protozoan sources of spontaneous coliform occurence in chlorinated drinking water.
Project summary. United States Environmental Protection Agency, Project No. EPA/600/52-98/019. Cincinnati, OH 45268.

Schwartz, T., Hoffmann, S and Obst, U (1998)
Formation and bacterial composition of young, natural biofilms obtained from public bank-filtered drinking water systems. Water resources, **32**:2787

Senthilnathan, P., Li, D. H. and Ganczarezyk, J. J. (1989)
Image analysis of biomass immobilized on micro-carriers.
Proceedings of the 44th Industrial Waste Conference, 175-181. Purdue University, West Lafayette, Ind.

Servais, P., Billen, G., Laurent, P., Levi, Y. and Randon, G. (1992).
Studies of BDOC and bacterial dynamics in the drinking water distribution system of the northern Parisian suburbs.
Review of Scientific instruments. **5**: 69-89.

Sessa, R., Pietro, M., Zamparelli, M., Schiavoni, G and Del Piano, M (2000)
Biofilm formation on the surface of ceramic tiles. The New Microbiologica: **23** (4) 407 - 413

Severin, E., Stellmach, J. and Nachtigal, H. M. (1985)
Fluorimetric assay of redox activity in cells.
Analytica Chimica Acta, **170**: 341-346.

Shahamat, M., Mai, U., Paszko-Kova, C., Kessel, M. and Colwell, R. R. (1993)
Use of autoradiography to assess viability of *Helicobacter pylori* in water. Applied and Environmental Microbiology, **59**: 1231-1235.

Siebel, M. A. and Characklis, W. G. (1991)
Observations of binary population biofilms.
Biotechnology and Bioengineering, **37**: 778-789.

Sieracki, M. E., Johnson, P. W. and Sieburth, J. (1985)
Detection, enumeration, and sizing of planktonic bacteria by image-analyzed epifluorescence microscopy.
Applied and Environmental Microbiology, **49**: 799-810.

Sierra, M. L., Sheridan, J. J. and Mcguire, L. (1997)
Microbial quality of lamb carcasses during processing and the acridine orange direct count technique (a modified DEFT) for rapid enumeration of total viable counts.
International Journal of Food Microbiology, **36**: 61-67.

Sleightholme, V. and Roberts, R. (1994)
Viable non-culturable *Vibrio cholerae* 01: a short review.
PHLS Microbiology Digest, **11**:(2) 77.

Smith J., Howington, J. P. and McFeters, G. A. (1994)
Survival, physiological response, and recovery of enteric bacteria exposed to a polar marine environment.
Applied and Environmental Microbiology, **60**: 2977-2984.

Steen, H., Jernaes, M. W., Skarstad, K. and Boye, E. (1994)
Staining and measurement of DNA in bacteria.
Methods in Cell Biology, **42**: 477-487.

Steinert, M., Birkness, K., White, E., Fields, B. and Quinn, F. (1998)
Mycobacterium avium bacilli grow saprozoically in coculture with *Acanthamoeba polyphaga* and survive within cyst walls.
Applied and Environmental Microbiology, **64**: 2256-2261.

Steinert, M., Hentschel, U and Hacker, J (2002)
Legionella pneumophila: an aquatic microbe goes astray. FEMS Microbiol. Rev. **26** (2) 149 - 162

Stellmach, J. (1984)
Fluorescention of fluorescent formazan by unstimulated and phorbol ester or digitonin-stimulated Ehrlich ascites tumor cells.
Histochemistry, **80**: 137-143.

Stevenson, L.H., Chrzanowski, T. H. and Erkenbrecher, C. W. (1979)
The adenosine triphosphate assay: concentrations and misconceptions.
In native aquatic bacteria: enumeration, activity and ecology, edited by Costerton, J. W., ASTM Spec. Tech. Publ., 99-116. University of Calgary, Md.

Stewart, P. S., Grab, L. and Diemer, J. A. (1995)
Biofilms structural heterogeneity visualized by three characterization of fungal biofilms within a municipal water distribution system, microscopic methods. Water Research, **29**:(8) 2006-2009.

Stewart, P.S., Grab, L. and Diemer, J. A. (1998)
Analysis of biocide transport limitation in an artificial biofilm system. Journal of Applied Microbiology, **85**: 495-500.

Stohr, M., Eipel, H., Goerttler, K. and Wogt-Schaden, M (1977)
Extended application of flow microfluorometry by means of dual excitation. Histochemistry, **51**; 305-313.

Strange, R. E., Wade, H. E., and Dark, F. A. (1963)
Effect of starvation on adenosine triphosphate concentration in *Aerobacter aerogenes*.
Nature, 4888 : 55-57.

Surman, B. S., Walker, J. T., Goddard, D. T., Morton, L. H. G., Keevil, C. W., Weaver, W., Skinner, A., Hanson, K., Caldwell, D. and Kurtz, J. (1996)
Comparison of microscope techniques for the examination of biofilms. Journal of Microbiological Methods, **25**: 57-70.

Sweet, R.G.(1965)
High frequency recording with electrostatically deflected ink-jet. Reviews of Scientific Instruments, 36: 131-136.

Szewzyk, U., Szewzyk, R., Manz, W and Schleifer, KH (2000)
Microbiological safety of drinking water. Annual Review of Microbiology, **45**: 81-127

Tabor, P. S. and Neihof, R. D. (1982)
Improved method for determination of respiring individual microorganisms in natural waters.
Applied and Environmental Microbiology, **43**: 1249-1255.

Tanious, F. A., Veal, J. M., Buczak, H., Ratemeyer, L. S. and Wilson, W. D. (1992)
DAPI (4′,6′-diamidino-2-phenylindole) binds differently to DNA and RNA: minor-groove binding at AT sites and intercalation at AU sites. Biochemistry, **31**: 3103-3112.

Thomas, J. C., Desrosiers, M., St-Pierre, Y., Lirette, P., Bisaillon, J. G., Beaudet, R. and Villemur, R. (1997)
Quantitative flow cytometric detection of specific microorganisms in soil samples using rRNA targeted fluorescent probes and ethidium bromide. Cytometry, **27**:(3) 224-232.

Tison, D. L. and Seidler, R. J. (1983)
Legionella incidence and density in potable drinking water supplies. Applied and Environmental Microbiology, **45**: 337-339.

Traganos, F., Darzynkiewicz, Z., Sharpless, T. and Melamed M. R. (1977)
Simultaneous staining of ribonucleic and deoxyribonucleic acid in unfixed cells using acridine orange in a flow cytofluorimentric system. Journal of Histochemistry, **25**: 46-56.

Tsuji T., Kawasaki, Y., Takeshima, S., Sekiya, T. and Tanaka, S. (1995).
A new fluorescence staining assay for visualizing living microorganisms in soil.
Applied and Environmental Microbiology, **61**: 3415-3421.

Tuomi, P., Torsvik, T., Heldal, M. and Bratbak, G. (1997)
Bacterial population dynamics in a meromictic lake.
Applied and Environmental Microbiology, **63**:(6) 2181-2188.

Turpin, P. E. and Maycroft, K. A. (1993)
Viable but non-culturable salmonellas in soil.
Journal of Applied Bacteriology, **74**: 421-427.

Ullrich S., Karrasch, B., Hoppe, H., Jeskulke, K. and Mehrens, M. (1996)
Toxic effects on bacterial metabolism of the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride.
Applied and Environmental Microbiology, **62**: 4587-4593.

Van Dilla, M. A., TruJillo, T. T., Mullaney, P. F. and Coulter, J. R. (1969)
Cell microfluorometry: a method for rapid fluorescence measurement. Science, **163**: 1213-1214.

Vesey, G., Slade, J. S., Bryne, M., Shepherd, K. and Fricker, C. R. (1993)
New techniques for the detection of protozoan parasites in water.
In New Techniques in Food and Beverage Microbiology, edited by Kroll, R. G., Gilmour, A. and Sussman, M. Society for Applied Bacteriology, Technical Series 31, Blackwell Scientific Publications, 101-114.

Vieira, M. J., Melo, L. and Pinhiiro, M. M. (1993)
Biofilms formation: hydrodynamic effects on internal diffusion and structure. Biofouling, **7**: 67-80.

Vogel, J and Isberg, RR (1999)
Cell Biology of *Legionella pneumophila.* Current Opinion in Microbiology **2**: 30 - 34

Walker, JT., Roberts, AD., Lucas, VJ., Roper, MA and Brown, RG (1999)
Quantitative assessment of biocide control of biofilms including *Legionella pneumophila* using total viable counts, fluorescence microscopy and image analysis. Methods in Enzymology **310**: 629 - 637

Wallner, G., R. Erhart, E. and Amann, R. (1995)
Flow cytometric analysis of activated sludge with rRNA-targeted probes. Applied and Environmental Microbiology, **61**: 1859-1866.

Wallner, G., Fuchs, B., Spring, S., Beisker, W. and Amann, R. (1997)
Flow sorting of microorganisms for molecular analysis.
Applied and Environmental Microbiology, **63**:(11) 4223-4231.

Watkins, J., Kemp, P. and Shepherd, K. (1995)
Analysis of water samples for *Cryptosporidium* including the use of flow cytometry.
In Protozoan Parasites and Water, edited by Betts, W. B., Casemore, D., Fricker, C., Smith, H. and Watkins, J. Royal Society of Chemistry, 115-121.

Watkins, J., and Xiangrong Jian. (1997)
Cultural methods of detection: recent advances and successes.
In The Microbiological Quality of Water, edited by Sutcliffe, D. W. 19-27. Freshwater Biological Association, 1997.

Watson, J. (1991)
Introduction to flow cytometry.
Cambridge University Press, U.K. ISBN 0 521 38061 8.

Weiss, M.J., and Chen, L. B. (1984)
Rh123: A lipophilic mitochondrial specific vital dye. Kodak Laboratory. Chemical and Pharmaceutical Bulletin, **55**: 1-4.

White, D. C., Bobbie, R. J., Herron, J. S., King, J. D. and Morrison, S. J. (1979)
Biochemical measurements of microbial mass and activity from environmental samples.
In Native Aquatic Bacteria : Enumeration, Activity and Ecology. Edited by Costerton, J. W.), ASTM Spec. Tech. Publ., 69-81, University of Calgary, Md..

Wilkins,M.F., Morris, C. W., and Boddy, L. (1994)
A comparison of Radial Basis Function and backpropagation neural networks for identification of marine phytoplankton from multivariate flow cytometry data.
Cabios, **10**: 285-294.

Wilkins, M., Boddy, L., Morris, C. W. and Jonker, R. R. (1999)
Identification of phytoplankton from flow cytometry data by using radial basis function neural networks.
Applied and Environmental Microbiology, **65**: 4404-4410.

Winding, A., Binnerup, S. and Sørensen, J. (1994)
Viability of indigenous soil bacteria assayed by respiratory activity and growth. Applied and Environmental Microbiology, **60**: 2869-2875.

Wood, P., Jones, M. and Bhakoo, M. (1996)
A novel strategy for control of microbial biofilms through generation of biocide at the biofilm-surface interface.
Applied and Environmental Microbiology, **62**: 2598-2602.

Xavier-Aabad, F., Pinto, R. M. and Bosch, A. (1998)
Flow cytometry detection of infectious rotavirus in environmental and clinical samples.
Applied and Environmental Microbiology, **64**:(7) 2392-2396.

Xu, H. S., Rober, N., Singleton, F. L., Atwell, R. W., Grimes, D. J. and Colwell, R. R. (1982).
Survival and viability of non-culturable *Escherichia coli* and *Vibrio chloerae* in the estuarine and marine environment.
Microbial Ecology, **8**: 313-323.

Yamaguchi, N. and Nasu, M. (1997)
Flow cytometric analysis of bacterial respiratory and enzymatic activity in the natural aquatic environment.
Journal of Applied Microbiology, **83**: 43-52.

Yu, F. P. and McFeters, G. A. (1994)
Physiological responses of bacteria in biofilms to disinfection.
Applied and Environmental Microbiology, **60**: 2462-2466.

Yu, W., Walter, K., Dodds, M., Banks, K., Skalsky, J. and Strauss, E. A. (1995)
Optimal staining and sample storage time for direct count microscopic enumeration of total and active bacteria in soil with two fluorescent dyes.
Applied and Environmental Microbiology, **61**: 3367-3372.

Zottola, E. A. and Sasahara, K. A. (1994)
Microbial biofilms in the food processing industry- should they be a concern? International Journal of Food Microbiology, **23**: 125-148.

Zweifel, U. L. and Hagstrõm, Å. (1995)
Total counts of marine bacteria include a large fraction of non-nucleoid-containing bacteria (ghosts).
Applied and Environmental Microbiology, **61**: 2180-2185.

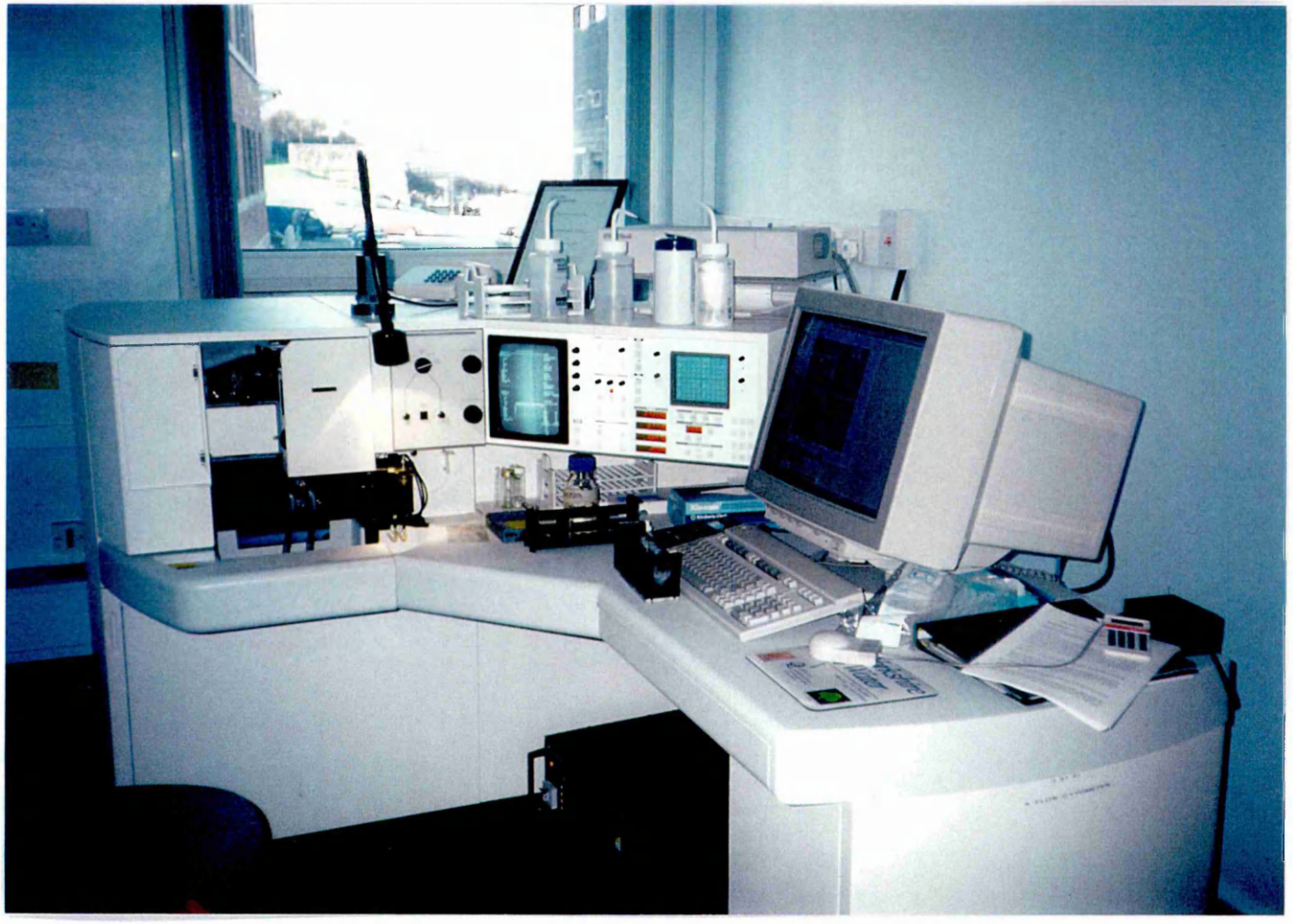
Appendix A. **Table-A1** Determinations of Bacteria in Water Distribution Systems by Direct Counting and Plate Counting Methods

DAPI DC for Direct Counting. YEA Plate for 1 day 37 °C and 3 days 22 °C. R2A Plate for 7 days 22 °C.

No.	1	2	3	4	5	6	7	8	9	10	Average	Correlation	Ratio
Sample 1													
YEA 1D cfu/ml	0	0	0	0	0	0	0	0	0	0	0		
YEA 3D cfu/ml	0	11	23	18	17	3	0	0	0	1	7.3	0.5583	YEA3d/DAPI
R2A 7D cfu/ml	29	110	808	544	608	0	1	8	0	19	212.7	0.9570	R2a/YEA3d
DAPI DC No/ml	248000	302000	372000	134000	268000	260000	220000	97600	5760	48500	195586	0.4884	R2A/DAPI
Sample 2													
YEA 1D cfu/ml	1	0	0	0	0	1	0	0	0	2	0.4		
YEA 3D cfu/ml	0	0	0	0	2	2	0	0	0	3	0.7	-0.2492	YEA3d/DAPI
R2A 7D cfu/ml	80	0	304	30	49	0	0	1	0	0	46.4	-0.2297	R2A/YEA3d
DAPI DC No/ml	290000	101000	326000	151000	216000	16600	50100	11200	12500	18700	191310	0.7984	R2A/DAPI
Sample 3													
YEA 1D cfu/ml	0	0	0	0	0	0	0	0	0	0	0		
YEA 3D cfu/ml	1	1	3	0	2	0	0	0	0	0	0.7	0.3131	YEA 3d/DAPI
R2A 7D cfu/ml	86	1	384	266	164	8	0	0	114	18	104.1	0.6646	R2A/YEA3d
DAPI DC No/ml	370000	257000	299000	247000	27600	280000	78400	81600	31200	8640	168044	0.2995	R2a/DAPI
Sample 4													
YEA 1D cfu/ml	0	0	0	0	1	0	1	0	1	0	0.3		
YEA 3D cfu/ml	0	0	4	0	3	0	0	0	0	0	0.7	0.4320	YEA 3d/DAPI
R2A 7D cfu/ml	56	1	184	264	276	61	1	0	16	8	86.7	0.6386	R2A/YEA3d
DAPI DC No/ml	403000	209000	257000	118000	303000	232000	30400	6400	35000	7680	160148	0.4318	R2A/DAPI
Sample5													
YEA 1D cfu/ml	0	0	0	0	0	0	0	0	0	0	0		
YEA 3D cfu/ml	0	1	0	0	3	2	0	2	0	7	1.5	-0.4258	YEA 3d/DAPI
R2A 7D cfu/ml	180	298	464	216	584	128	376	2	94	145	248	-0.1122	R2A/YEA3d
DAPI DC No/ml	241000	226000	461000	176000	273000	134000	62400	16000	155500	24000	176890	0.6481	R2A/DAPI
Sample 6													
YEA 1D cfu/ml	3	0	0	0	0	0	0	0	0	1	0.4		
YEA 3D cfu/ml	6	5	10	0	14	0	0	0	0	4	3.9	0.3351	YEA 3d/DAPI
R2A 7D cfu/ml	270	348	944	752	488	25	0	0	38	173	304	0.5709	R2A/YEA3d
DAPI DC No/ml	247000	79200	250000	179000	249000	168000	130000	304000	22100	13400	164170	0.3126	R2A/DAPI

Continued Table-A1

No.	1	2	3	4	5	6	7	8	9	10	Average	Correlation	Ratio
Sample 7													
YEA 1D cfu/ml	0	0	0	0	1	0	0	0	7	0	0.8		
YEA 3D cfu/ml	0	4	3	0	2	5	3	3	70	1	9.1	-0.3136	YEA 3d/DAPI
R2A 7D cfu/ml	508	970	1504	1024	636	1000	400	325	688	970	803	-0.1021	R2A/YEA 3d
DAPI DC No/ml	139000	350000	370000	134000	431000	125000	38400	35200	29300	63800	171570	0.4757	R2A/DAPI
Sample 8													
YEA 1D cfu/ml	0	0	0	0	0	0	0	0	0	0	0		
YEA 3D cfu/ml	112	36	18	0	16	1	0	1	0	0	18	0.3786	YEA 3d/DAPI
R2A 7D cfu/ml	802	1000	912	576	384	100	53	5	1	99	393	0.6059	R2A/YEA 3d
DAPI DC No/ml	245000	410000	413000	242000	381000	46400	64000	12800	135000	37900	198710	0.8639	R2A/DAPI
Sample 9													
YEA 1D cfu/ml	0	0	0	3	1	0	0	0	0	0	0.4		
YEA 3D cfu/ml	13	0	1	2	2	0	1	0	0	2	2.1	0.5920	YEA 3d/DAPI
R2A 7D cfu/ml	170	98	240	88	290	56	23	0	6	46	102	0.3346	R2A/YEA 3d
DAPI DC No/ml	353000	142000	355000	140000	250000	91200	52000	24000	22600	9600	143940	0.8744	R2A/DAPI



Appendix-B Plate-1 FACS VANTAGE Flow Cytometry (with sorting function)
Light detectors: FSC (low angle light scatter), SSC (wide angle light scatter), Fluorescence detectors: FL1 (Green, 530nm), FL2 (Orange, 550nm), FL3 (red, 590nm), FL4 (UV blue, 390nm).

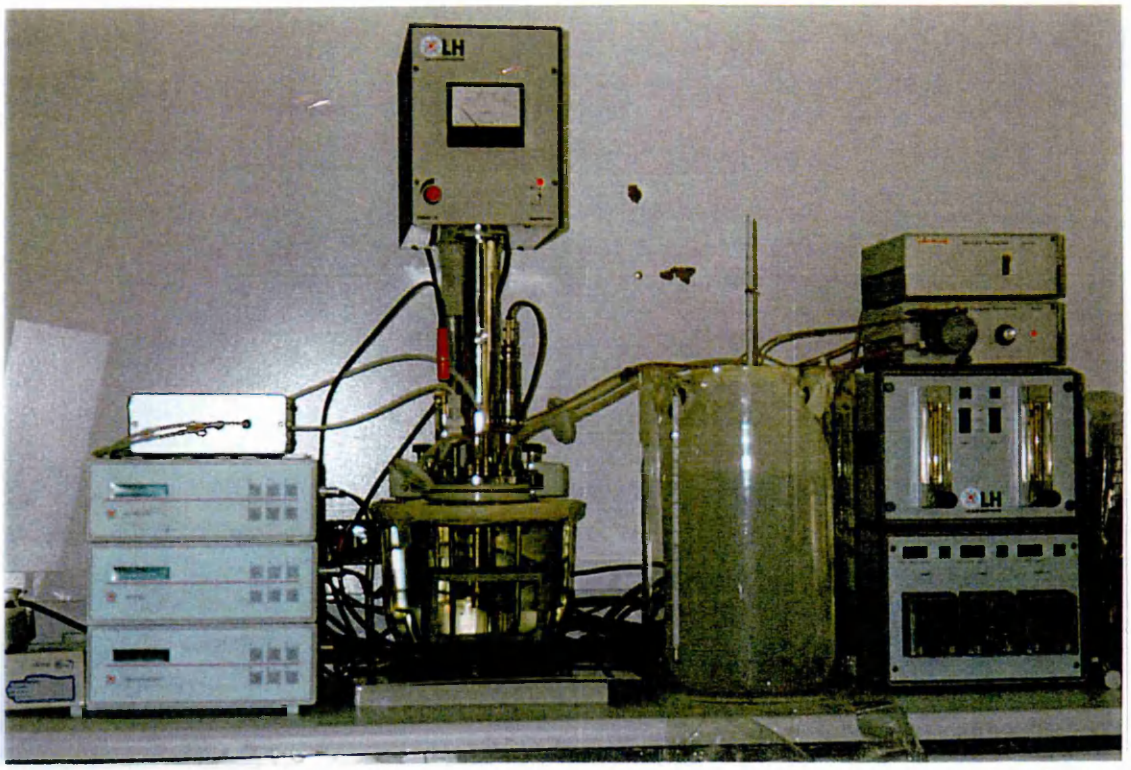
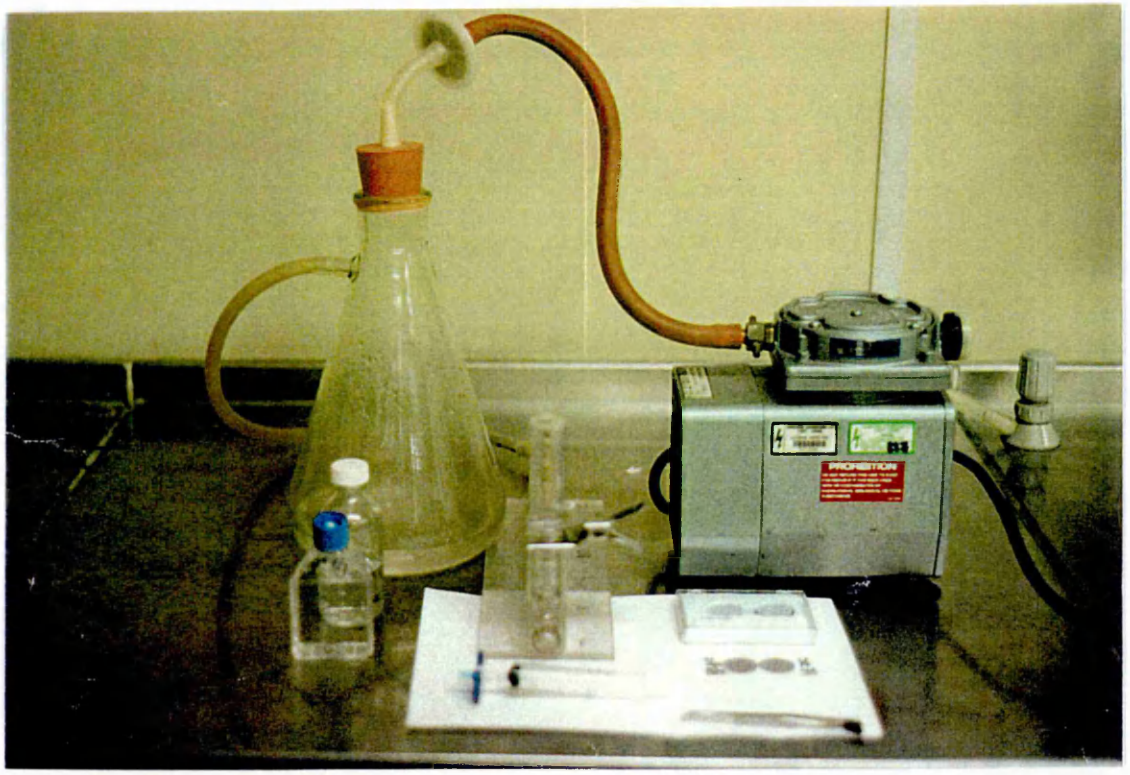


Plate-2a Biofilms formation system.

Plate-2b Filtration device for the direct counting.



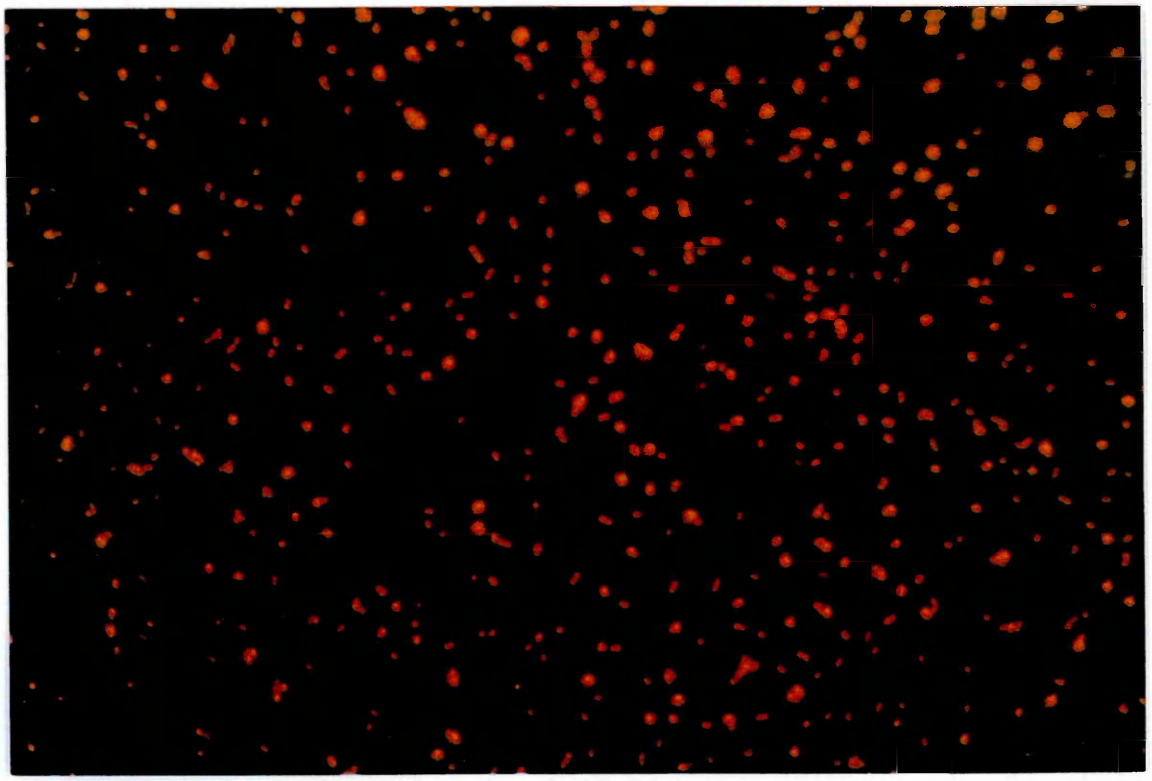
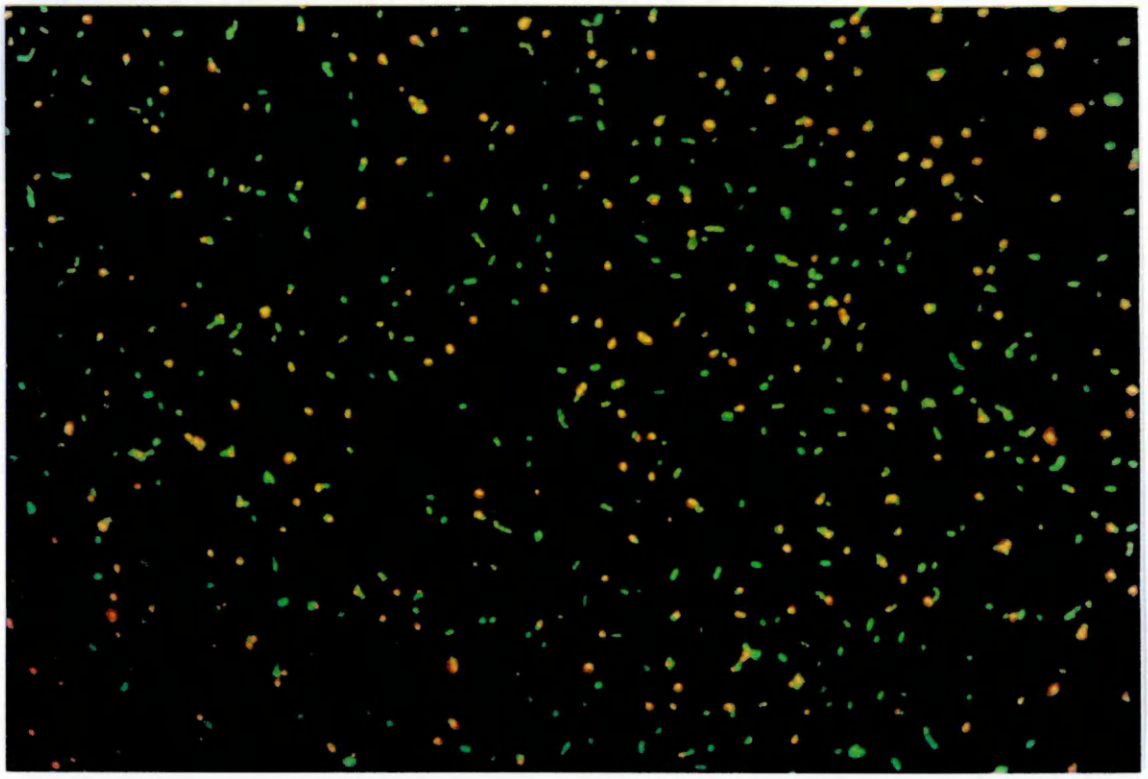


Plate-3 CTC-Rh123 dual viable staining of *E. coli*
Top: Red cells stained by CTC and photo by EFM (570nm filter).
Bottom: Green cells stained by Rh123 and photo by EFM (510 nm filter).



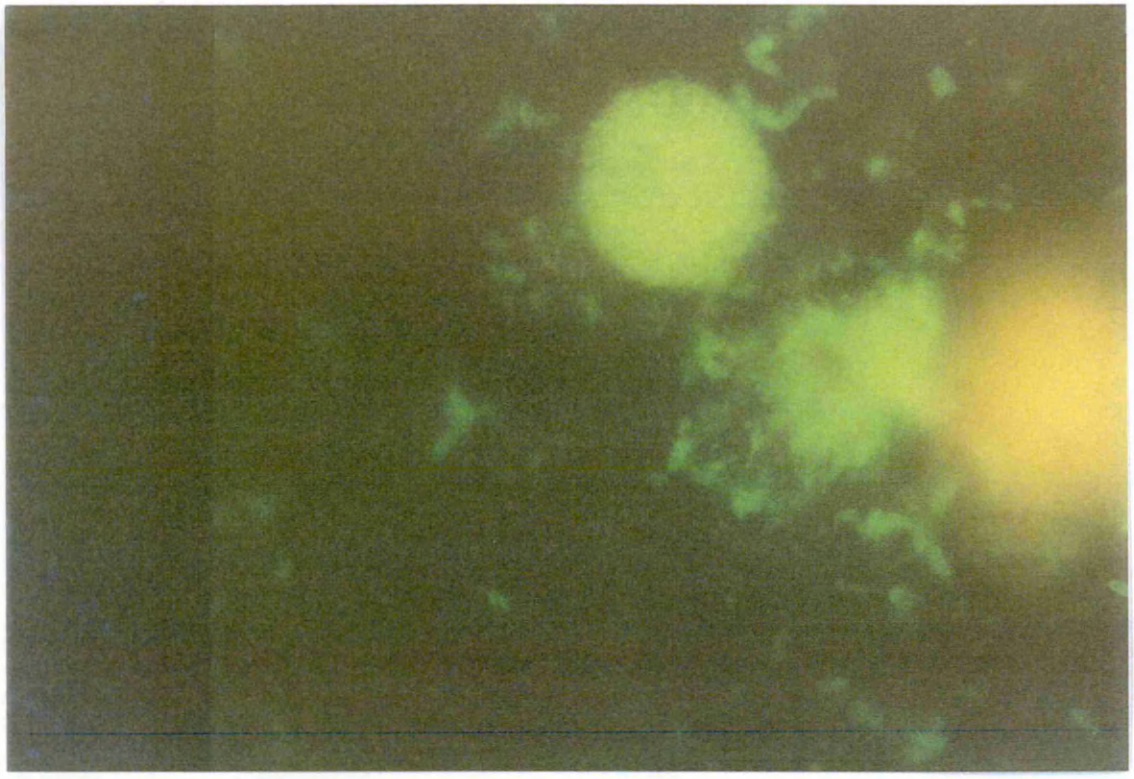
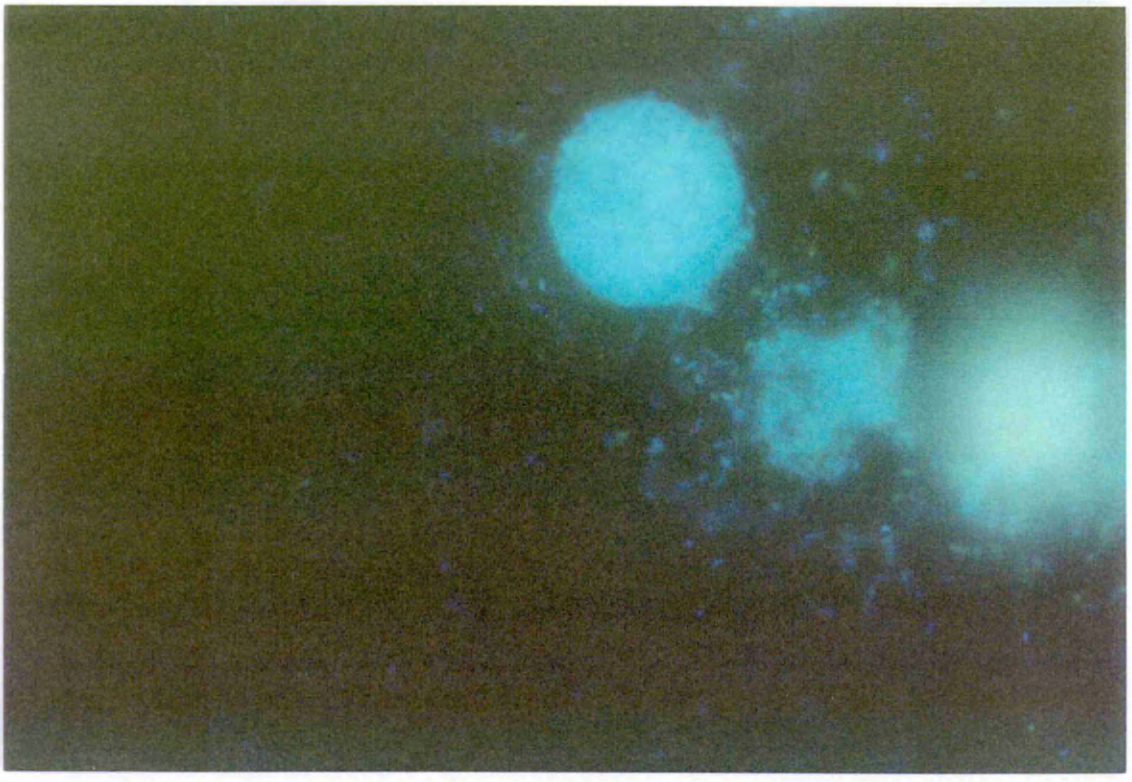


Plate-4 *L. pneumophila* and *Amoeba* cysts stained by mAb-FITC and DAPI.
Top: Green cells and cysts by mAb-FITC staining (Photo by EFM 510nm filter).
Bottom: Blue cells and cysts stained by DAPI (Photo by EFM with 410nm filter).



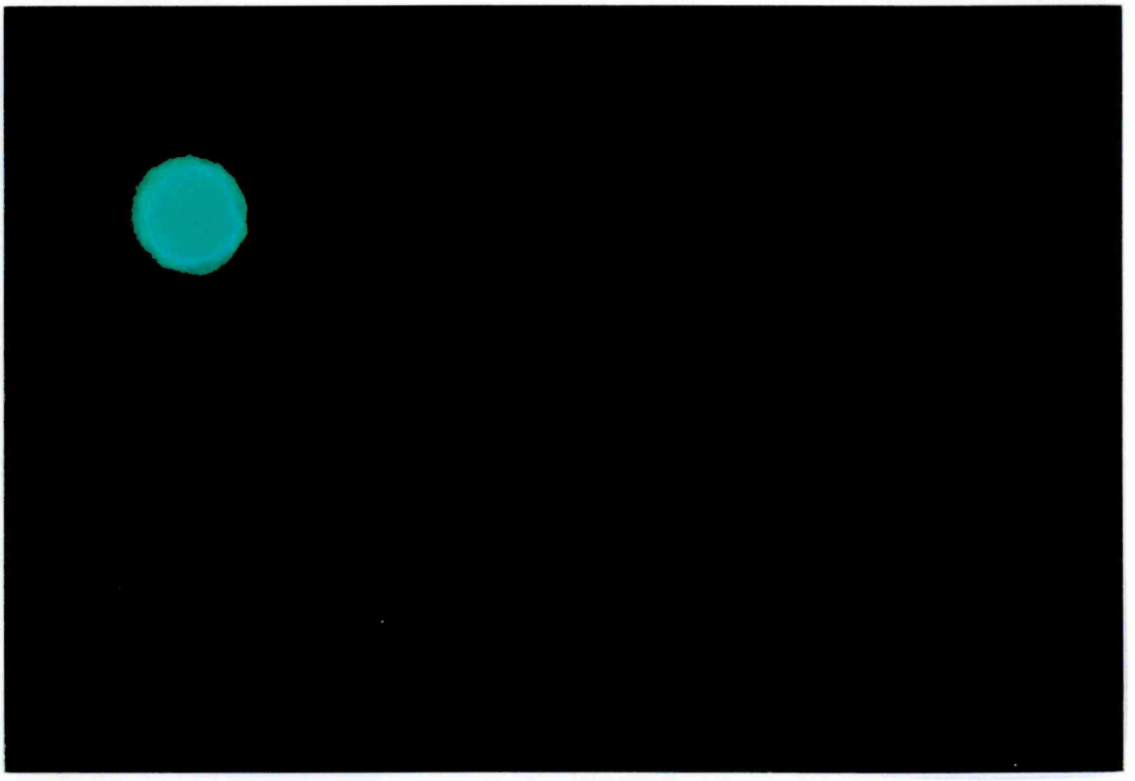
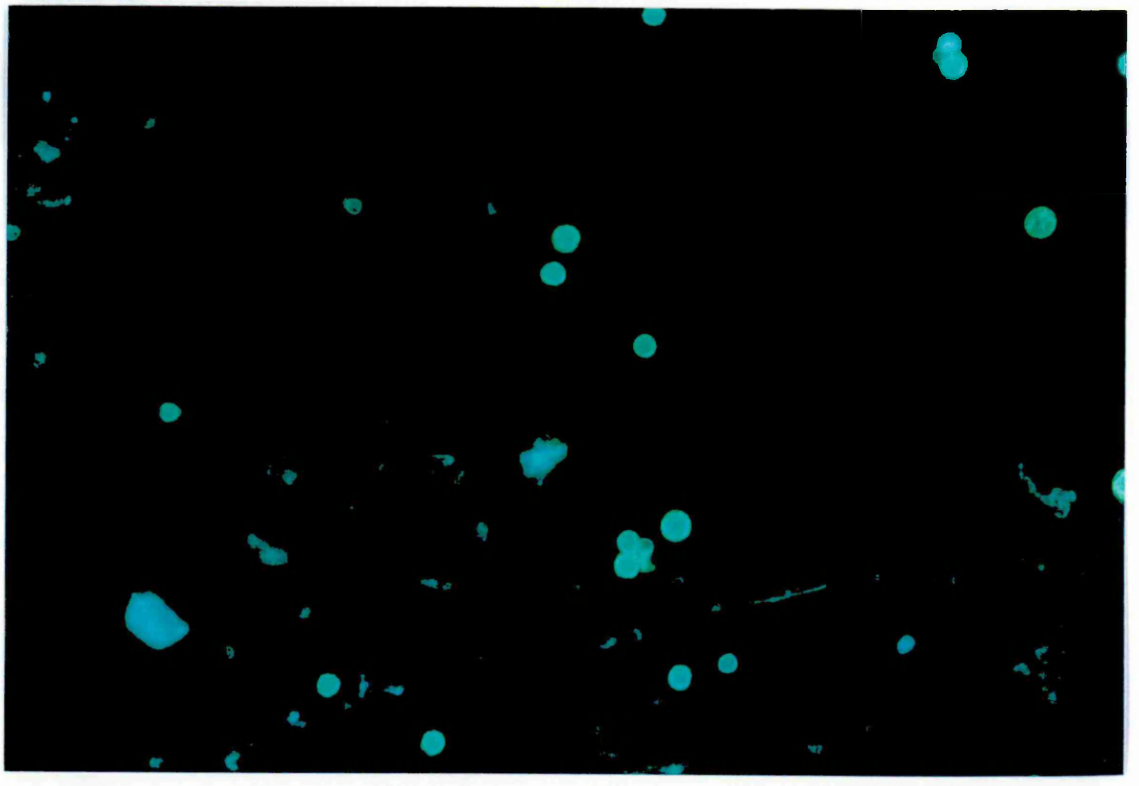


Plate-5 *Amoeba* cysts in biofilm stained by DAPI.
Top: Blue cys with thick wall stained by DAPI (Photo by EFM).
Bottom: Amoeba cysts stained by DAPI in biofilm (Photo by EFM).



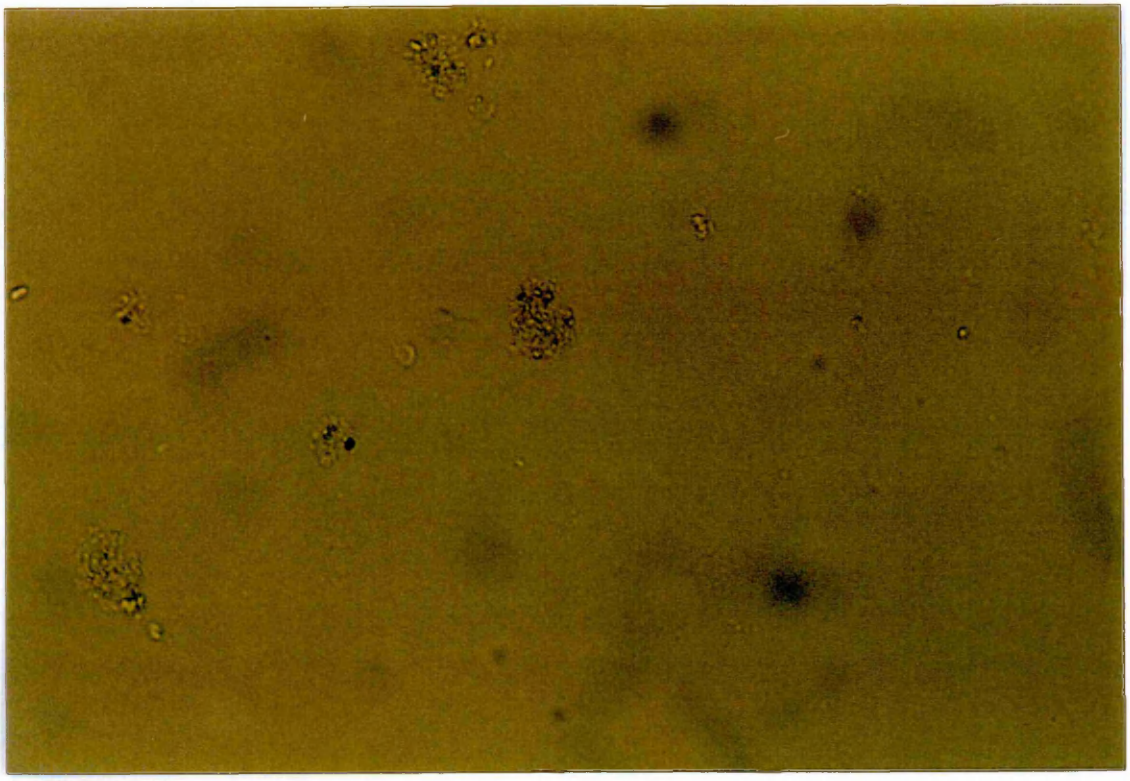
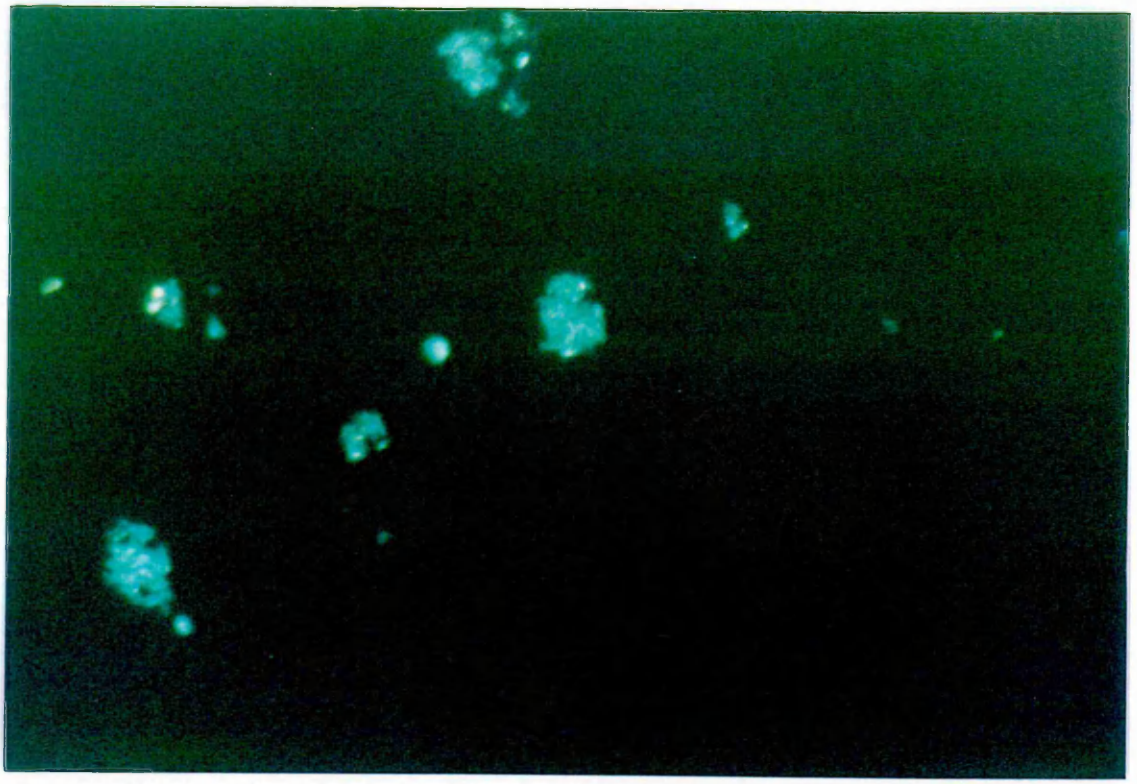
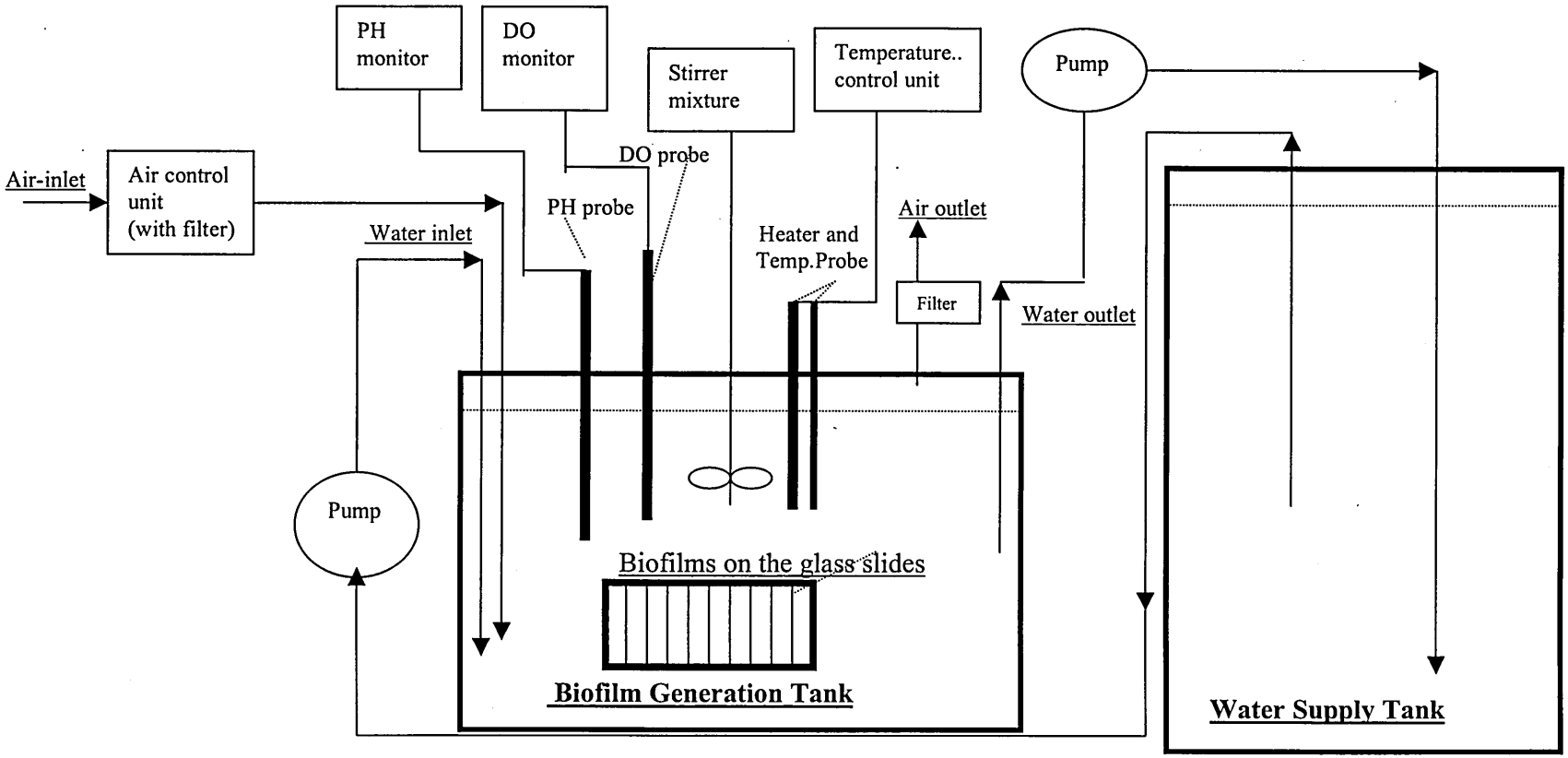


Plate-6 FCM sorting *Amoeba* stained by DAPI.

Top: Amoeba sorted on the slide by FCM (Photo by Light Microscope).
Bottom: Amoeba sorted on the slide by FCM (Photo by EFM).



Appendix C Diagram of Biofilm Generator



The following paper was produced as a consequence of this research work. The reference is given below together with a copy of the paper

Watkins, J., and Xiangrong Jian. (1997) Cultural methods of detection: recent advances and successes. In The Microbiological Quality of Water, edited by Sutcliffe, D. W. 19-27. Freshwater Biological Association, 1997.

Cultural methods of detection for microorganisms: recent advances and successes

JOHN WATKINS AND XIANGRONG JIAN
Yorkshire Environmental, Alcontrol UK, Bradford Laboratory, George Street, Bradford, BD1 5PZ, UK

Most microbiological methods require culture to allow organisms to recover or to selectively increase, and target organisms are identified by growth on specific agar media. Many cultural methods take several days to complete and even then the results require confirmation. Alternative techniques include the use of chromogenic and fluorogenic substances to identify bacteria as they are growing, selective capture using antibodies after short periods of growth, molecular techniques, and direct staining with or without flow cytometry for enumeration and identification. Future microbiologists may not use culture but depend on the use of specific probes and sophisticated detection systems.

Introduction

In the beginning there was the Most Probable Number (MPN) or multiple tube technique for the solation of coliforms and *Escherichia coli* from water. The test was based upon a nutrient nedium (MacConkey Broth) containing lactose and bile salts as an enrichment medium (Anon 1956). This was replaced at a later date by the introduction of the chemically-defined medium, Minerals Modified Glutamate Medium (PHLS 1969), as the standard for water testing. Both nedia detected bacteria by the fermentation of lactose in the presence of a surfactant, with the production of acid and gas. Incubation was at 37°C for up to 48 h. A lack of total specificity in he medium necessitated the requirement of subculture and confirmation, and this took a further 48 h. Hence a confirmed result took 4 days and this has always been seen as unsatisfactory. In addition the test was labour intensive and open to interpretation by, in particular, the failure to produce gas. The final result was based on the analysis of 100 ml of water.

In 1953, Windle-Taylor *et al.* described a membrane filtration test for the analysis of drinking water and by the mid-1970s this had gained wide acceptance. The isolation medium was still based on surfactant and lactose but now typical colonies could be identified and counted. Membranes could be read in as little as 14 h although a presumptive result took 18 h to obtain. The medium still lacked specificity and confirmation taking a further 24 h was still required. Nevertheless, results became available at the beginning of the next working day. The method required less preparation but was still labour intensive. It was also open to interpretation. Colonies which failed to ferment lactose, even though they might be indicators, were not counted, and any which failed to confirm by acid, gas or indole production were discarded. Newly developed biochemical testing kits designed for the medical microbiologist were of some help in sorting out problems, although they were not defined as part of the testing procedure. We still manage to confirm the occasional strain of *Yersinia pestis*. Membrane filtration is based on the analysis of 200 ml of water, albeit for two separately identified tests.

Plate counts (Anon 1994) are a standard method for detecting microorganisms in water. These rely on the growth of bacteria in a nutritionally rich culture medium. Such bacteria are under stress from being in nutritionally deplete water and may be additionally damaged through water treatment or disinfection. It has long been recognised that cultural methods only detect 0.01 to 1% of the total bacterial population present in any water sample.

In-situ testing

Colonies that grow on selective media contain a wide range of enzymes which can be utilised for the purpose of biochemical confirmation. Dufour & Cabelli (1975) described an *"in-situ"* test procedure for differentiating coliforms within the coliform group. These tests were based on urease, oxidase and indole, all conducted on the colony on the membrane. The same principle was applied to clostridia (Bisson & Cabelli 1979) and *Aeromonas hydrophila* (Rippey & Cabelli 1979), and can be seen in the confirmation of enterococci by the hydrolysis of aesculin (Anon 1983). This type of test may reduce the total analysis time to 24 h. Biochemical test kits have also been produced with an incubation time of 2 to 4 h. We still, however, rely on presumptive counts for any decisions we may take about water quality.

Utilisation of specific enzymes

The need for less labour intensive analysis, together with utilisation of specific enzymes for easier interpretation and an earlier confirmed result, has lead to a return to the principles of the multiple tube technique. There is a range of chromogenic substrates which can be incorporated into media to give enzyme-based colour changes (Table 1). The enzyme β-galactosidase cleaves lactose into glucose and galactose, and is an essential stage in lactose fermentation. Substrates such as ortho-nitrophenol-β-D-galactopyranoside, when cleaved by the enzyme, produce the yellow colour of ortho-nitrophenol. The enzyme β-galactosidase is found in coliforms, including *E. coli*, and therefore a simple colour test for coliforms becomes available. An additional enzyme, β-glucuronidase, is found in *E. coli*, *Salmonella* spp. and *Shigella* spp. An additional substrate, methyl umbelliferyl-β-D-glucuronide, can also be added to the medium. The substrate is broken down by the enzyme and free methyl umbelliferone can be demonstrated by fluorescence in the medium under ultra-violet light. Edberg *et al.* (1988) developed the combination of the two substrates in a chemically-defined medium, providing the basis for a qualitative detection system for coliforms and *E. coli*. The test, originally a 24 h test and now designed for 18 h incubation, provides a confirmed result, is less labour intensive than membrane filtration, and uses only 100 ml of water. Although originally only qualitative, its use has been validated through extensive trials (Edberg *et al.* 1989; Cowburn *et al.* 1994) and was found to give comparable results to standard methods. The test method has now been modified to provide quantitative results in the form of the Quantitray (Idexx, USA) and national trials are now being run to validate this.

Table 1. *A list of some of the chromogenic and fluorogenic substrates available commercially.*

Para-nitrophenol-β-D-galactopyranoside (PNPG)
Ortho-nitrophenol-β-D-galactopyranoside (ONPG)
4-methylumbelliferyl-β-D-galactopyranoside (MUGAL)
4-methylumbelliferyl-β-D-glucuronide (MUG)
8-hydroxyquinoline-β-glucuronide (BCIG)
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG)
4-methylumbelliferyl phosphate (MUP)
5-bromo-4-chloro-3-indolyl phosophate (BCIP)
4-methylumbelliferyl-β-glucoside

There are other chromogenic substrates. Sartory & Howard (1992) described the use of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide as a marker for β-glucuronidase in the isolation of *E. coli* from water, in a single membrane filtration test incubated at 37°C. The principle has now been adopted by a number of manufacturers and a wide variety of media are now available containing chromogenic substrates. Some of these are listed in Table 2. Although the test still takes 18 h, analysis is reduced to a single membrane and the results obtained are confirmed results. Like Colilert, the test volume has been reduced to 100 ml.

Table 2. *Some examples of commercially available chromogenic and fluorogenic media.*

Product Name	Type of medium	Manufacturer
Colilert	Liquid	Indexx, USA
Colilert (Quantitray)	Liquid	Indexx, USA
Enterolert	Liquid	Indexx, USA
Colisure	Liquid	Millipore, USA
Coliform	Test kit	Millipore, USA
CHROMagar ECC	Agar	Chromagar, France
CHROMagar Urinary Paths	Agar	Chromagar, France
CHROMagar *Candida*	Agar	Chromagar, France
Fluorocult	Liquid	Merck, Germany
Chromocult Coliform	Agar	Merck, Germany
Chromocult *Enterococcus*	Agar	Merck, Germany
Microsure *E. coli*	Agar	Gelman, USA
Coli 1 D	Agar	Biomerieux, France
EMX Agar	Agar	Biotest, Germany
CL-EC-MF Agar	Agar	Biolife, Italy

A number of questions have been raised about the ability of environmentally or disinfectant-damaged organisms to grow on selective media with high levels of nutrients, and to express the enzymes β-galactosidase and β-glucuronidase. The addition of sodium pyruvate to isolation media (Sartory 1995) will improve the recovery of organisms. In trials at Yorkshire Environmental as part of a national trial comparing the method of Sartory & Howard (1992) with conventional membrane filtration, a significant number of samples failed on the test medium (m-LGA) with 100 ml of treated water, but were found to be satisfactory by conventional membrane filtration on the same sample (Anon 1983). In addition, there was an increase in the number of coliforms isolated on the new medium compared with the numbers isolated by the standard method when both were positive. Pyruvate was not the only factor in m-LGA that was different but an increased sensitivity was noted.

The expression of the enzyme β-glucuronidase by *E. coli* is variable (Lewis & Mak 1989; Clarke *et al.* 1991; Schets *et al.* 1993). Environmentally-derived *E. coli* from surface waters was found to be poor at expressing β-glucuronidase. This was one point highlighted in a report prepared for the Department of the Environment, on the comparison of a number of media with simulated and environmental samples done by the Public Health Laboratory Service. An additional problem may be environmental coliforms which possess β-galactosidase but fail to ferment lactose on primary isolation. Such isolates would be positive by chromogenic substrate analysis but negative by lactose fermentation. Increasing the sensitivity of the detection method, whilst important in detecting faecal contamination, will apparently lead to a decrease in water quality and in water quality statistics. It is therefore important that new methods are comparable with existing methods in their sensitivity and that this is properly validated. It is equally important to understand that different sources of water may give varying results with chromogenic and fluorogenic media. Trials in any water company are therefore important to assess the sensitivity of any new test method with a cross-section of waters. What then becomes unclear is to what extent the trials should be conducted. How many different water types should be examined and how many samples should be tested for each water type? To compound matters further, potable water analysis produces a low failure rate. Many thousands of samples may need to be analysed to produce a significant number of

positive samples for statistical analysis. The question of the analysis of a single 100 ml water sample instead of 200 ml of water must also be considered carefully, in that any apparent improvement in water quality statistics might be interpreted as being due to a change in the test procedure or a reduction in the test volume.

It is not difficult to produce new media using the widely available chromogenic substrates. For example, detection of *Clostridium perfringens* is based on the production of hydrogen sulphide from the reduction of sulphite, producing black colonies on isolation media. This feature is variable (Oldham 1995; Rushby *personal communication*) and typical colonies are often colourless. Adding BCIP for phosphatase in the selective medium will give blue colonies or MUP will give fluorescent colonies.

Whilst these newer test methods are designed to be less labour intensive (more cost effective) and provide confirmed results, they still require 18 h incubation to produce a result. In some respects this is convenient because samples are analysed on one afternoon and results are available at the beginning of the following working day. Reduction of the analytical time to provide results on the same working day, in the form of a 6 to 8 h test (Sidorowicz & Whitmore 1995), may seem desirable but is not practicable. Without altering current sampling regimes, results would be available about midnight. Reduction of the analysis time to 1 to 2 h would provide same-day results but there is insufficient time for culture. As an alternative, assessment for viability now becomes important. Raw waters contain coliforms and *E. coli*. Treatment is designed to remove some and render the remainder non-viable (non-culturable). These organisms will not be detected by standard cultural techniques but may be detected by viability assessment and their significance needs to be clearly established.

Alternative methods of detection

Newer techniques for detecting microorganisms in drinking water have been reviewed by Sidorowicz & Whitmore (1995). Some of these have limited culture as part of the technique. The short-term culture of target organisms in non-selective culture media (eliminating the need for pre-enrichment) may be followed by a method of labelling cells specifically and a highly sensitive method of detection.

A number of DNA-specific fluorochromes are available for the detection of microorganisms. Some of these can differentiate between viable and non-viable bacteria. Acridine orange is commonly used to detect and count bacteria in water by direct microscopy (Fry 1988). Alternatives are 4'6'-diamidino phenylindole (DAPI) and the bis-benzamide derivative Hoechst 33342, used by Monger & Landry (1993) to detect bacteria in fresh and marine waters. Rhodamine 123 (Kaprelyants & Kell 1993; Morgan *et al.* 1993) and fluorescein diacetate (Jorgensen *et al.* 1992) have been used to determine viable biomass in water and waste water treatment. A tetrazolium salt, 5-cyano-2,3-ditolyltetrazolium chloride (CTC), has been used to detect viable bacteria in culture (Kaprelyants & Kell 1993) and in secondary treated effluents (Rodriguez *et al.* 1992). It has also been used to count planktonic and sessile respiring bacteria in drinking water (Schaule *et al.* 1993). Bovill *et al.* (1994) also reported the use of CTC for detecting metabolic activity in heat-stressed cells. Deere *et al.* (1995) used *bis*-(1,-3-dibutylbarbituric acid) trimethine oxonol for assessing bacterial viability, and Porter *et al.* (1995) describe the use of a range of viability dyes for analysis of indigenous bacteria from soil. The incorporation of additional specific labels in the form of fluorescent antibodies enables specific bacterial species to be detected and their viability assessed.

The fluorochromes listed in Table 3 can be used to study bacteria in water. When results of direct counting are compared with plate counts on nutrient media for the formation of biofilms, two very different pictures emerge. Figure 1 shows the accumulation of bacteria in a biofilm

over a period of 2 weeks. Counts were made using yeast extract agar (3 days at 22°C; Anon 1994), R2A agar (7 days at 20°C; Reasoner & Geldreich 1985) and staining with DAPI. Values obtained by direct counting are always higher than cultural techniques but after 9 days there is a significant increase in the biofilm that can only be detected by DAPI staining.

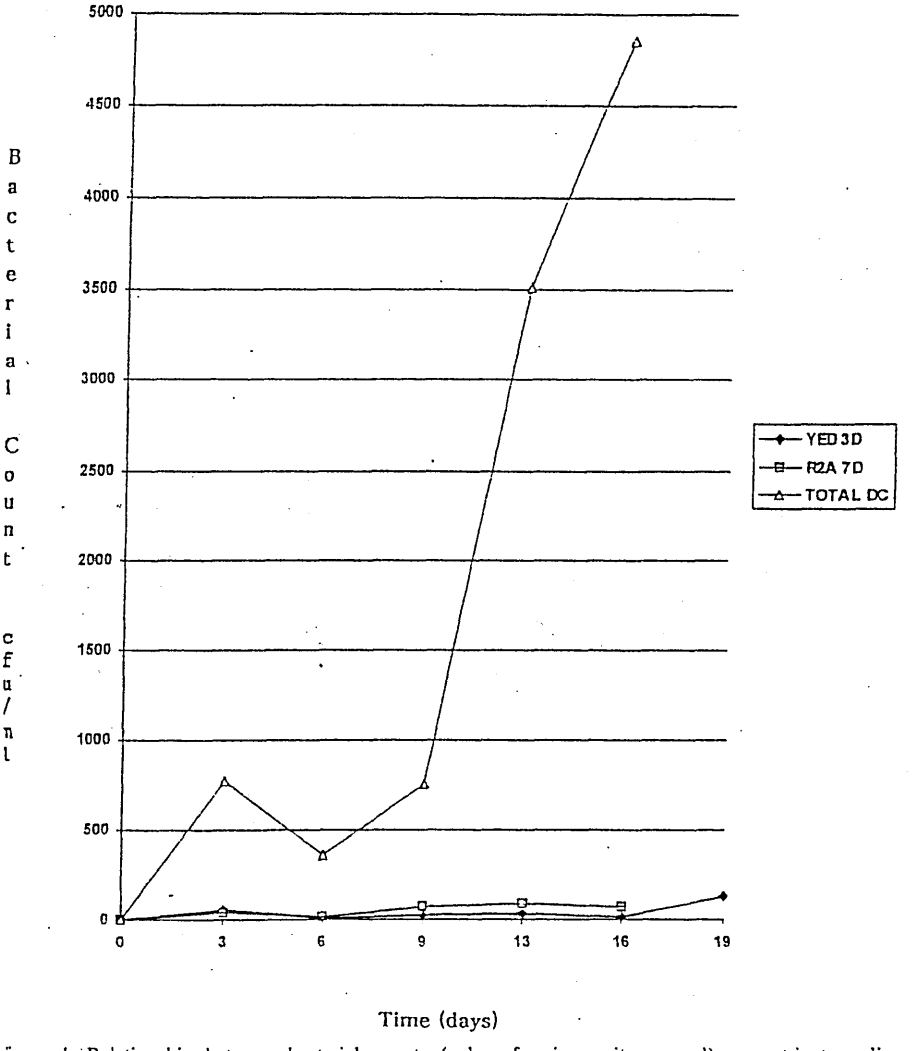


Figure 1. Relationship between bacterial counts (colony-forming units per ml) on nutrient media yeast extract for 3 days (♦, YED 3D) and R2A agar for 7 days (□, R2A 7D)) and total count using DAPI (△, Total DC).

Table 3. *Some examples of fluorochromes with their fluorescent properties.*

Reagent	Wavelength Excitation	Emission
Acridine orange	487	510
Fluorescein isothiocyanate	488	530
Phycoerythrin (PE)	488	575
Diamidino phenylindole (DAPI)	345	455
Propidium iodide (PI)	493	639
Rhodamine 123	505	543
Fluorescein diacetate (FDA)	505	530
Trimethine oxonol	535	560
Hoechst 33343	350	500
Cyanoditolyl tetrazolium chloride (CTC)	530	600

Immunological methods

Immunofluorescence, based on detection by labelling antibodies with a fluorochrome (direct immunofluorescence) or the detection of an antibody antigen complex with a labelled anti-species (indirect immunofluorescence), has been used widely to detect and serotype specific microorganisms. The development of mouse monoclonal antibodies has further enhanced this technique. Immunofluorescence has been used to detect a wide variety of microorganisms in food and water and provides a quick way of establishing their presence. Colbourne & Dennis (1989) demonstrated *Legionella pneumophila* serogroup 1 in waters abstracted for drinking. Brayton *et al.* (1987) described the enumeration of *Vibrio cholera* in tropical waters using immunofluorescence as a more sensitive tool than culture. More recently Pyle *et al.* (1995) have described a combined immunofluorescence and fluorogenic probe to detect and assess the viability of *E. coli* O157:H7 in water.

Immunofluorescence is one of many methods to be used for the detection of bacteria. Enzyme-linked immunosorbant assay (ELISA) has also been used, after a short period of growth, to detect *Salmonella* spp. in foods. However, with the exception of the detection of rotavirus, the technique is not used in the water industry. The test is relatively insensitive, requiring ca. 10^4 cells for a positive response.

Immunocapture using latex-coated magnetisable beads has shown more promise. Used primarily for the isolation of bacteria from food (Cudjoe *et al.* 1994), the technique has been adapted for the isolation of *E. coli* O157:H7 from food (Wright *et al.* 1994) and water, and has been used to concentrate *Cryptosporidium* from water. The principle is based on using antibody-coated magnetisable particles to capture target organisms from a heterogenous mixture of microorganisms. Once the organisms are captured, the beads can be separated from the mixture using a magnet. The captured organisms can be subjected to conventional culture or detected by microscopy, ELISA, molecular techniques or ATP assay. The period of culture by pre-enrichment before detection can be substantially reduced to ca. 4 to 5 h incubation, where only low numbers of organisms are present. With rapid detection techniques, organisms can be detected in as little as 8 hours.

Flow cytometry

Flow cytometry was originally developed for use in the study of eukaryotic cells and, in particular, leucocytes in mammalian blood. More recently attention has been turned to the study of microorganisms. Flow cytometry has been used in the study of the bacterial cell cycle (Skarstad *et al.* 1983) and its potential as a tool for microbial ecology has been recognised by

Edwards *et al.* (1992) who review the principles of flow cytometry. Diaper & Edwards (1994) reported the use of flow cytometry for the detection and enumeration of viable *Staphylococcus aureus* during survival in a lakewater microcosm, and Porter *et al.* (1995) were able to enumerate and sort mixtures of *S. aureus* and *E. coli* labelled with fluorescent antibody.

Flow cytometry can be used as a rapid and sensitive method for the analysis of bacterial populations and for detecting small numbers of target microorganisms within a heterogenous population (Watkins *et al.* 1995). There is a wide range of fluorescent probes available (Table 3) for both total and viable counts, and for specific detection using labelled monoclonal or polyclonal antibodies. In comparing flow cytometric counting with direct epifluorescence counting on the same suspension, results would appear to be reproducible under the correct operating conditions (Table 4). Detection of small numbers of target organisms was demonstrated by Pinder *et al.* (1994), counting *Aeromonas salmonicida* in survival studies and detecting *Salmonella typhimurium* in a mixed *Salmonella* population. Industrial applications include analysis of yeast in wine and fruit preparations, total viable counts on vegetables, and antibiotic sensitivity testing (Brailsford & Gatley 1994). Studies on drinking water, by comparing propidium iodode (PI) staining and counting by epifluorescence, and PI staining and counting by flow cytometry, have given good comparative results (Table 5).

Table 4. *Comparison of counts of* Staphylococcus aureus *stained with CTC and DAPI, obtained by flow cytometry (FCM) and by sorting and direct counting (EFM).*

CTC Stain		DAPI Stain	
FCM	EFM	FCM	EFM
68	68	8	6
46	44	7	6
41	39	12	12
65	62	15	10
256	257	31	33

Table 5. *Comparison of counts on drinking water samples stained with propidium iodide and enumerated by direct epifluorescence microscopy (EFM) and flow cytometry (FCM).*

Sample Number	Total Count (cells per ml)	
	FCM	EFM
16113433	880,000	760,000
16113343	70,000	28,000
16113435	168,000	117,000
16113436	560,000	504,000
16113444	284,000	212,000

There is a wide range of applications for rapid-flow cytometric analysis following a short period of pre-enrichment. *Salmonella* and *Listeria monocytogenes* could be analysed simultaneously from the same broth using antibodies conjugated with different fluorochromes. The incubation period could be as little as 4 h, and with a sort facility target organisms can be selected for culture, ELISA, or confirmation with molecular probes. Rapid detection of *Legionella pneumophila* from environmental samples also becomes easy and determination of viability is easy. Having a short period of culture followed by flow cytometric analysis and confirmation, immunological or molecular techniques will reduce analysis time down to less than 8 h.

Discussion

A number of rapid methods are available for the detection and enumeration of microorganisms. Most of these have been developed for, and are used in, the food industry. In the water industry, current attention is focused on chromogenic and fluorogenic substrates as a way of getting confirmed results after primary incubation. A similar trend is developing in clinical microbiology. To be effective in water analysis, a reduction to 8 h for the time between sampling and result is not really applicable. A reduction to between 1 and 2 h is a possibility but culture becomes impossible and we have to rely on enzyme-based detection methods. There are substrates for the enzymes β-galactosidase and β-glucuronidase which emit light when broken down. Detection systems for light emission can detect single cells on a membrane and can therefore detect faecal indicators without growth.

Direct comparisons with cultural techniques are important if meaningful comparisons are to be made about water quality. The technology is available and being developed, and perhaps one day microbiologists will have to accept a change in culture, throwing away the humble Petri dish for rapid detection of microorganisms "*in-situ*".

The authors wish to thank Alcontrol UK for permission to publish this article. The opinions expressed here are those of the authors and not necessarily those of the company they represent. For Harry Fennell and Frank Jones.

References

Anon, (1956). *The Bacteriological Examination of Water Supplies.* Report No. 71. Ministry of Health, HMSO, London.

Anon, (1983). *The Bacteriological Analysis of Drinking Water Supplies 1982.* Reports on Public Health & Medical Subjects No. 71. Department of the Environment, HMSO, London.

Anon, (1994). *The Microbiology of Water 1994. Part 1 – Drinking Water.* Reports on Public Health & Medical Subjects No. 71. HMSO, London.

Bisson, J. W. & Cabelli, V. J. (1979). Membrane filter enumeration method for *Clostridium perfringens. Applied & Environmental Microbiology*, 37, 55-66.

Bovill, R. A., Shallcross, J. A. & Mackey, B. M. (1994). Comparison of the fluorescent redox dye 5-cyano-2,3-ditolyltetrazolium chloride with p-iodonitrotetrazolium violet to detect metabolic activity in heat-stressed *Listeria monocytogenes* cells. *Journal of Applied Bacteriology*, 77, 353-358.

Brailsford, M. A. & Gatley, S. (1994). Industrial application of flow cytometry for the rapid detection of microorganisms. In *New Techniques in Food and Beverage Microbiology* (eds R. G. Kroll, A. Gilmour & M. Sussman), pp. 131-145. Blackwell Scientific Publications, Oxford.

Brayton, P., Tamplin, M., Huq, A. & Colwell, R. R. (1987). Enumeration of *Vibrio cholera* O1 in Bangladesh waters by fluorescent-antibody direct viable count. *Applied & Environmental Microbiology*, 53, 2862-2865.

Clarke, D. L., Milner, B. B., Stewart, M. H., Wolfe, R. L. & Olson, B. H. (1991). Comparative study of commercial 4-methylumbelliferyl-β-D-glucuronide preparations with the standard methods membrane filtration faecal coliform test for the detection of *Escherichia coli* in water samples. *Applied & Environmental Microbiology*, 57, 1528-1534.

Colbourne, J. S. & Dennis, P. J. (1989). The ecology and survival of *Legionella pneumophila. Journal of the Institution of Water Engineers & Scientists*, 3, 345-350.

Cowburn, J. K., Goodall, T., Fricker, E. J., Walter, K. S. & Fricker, C. R. (1994). A preliminary study of the use of Colilert for water quality monitoring. *Letters in Applied Microbiology*, 19, 52-54.

Cudjoe, K. S., Patel, P. D., Olsen, E., Skjerve, E. & Olsvik, O. (1994). Immunomagnetic separation techniques for the detection of pathogenic bacteria in foods. In *New Techniques in Food and Beverage Microbiology* (eds R. G. Kroll, A. Gilmour & M. Sussman), pp. 131-145. Blackwell Scientific Publications, Oxford.

Deere, D., Porter, J., Edwards, C. & Pickup, R. W. (1995). Evaluation of the suitability of *bis*-(1,3-dibutylbarbituric acid) trimethine oxonol. (biBA-C_4(3)-), for the flow cytometric assessment of bacterial viability. *FEMS Microbiology Letters*, 130, 165-170.

Diaper, J. P. & Edwards, C. (1994). Survival of *Staphylococcus aureus* in lakewater monitored by flow cytometry. *Microbiology*, 140, 35-42.

Dufour, A. P. & Cabelli, V. J. (1975). Membrane filter procedure for enumerating the component genera of the coliform group in sea water. *Applied & Environmental Microbiology*, 29, 826-833.

Edberg, S. C., Allen, M. J. & the National Collaborative Study. (1988). National field evaluation of a defined substrate

method for the simultaneous enumeration of coliforms and *Escherichia coli* from drinking water: comparison with the standard multiple tube fermentation method. *Applied & Environmental Microbiology*, **54**, 1595-1601.

Edberg, S. C., Allen, M. J., Smith, D. B. & the National Collaborative Study. (1989). National field evaluation of a defined substrate method for the simultaneous enumeration of total coliforms and *Escherichia coli* from drinking water: comparison with presence-absence techniques. *Applied & Environmental Microbiology*, **55**, 1003-1008.

Edwards, C., Porter, J., Saunders, J. R., Diaper, J. *et al.* (1992). Flow cytometry and microbiology. *Society for General Microbiology Quarterly*, **November 1992**, 105-108.

Fry, J. C. (1988). Determination of biomass. In *Methods in Aquatic Bacteriology* (ed. B. J. Austin), pp. 27-72. Wiley, New York.

Jorgensen, P. E., Eriksen, T. & Jensen, B. K. (1992). Estimation of viable biomass in wastewater and activated sludge by determination of ATP, oxygen utilisation rate and FDA hydrolysis. *Water Research*, **26**, 1495-1501.

Kaprelyants, A. S. & Kell, D. B. (1993). Dormancy in stationary-phase cultures of *Micrococcus luteus;* flow cytometry analysis of starvation and resuscitation. *Applied & Environmental Microbiology*, **59**, 10, 3187-3196.

Lewis, C. M. & Mak, J. L. (1989). Comparison of membrane filtration and autoanalysis Colilert presence absence techniques for the analysis of total coliforms and *Escherichia coli* in drinking water samples. *Applied & Environmental Microbiology*, **55**, 3091-3094.

Monger, B. C. & Landry, M. R. (1993). Flow cytometric analysis of marine bacteria with Hoechst 33342. *Applied & Environmental Microbiology*, **59**, 905-919.

Morgan, J. A. W., Rhodes, G. & Pickup, R. W. (1993). Survival of nonculturable *Aeromonas salmonicida* in lake water. *Applied & Environmental Microbiology*, **59**, 874-880.

Oldham, N. S. (1995). *A comparison of methods for the detection of* Clostridium perfringens *in raw water samples.* Internal project report, LabServices, Bradford.

Pinder, A. C., Edwards, C., Clarke, R. G., Diaper, J. P. & Poulter, S. A. G. (1994). Detection and enumeration of viable bacteria by flow cytometry. In *New Techniques in Food and Beverage Microbiology* (eds R. G. Kroll, A. Gilmour & M. Sussman), pp. 131-145. Blackwell Scientific Publications, Oxford.

Porter, J., Pickup, R. & Edwards, C. (1995). Flow cytometric methods for the detection and viability assessment of bacteria in soil. (In press).

Public Health Laboratory Service. (1969). A minerals-modified glutamate medium for the enumeration of coliform organisms in water, by the Public Health Laboratory Service Standing Committee on the Bacteriological Examination of Water Supplies. *Journal of Hygiene*, **81**, 367-374.

Pyle, B. H., Broadway, S. C. & McFeters, G. A. (1995). A rapid, direct method for enumerating respiring enterohaemorrhagic *Escherichia coli* O157:H7 in water. *Applied & Environmental Microbiology*, **61**, 2614-2619.

Reasoner, D. J. & Geldreich, E. E. (1985). A new medium for the enumeration and sub-culture of bacteria from potable water. *Applied & Environmental Microbiology*, **49**, 1-7.

Rippey, S. R. & Cabelli, V. J. (1979). Membrane filter procedure for the enumeration of *Aeromonas hydrophila* in fresh water. *Applied & Environmental Microbiology*, **38**, 108-113.

Rodriguez, G. G., Phipps, D., Ishiguro, K. & Ridgway, H. F. (1992). Use of a fluorescent redox probe for direct visualisation of actively respiring bacteria. *Applied & Environmental Microbiology*, **58**, 1801-1808.

Sartory, D. P. (1995). Improved recovery of chlorine-stressed organisms with pyruvate supplemented media. *Water Science & Technology*, **31**, 255-258.

Sartory, D. P. & Howard, L. (1992). A medium detecting β-glucuronidase for the simultaneous membrane filtration enumeration of *Escherichia coli* and coliforms in drinking water. *Letters in Applied Microbiology*, **15**, 273-276.

Schaule, G., Flemming, H. C. & Ridgway, H. F. (1993). Use of 5-cyano-2,3-ditolyl tetrazolium chloride for quantifying planktonic and sessile respiring bacteria in drinking water. *Applied & Environmental Microbiology*, **59**, 11, 3850-3857.

Schets, F. M., Medema, G. J. & Havelaar, A. H. (1993). Comparison of Colilert with Dutch standard methods for *Escherichia coli* and total coliforms in water. *Letters in Applied Microbiology*, **17**, 17-19.

Sidorowicz, S. V. & Whitmore, T. N. (1995). Prospects for new techniques for rapid bacteriological monitoring of drinking water. *Journal of the Institute of Water & Environment Management*, **9**, 92-98.

Skarstad, K., *et al.* (1983). Cell cycle parameters of slowly growing *E. coli* studied by flow cytometry. *Journal of Bacteriology*, **154**, 656-662.

Watkins, J., Kemp, P. & Shepherd, K. (1995). Analysis of water samples for *Cryptosporidium* including the use of flow cytometry. In *Protozoan Parasites and Water* (eds W. B. Betts *et al.*), pp. 115-121. Royal Society of Chemistry.

Windle Taylor, E., Burman, N. P. & Oliver, C. W. (1953). Use of the membrane filter in the bacteriological examination of water. *Journal of Applied Chemistry*, **3**, 233.

Wright, D. J., Chapman, P. A. & Siddons, C. A. (1994). Immunomagnetic separation as a method for isolation *Escherichia coli* O157:H7 from food samples. *Epidemiology & Infection*, **113**, 31-39.