2011

Biofilm Formation and Control in a Novel Warm Water Distribution System

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BIOFILM FORMATION AND CONTROL IN A NOVEL WARM WATER DISTRIBUTION SYSTEM

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Biofilm Formation and Control in a Novel Warm Water Distribution System

A thesis submitted in fulfilment of the

requirement for the degree of:

DOCTOR OF PHILOSOPHY

University of Plymouth

School of Biomedical and Biological Sciences

Faculty of Science and Technology

By Paul Lewis Waines

June 2011

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Abstract

Investigations were carried out to assess biofilm formation within a model warm water distribution system (test rig) under a variety of conditions. Analysis methods included ATP-/ culture-based analysis, SEM and confocal microscopy. Molecular-based community analysis was carried out using PCR/DGGE.

High pH (9.53-10.08), induced by the presence of a sacrificial anode within the water heater, had a profound inhibitive effect on the culturability of biofilm bacteria on copper (Cu) pipe within the test rig. Concurrent investigations into the effect of stagnation (varied periods of non-flushing) appeared to contradict the widely held view that stagnation is conducive to biofilm formation, with greater flushing frequencies resulting in increased biofilm. It was concluded that a higher frequency of nutrient-delivering events were largely responsible for this and that in systems where lengthier stagnation periods were employed, factors such as low oxygen and reduced nutrient levels inhibited biofilm formation on previously uncontaminated Cu pipe.

Thermal purging (TP) over a 28 day period of 30 second, 12 hourly flushing at 41 °C and three-daily one minute purging with 70 °C water resulted in a 99% reduction in the culturability of biofilm bacteria on both Cu and LLDPE. However, confocal microscopical analysis of bacterial numbers indicated that 25.06% (Cu) and 21.55% (LLDPE) of the initial bacterial population remained viable. A large proportion of non-viable biofilm bacteria were also observed. Further work is therefore required in order to optimize TP within the test rig.

Biofilm formation on a range of different materials; Cu, stainless steel, PEX, and EPDM, showed significantly greater biofilm development on EPDM in comparison to the other materials. Preliminary investigations of LLDPE and tap outlet fittings showed that laminar flow outlet fittings may act as reservoirs for the development and subsequent dissemination of biofilm.

Molecular bacterial community structural studies of test rig biofilms clearly showed that biofilm community composition was significantly affected by both temporal and environmental factors, and varied at points within the same system. Sequencing did not provide a great insight into the composition of the bacterial communities within the test rig, and further work is required to gain a more complete picture of bacterial community diversity within the test rig.

These studies show that biofilm formation within the test rig is greatly influenced by a wide variety of factors. The test rig's unique design necessitates a cautionary approach when making comparisons with, for example, larger water distribution systems

Contents

Chapter 3 An Investigation into the Effects of Stagnation and High pH on Biofilm Formation in a Model Distribution System

List of abbreviations

ALFO	Antimicrobial Laminar Flow Outlet
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BCYE	Buffered Charcoal Yeast Extract
BLAST	Basic Local Alignment Search Tool
CAB	Culturable Aerobic Bacteria
CFC	Cetrimide Fucidin Cephalosporin (supplement for *Pseudomonas* agar base)
CFU	Colony Forming Units
(C)LSM	(Confocal) Laser Scanning Microscope
CTC	5-Cyano-2,3-Tolyl-Tetrazolium Chloride
CTU	Co-migrating Taxonomic Unit
dNTP	Deoxynucleotide Triphosphate
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
DVS	Dart Valley Systems Ltd.
EDTA	Ethylenediaminetetraacetic Acid
EM	Electron Microscopy
EPDM	Ethylene Propylene Diene Monomer (M-class) rubber
EPS	Extracellular Polymeric Substances
FISH	Fluorescence *in situ* Hybridization
FS	Flow Straightener
GFP	Green Fluorescent Protein

GVPN	Glycine Vancomycin Polymyxin Nalidix Acid (supplement for BCYE agar)
IMS	Industrial Methylated Spirits
ID	Inner Diameter
LFO	Laminar Flow Outlet
LLDPE	Linear Low Density Polyethylene
MDPE	Medium Density Polyethylene
OD	Outer Diameter
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
PE	Polyethylene
PEX	Cross-linked Polyethylene
PVC	Polyvinyl Chloride
MIC	Microbially Induced Corrosion
R2A	Reasoner 2A (low nutrient agar)
RISA	Ribosomal Intergenic Spacer Analysis
RNA	Ribonucleic Acid
SG	Sealing Gasket
SEM	Scanning Electron Microscopy
smp	'Sample' (see section 2.8.3)
SPSS	Statistical Package for the Social Sciences
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
TE	Tris EDTA
TMV	Thermal Mixing Valve
TP	Thermal Purging

UV	Ultra Violet
WRAS	Water Regulations Advisory Scheme

Commonly used abbreviations for bacterial genera

P.	*Pseudomonas*
L.	*Legionella*
M.	*Mycobacterium*

List of Tables

List of Figures

Acknowledgements

I would like to extend my sincere thanks to all those who have helped and supported me throughout my studies.

Firstly, I would like to thank my Director of Studies, Dr. Graham Bradley, for giving me the opportunity to undertake this project, and also for his patience, guidance, encouragement and support throughout. I would also like to thank Dr. John Moody, and Dr. Roy Moate for their interest in the project and academic support.

I am deeply indebted to Dart Valley Systems Limited and the University of Plymouth, for funding this research. I would particularly like to thank Mr. Mike Allen, former Managing Director of Dart Valley Systems Limited. This work could not have taken place without your intellectual input, initiative, support and continued enthusiasm. I would also like to thank Mr. Colin Richards and Mr. Ben Cornish for their technical assistance and advice.

I also extend a heartfelt thanks to members of the Faculty of Science and Technology technical staff (and former colleagues, past and present) who have helped me at various times throughout this project, either through direct action, or simply through their friendship and words of encouragement: Andy Atfield, Dr. Laura Biggs, Peter Bond, Chris Boxley, Chris Coleman, Lynn Cooper, Nick Crocker, Matthew Emery, Dr. Andy Fisher, Glenn Harper, Angela Harrop, Sarah Jamieson, Dr. Michele Kiernan, Wendy Locock, Dr. Mark Montgomery, Luke Peakman, Rick Preston, and Will Vevers.

I would like to thank my friend and colleague Lindsey Glew for all those discussions on the vagaries of biofilm research. Also to Dr. Daniel Merrifield, Debbie Petherick, Annie Treeby and in particular Professor Simon Davies for their friendship, advice and support.

Thanks also to all my family, and in particular my parents, brother David and sister Polly, for the unconditional love and encouragement that you have always shown me.

Finally, I would like to thank my wife Emma and my boys George and Archie for your continuing patience, love and understanding.

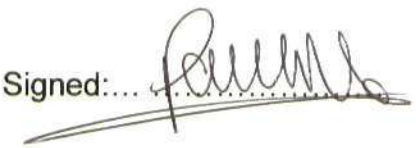
Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

The study was jointly funded by Dart Valley Systems Ltd. (Paignton, Devon, U.K.) and the University of Plymouth.

Relevant scientific workshops and conferences were frequently attended, work was often presented and three manuscripts are soon to be submitted for peer review.

Word count (chapters 1-6 inclusive): 45,462

Signed:... 

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Chapter 1 Introduction

1.1 The biofilm defined

For many years, the research approach adopted by microbiologists was largely based on the growth and subsequent analysis of microorganisms in pure culture, this in spite of the fact that the first observations of bacteria were as biofilms (Costerton 2007). Whilst this approach has formed the bedrock of current knowledge, and produced many breakthroughs in a number of related fields, the increasing appreciation of the ubiquity of biofilms in the environment has led to huge advances in the fields of biofilm research and microbial ecology over the last thirty years. The rapidity of these advances has led to many revisions of the definition of the term 'biofilm', in order that they may be adequately described. Based on current knowledge, such a definition is proposed below:

'A microbial biofilm is formed by microorganisms (bacteria, yeasts, protozoa, diatoms, viruses and fungi) through attachment to a suitably conditioned biotic or abiotic surface, and subsequent enclosure (either complete or partial) in a hydrated matrix of extracellular polymeric substances (EPS) which they themselves produce in response to local environmental conditions, forming as a result of changes in gene expression which also bring about profound phenotypic and physiological changes in comparison to the planktonic form.'

Ubiquitous in both natural and man-made environments, biofilms may exert either harmful or beneficial effects depending on the type(s) of microorganisms

present (Brading *et al.* 1995). The tendency for microorganisms to preferentially form biofilms in nature has prompted much investigation into the benefits to the microorganisms that this mode of existence implies when compared to growth in comparison to the planktonic state (Mueller 1996), and it would appear that one of the main 'drivers' for biofilm formation is physiological, genetic, and ecological efficiency (Costerton 2007).

The benefits to microorganisms of existing in the biofilm state include:

1. Increased resistance to harsh environmental conditions, including predation from protozoa (such as *Acanthamoeba*, *Tetrahymena* ssp., and *Hartmanella* spp.) and parasites (such as *Bdellovibrio* and bacteriophages) (Barker and Brown 1994), as well as increased resistance to antimicrobials (Lewis 2001; O'Toole and Stewart 2005). Resistance to many conventional control strategies such as chemical and mechanical removal has also been extensively reported (Simões *et al.* 2010).
2. More efficient nutrient-streaming strategies, through the establishment of metabolically co-operative consortia. In oligotrophic environments, the formation of multispecies biofilms facilitates the exploitation of nutrient opportunities such as the utilization of the metabolites of neighbouring bacteria and the generation of energy from metal surfaces (Costerton 2007).
3. Increased opportunity for horizontal gene transfer, and the acquisition of desirable genetic traits. The biofilm itself provides a stable environment in which transfer of plasmid-borne and somatic (non-

plasmid borne) genes can occur (Costerton 2007), and this genetic diversification offers greater protection from adverse environmental conditions (Boles *et al.* 2004).

4. Increased opportunity for communication between individual cells by such mechanisms as nanowires (Gorby *et al.* 2006) and vesicle formation, or between groups by mechanisms such as quorum sensing.

1.2 Biofilm formation in large scale water distribution systems

A water distribution system (WDS) may be described as a series of conduits from the point of entry of water into a building, to the extraction points (Mouchtouri *et al.* 2007), and the variety of microenvironments which result means that WDS biofilms are notoriously difficult to control and almost impossible to eradicate.

Biofilm formation is a complex, multi-stage process which is initiated by the conditioning of a surface via the accumulation of nutrients. Surface conditioning occurs rapidly, being brought about by the assumption of a net negative charge at that surface, and resulting in the adhesion of molecules of potential nutritional value to bacteria (Brading *et al.* 1995). Investigators have shown that materials with diverse surface properties are rapidly conditioned by adsorbing organic nutrients when exposed to waters with low organic concentrations, such as are found in water distribution systems, and different substrata may accumulate conditioning films of different composition because of their differing surface properties such as potential, charge and critical surface tension (Characklis 1990). Bacterial attachment to a conditioned surface is affected by a variety of

factors including the characteristics of the bacteria themselves, the target material surface and environmental factors (An and Friedman 1998). These environmental factors may include water temperature, pH, pipe and plumbing component materials, biocide concentration, microbially available nutrients, and the hydrodynamics of the distribution system (Camper *et al.* 1999). Brading *et al.* (1995) suggested that the overall stability of the biofilm may also be influenced by its diversity, with mixed culture biofilms being inherently less stable, due to possible species-species/ EPS-substratum incompatibility. In contrast, Simões *et al.* (2009) found that multispecies biofilms (in this case consisting of *B. cereus* and *Ps. fluorescens*) exhibit an increased resistance to both chemical and mechanical removal. It is postulated that the higher proportion of viable cells when compared to single species biofilms grown under the same conditions, is a significant factor in increasing resistance (Simões *et al.* 2009). The principle of species diversification within an ecosystem community increasing the resistance of those species to environmental stresses, known as the 'insurance hypothesis', has been shown to apply to biofilm communities through the demonstration of rapid genetic diversification of *Pseudomonas aeruginosa* during the early stages of biofilm formation (Boles *et al.* 2004).

1.2.1 Biofilms and WDS-related infections

Biofilms act as potential reservoirs for a number of human pathogens, particularly *Pseudomonas, Legionella, and Mycobacterium* spp., and this can have serious public health implications (Momba *et al.* 2000). Fungi and viruses, such as *Aspergillus fumigatus* and rotavirus, have also been implicated in a

number of infection outbreaks (Ortolano *et al.* 2005). Since the majority of viruses observed in biofilms are thought to be bacteriophagous in nature, (Lehtola *et al.* 2004), it is assumed that a lack of suitable host outside the biofilm would mean that their ability to take on the role of potential human pathogen in a WDS is severely restricted. Table 1.1 (overleaf) presents an overview of microorganisms which have been associated with waterborne nosocomial infections.

The fact that biofilms are a potential reservoir for pathogenic microorganisms is of significant importance in a clinical setting, and numerous studies cite the hospital water supply as a major source of nosocomial infections (Bert *et al.* 1998; Ferroni *et al.* 1998; Squier *et al.* 2000; Anaissie *et al.* 2002; Kusnetsov *et al.* 2003; Suman *et al.* 2008; Feazel *et al.* 2009), particularly in situations where the WDS is old and/or poorly designed. *Legionella pneumophila, Pseudomonas* spp., and *Mycobacterium* spp. are well documented as the causative agents of a variety of such infections among immune-compromised individuals

Table 1.1 Summary of microorganisms associated with waterborne nosocomial infection outbreaks. Adapted from a review by Anaissie *et al.* (2002), with alternative supporting references where appropriate.

Microorganism	Site(s) of infection	Reference
Bacteria		
Aeromonas hydrophila	Blood	(Picard and Goullet 1987)
Campylobacter jejuni	Stools	(Rautelin *et al.* 1990)
Legionella pneumophila	Lungs	(Patterson *et al.* 1997; Brulet *et al.* 2008; Shachor-Meyouhas *et al.* 2010)
Mycobacterium avium	Disseminated	(Dumoulin *et al.* 1988; von Reyn *et al.* 1994)
Mycobacterium fortuitum	Disseminated respiratory tract, sputum, sterna wound infection wound	(Hector *et al.* 1992; Kauppinen *et al.* 1999)
Mycobacterium kansasii	Abscess, blood, bone, sputum, stomach, urine	(Wright *et al.* 1985; Picardeau *et al.* 1997)
Mycobacterium xenopi	Various, spine	(Wright *et al.* 1985)

Table 1.1 Summary of microorganisms associated with waterborne nosocomial infection outbreaks (cont'd).

Microorganism	**Site(s) of infection**	**Reference(s)**
Pseudomonas aeruginosa	Blood, lungs, peritoneum, sinuses, trachea, urine	(Bert *et al.* 1998; Ferroni *et al.* 1998; Blanc *et al.* 2004; Trautmann *et al.* 2005; Suman *et al.* 2008; Inglis *et al.* 2010)
Pseudomonas mesophilica	Febrile illness	(Gilchrist *et al.* 1986)
Sphingomonas paucimobolis	Blood	(Perola *et al.* 2002)
Stenotrophomonas maltophila	Blood, peritoneum, respiratory tract, skin, stools, throat, trachea, urine	(Denton *et al.* 1998; Weber *et al.* 1999)
Fungi		
Aspergillus fumigatus	Lungs	(Anaissie *et al.* 2003)
Exophiala jeanselmaei	Disseminated	(Nucci *et al.* 2001)
Fusarium solani	Disseminated	(Anaissie *et al.* 2001)
Viruses		
Unnamed enteric virus	Stomach/ digestive system	(Schvoerer *et al.* 1999)

1.2.1.1 *Legionella*

Legionella is a Gram-negative, aerobic aquatic member of the family Legionellaceae, which comprises 57 known species, 17 of which have been linked to outbreaks of pneumonia-like infections collectively referred to as Legionellosis (Steinert *et al.* 2002). In 1976, *Legionella pneumophila* caused the first documented outbreak of Legionnaires disease in Philadelphia, and is the major cause of both community-acquired and nosocomial Legionellosis. It has been extensively reviewed both microbiologically and clinically (for examples, see Steinert *et al.* (2002), Cianciotto *et al.* (2006) and Declerck (2009)). Perola *et al.* (2005) reported that up to 68% of hospital plumbing systems have been reported as being colonized with *Legionella pneumophila*, while Ortolano *et al.* (2005) state that under-diagnosis and under-reporting are high, with only 2-10% of cases believed to be accurately reported. Leoni *et al.* (2005) investigated the prevalence of *Legionella* species in hot water distribution systems in the city of Bologna (Italy) and detected them in 40% of the 137 distribution system samples taken. The highest colonization was found in the hot water systems of five hospitals, where 93.7% of 32 samples were positive for *L. pneumophila*.
In the natural environment, *L. pneumophila* demonstrates a close reliance on biofilms as well as several protozoan species for its survival, such as *Acanthamoeba polyphaga* and *Hartmanella* spp., both of which have previously been observed in tap water systems (Steinert *et al.* 2002). Conversely, there have been numerous studies that indicate protozoan-independent growth, whereby *L. pneumophila* uses biofilms and sediments for shelter and growth where the water temperature is between 25 °C and 45 °C (van der Kooij *et al.* 2002; Temmerman *et al.* 2006). Garcia *et al.* (2007) compared the resistance of

L. pneumophila grown in either the presence or absence of *Acanthamoeba*, clearly demonstrating that co-culture with the protozoa conferred considerable resistance to sodium hypochlorite disinfection as well as increasing the resuscitation rate of viable but non-culturable (VBNC) cells. Because debris has a tendency to accumulate in the periphery of water distribution systems, Temmerman *et al.* (2006) studied the ability of *L. pneumophila* to exhibit necrotrophy i.e. the ability to exist on a variety of heat-killed bacterial cells, including those of a pre-established biofilm. An increase in cell numbers, quantified using a variety of methods, was noted. However, this was shown to be lower than when *L. pneumophila* was co-cultured with *Acanthamoeba*. *L. pneumophila* therefore demonstrates versatility in terms of the survival strategies it employs, and perhaps the most dramatic illustration of this versatility is the ability of this aquatic bacterium to invade and grow within macrophages (Steinert *et al.* 2002).

Legionella is fastidious in terms of its requirements for culture in the laboratory (Bopp *et al.* 1981), a trait which is perhaps surprising when one considers its occurrence in nature within a range of freshwater (i.e. generally nutrient-poor) environments. It has been suggested that these complex nutritional requirements preclude it from being a primary colonizer of surfaces, and that it is instead incorporated into microbial consortia which are capable of providing nutritional benefits (Declerck 2009). Indeed, incorporation into multispecies biofilms on a variety of materials has been observed in numerous studies (Schofield and Locci 1985; Murga *et al.* 2001; Declerck *et al.* 2009; Moritz *et al.* 2010), where prolonged survival is possible through entry into the VBNC state (Declerck *et al.* 2009).

1.2.1.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, aerobic bacillus with unipolar motility, and is often found in biofilms in plumbing systems, due to its ability to exist in low nutrient (oligotrophic) environments. The readiness with which *P. aeruginosa* forms biofilms has made it a model microorganism for studies of this type.

It is well established as a waterborne pathogen in hospitals, and has been proven to be the causative organism in a wide variety of infections (see Table 1.1). Anaissie *et al.* (2002) reported that an estimated 1400 deaths occur each year in the United States as a result of waterborne nosocomial pneumonias caused by *Pseudomonas aeruginosa* alone. Ferroni *et al.* (1998) stated that around 11% of all hospital-acquired urinary tract infections were attributable to *P. aeruginosa*, although they also stated that tap water has rarely been reported as the source of such outbreaks.

1.2.2 The role of EPS in biofilm formation

The production of EPS by microorganisms is fundamentally important to the process of biofilm formation. Whilst this has been recognized since the early 1980s (Costerton *et al.* 1981), detailed investigations into its exact nature and function form a relatively small proportion of biofilm research to date. Composed of a wide variety of polysaccharides (e.g. alginate), lipids and proteins (e.g. extracellular enzymes), it plays a major role in determining the living conditions of the microorganisms enclosed within. The exact composition of the EPS varies greatly depending on not only the species of microorganisms present

within a particular biofilm, but also on the environment in which the biofilm is being formed (Beech 2004). This complexity of composition means that EPS demonstrates a multiple functionality which is summarized in Table 1.2 (overleaf). It is possible that EPS performs more functions than those listed, but the exact biochemical characterization of EPS is made challenging by its close association with cells and non-biofilm associated macromolecules (Flemming and Wingender 2001).

In terms of biofilm formation in WDSs, EPS contributes considerably to the overall biomass present. Its complexity and the integral role it plays in biofilm formation and function mean that gaining an understanding of its exact role in such situations would appear crucial in minimizing potentially hazardous microbial contamination. The interaction of EPS with metal surfaces such as copper has been shown to play a role in the colonization and biodeterioration of such surfaces, due to its ability to readily bind metal ions (Angell and Chamberlain 1991). However, it is not clear exactly which types of macromolecule are involved in this process (Beech 2000).

1.2.3 Hydrodynamic effects on biofilm formation within WDSs

The hydrodynamics of aqueous environments have a significant effect on biofilm development and activity and are in turn influenced by biofilms in various industrial systems (Stoodley *et al.* 1994). Hydrodynamic conditions influence substrate concentration, which in turn influences cell growth and subsequent biofilm development, and it is a combination of these effects that influences the physical properties of a biofilm, such as its thickness, adhesiveness and porosity.

Table 1.2 Summary of EPS components and their functionality (adapted from Flemming *et al.* (2007)).

Nature of component	Role within biofilm
Polysaccharides	Structural/ enzyme binding
Charged/ hydrophobic polysaccharides	Sorption of organic compounds/ ion exchange
Extracellular enzymes	Polymer degradation (for nutritional purposes)
Membrane vesicles	Cell-to-cell communication
Nucleic acids (extracellular DNA)	Genetic information, structural
Membrane vesicles containing nucleic acids, enzymes etc.	Export of cell components
Potentially all components	Nutrient source

Within a water distribution system, water flow may be *laminar, transitional, turbulent* or *stagnant* (Munson *et al.* 2002) at different points within the same system. Laminar flow (Figure 1.1 (a), overleaf) describes the smooth flow of water through a pipe with no lateral mixing, whereas turbulent flow describes flow which is more erratic and irregular (Brading *et al.* 1995). Transitional flow describes the point at which flow may be either laminar or turbulent, and all three flow types are estimated using a dimensionless parameter known as the Reynolds number, whereby a Reynolds number of approximately 2100 or less represents laminar flow, and a value of 4000 or more represents turbulent flow, with any value between these figures representing transitional flow (Munson *et al.* 2002).

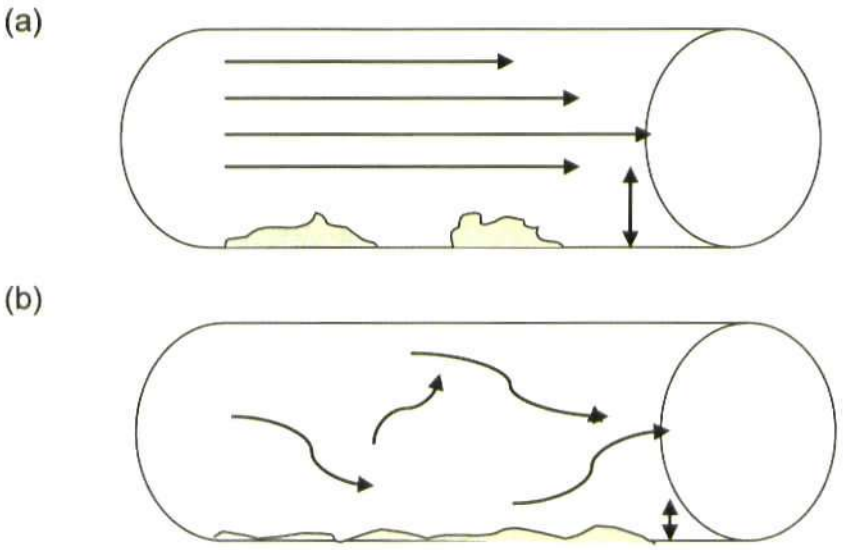


Figure 1.1 Diagrammatic representation of (a) laminar flow and (b) turbulent flow. Single-headed arrows represent direction and relative fluid velocity at particular positions within the pipe. Double-headed vertical arrows represent the relative thickness of the boundary layer at the pipe surface under the two types of flow. Molecular diffusion and bacterial motility are governing factors in determining transfer of particles from bulk flow to the substratum. In (b), particles suspended within the fluid are transported towards the pipe wall primarily by dynamic forces. Biofilm presence and thickness is also illustrated. (adapted from Brading *et al.* (1995), and Costerton (2007)).

Turbulent and laminar flow are also characterized by the existence of a boundary layer next to the surface of the water pipe. In the case of laminar flow this layer may extend far into the pipe, whereas in the case of turbulent flow the boundary layer may be comparatively thin. The boundary layer is characterized by the predominance of laminar flow in both cases, although in turbulent flow this may often be interrupted, and immediately adjacent to the surface the water actually has a velocity of virtually zero. At this point bacterial motility and diffusion are important factors in determining whether bacterial cells come into contact with a surface (Brading *et al.* 1995).

When considering biofilm formation in water distribution systems, it is important to remember that the fluid flow characteristics are not likely to be constant throughout, due to the existence of obstructions, bends and 'dead legs'. Characklis (1990) states that microbial transport is 'influenced by the

macroscopic geometry of the experimental system', whereby fluid forces within the various environments are quite complex and influence transport rates. The resulting microenvironments (e.g. crevices) will also determine the type of microbial activity in the biofilm.

1.2.3.1 Effect of stagnation on biofilm formation in WDSs

Stagnation, particularly in distal portions of large distribution systems, is widely believed to predispose water systems to colonization by *Legionella* (Wadowsky *et al.* 1982; Ciesielski *et al.* 1984) and other biofilm-forming bacteria of clinical significance, such as *Pseudomonas aeruginosa* (Blanc *et al.* 2004). The transport of particles from the bulk fluid to the substratum in this case is by sedimentation, Brownian motion, or by motility (Characklis 1990). Areas of obstruction, such as taps, tap aerators and showerheads, have been shown to harbour bacteria implicated in nosocomial out breaks (Bollin *et al.* 1985; Zacheus and Martikainen 1994; Weber *et al.* 1999; Blanc *et al.* 2004).
Liu *et al.* (2006) conducted an investigation into the effects of stagnation, laminar flow and turbulent flow on numbers of *Legionella pneumophila* in an experimental system, partly because the effect of stagnation had not previously been rigorously assessed (Liu *et al.* 2006). This experimental system consisted of three parallel pipes each subjected to a different flow regime over a five week period, and was inoculated using contaminated filter cartridges obtained from a local hospital building. The authors were unable to demonstrate that stagnant conditions promoted growth of *Legionella pneumophila*, with plate counts high under both turbulent and laminar flow conditions, and highest under turbulent flow. The results obtained for stagnant flow apparently contradict a number of

earlier studies which state that stagnation favours *Legionella* replication and biofilm formation in general (Ciesielski *et al.* 1984; Harper 1988; Whitehouse *et al.* 1991; Singh and Coogan 2005). Significantly, it is not apparent that any analysis of the general biofilm community over this period was carried out. In an oligotrophic environment at a mean temperature of 24 °C such as was described in this study, it is entirely possible that (a) the *Legionella* are losing out on essential nutrients (and other requirements for growth) due to competition from other microorganisms more suited to the particular environment described, or (b) a proportion of the *Legionella* are surviving within the biofilm in the Viable But Non-Culturable (VBNC) state. The short length of the study may also be relevant. Ciesielski *et al.* (1984) showed in a hospital setting that *Legionella pneumophila* contamination was significantly reduced by a corresponding reduction in stagnation. This was done by comparing four hospital hot water storage tanks, fed by the local municipal water supply. Two systems were brought on-line after a period of stagnation and kept so continuously for a year. Due to frequent demand, these tanks were frequently diluted with cold water (thus maintaining a below optimum temperature for the *Legionella*). The other two tanks were kept off-line (i.e. stagnant) and stored at a mean ambient temperature of 29 °C. Monthly plate counts of tank-derived water samples revealed a sharp drop in *Legionella* numbers in the on-line tanks immediately after they were brought back on line, and a corresponding nosocomial outbreak is mentioned, presumably due to biofilm sloughing due to increased *shear stress*, a force due to the friction between the flow and the pipe surface (Liu *et al.* 2006).

1.2.3.2 Effect of shear stress on biofilm formation in WDSs

The concept of shear stress is linked to the flow types previously described, in that laminar flow subjects biofilms to *low* shear stress and, conversely, turbulent flow subjects them to *high* shear stress. It can affect many biofilm characteristics, such as biofilm thickness, density, community diversity and shape (Rickard *et al.* 2004; Ramasamy and Zhang 2005), and cause detachment via sloughing or erosion events which can have serious implications in a hospital water system, where tap water effluent can subsequently be contaminated. These large-scale detachment events appear to be caused more by sudden changes in shear stress, and it is possible that the observations of Ciesielski *et al.* (1984) are an illustration of this phenomenon. Stoodley *et al.* (1999) conducted a series of experiments looking at the effect of fluctuations in shear stress on biofilms grown in pipes and suggested that the detachment rate was closely related to changes in shear frequency and magnitude, with the ultimate effect being mechanical failure and detachment. Peyton (1994), investigated the effects of shear stress on *P. aeruginosa* biofilm thickness, and found that shear stress had no significant effect on either thickness or volumetric density, while other researchers have reported that constant high shear stresses give rise to denser, smoother biofilms which are better equipped to avoid sloughing events (Vieira *et al.* 1993; Liu and Tay 2001b; Stoodley *et al.* 2002).

It has been shown that the reduction in detachment rate that is observed through increased shear stress has a biological basis, and that the microorganisms involved adjust their metabolic processes accordingly (Liu and Tay 2001), in order that they may become more strongly adhered to a surface.

This observation is supported by Melo and Vieira (1999), who showed that substrate consumption decreases under high shear conditions, while bacteria in biofilms formed under lower shear forces contain a greater amount of active biomass concentrated in the upper region of the biofilm, suggesting that only a proportion of cells in thinner denser biofilms are active. Beyenal and Lewandowski (2002) demonstrated that biofilms grown at low flow velocities (low shear) exhibit low density and high nutrient diffusivity, but are unable to resist high shear stress, whereas the thinner, denser biofilms seen at higher flow rates have lower effective diffusivity, an observation which goes some way to explaining the low substrate consumption rates previously reported.

As previously mentioned, Peyton (1994) observed no effect of shear stress. It was found that substrate loading rate was far more significant in terms of determining the thickness, roughness and density of a monoculture biofilm (in this case *Pseudomonas aeruginosa*). It was also noted that for mixed culture biofilms under identical conditions, these three characteristics tend to be greater due to spatially significant variations (Peyton 1994). An earlier paper by Peyton and Characklis (1993), again using a monoculture of *P. aeruginosa* and looking at the relationship between shear stress and substrate loading rate, concluded that detachment rate is directly related to growth rate, and that factors which limit growth rate (in this case, substrate loading rate) will, in turn, limit the detachment rate.

1.2.4 Effect of materials on biofilm formation in WDSs

1.2.4.1 Pipe materials

In any WDS most of the microbial growth occurs on the inner surface of the pipe itself, although growth will also occur on sediments (Camper *et al.* 1999). Therefore, the characteristics of the pipe material used in a particular plumbing system can have a considerable influence on the rate and extent of biofilm formation and regrowth, although it is important to remember that in any one system, a wide variety of factors will determine the way in which a biofilm develops. Some of these factors have already been described. Characteristics such as roughness and material stability may greatly influence the density of bacteria, and the microbial community may also be affected (Niquette *et al.* 2000). To date, no surface that is resistant to biofilm formation has been discovered (Kaplan 2005).

Along with the potential health problems associated with biofilm formation in the hospital plumbing environment, biofilms can exhibit deleterious effects on the pipes themselves through corrosion. An example of this corrosive effect is the pepper pot pitting observed in copper pipes, which has been directly related to formation of microbially diverse biofilms (as well as the presence of humic acids in potable water) and is thought to be due to the establishment of localized chemical gradients and metal loss (Walker *et al.* 1998). Whilst copper is thought to be highly resistant to biofilm formation, there have been instances of rapid pipe failure, and high uptake of copper into the water has been observed when pH levels are below 7 (Percival *et al.* 1998a).

It is therefore desirable to reduce and eliminate corrosion and biofilm formation through careful selection of pipe materials, and there have been numerous studies investigating biofilm formation in a variety of metals and plastics (Lehtola *et al.* 2004). It has been demonstrated that plastic materials such as medium- and high-density polyethylene (M/HDPE), unplasticised polyvinyl

chloride (uPVC), chlorinated PVC, polybutylene and cross-linked polyethylene (PEX), support lower numbers of bacteria than traditionally-used forms of iron and steel (Rogers *et al.* 1994; Kerr *et al.* 1999; Niquette *et al.* 2000), although in recent years, stainless steel has become increasingly widely used within WDS and is the subject of a number of investigations focussing on the influence of factors such as grade selection (Arnold and Bailey 2000; Kielemoes *et al.* 2000), shear stress (Azevedo *et al.* 2006) and copper-alloying (Kielemoes and Verstraete 2001; Kawakami *et al.* 2010) on biofilm formation thereupon. Cast iron has been shown to harbour an average of 97% more viable microorganisms than medium density polyethylene (MDPE) and uPVC (Kerr *et al.* 1999), and previous work has shown corrosion of iron to be a major contributory factor to greater biofilm formation, due to fact that many of the products released during iron corrosion may be utilised by microorganisms and can also lead to a reduction in chlorine levels in tap water (Niquette *et al.* 2000). Other studies have confirmed the apparent relative toxicity of copper towards microorganisms in comparison with other materials and thus its apparent suitability and common use as a pipe material in this respect (Rogers *et al.* 1994; van der Kooij *et al.* 2005; Lehtola *et al.* 2006). However, it is worth noting that in their 2004 study, Lehtola *et al.* found that after running a pilot distribution system for 200 days, the number of bacteria attached to both copper and plastic pipes was the same. This could possibly have been due to an increased presence of copper-resistant bacteria, and the observed differences in microbial community between the two materials may support such a hypothesis (Lehtola *et al.* 2004). Copper-resistant bacteria have previously been found in large numbers associated with mixed population biofilms (Percival *et al.* 1998b). Lehtola *et al.* (2004) state that the polyethylene used in this study has a

tendency to leach microbially available phosphorus into the water, and speculate that this is a contributory factor to the observed high level of microbial growth in the plastic pipe. This was supported in studies by van der Kooij *et al.* (2002) and van der Kooij *et al.* (2005), which demonstrated that plastics such as PEX readily supported biofilm growth (and growth of *Legionella* within those biofilms) at higher levels than copper and stainless steel. The chemical interactions between WDS pipes and the water they contain therefore potentially influence biofilm formation, and these interactions have been reported to significantly affect the efficacy of disinfectants such as chlorine (Momba 2004). In general, available literature suggests that, in the short term, copper is consistently more effective at slowing microbial growth.

1.2.3.2 Fittings

Taps and showerheads are easily contaminated with potentially pathogenic bacteria such as *P. aeruginosa, Legionella* spp. and *Mycobacterium avium* (Feazel *et al.* 2009), and the generation of aerosols containing such bacteria at these outlets was shown to be an area of concern over 25 years ago (Colbourne *et al.* 1984; Bollin *et al.* 1985). Despite this, it would appear that research into this specific area of the water system has been somewhat sporadic. Non-touch (infra-red operated) taps are now commonplace in hospitals, as their aim is to promote hygiene by reducing contamination (van der Mee-Marquet *et al.* 2005). They are also considered to be more cost-effective in terms of water consumption (Halabi *et al.* 2001). However, the materials from which they (and other WDS components such as rubber-coated valves) are made, have been shown to promote bacterial growth of coliforms, *Legionella*,

and *P. aeruginosa* (Kilb *et al.* 2003; van der Mee-Marquet *et al.* 2005). Halabi *et al.* (2001) analysed biofilm samples from 38 non-touch taps in a hospital in Southern Austria- 23 with temperature selection and 15 without. *P. aeruginosa* was found in 74% of biofilm samples taken from non-touch taps without temperature selection, as opposed to just 7% of those with temperature selection. A comparison of these taps with adjacent conventional taps showed a considerably lower contamination in the latter after a three month period. These findings led to an investigation into the relative contamination levels of the individual non-touch tap components, whereupon the magnetic valve, mixing device and the outlet were found to be the most contaminated parts. It is explained that the materials used in the construction of these components are known to support bacterial growth, and that these particular taps allow the stagnation of water at a temperature of 35 °C, which is fairly close to the optimum growth temperature for the organisms in question. *Legionella* spp. were found in all of the non-touch taps and only three of the conventional taps, although at lower numbers than *P. aeruginosa*, and there is no mention made of corresponding nosocomial infections. It is postulated that the lack of stagnation associated with conventional taps is a contributory factor in reducing the numbers of resident bacteria. Leprat *et al.* (2003) supported Halabi *et al.*'s 2001 study, stating that they had conducted a similar investigation into the incidence of *P. aeruginosa* on three non-touch taps without temperature selection in comparison to the incidence observed on conventional taps. All were contaminated, and genotypic analysis proved that the contamination did not originate in the central distribution system. Thus, the non-touch taps were proven to be the source of infection and a recommendation made that evaluation of non-touch systems in hospitals is necessary.

1.2.4 Role of quorum sensing in biofilm formation/ differentiation

Genetic analysis of biofilm formation has led to the proposal that extracellular signals and quorum-sensing regulatory systems are essential for differentiated biofilms (Kjelleberg and Molin 2002). Davies *et al.* (1998) were the first to demonstrate the role of quorum-sensing chemicals in the differentiation of *P. aeruginosa* biofilms, and *P. aeruginosa* has been used as a model organism in many subsequent studies. This research led to an increased interest in the role of genomic functions in differentiation of biofilms, and the subsequent proposal of a model describing the involvement of quorum-sensing molecules in differentiation after the initial processes associated with biofilm formation (adhesion, EPS secretion etc.) (Kjelleberg and Molin 2002).

It is possible that quorum sensing mediates controlled release of extracellular DNA, which functions as an extracellular component in a number of ways (Allesen-Holm *et al.* 2006; Lappann *et al.* 2010). Shrout *et al.* (2006) showed that the use of quorum sensing is dependent on nutritional conditions, and that the swarming motility of *P. aeruginosa* in the initial stages of biofilm formation was particularly affected. This appears to contradict the suggestion by Davies *et al.* (1998) that the cell density-dependent nature of quorum sensing precludes it from involvement in the early stages of biofilm formation. The exact role of quorum sensing in biofilm formation and differentiation therefore remains unclear, both in general terms and within WDSs. The prevalence of *P. aeruginosa* as a nosocomial pathogen highlights the need for further understanding of this area, in order that methods of interfering with this cell-to-cell communication may be developed. However, it remains to be seen whether

such methods could be adapted for use in such complex conditions as are found in large-building water distribution systems.

1.3 Methods of biofilm control in WDS

Avoiding the presence and growth of pathogenic microorganisms is of paramount importance in the preservation of water quality (Manuel *et al.* 2010), and the problem of controlling biofilms in WDSs has been subject to a wide variety of approaches, either individually or in combination.

It has been suggested that control of biofilms should involve an integrated approach using a combination of several types of action (Kerr *et al.* 1999), as no single approach appears to be entirely successful at eliminating them.

Biofilms have been shown to exhibit increased resistance to biocides (Costerton *et al.* 1999). There are two widely accepted mechanisms of resistance, the first being the failure of a biocidal agent to penetrate far enough into a biofilm to kill all the resident microorganisms, although with antibiotics this is not the case as they have been shown to be able to penetrate deep into biofilms (Spoering and Lewis 2001). This resistance to biocides is thought to be due to the ability of the biofilm matrix to resist and/or neutralize such agents (De Beer *et al.* 1994). The second mechanism (which is more specifically focussed on resistance to antibiotics) being due to nutrient limitation deep within a biofilm. The resulting change in physiological state means that these cells, which represent a small proportion of the total and are termed 'persister cells', are less susceptible to antibiotics (Costerton *et al.* 1999; Spoering and Lewis 2001). This slow growth in low nutrient conditions, coupled with the spatial/ community heterogeneity exhibited by biofilms, would suggest that in a water distribution system,

antibiotic/ antiseptic treatment may not be a particularly effective biofilm control strategy. Any proposal to treat a WDS with antibiotics would presumably be prohibited by factors such as high costs and the potentially elevated public health risk. The use of hot water to control biofilms in hospital water distribution systems is based around the principle of delivering water at a temperature of between 70 °C and 80 °C to all points of a distribution system for a limited time. However, many large building complexes will not sustain long flushes without a drop in water temperature, and so only short-term benefits are seen (Perola *et al.* 2005). Also, a flushing method is laborious and must be repeated regularly (Darelid *et al.* 2002). Table 1.3 (overleaf) summarizes the heat resistance of *Legionella pneumophila* serogroup 1, this serogroup being responsible for the majority of legionellosis cases. Maintaining the circulating water temperature constantly above 55 °C has been shown to be effective in controlling outbreaks of Legionnaire's disease over a 10 year period in Ryhov Hospital, Sweden, even though *Legionella* was isolated from 12% of cultured hospital water samples over the same period (Darelid *et al.* 2002). Perola *et al.* (2005) suggested that flushing times should be extended from five minutes to 30 minutes to achieve adequate flushing temperatures at distal sites, as neither the superheat and flush method, nor the installation of a heat-shock unit proved capable of eradicating *Legionella*. In their study, the temperature of water returning the heat exchanger was only 50.5-53 °C, and *Legionella* was also detected at this point. Stout *et al.* (1998) compared copper-silver ionisation, which generates ions that cause cell lysis, with the superheat and flush method at an acute-care facility in Pittsburgh. The heat and flush procedure was performed intermittently for 20-30 minutes over a 13 year period, and the copper-silver ionisation performed over a subsequent 36 month period, with the

results being compared, retrospectively. Whilst the authors recognise that conditions may have been different during the period when ionization was trialled, it appears that this method is more effective as average annual nosocomial Legionellosis infections dropped from six to two in this period.

Table 1.3 Summary of heat resistance of *Legionella pneumophila* serogroup 1 in tap water (based on data obtained from Dennis *et al.* (1984)* and Stout *et al.* (1986)**).

Temperature	Disinfection time (minutes)
50*	111*
54*	27*
58*	6*
60**	2.6**
70**	1.2**
80**	0.4**

As the prevention or eradication of biofilms in an environment as complex as a large scale water system is difficult given the wide range of adaptations exhibited by biofilm-bound bacteria to enhance their survival (Manuel *et al.* 2010), effective biofilm management and the subsequent minimization of biofilm detachment would appear to be a more appropriate and cost-effective approach until new technologies become available.

1.4 Thesis research objectives

The aim of this research project may be broken down into several objectives:

- To investigate the effects of high pH and stagnation on the formation of microbial biofilm on copper.
- To investigate the effect of regular thermal purging on biofilm formation on copper and linear low density polyethylene (LLDPE), over the short term.
- To investigate the ability of different materials and tap outlet fittings to support biofilm development over the longer term.
- To investigate changes in the biofilm-associated bacterial community, using conventional molecular microbial ecology techniques.
- To provide tentative recommendations for future strategies for biofilm control and management, depending on results obtained, and to identify possible areas of future research.

Chapter 2 General Materials and Methods

2.1 Introduction

2.1.1 Biofilm analysis methods

Biofilms are complex and tend to be heterogeneous, both in terms of structure and population. Analytical approaches are therefore many and varied, but can be divided into three categories as follows (adapted from Flemming *et al.* (2006)):

1. **Analysis of structure and architecture (microscopy and associated methods)**
2. **Analysis of intra- and extracellular processes**
    - Flow cells with CLSM (Pereira *et al.* 2002)
    - Molecular biology e.g. reporter gene analysis
    - GFP tagging of micro-organisms
    - Use of microelectrodes to measure, for example, oxygen diffusivity
3. **Analysis of the physical, biological and chemical properties of the biofilm**
    - Chemical analysis e.g. carbohydrate, protein, lipid analysis of EPS
    - Biochemical analysis
        - Proteomics
        - Enzymes
        - ATP measurement (van der Kooij *et al.* 2002; Lehtola *et al.* 2006)
    - Population analysis
        - Classic microbiology
        - Fatty acid analysis (Walker *et al.* 1993; Lehtola *et al.* 2004)
        - Molecular microbial ecology
    - Physico-chemical properties
        - Mechanical stability i.e. studies of hydrodynamic effects etc.

2.1.2 Analysis of structure and architecture (microscopy and associated methods)

Studying biofilms is a complex task, based on the experience and judgement of the individual. When studying biofilms themselves, as opposed to the parameters which may affect them, the decision must be made to investigate non-destructive or disruptive techniques, as disruptive techniques will reduce or eliminate microbial relationships, complex structures and organization which are essential features of these systems (Lawrence *et al.* 1998). The information that may be gained from various microscopy techniques include quantification (e.g. depth measurements), identification of individual species, and assessment of viability (Surman *et al.* 1996), although with advances in technology, the variety and amount of information that can be gathered is increasing. Surman *et al.* (1996) published a comprehensive comparison of a variety of microscopy techniques; environmental scanning electron microscopy (ESEM), confocal laser scanning microscopy (CLSM), atomic force microscopy (AFM), Hoffman modulation contrast microscopy (HMC), and differential interference contrast microscopy (DIG) in order to ascertain the most appropriate method(s) for investigating a biofilm derived from a naturally occurring aquatic consortium, and grown on solid supports in a continuous culture system. Each technique was demonstrated to have both advantages and disadvantages, both in terms of material considerations such as cost, and also in terms of the quality of the data obtained. The authors concluded that, given the heterogeneous nature of biofilms, it is preferable to use as many techniques as are available to gain an accurate picture of biofilm structure and organisation (Surman *et al.* 1996).

For example, EM techniques, while allowing the visualisation of complex structures at high magnification, require a harsh method of sample preparation which may result in the hydrated portion of the biofilm being condensed by 99% (Surman *et al.* 1996). This problem has been partially circumvented by the development of ESEM, during which samples are viewed in a moist chamber and without the use of a conductive coating, thus allowing improved visualisation of biofilms. However, while ESEM was originally thought to be performed optimally without treatment of the biofilm, Priester *et al.* (2007) demonstrated the use of conventional SEM staining techniques to improve the visualisation of *P. aeruginosa* biofilms using ESEM. This was due to the fact that EPS is not electron dense and therefore can lead to poorly defined images. ESEM images of untreated biofilms can also exhibit a great deal of variability depending on the electron density of the substratum and the degree of interaction with the biofilm.

One of the most versatile and effective approaches is confocal laser scanning microscopy (CLSM), as it permits the quantitative analysis of multiple parameters in time and space within biofilms (Lawrence *et al.* 1998) and also enables the acquisition of images with minimum disruption and artefacts (Walker *et al.* 1998). CLSM has been widely used in the study of biofilms, and in a variety of different ways. For example, (Packroff *et al.* 2002) studied the interaction of protozoa with biofilm communities via the use of novel fluorescent compounds, and were able to identify and distinguish them within a biofilm. Walker *et al.* (1998) investigated the relationship between the presence of WDS-derived biofilms and the occurrence of pepper-pot pitting in copper plumbing tubes. Using CLSM, a halo of low pH was seen around the bacteria, thought to occur as a result of metabolic activity, although the authors

acknowledge that this may be due to presence of exopolymers. Using this technique it was also possible to confirm the heterogeneity of the biofilm, both in terms of population composition and gross internal structure. A study by Kuehn *et al.* (1998) sought to develop a method suitable for routine measurements of biofilm structures under *in situ* conditions.

The fusion of SEM and *in situ* hybridization has recently been demonstrated using *E. Coli* and combining these two technologies potentially offers new possibilities regarding the elucidation of the spatial distribution of target bacteria in multispecies biofilms (Kenzaka *et al.* 2009).

2.1.3 Analysis of the intra- and extracellular properties of the biofilm

2.1.3.1 Molecular analysis of biofilms

The recent advances in molecular techniques have improved understanding of biofilms in a number of ways. From an ecological viewpoint, researchers have been able to gather information on the non-culturable microorganisms which, in many cases, constitute the majority of the microbial population in a given sample. Novel molecular techniques have also facilitated studies involving the expression of particular genes, for example the identification of genes involved in quorum sensing by *P. aeruginosa* (Whiteley *et al.* 1999). Figure 2.1 (overleaf) gives an overview of molecular investigative strategies commonly employed in the molecular study of biofilms.

Techniques for cloning and sequencing the 16S rRNA gene are commonly used in molecular ecological studies of biofilms, and involve extraction of nucleic acids from the biofilm, amplification and cloning of the 16S rRNA genes,

followed by sequencing and identification. Martiny *et al.* (2003) used these techniques, along with CLSM, to investigate long-term succession of structure and community diversity in a biofilm formed in a drinking water distribution system over three years. Therefore the possible bias associated with culture-based investigation was avoided. DNA extraction, PCR, RFLP analysis, cloning and sequencing of 16S rRNA fragments were all performed according to standard methods, with a correlation being found between the population profiles generated via these methods and the age of the sample i.e. a succession effect was observed, resulting in a decrease in the diversity of the population as the biofilm matured. This molecular data was also complemented by the associated CLSM-based data on the development of biofilm structure over time, and it is worth taking note of the length of time over which this study was conducted, and the possible implications for similar studies of shorter length. Using similar techniques, Williams *et al.* (2004) explored phylogenetic diversity of bacteria in a model drinking water system. In this study, DNA extraction was carried out from cultured bacteria and it is acknowledged that this approach may introduce a degree of bias. It is also pointed out that PCR reactions carried out on DNA originating directly from mixed samples may carry a degree of bias and measures taken to reduce this are briefly described. Williams *et al.* (2005) presented details of an investigation into the community structure of 10 week old biofilms that had been exposed to a variety of chlorine-based disinfectants, and ascertained that community structure was affected by the disinfection regime employed, with a *Legionella*-like sequence being identified in the system that contained no active chlorine, and *Mycobacterium* spp. predominating in the system treated with chloramines. It is not clear why *Mycobacterium* should exhibit an increased resistance to chloramines, and the

author proposes further studies in order to ascertain whether chloramines are being actively degraded (Williams et al. 2005). The use of these molecular tools thus allows the detection of pathogens, within water systems with far more sensitivity than traditional culture-based methods (Berry *et al.* 2006), and has been particularly described in a clinical setting, when describing specific nosocomial outbreaks of *L. pneumophila* and *P. aeruginosa* (Ferroni *et al.* 1998; Perola *et al.* 2005).

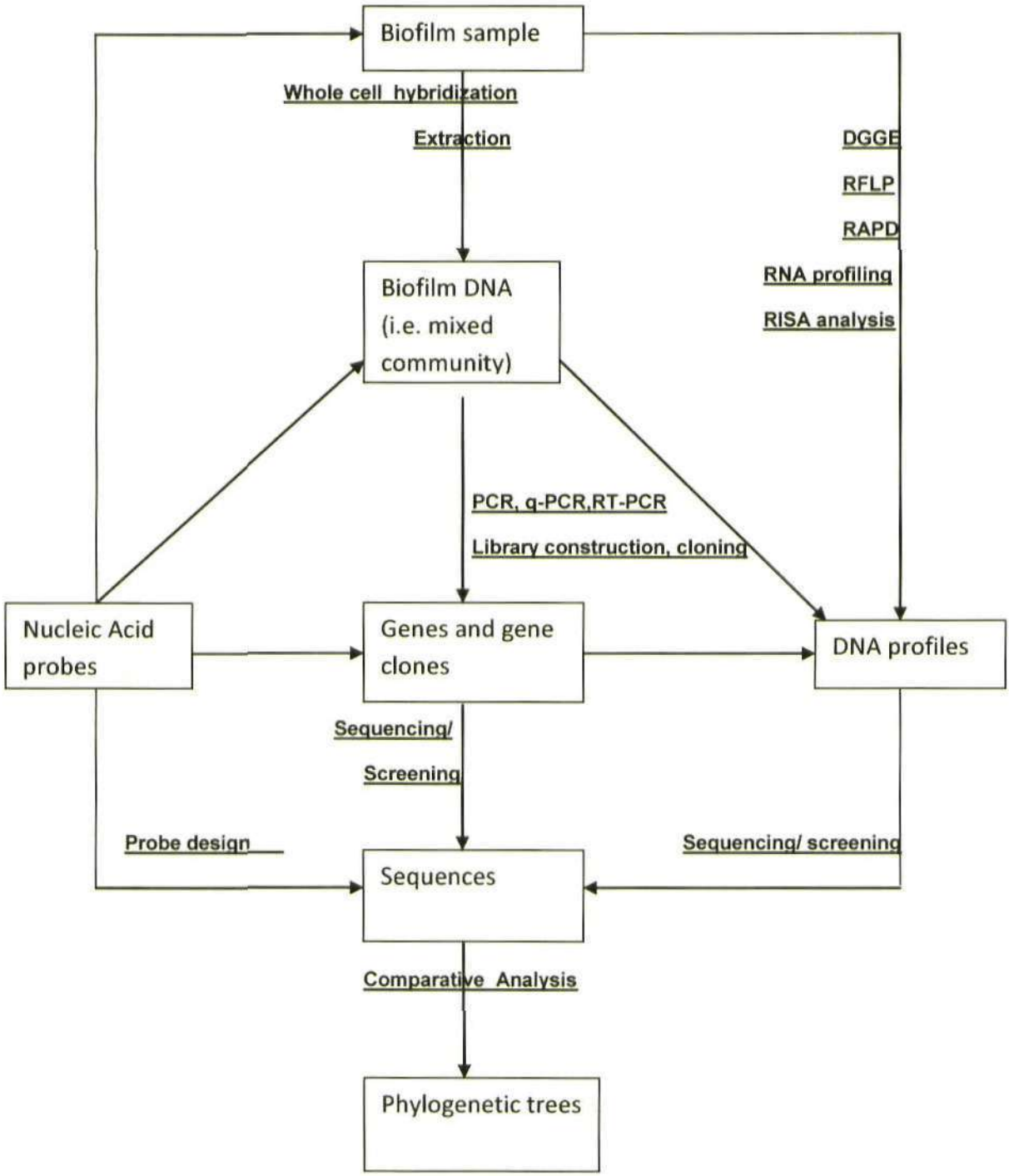


Figure 2.1 Overview of molecular methods employed in molecular ecology study of biofilms isolated from environmental samples. DGGE= Denaturing Gradient Gel Electrophoresis, RFLP= Restriction Fragment Length Polymorphism, RAPD= Randomly Amplified Polymorphic DNA, RISA= Ribosomal RNA (rRNA intergenic spacer analysis, (RT)-PCR= (Reverse Transcriptase) Polymerase Chain Reaction (Adapted from Flemming *et al.* (2006)).

2.1.4 The use of model water distribution systems in biofilm research

A model experimental system is described by Rosenblueth and Wiener (1945) as 'a simple, idealized system which is accessible and can be easily manipulated'. The employment of model systems in all scientific disciplines is commonplace, and this approach extends to WDS biofilm research, allowing researchers the flexibility to manipulate a number of parameters e.g. water chemistry/ materials, and conduct scientific investigations in a controlled manner, using small-scale, simplified model WDSs. A brief review of the literature reveals a diverse range of approaches to model WDS design and utilization, in terms of design, complexity and experimental approach. For example, Zacheus *et al.* (2000) employed a simple 'basin-mounted slide' system to investigate the effect of ozonation on biofilm formation on PVC and polypropylene, whilst Lehtola *et al.* (2005) conducted long term disinfection experiments using 100 m lengths of polyethylene and copper pipes which received water from a custom-built pilot-scale waterworks. Moritz *et al.* (2010) utilized a stainless steel water tank, perfused with tap water and inside which was suspended coupons of test materials. In a similar study by Bressler *et al.* (2009), 80 ml glass 'reactors', containing the elastomeric material under investigation, and Tygon tubing were continuously supplied with ambient temperature drinking water. More sophisticated systems were employed by Percival *et al.* (1999) to investigate biofilm formation on stainless steel 304 and 316, and by Liu *et al.* (2006) to investigate the effect of different flow regimes on *Legionella* growth on PVC. These systems incorporated plumbing technologies such as copper piping, storage tanks, recirculation of water, and ball valve-

regulated flow of potable water. It would appear that, generally speaking, the 'biofilm reactor' approach is a more popular choice when it comes to modelling WDSs, and it is presumed that one of the main reasons for this is the comparatively low cost of setting up such systems, as well as their ease of use. However, it is worth bearing in mind that the conditions within are likely to be far removed from those encountered within a full scale, highly complex WDS.

2.2 Description of test rig

2.2.1 Design and manufacture

The model warm water distribution system, or 'test rig' was manufactured by Research and Development staff at Dart Valley Systems (DVS) Ltd., Paignton, U.K. Aspects of the design were subject to discussion between DVS staff and University of Plymouth staff directly involved with the project. All materials used in the manufacture of the test rig and associated services (heater, storage tank etc.) were sourced and supplied by DVS Ltd.

A schematic diagram of the test rig and associated services is shown in Figure 2.2 (page 38). The test rig was mounted on a bench in a temperature-controlled laboratory, where the ambient temperature was maintained at 16 °C (Figure 2.3, page 39). Services supplying water to the test rig were housed directly underneath the bench (Figure 2.4, page 39). The test rig was supplied by separate hot and cold feeds. A standard 15 litre unvented under-sink heater (Ariston, UK) supplied hot water at a set temperature of 75 °C, and received water via a mains water supply-fed polypropylene 20 litre unheated water

storage/ pump assembly running at a pressure of 2.4 bar (Northern Pump Suppliers Ltd., U.K.).

2.2.2 System arrangement and design

The test rig consisted of eight vertical pipes or 'systems', plus associated copper plumbing. The hard plumbing consisted of 15 mm outer diameter copper pipe, with an inner diameter of 13 mm. A 'system' constituted the primary area from which samples were taken for biofilm analysis and was comprised of the following elements; thermal mixing valve (TMV), thermal purging solenoid, pressure dumping valve, sample inserts, seven chrome ball valves, and purging solenoid.

The experimental portion of each system was divided into six removable pipe sections, or 'inserts'. Each consisted of 15 mm outer diameter copper pipe, unless stated otherwise. Inserts were 79 mm in length, and separated by stainless steel/ chrome ball valves (John Guest Ltd., U.K.), which facilitated insert isolation and removal, whilst leaving the rest of the system as undisturbed as possible.

A TMV at the top of each system ensured that water was supplied at a temperature of 41 $^{\circ}$C. This temperature was recommended by DVS as representative of that which would be used in a typical commercial installation.

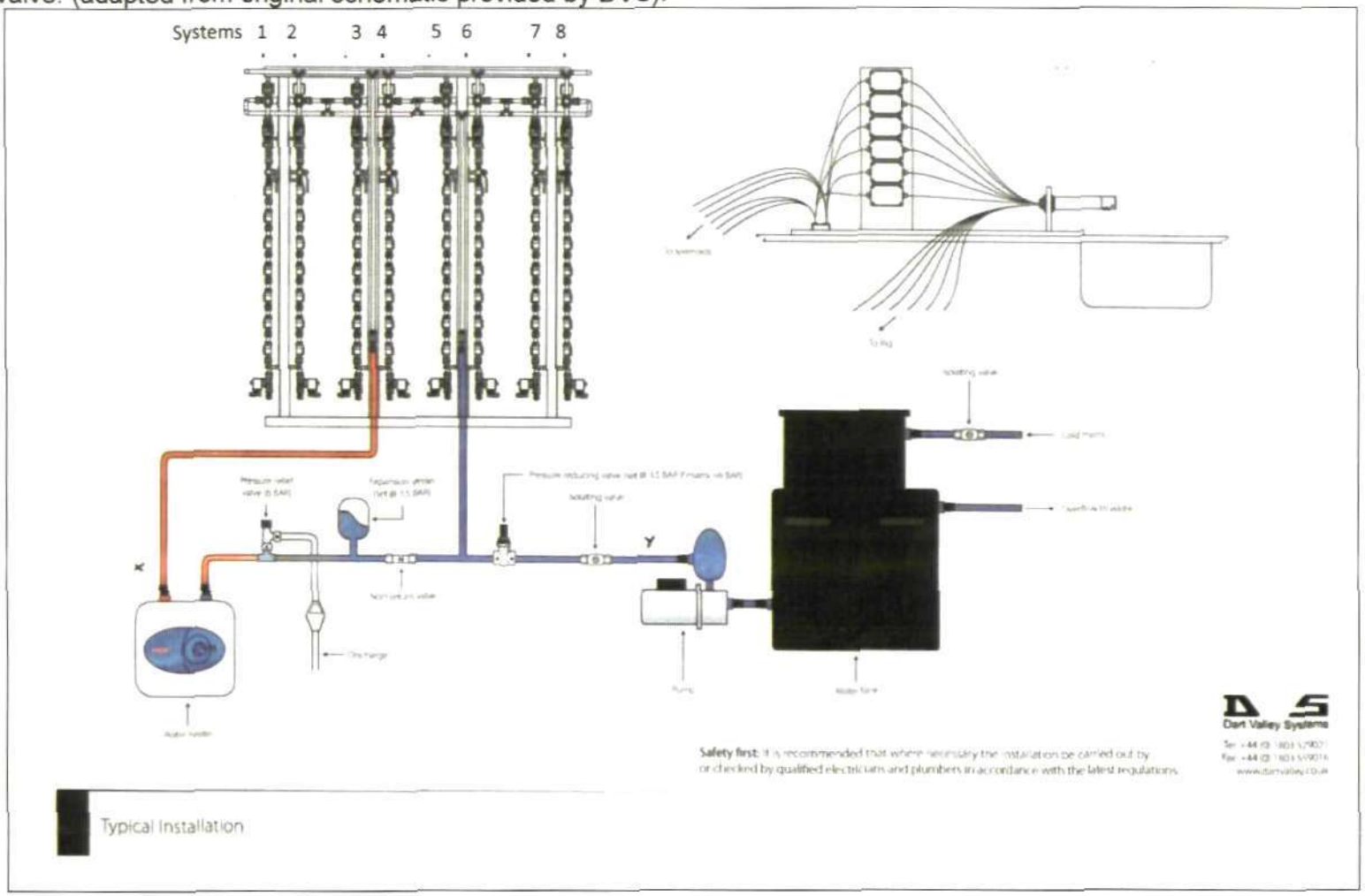
A solenoid was installed at the top of each system in order to facilitate the thermal purging procedure described in chapter 4, whilst a solenoid at the base of each system facilitated either manual or automatic flushing. Manual flushing was performed using a key switch as shown in Figure 2.5 (page 40). The flow

rate from each system was set during manufacture to be 6 litres min^{-1}. This flow rate is typical of a commercial installation.

For safety reasons it was possible to isolate each system from the incoming water supply prior to insert removal, using ball valves.

Each system was connected to infra-red operated Aquarius Surgeons Scrub-Up taps (DVS Ltd., Paignton, U.K.). Connection was via polypropylene fittings (John Guest Ltd., U.K.) and identical lengths (1800 mm) of 12 mm outer diameter linear low density polyethylene (LLDPE) tubing (6 mm inner diameter).

Figure 2.2 Schematic diagram of test rig. Extra sampling points are included at points **x** and **y**. EV=expansion vessel; PRV= pressure release valve. (adapted from original schematic provided by DVS).



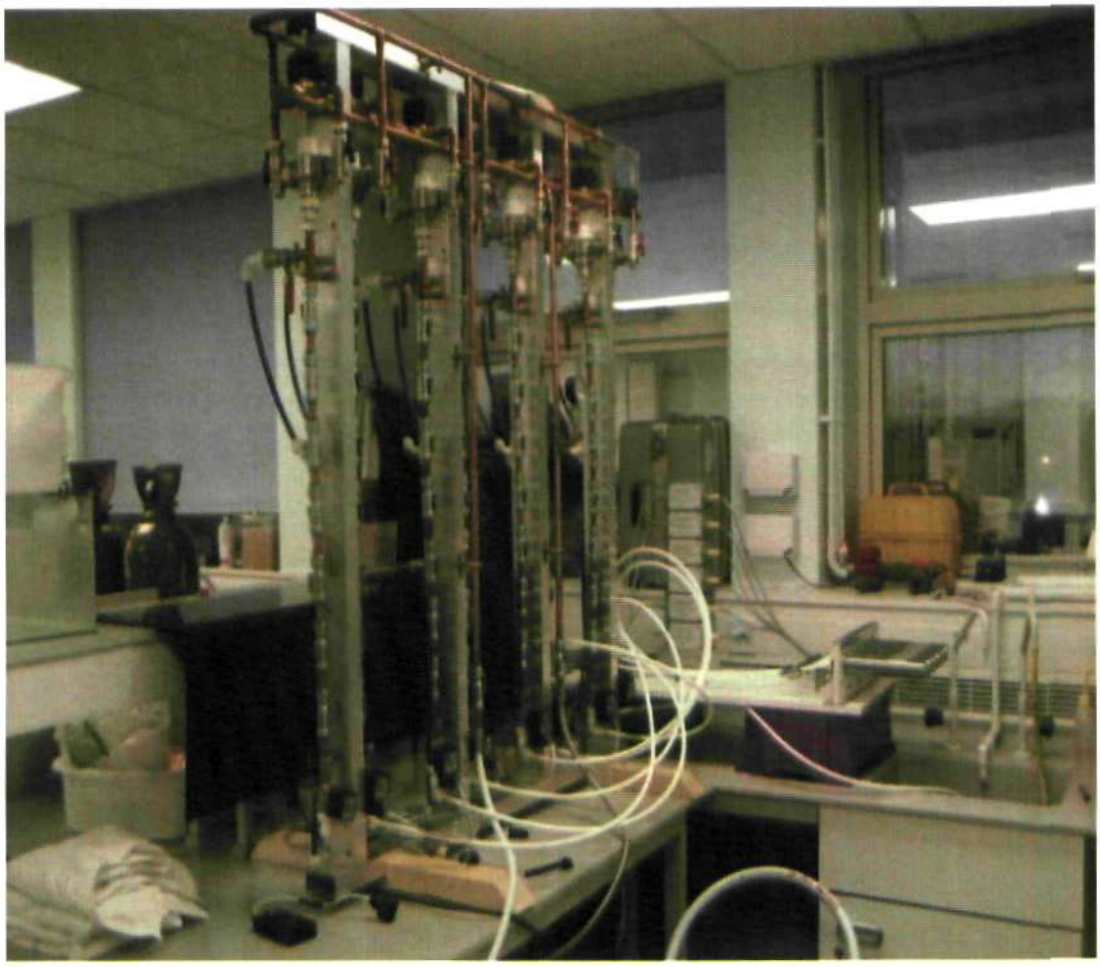


Figure 2.3 Model warm water distribution system or 'test rig' used during current research project.

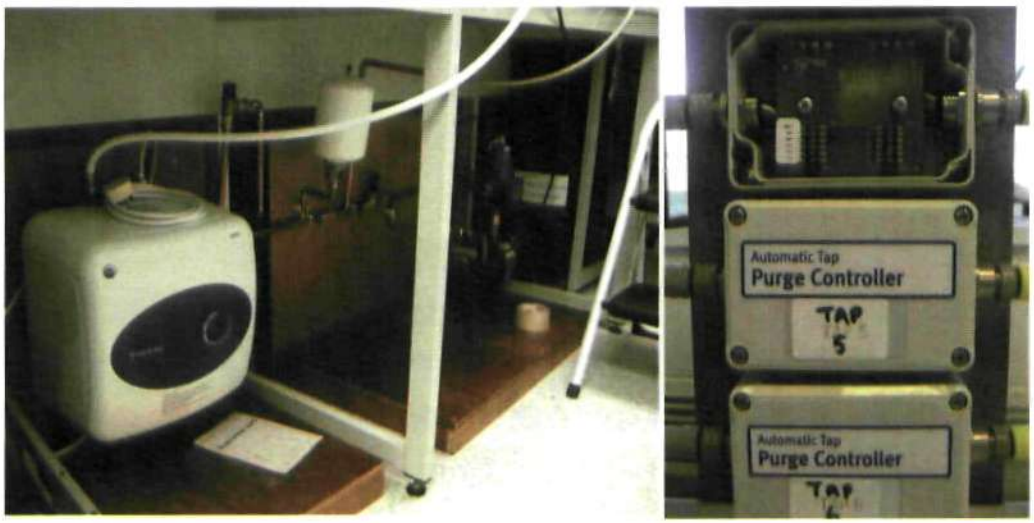


Figure 2.4 Detail of services supplying test rig, including (left) heater and storage tank, and (right) automatic tap purge controller.

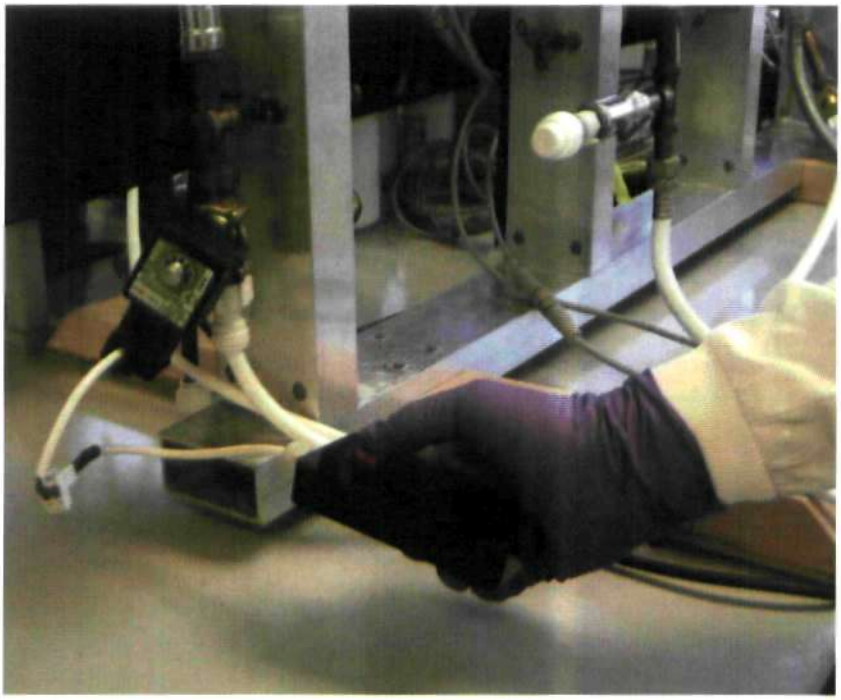


Figure 2.5 Manual flushing of test rig system one using key switch.

2.2.3 Description of automatic flushing technology

Auto-flushing technology facilitated automatic flushing of the rig. When manual flushing was not employed, the solenoids at the base of each system and their corresponding no-touch (infra-red operated) taps were connected to individual control boxes (shown in Figure 2.4) which could be used to set flushing time and frequency. Flushing time was 30 seconds and flushing frequency was set according to the requirements of particular trials.

2.3 Experimental methods

An overview of the experimental methods employed during this research project is presented in Figure 2.6 (overleaf).

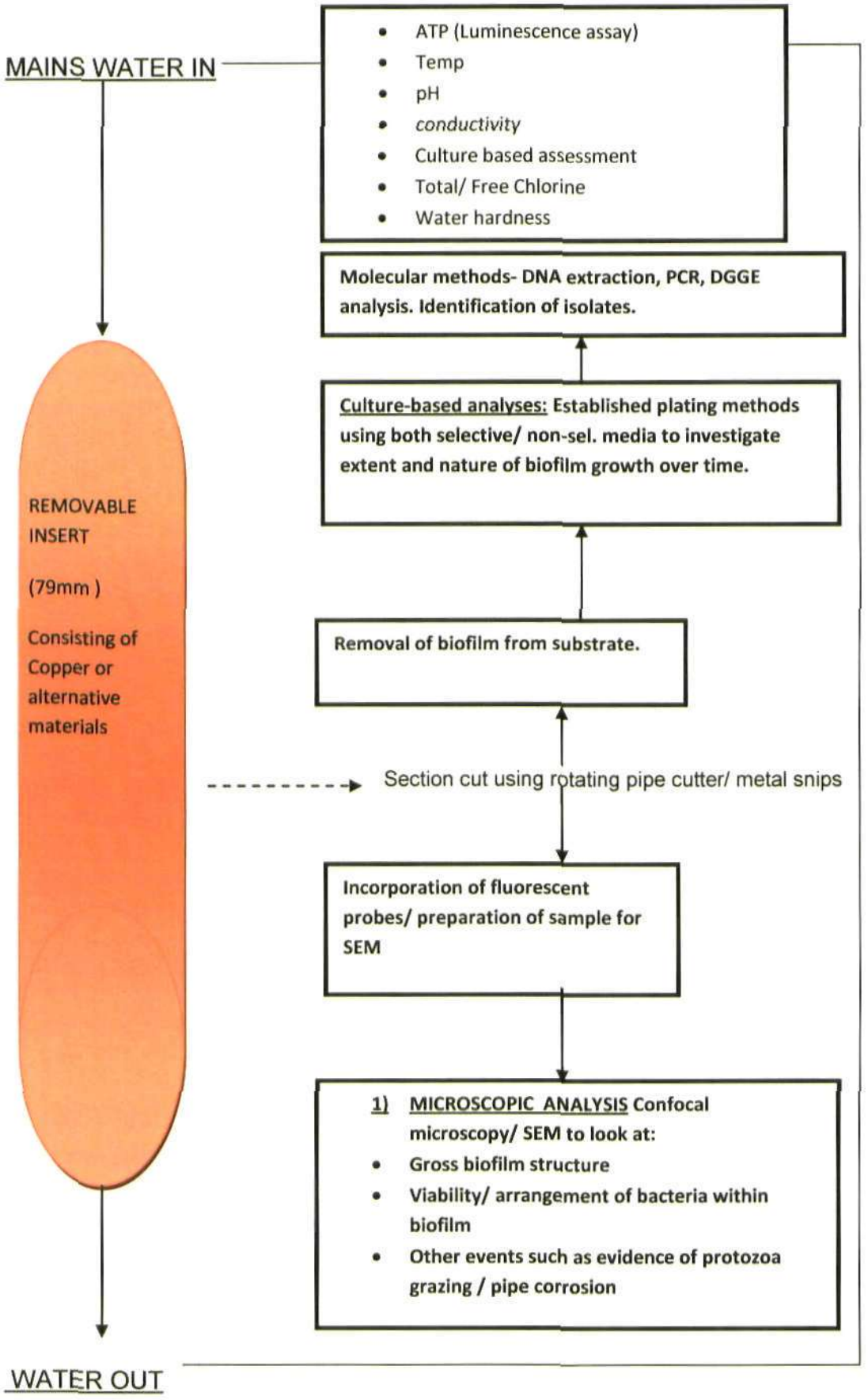


Figure 2.6 Summary of experimental methods.

2.4 Sampling regime

2.4.1 Removal of inserts from test rig

Sampling was undertaken on a four-weekly basis during trials. On each sampling day, inserts were removed from the rig in a way which helped to maintain reproducibility and personal safety (Figure 2.7, below).

Figure 2.7 Removal of copper insert from test rig system. The chrome ball valves above and below the insert of interest are in the 'closed' position, as indicated by white arrows.

Sampling of inserts from systems was carried out sequentially i.e. beginning with system one and working along the test rig. Inserts were processed immediately and individually according to the requirements of the trial. At all times, care was taken to retain water within the inserts, so as to minimise

biofilm disturbance and desiccation. Nitrile gloves were used for manual handling and were regularly rinsed with 70% industrial methylated spirits (IMS).

The exact sequence of copper insert removal is shown in Table 2.1 (below).

Table 2.1 Description of stages involved in removal of inserts from test rig for analysis.

Step number	Action
1	Disconnect solenoid at base of test rig system
2	Shut off hot/ cold feed to each system, using taps located immediately prior to TMV
3	Dump pressure from system to facilitate insert removal
4	Isolate system by closing top chrome ball valve
5	Isolate insert of interest by closing valves either side
6	Using insert removal tool, remove portion of system below the insert of interest
7	Remove insert of interest from the **upper** ball valve. The insert is now sitting in the **lower** ball valve and can be removed from the test rig.
8	Replace the lower part of the system, reopen appropriate valves, and replace solenoid at base of system
9	Using parafilm, seal the top (open) end of the insert. The insert can then be removed from *the ball valve for downstream processing*
10	If any delay is anticipated before insert processing, parafilm is used to seal both ends of the insert.

2.4.2 Cutting of inserts for downstream biofilm analysis

After removal from the test rig, the insert to be sampled was secured vertically in a bench-top vice and the insert exterior cleaned with 70% IMS using laboratory tissue. A rotating pipe cutter was sterilized with 70% IMS using laboratory tissue, and used to cut portions of insert for downstream processing as shown in Figure 2.8. The nature of the different materials being used meant that certain changes to this cutting procedure had to be made, and these changes are described in detail where appropriate.

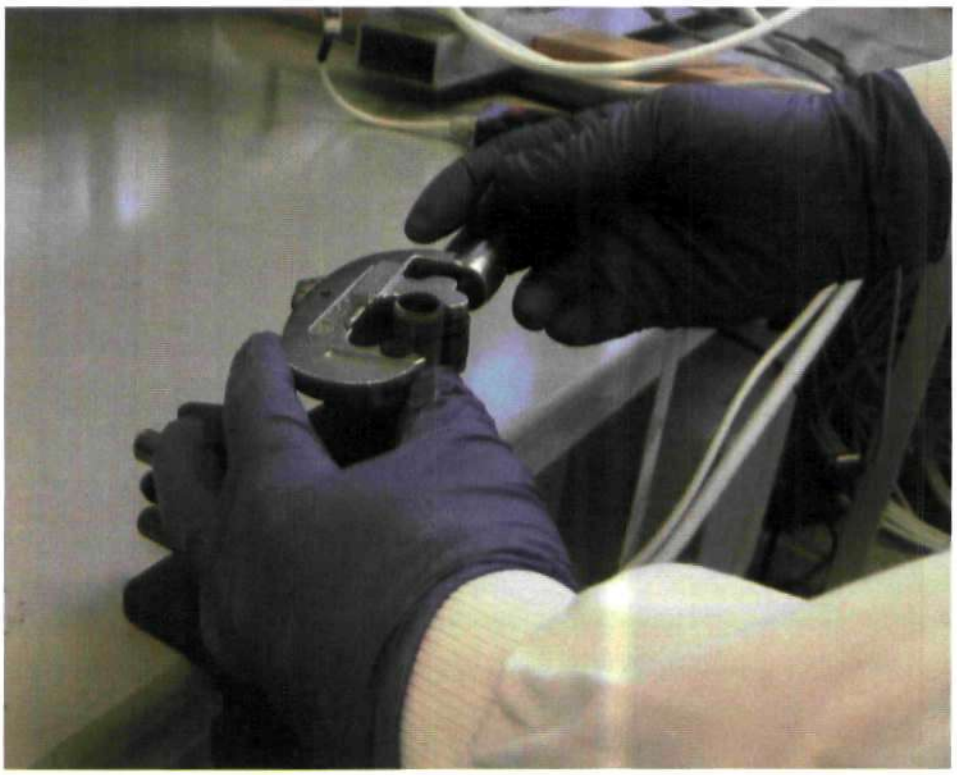


Figure 2.8 Use of rotating pipe cutter to cut samples for downstream processing.

2.4.3 Collection of water samples from test rig

Water samples were collected in 1 litre conical flasks, starting with system one and working sequentially across the test rig (left to right). Prior to sample

collection, flasks were washed in 2% Decon 90 detergent (Decon laboratories, U.K.), rinsed thoroughly with deionised water, and autoclaved at 121 °C for 15 minutes. Contamination by airborne contaminants was prevented by covering the flask with aluminium foil prior to autoclaving.

Water samples were collected from each system by first disconnecting the auto-flushing solenoid at the base. Each system was then separated from the LLDPE piping/ no-touch tap assembly and a (30 mm) length of virgin, pre-autoclaved, LLDPE piping connected in its place. The key switch was then used to manually flush the system, and effluent was collected in an appropriately labelled conical flask for a period of 10 seconds.

As well as water samples being collected from each system at every sampling point, water samples were also collected from the hot feed, cold feed and mains water supply. In each case, water was run to waste for 30 seconds before collection in order to eliminate standing water in the pipes.

2.5 Culture based analysis

All microbiological media, reagents and selective supplements used were supplied by Oxoid Limited (Basingstoke, U.K), unless otherwise stated.

2.5.1 Culture-based analysis of inserts

An insert portion of approximately 20 mm was cut as described in section 2.4.2. The exact length was measured and recorded, in order that the surface area of the inner surface could later be calculated. A sterile cotton swab (Fisher Scientific, Loughborough, UK) was moistened in sterile bacteriological (0.85%)

saline, and the entire inner surface of this section swabbed thoroughly for a minimum of 20 seconds. Each swab was stored at 4 °C after use until all swab samples had been collected.

For culture based analysis, the swab head was removed using scissors which were flame-sterilized using 70% IMS immediately prior to use. The swab head was placed directly into 1 ml of sterile bacteriological (0.85%) saline, and the sample mixed continuously for one minute using a vortex mixer. Using metal forceps which were flame-sterilized using 70% IMS immediately prior to use, the swab head was then removed and discarded.

Serial tenfold dilutions of each sample were prepared in 1.5 ml microcentrifuge tubes using sterile bacteriological saline as the diluent, to a final volume of 1 ml. Triplicate spread plates were prepared of three appropriate dilutions, using 100 µl of sample and R2A agar. R2A agar is a low nutrient agar designed for the recovery of bacteria from drinking water, and was first developed by Reasoner and Geldreich in 1985.

The exact dilutions used were dependent on the materials under investigation, and expected bacterial numbers. Incubation of plates was carried out for two weeks at 20 °C.

In order to enumerate culturable *Pseudomonas* spp. associated with insert-bound biofilms, 100 µl of the appropriate dilutions was spread plated onto triplicate CFC agar plates. *Pseudomonas aeruginosa* NCIMB 8126 was streaked onto a single CFC agar plate as a positive control. Plates were incubated at 20 °C for five days.

At the end of each trial, attempts were made to culture and enumerate *Legionella* spp. associated with insert-bound biofilms, using a protocol based upon the Health Protection Agency (HPA) National Standard Method for

detection of *Legionella* spp. in biofilms and sediments (2006). 100 µl samples were taken from the lowest dilutions and these samples pooled according to the experimental conditions employed. A single serial tenfold dilution of this combined sample was then prepared using bacteriological saline as the diluent, to a final volume of 1 ml. This was subdivided into two 500 µl sub-samples, one of which was incubated at 50 °C for 15 minutes. The other sub- sample was acid-treated by addition of 500 µl acid buffer pH 2.2 (39 ml 0.4M hydrochloric acid/ 250 ml 0.4M potassium chloride; pH adjusted with 1M potassium hydroxide), followed by incubation at room temperature for five minutes. 100 µl of each sub-sample was then spread plated onto BCYE-GVPN agar. *Legionella pneumophila* NCIMB 12821 was streaked onto a single plate of the same media as a positive control, and also to aid in the identification of suspect colonies. Plates were incubated at 37 °C in a sealed box containing wet tissue for 7-14 days, during which time plates were checked every three days for evidence of colony development.

2.5.2 Culture-based analysis of effluent

Water samples were collected from the test rig as described in section 2.4.3. 100 µl aliquots were taken from each collected water sample and serial tenfold dilutions made in 1.5 ml microcentrifuge tubes, to a final volume of 1 ml, using sterile bacteriological saline as the diluent. Triplicate spread plates were prepared of three appropriate dilutions, using 100 µl of sample and R2A agar. Incubation of plates was carried out for two weeks at 20 °C. Where lower counts were expected, water samples were filtered onto 0.45 µm cellulose nitrate filters

(Whatman, U.K.) using a filtration manifold / vacuum pump assembly (Fisher Scientific Ltd., Loughborough, U.K.).

2.6 SEM analysis of inserts and materials

Sections of each insert to be analyzed approximately 10 mm in length were cut as described in section 2.4.2 and gently placed in 50 ml polypropylene flip-top bottles containing 5 mls fixative (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer- Sigma Aldrich Ltd., Poole, UK), using fine laboratory forceps which had been flame sterilized using 70% I.M.S. Fixed samples were stored at 4 °C until processing.

Inserts were gently rinsed by placing in 10 ml 0.1M sodium cacodylate buffer for 15 minutes, and a pair of metal shears used to cut the insert longitudinally, before cutting small pieces as shown in Figure 2.9. These pieces, measuring approximately 5 mm x 10 mm, were labelled on the reverse (i.e. non-imaged) side using a diamond pen, before serial dehydration in 5mls of 30%, 50%, 70%, 90% and 100% ethanol (15 minutes in each).

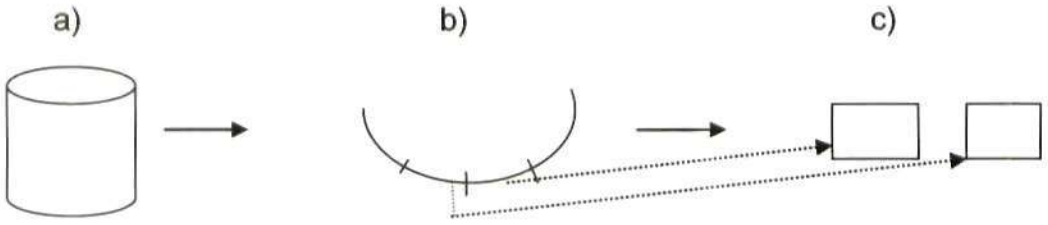


Figure 2.9. Production of cut copper insert samples for Scanning electron-/ confocal microscopy analysis. (a) shows direction of initial cut (b) pipe is opened out to facilitate further cutting (c) small sections (approx 5mm x 10mm) are cut and used for imaging purposes.

Samples were then placed into fresh 100% ethanol before drying using an Emitech K850 critical point drier, with ethanol as the intermediate fluid and

carbon dioxide as the transitional fluid. Samples were then mounted and sputter-coated using an Emitech K550 gold sputter coater. Imaging was carried out using either a JEOL 5600 scanning electron microscope unless otherwise stated. A minimum of five images were taken of each sample in order to obtain representative qualitative data.

2.7 Confocal laser scanning microscopy analysis of inserts

As mentioned previously, 10 mm pieces of insert were stored in sterilized milli-Q water at 4 °C until required for confocal laser scanning microscopy. A sample was then cut as described in section 2.4.2, and a small piece of lens tissue gently applied to one edge to remove excess water and improve staining efficiency.

2.7.1 LIVE/DEAD staining of biofilm

LIVE/DEAD BacLight stain (Invitrogen, UK) was employed to qualitatively assess bacterial cell viability The two staining solutions, comprising Syto9 and propidium iodide, were prepared and combined according to the manufacturer's instructions, yielding final concentrations of 6 µM and 30 µM, respectively. 30 µl of the mixture was applied to the inner surface and the sample incubated at room temperature in a 25 mm foil-wrapped petri dish for 30 minutes. The sample was then removed using forceps and gently placed in a 25 mm plastic petri dish containing 3 ml sterile bacteriological (0.85%) saline, which acted both as a rinsing agent and support medium during observation. In order to prevent sample movement during observation, each sample was immobilized by using

two flat pieces of copper pipe, of approximately equal size to the samples being analyzed. Observation of all fluorescently-labelled samples, was carried out using an LSM 510 confocal laser scanning microscope (Carl Zeiss Ltd., Germany), equipped with a x40 water dipping objective and a x63 oil immersion objective.

2.7.2 Image collection and analysis of confocal images

2.7.2.1 Pipe material curvature and its effect on image acquisition.

Visualization of a complete, fully-focussed image was extremely challenging. This was due to the fact that only a small portion of the available surface was in the focal plane at any one time. Thus, an incomplete image was observed, with the degree of curvature dictating the proportion of the surface which was immediately visible in the image window. This had implications when collecting images for later analysis, in terms of time taken and image optimization.
The step-wise protocol employed for assessment of coverage of pipe surface by biofilm is described below:

a) The objective was roughly focussed on an approximately central part of the pipe piece under observation.

b) Settings were then adjusted in order that a stack of images (Z-stack) could be produced ('Mark First' and 'Mark last' function).

c) A three-dimensional projection of each stack was then produced, using the *y* axis as the axis of rotation. Settings were such that a projection of optimal quality was obtained. This approach effectively

'stitched together' a stack of incomplete images, and a complete image of the sample surface was obtained.

d) The resulting '.lsm' file was then opened using the image analysis software package 'Image J' (http://rsbweb.nih.gov/ij/, equipped with the '.lsm reader' plug-in).

e) The 'plan view' of the sample was saved as a .jpeg file (Figure 2.10, overleaf) This was necessary for downstream processing of the image. Before saving, it was necessary to combine two images, relating to each of the two channels used in detection/ viewing of the two stains used in the LIVE/DEAD staining kit. The 'subtract background' option was also employed, in order to assist in visualisation of the cells.

f) The '.jpeg' version of the image was then reopened in Image J and duplicated. Duplication of the image allowed reference from the processed image back to the original. This image was then converted to an 8-bit 'greyscale' image, and thresholded to a pre-determined level (Figure 2.11, overleaf).

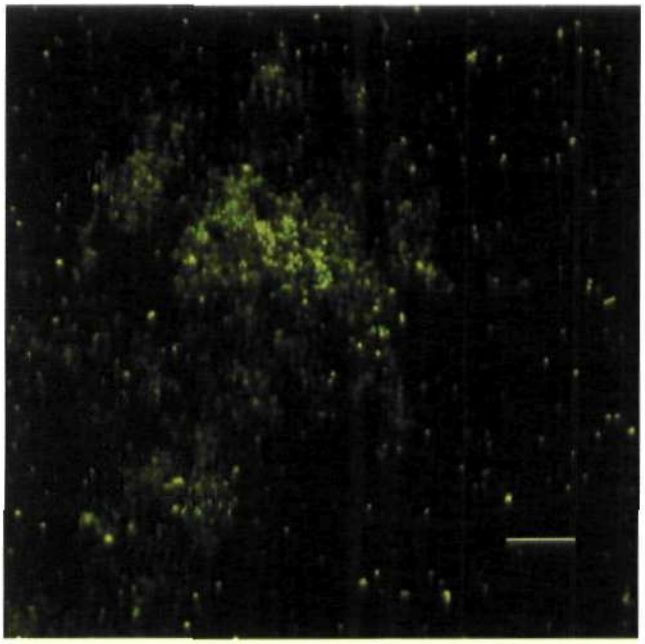


Figure 2.10 Example image of biofilm obtained using confocal microscopy, according to the procedure described in section 2.7.2.1 (a)-(f), stained using Syto9 nucleic acid stain, and visualized using x40 water dipping objective (bar- 10 µm).

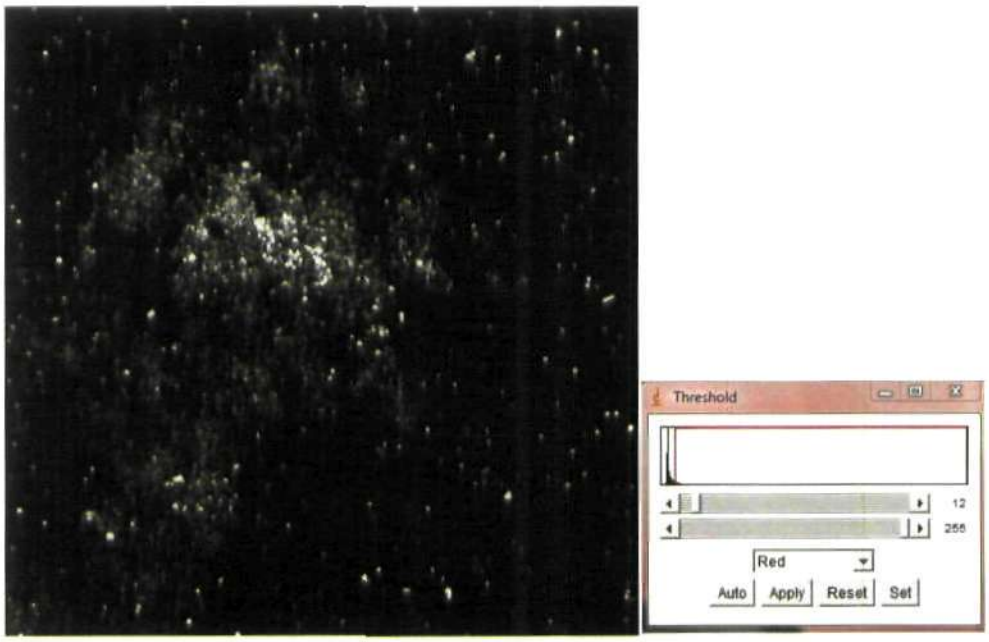


Figure 2.11 8-bit greyscale image of Figure 2.10 (bar- 10 µm).

g) Images were then converted to binary (black and white) format (see Figure 2.12, below). In the binary format, biofilm and associated bacterial cells are represented as black pixels set against a white background.

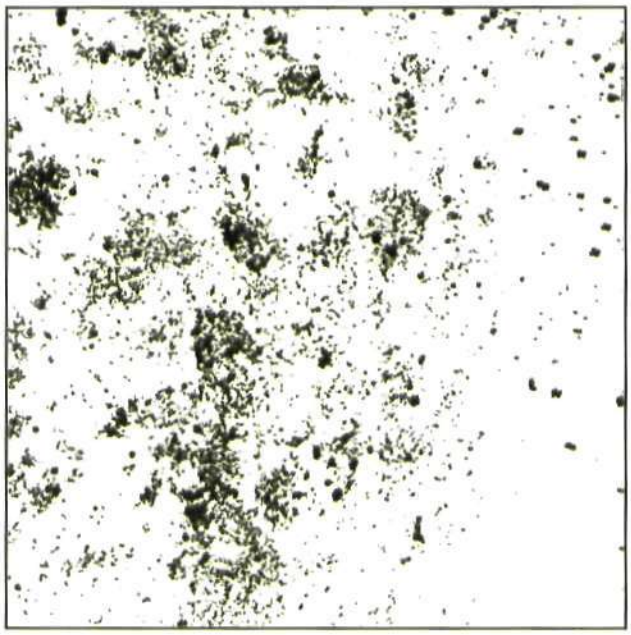


Figure 2.12 Binary image after thresholding of Figure 2.11

h) The 'set measurements' function was selected, and the desired measurement/ results display parameters defined.

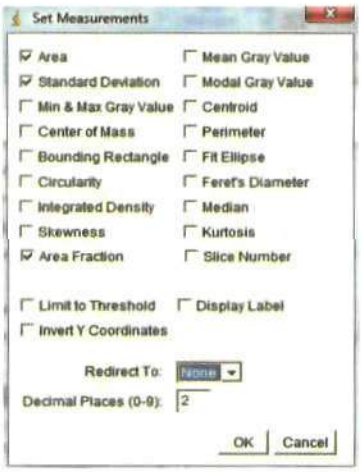


Figure 2.13 Screenshot of 'set measurements' function in Image J

i) The 'Analyze particles' function was selected, and the desired measurement/ results display parameters defined as shown in Figure 2.14, below.

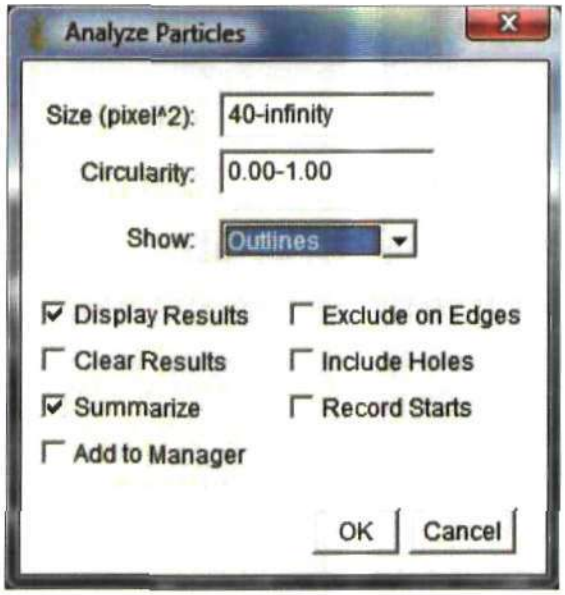


Figure 2.14 Screenshot of 'Analyze Particles' function in ImageJ

j) Figure 2.15 (below) shows an example results summary table, which was generated upon clicking 'Ok' in the 'Analyze Particles' window.

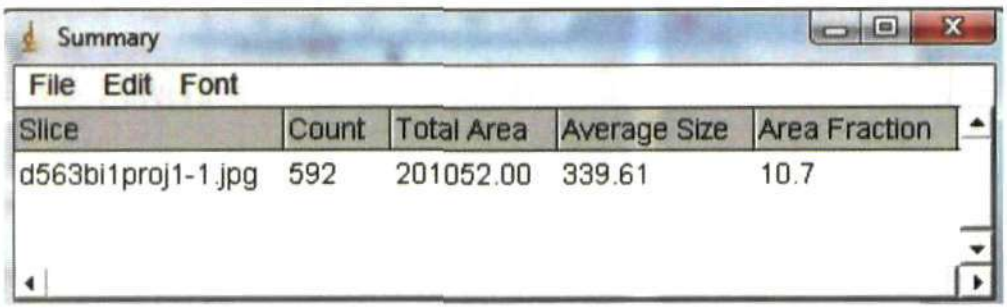


Figure 2.15 Screenshot of typical results summary generated from binary image using Image J

'Count'- refers to the total number of black biofilm particles in the image

'Total Area' refers to the total area (in pixels) occupied by black pixels.

'Average Size'- refers to the average size (in pixels) of black 'biofilm particles' in the image.

'Area Fraction'- is a percentage value and refers to the proportion of the image which is occupied by black pixels.

l) Summary tables were then exported into Microsoft Excel for further analysis.

For each system, five images were collected and analyzed according to the steps described above. Whilst collection of more images would have been preferable, it was felt that the time taken to collect and process images, coupled with the fact that other biofilm analysis techniques were also being employed, precluded the collection of more data by this method.

2.8 ATP analysis

2.8.1 Sample collection- inserts

Samples for analysis of surface-associated adenosine triphosphate (ATP) were collected in the same way as was done for culture-based analysis. An insert portion of approximately 20 mm was cut as described in section 2.4.2 The exact length was measured and recorded, in order that the surface area of the inner surface could later be calculated. A sterile cotton swab (Fisher Scientific, Loughborough, UK) was moistened in sterile bacteriological (0.85%) saline, and the entire inner surface of this section swabbed thoroughly for a minimum of 20 seconds. Afterwards each swab was stored at 4 $^{\circ}$C until all swab samples had been collected.

The swab head was removed using scissors which were flame-sterilized using 70% IMS immediately prior to use, and placed directly into a sterile 1.5 ml

microcentrifuge tube containing 1ml of bacteriological (0.85%) saline. The sample was mixed continuously for one minute using a vortex mixer. Using metal forceps, which were flame-sterilized using 70% IMS immediately prior to use, the swab head was then removed and discarded.

Samples were then placed in a bench-top microcentrifuge and pulse-spun in order to loosely pellet any suspended copper which may have interfered with the activity of the luciferase enzyme used in the measurement of ATP. The supernatant was then aspirated using a 200 µl micropipette and placed into a fresh, sterile, labelled 1.5 ml microcentrifuge. A further spin was carried out in order to pellet the suspended biofilm material (10000g, 10 minutes), and the supernatant carefully aspirated and discarded. The pelleted cells were resuspended in 100 µl of bacteriological (0.85%) saline and frozen at -20 °C for 24 hours. After this period, they were transferred to -80 °C until processing.

2.8.2 Sample collection- water

The effluent from each experimental system was analyzed for ATP, as well as samples from the hot feed, cold feed and mains supply water. This was done at each monthly sampling session.

50 mls of sample water was pipetted into two 50 ml polypropylene falcon tubes (total volume 100 mls), and centrifuged in a benchtop centrifuge (30000g, 15 minutes) to pellet the cells. After centrifugation, a 10 ml pipettor was used to remove 49 mls of supernatant from each tube, and the cells resuspended in the remaining 1 ml. The two 1 ml aliquots were then combined in a single sterile 2 ml microcentrifuge tube and spun in a bench-top microcentrifuge (10000g, 10 minutes). After centrifugation, the supernatant was carefully aspirated from

each tube and discarded. The pelleted cells were resuspended in 100 µl of bacteriological (0.85%) saline and frozen at -20 °C for 24 hours. After this period, they were transferred to
-80 °C until processing.

2.8.3 ATP bioluminescence assay

ATP was quantified using the ATP biomass kit HS (Biothema AB, Sweden), in conjunction with a Pi-102 tube luminometer (Hygiena International Ltd., Watford, U.K.).

The ATP Biomass kit HS consisted of four components:

a) ATP reagent (consisting of lyophilised luciferase/ luciferin)
b) Diluent 'B'- for reconstitution of ATP reagent
c) ATP standard- 10^{-7} mol/L ATP
d) Extractant BS- for extraction of ATP

The ATP Bioluminescence assay procedure was carried out with amendments to the manufacturer's instructions, as detailed below:

a. Samples to be analyzed were removed from the freezer and processed individually, after gentle thawing on ice.
b. 25 µl extractant BS was pipetted into each of three 3 ml assay tubes
c. After gently mixing the sample with a 200 µl pipettor, 25 µl of sample was pipetted into each assay tube. Tubes were placed on ice for one minute.

d. One assay tube, containing the lysate, was placed in the tube luminometer.

e. 200 µl of ATP reagent was added to the tube, the contents quickly mixed without pipetting, and a value obtained. This value corresponded to r.l.u. or 'relative light units'.

f. 10 µl of ATP standard was then added as soon as possible, and a second value obtained.

g. The assay was repeated for the other two replicates

h. Spent tubes were discarded, and the next sample removed from the freezer for processing.

Calculation of sample ATP content was according to the equation

$$ATP_{smp} = I_{smp} / (I_{smp+std} - I_{smp})$$

Where

I_{smp}= rlu value of sample (obtained at step (e) above)

$I_{smp+std}$= rlu value of sample/ standard mixture (obtained at step (f), above)

Further calculations (in terms of insert surface area/ correction of values to include sample volume etc.) were made as appropriate.

2.9 Analysis of water parameters

Monthly monitoring of the following water parameters was carried out as described below during trials, and any notable variations in the values obtained are reported where appropriate.

2.9.1 pH and conductivity

Triplicate 50 ml sub-samples of mains water, cold feed, hot feed and effluent (from systems one to eight) were collected, and pH data collected using an HQ40d Dual Input Multi Parameter Digital Meter (Hach, Loveland, CO., USA). Conductivity of triplicate 50 ml sub-samples was measured using a CMD8500 conductivity meter (Walden Precision Apparatus Ltd., Cambridge, UK).

2.9.2 Total/ Free Chlorine

Total/ free chlorine of both mains and cold feed water was measured using DPD total and free chlorine reagent in conjunction with a DR2800 portable spectrophotometer (Hach, Loveland, CO.,USA), according to the manufacturer's instructions.

2.9.3 Water hardness

Water hardness of the incoming water (hot feed, cold feed and mains water supply) was measured at each monthly sampling using a commercially available kit (Hach, Loveland, CO, USA) in conjunction with a DR2800 portable spectrophotometer (Hach, Loveland, CO.,USA), and according to the manufacturer's instructions.

Chapter 3 An Investigation into the Effects of Stagnation and High pH on Biofilm Formation in a Model Distribution System

3.1 Introduction

The formation of microbial biofilms within water distribution systems (WDSs) represents a significant contamination risk, as the vast majority of microorganisms present are likely to exist in this state (Flemming 2002), given the numerous advantages that the biofilm mode of existence holds over a planktonic lifestyle. Both biofilm accumulation and the subsequent detachment of single cells and biofilm aggregates are affected by a wide variety of factors, such as substrate concentration (Wimpenny 2000), material choice (Kerr *et al.* 1999; Zacheus *et al.* 2000; Lehtola *et al.* 2005), temperature and hydrodynamic effects (Melo and Vieira 1999; Liu and Tay 2001b; Liu *et al.* 2006), and many studies have attempted to unravel the effects of these factors when applied to biofilms both individually and in combination.

Hydrodynamic effects may vary throughout a WDS, depending upon its size and complexity, with these effects ranging from stagnant conditions to laminar/ turbulent flow (Manuel *et al.* 2010). Each of these flow regimes has been shown to exert significant effects on structural aspects of the biofilm and, in turn, on the water quality from a microbiological viewpoint (Peyton 1994; Stoodley *et al.* 1999; Lehtola *et al.* 2006; Manuel *et al.* 2010). In general, there appears to be an inverse relationship between biofilm porosity and shear stress. Higher shear stress leads to a decrease in porosity, increased cell numbers and enhanced selection for bacteria with greater adhesive tendencies (i.e. those that exhibit

greater extracellular polymer production), whilst lower shear stress is considered to lead to the formation of more porous, less adhesive, biofilms (Percival *et al.* 1999; Flemming 2002; Manuel *et al.* 2010).

Stagnation has previously been shown to adversely affect water quality from a microbiological viewpoint in WDS (Wingender and Flemming 2003), as well as dental unit water systems (Walker and Marsh 2007), as increased biofilm accumulation and detachment are common observations whenever stagnation occurs (Ciesielski *et al.* 1984; Percival *et al.* 1999; Lehtola *et al.* 2007; Manuel *et al.* 2010). Numerous authorities and publications emphasize the importance of avoiding stagnant conditions within WDS wherever possible, with the primary aim being to eliminate outbreaks of legionellosis (Liu *et al.* 2006; HSC 2008), as *Legionella* (as well as other waterborne pathogens) are well-documented residents of WDS biofilms (Declerck *et al.* 2009; Giao *et al.* 2009; Lau and Ashbolt 2009). However, in distribution systems of older design and layout it can be expensive and logistically challenging to solve this problem.

In this study, a model distribution system or 'test rig' was employed to study the effect of daily, weekly, monthly, and non-flushing (i.e. stagnation) on biofilm formation over 84 days. An investigation was also carried out into the effect of high pH on biofilm formation under the same hydrodynamic conditions over the same period. Maintenance of high pH values within WDSs has previously been suggested as a potential method of both controlling both corrosion and biofilm formation, and some studies have shown that high pH is capable of reducing bacterial viability and subsequent attachment (Martin *et al.* 1982; Camper 2000; Nandakumar *et al.* 2002), as well as reducing copper corrosion (Wagner and Chamberlain 1997).

3.2 Materials and methods

3.2.1 Test rig system set-up

The design and operation of the model warm water system, or 'test rig' used during this study is described in chapter 2, section 2.2, page 35.
This study was comprised of two trials, designated 'neutral pH' and 'high pH'. Each trial was 84 days duration. 'Neutral pH' refers to a pH range of 7.15 to 7.88, whilst 'high pH' refers to a pH range of 9.53 to 10.08, and these ranges were derived from the measurement of triplicate test rig effluent samples collected from each system throughout the study, according to the protocol described in chapter 2, section 2.9.1, page 59. Flushing conditions were identical during both trials and are described in Table 3.1 (overleaf). Copper inserts were used throughout the study.

During the 'high pH' trial the unvented water heater was used as per the manufacturer's instructions. During the 'neutral pH' trial the sacrificial anode was removed from the heater.

3.2.1.1 Utilization and relevance of sacrificial anode

The sacrificial anode is primarily composed of magnesium and is fitted as standard in unvented water heaters. Positioned in close proximity to the heating element, its role is to reduce corrosion in situations where dissimilar metals come into contact with each other (e.g. copper and stainless steel), even if these metals are only bridged by mains water. The net result is the preferential dissolution of this anode, and an increase in pH which is due to the presence of

elevated levels of magnesium salts such as magnesium (bi)carbonate and magnesium hydroxide/ oxide (Dr. Mike Foulkes, personal communication).

Table 3.1 Description of flushing conditions employed during 'neutral pH' and 'high pH' trials.

		Flushing conditions		
Trial duration	**System**	**duration**	**Temperature**	**Period between flushing i.e. stagnation period**
84 days	2, 4	30 seconds	41 °C	24 hours (daily)
84 days	5, 7	30 seconds	41 °C	7 days (weekly)
84 days	3, 6	30 seconds	41 °C	28 days (monthly)
84 days	1, 8	30 seconds	41 °C	84 days (non-flushing)

3.2.2 Culture-based analysis

3.2.2.1 Inserts

Culture-based analysis was carried out as described in chapter 2, section 2.5.1, page 45.

The tenfold dilution series and spread plating regime was adjusted for each material according to expected numbers of culturable aerobic bacteria (CAB). The non-selective agar R2A and the *Pseudomonas*-selective agar CFC were used at each sampling point throughout the trials (i.e. every 28 days), whilst samples were also plated onto *Legionella*-selective BCYE/GVPN agar after 84

days. *P. aeruginosa* NCIMB 8626, and *L. pneumophila* NCIMB 12821 were used as positive quality control organisms.

3.2.2.2 Effluent

Culture-based analysis of the effluent was carried out as described in chapter 2, section 2.5.2, page 47. The tenfold dilution series and plating regime was adjusted for each system accordingly, and R2A, CFC and BCYE/GVPN agars used as described in the previous section.

Culture-based analysis of the cold feed was also carried out at each sampling point, and in the same way as described in chapter 3.2.2.1.

3.2.3 ATP analysis

3.2.3.1 Inserts

ATP analysis of inserts was carried out according to the protocol described chapter 2, section 2.8.1, page 55. In the case of all non-copper pipe materials, samples were prepared for analysis as described in chapter 5, section 5.1.2.3.1, page 116. The exact length of all pipe samples was recorded in order that the inner surface area could be calculated in each case.

3.2.3.2 Effluent

ATP analysis of effluent from each system was carried out according to the protocol described in chapter 2, section 2.8.2, page 56.

The ATP content of the cold feed was also recorded in each case.

3.2.4 SEM analysis

Sample preparation and analysis of inserts taken from each system was carried out as described in chapter 2, section 2.6, page 48.

3.2.5 Confocal laser scanning microscopy analysis

CLSM analysis of copper inserts was carried out as described in chapter 2, section 2.7, page 49, using the nucleic acid stain Syto9 in the assessment of surface coverage.

3.2.6 Statistical analysis

Data analysis (paired T-tests, Kruskal-Wallis/ one-way ANOVA and *post hoc* LSD tests as appropriate) was carried out using Microsoft Excel and SPSS V17.0 (SPSS Ltd., Chicago, U.S.). A p-value of <0.05 was used to indicate a significant difference.

3.3 Results

3.3.1 Incoming water parameters

An overview of water parameter data collected during the current study are summarized in Table 3.2 (overleaf).

Table 3.2 Overview of incoming water parameters

	High pH			Neutral pH		
parameter	**mains water**	**cold feed**	**hot feed**	**mains water**	**cold feed**	**hot feed**
Temperature range, °C	11.5-14.5	15-17.5	70.5-74.5	10.0-14.5	15.5-17.5	68.5-74.5
pH range	7.44-8.31	7.02-7.40	9.64-10.18	7.60-8.24	7.27-7.94	8.01-8.21
Conductivity range, µS	147-207	177-202	179-215	154-193	164-199	167-221
Total chlorine concentration range, mgl^{-1}	0.60-0.65	0	0	0.61-0.65	0	0
Free chlorine concentration range, mgl^{-1}	0.51-0.58	0	0	0.53-0.60	0	0

3.3.2 Culture based analysis

3.3.2.2 Inserts

Figure 3.1 (overleaf) shows the results of culture-based analysis of inserts during exposure to neutral pH (pH range 7.15- 7.88) and it is clear that, over the course of the study, the greater the stagnation period, the fewer culturable aerobic bacteria (CAB) were recovered. A significantly greater number of culturable aerobic bacteria (CAB) were recovered after 28 days of daily flushing, in comparison to the other flushing regimes employed (mean value 4.85×10^2 cfu cm^{-2}, $p<0.05$). This remained the case after 84 days (mean value 2.06×10^3 cfu cm^{-2}, $p<0.05$). No statistically significant differences in the CAB numbers were observed between weekly (mean value 5.84 cfu cm^{-2}) and monthly (mean value 6.42 cfu cm^{-2}) flushed systems ($p>0.05$) after 28 days. However, after 84 days CAB numbers associated with weekly flushing (mean value 7.18×10^2 cfu cm^{-2}) had increased to a level significantly greater than those associated with monthly flushing (8.39 cfu cm^{-2}, $p<0.05$).

Only in the case of daily and weekly flushing were any post-28 day increases in CAB numbers shown to be significant ($p<0.05$). No CAB were isolated from the stagnant systems throughout the study. During exposure to high pH (pH range 9.53-10.08, with sacrificial anode in place within heater), no CAB were found to be present *at any point* during the trial. By way of a confirmatory check, all R2A plates were incubated under identical conditions

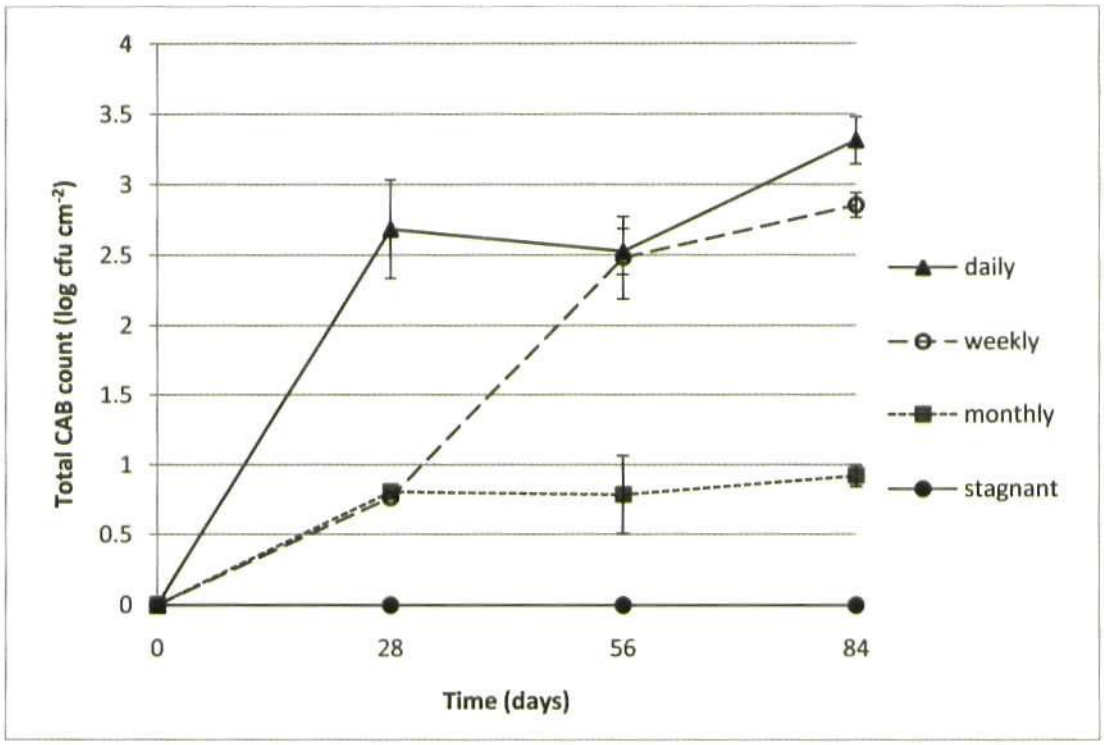


Figure 3.1 Total CAB counts associated with test rig copper inserts (n=3) over 84 days, under neutral pH conditions and varying flushing frequencies (error bars = SE).

for a further seven days, and this remained the case, although occasional pigmented pinprick colonies were observed. No culturable *Pseudomonas* spp., or culturable *Legionella* spp. were isolated at any point.

3.3.2.3 Effluent

Total CAB numbers observed in the test rig effluent under both neutral pH and high pH conditions are summarized in Table 3.3 (page 71). Under both neutral pH and high pH conditions no statistically significant differences in CAB numbers were observed between the flushing regimes employed at any point ($p>0.05$, data not shown). No statistical differences were observed under each pH condition when comparisons of each flushing condition and the incoming cold feed were undertaken ($p>0.05$ in all cases). No statistical comparisons

were subsequently made between equivalent flushing regimes under differing pH conditions due to the perceived influence of the incoming cold feed on effluent numbers.

3.3.3 ATP analysis

3.3.3.1 Inserts

Figure 3.2 (page 72) shows the results of ATP analysis of inserts during exposure to neutral pH (pH range 7.15- 7.88), and high pH (pH range 9.53-10.08).

During exposure to neutral pH, ATP levels were significantly greater after 28 days of daily flushing, in comparison to the other flushing regimes employed ($p<0.05$), whilst ATP levels observed in weekly/ monthly flushed systems were not significantly different from each other ($p>0.05$), but were significantly higher than levels observed in stagnant systems ($p<0.05$). After 84 days no statistically significant differences were observed between daily and weekly flushed systems ($p>0.05$). ATP levels observed under these flushing regimes were significantly higher than under monthly/ non-flushing regimes, and in each case the post-28 day increases in ATP levels were significant ($p<0.05$). Throughout the study, ATP levels remained low in the case of monthly/ non-flushed systems, although monthly flushing led to significantly greater levels than non-flushing, after 84 days.

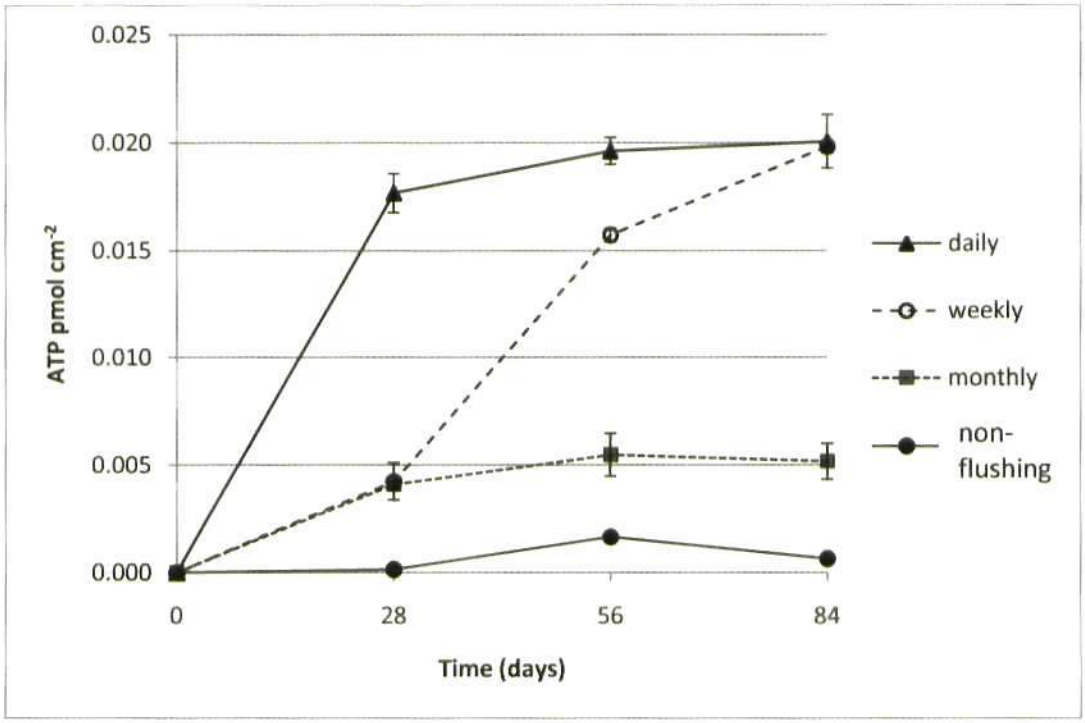
During exposure to high pH, daily flushing resulted in significantly higher ATP levels in comparison to other flushing regimes ($p<0.05$). After 84 days, weekly flushing yielded a marginally significant increase in ATP levels (in comparison to

28 day values). Changes in levels associated with monthly flushing/ stagnation were not significant when compared to 28 day values, whilst after 84 days of daily flushing ATP levels were subject to an overall drop post-28 days.

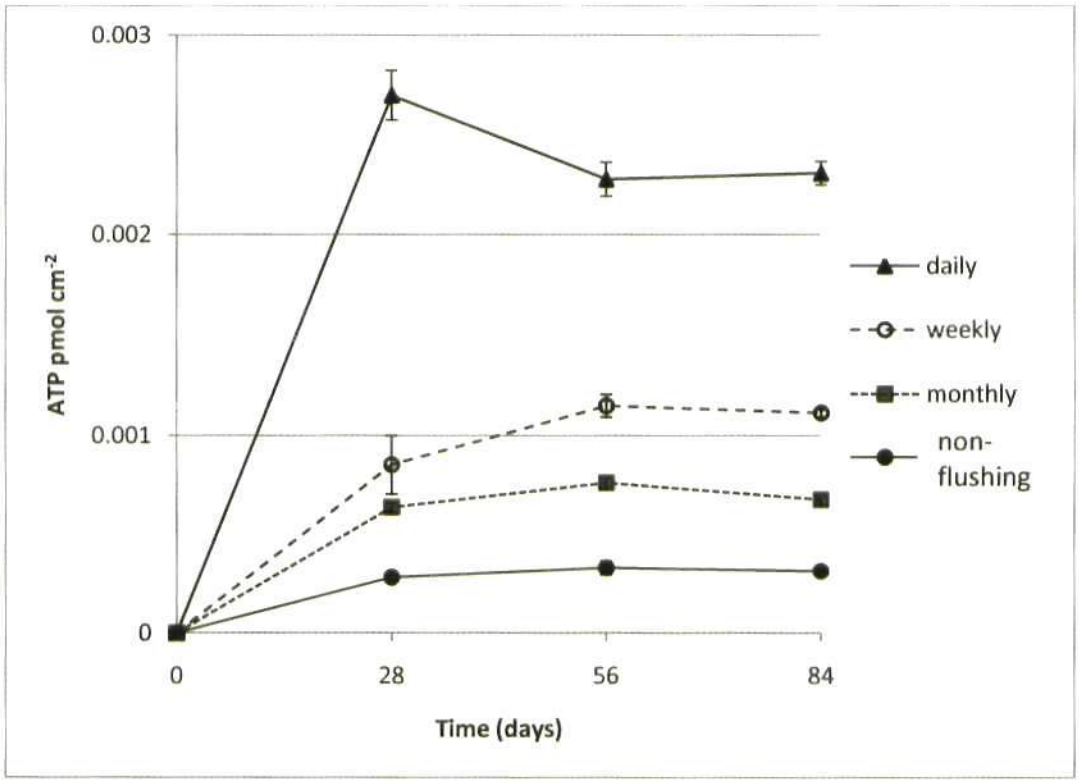
In all cases, ATP levels observed during employment of neutral pH were significantly higher in all flushing regimes, when compared to the corresponding values obtained when high pH conditions were employed ($p<0.05$).

Table 3.3 Summary of total CAB numbers observed under neutral pH and high pH conditions within cold feed and effluent.

	neutral pH		high pH	
	Mean cfu ml^{-1}	**Range cfu ml^{-1}**	**Mean cfu ml^{-1}**	**Range cfu ml^{-1}**
Cold feed	0.73×10^5	$0.37 - 1.55 \times 10^5$	3.71×10^4	$1.07 - 7.40 \times 10^4$
Daily flushing	1.07×10^5	$0.39 - 1.63 \times 10^5$	5.95×10^4	$4.30 - 7.50 \times 10^4$
Weekly flushing	1.03×10^5	$0.24 - 2.49 \times 10^5$	4.66×10^4	$2.87 - 6.33 \times 10^4$
Monthly flushing	1.22×10^5	$0.43 - 2.28 \times 10^5$	5.06×10^4	$3.40 - 6.66 \times 10^4$
Non-flushing	2.20×10^5	$0.47 - 4.5 \times 10^5$	5.45×10^4	$2.53 - 6.83 \times 10^4$



(a)



(b)

Figure 3.2 ATP levels associated with test rig copper inserts over 84 days under (a) neutral, and (b) high pH conditions and varying flushing frequencies (error bars = SE).

3.3.3.2 Effluent

ATP levels observed in the test rig effluent under both neutral pH and high pH conditions are summarized in Table 3.4 (page 76).

Under both neutral pH and high pH conditions no statistically significant differences in CAB numbers were observed between the flushing regimes employed at any point ($p>0.05$ in all cases). No statistical differences were observed under each pH condition when comparative ATP analysis of each flushing condition and the incoming cold feed were undertaken ($p>0.05$ in all cases).

No statistical comparisons were subsequently made between equivalent flushing regimes under differing pH conditions due to the perceived influence of the incoming cold feed on effluent ATP levels.

3.3.4 SEM analysis

Qualitative inspection of the virgin (uncontaminated) copper pipe surface (Figure 3.3, overleaf) showed it to be heterogeneous in terms of surface roughness and texture. A large amount of material apparently flaking from the surface was observed. Contact with high pH water (pH range 9.53-10.08) did not cause gross deterioration of the copper insert surface, and this was the case regardless of the flushing regime employed (Figures 3.4 and 3.5, page 77). Conversely, employment of neutral pH (pH range 7.15-7.88) appeared to facilitate deterioration of the copper pipe surface, in terms of surface roughness

and texture, and was clearly observed in all systems, regardless of flushing regime employed (Figure 3.6, page 78).

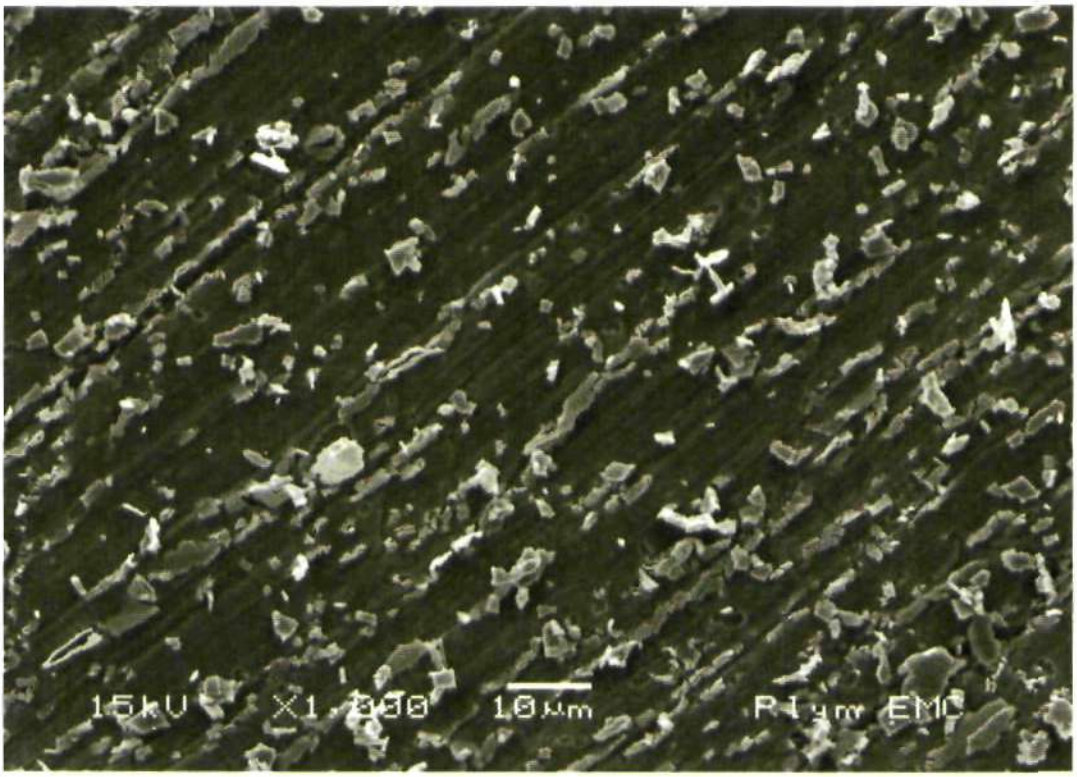


Figure 3.3 Scanning electron micrograph of interior surface of virgin (uncontaminated) copper pipe.

There was no evidence of bacterial colonization on the copper inserts which had been subjected to four weekly- and non-flushing, under high pH (Figure 3.4). Daily and weekly flushing yielded evidence of individual bacteria associated with the surface, but no evidence of biofilm formation was observed at any point (Figure 3.5). In contrast, during neutral pH exposure, daily- and weekly- flushed pipe surfaces exhibited microcolony formation after 28 days and subsequently throughout the trial. These microcolonies appeared to be mainly composed of small coccoidal cells arranged in a highly structured manner, with a number of discrete pores and channels permeating the microcolony structure (Figures 3.7 and 3.8).

Table 3.4 Summary of ATP levels observed under neutral pH and high pH conditions within cold feed and effluent.

	neutral pH		high pH	
	Mean ATP pmol ml^{-1}	**Range ATP pmol ml^{-1}**	**Mean ATP pmol ml^{-1}**	**Range ATP pmol ml^{-1}**
Cold feed	0.052	0.035-0.076	0.058	0.026-0.076
Daily flushing	0.058	0.038-0.073	0.059	0.038-0.076
Weekly flushing	0.053	0.037-0.081	0.039	0.037-0.079
Monthly flushing	0.047	0.017-0.074	0.032	0.017-0.061
Non-flushing	0.047	0.034-0.89	0.073	0.021-0.106

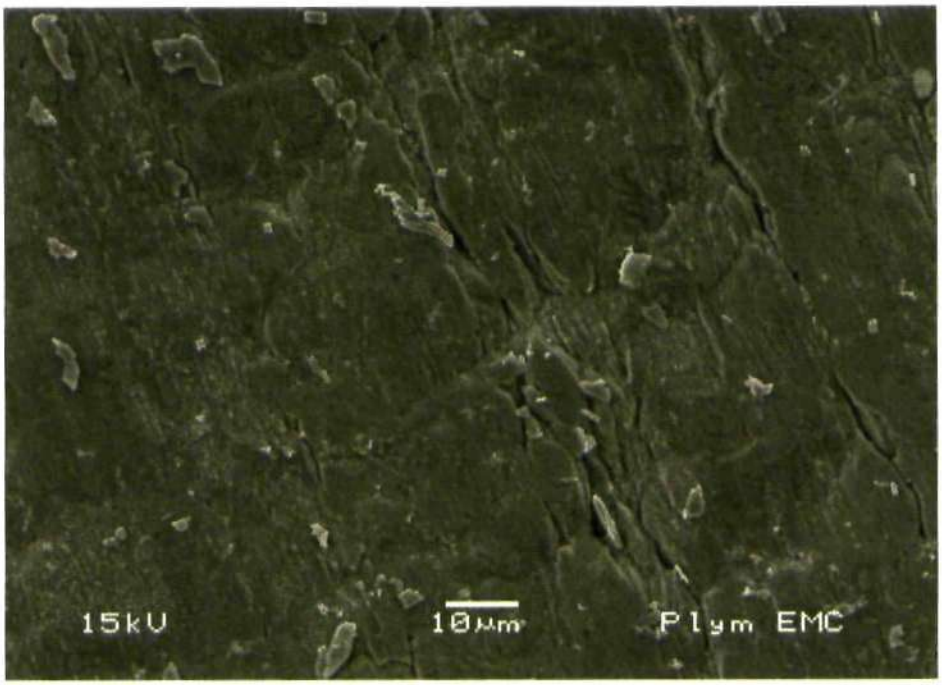


Figure 3.4 Scanning electron micrograph (x1000) of copper insert surface taken from a non-flushed system after 84 days high pH exposure. Points of interest are a complete lack of biofilm formation and general good condition of pipe surface, when compared to virgin copper pipe.

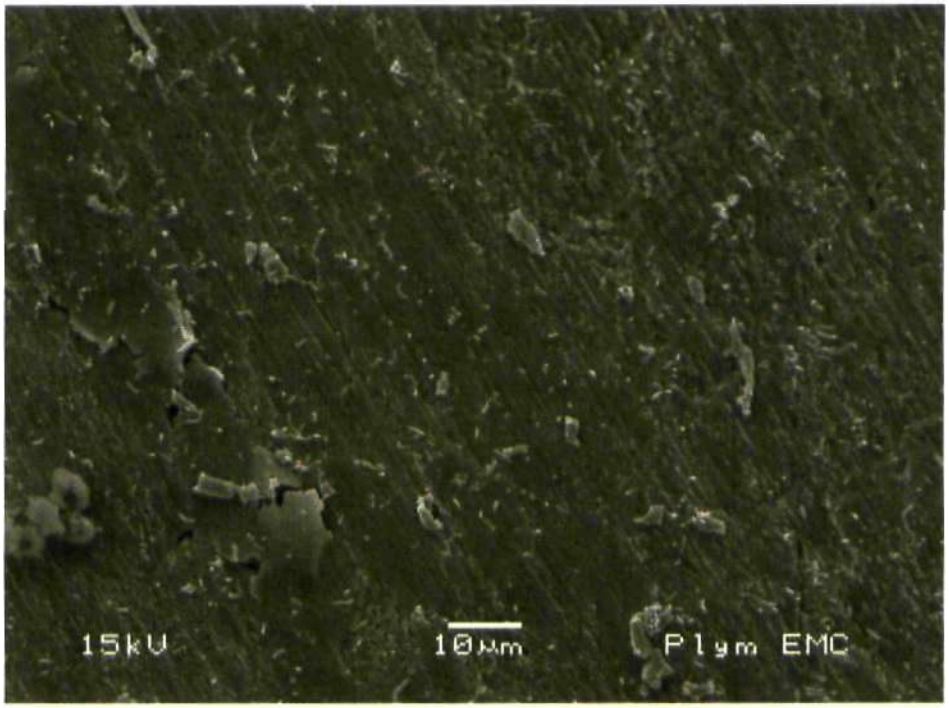


Figure 3.5 Scanning electron micrograph (x1000) of copper insert surface taken from a daily flushed system after 84 days of high pH exposure. Points of interest are presence of individual (non-biofilm-bound) rod- shaped bacteria and general good condition of the pipe surface, when compared to virgin copper pipe.

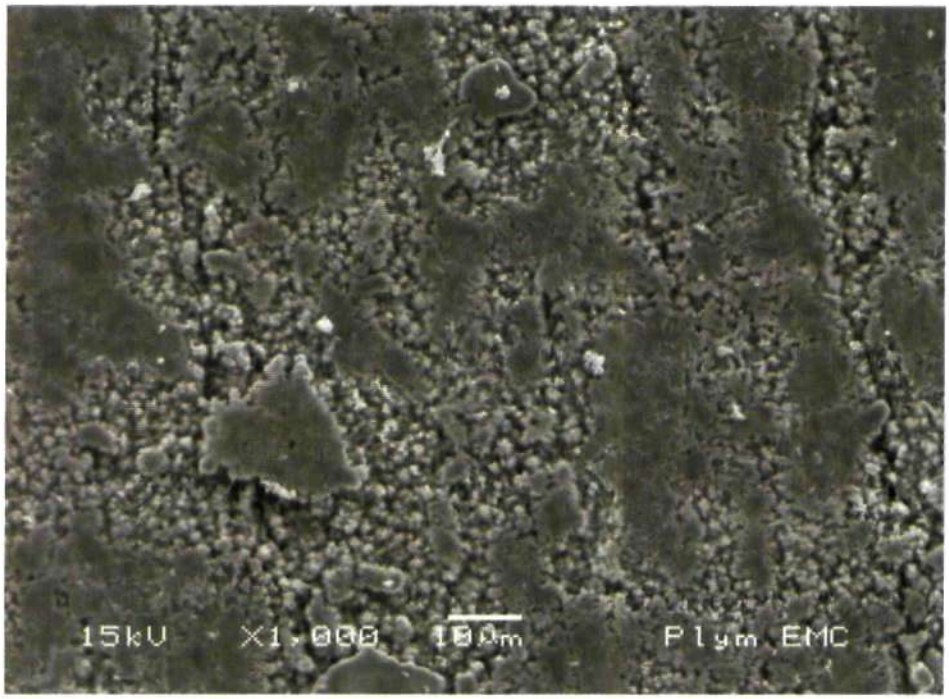


Figure 3.6 Scanning electron micrograph of copper insert surface taken from a non-flushed system after 84 days of 'neutral' pH exposure. Points of interest are a lack of biofilm formation and general poor condition of pipe surface, when compared to virgin copper pipe.

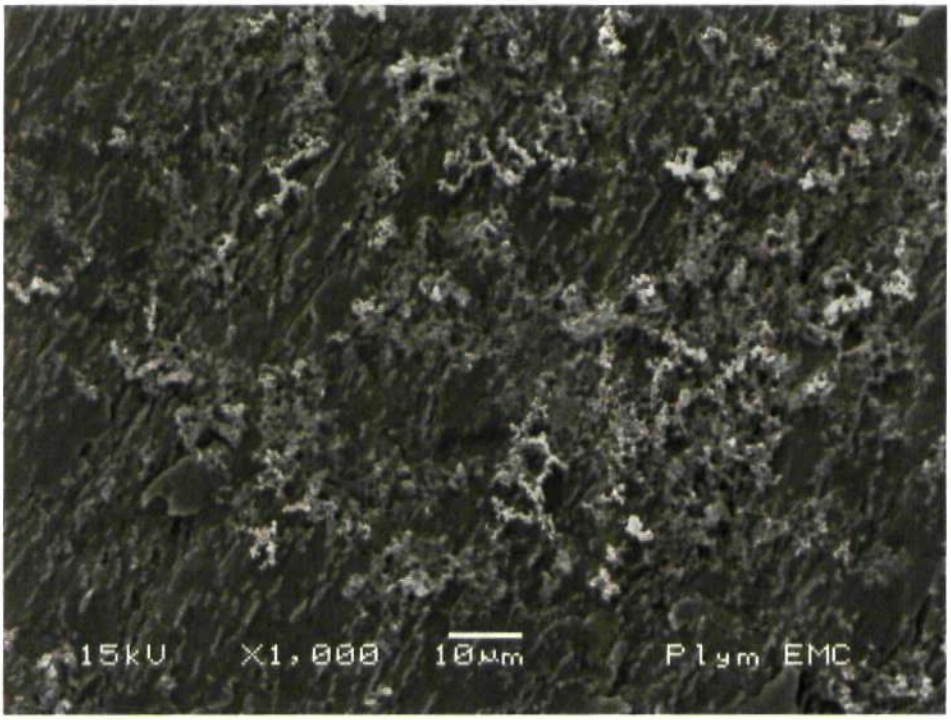


Figure 3.7 Scanning electron micrograph image of copper insert surface taken from a weekly flushed system after 56 days of 'neutral' pH exposure, demonstrating substantial microcolony formation.

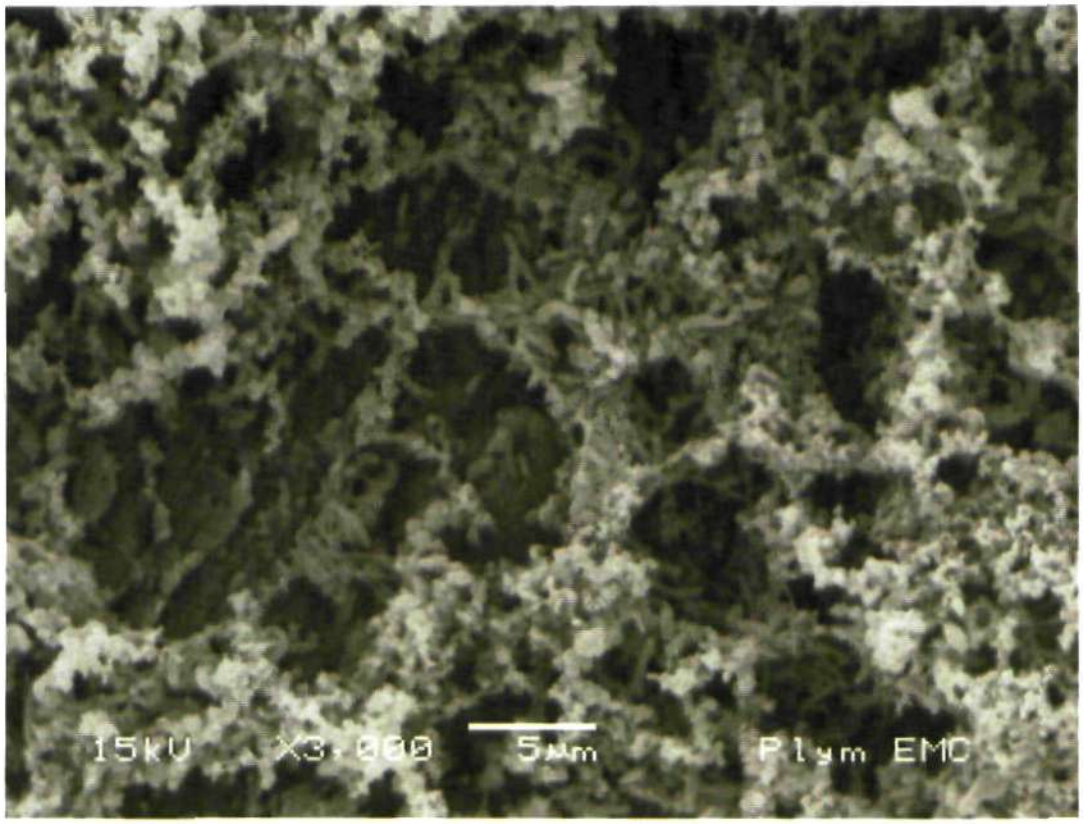


Figure 3.8 Scanning electron micrograph showing detail of biofilm observed on copper insert taken from weekly flushed system after 56 days of 'neutral' pH exposure, including possible presence of water channels.

3.3.5 Confocal laser scanning microscopy analysis

Confocal microscopical analysis was carried out after 84 days of exposure to neutral pH conditions. It was not attempted after exposure to high pH. This was due to the presence of large amounts of particulate matter derived from the sacrificial anode which adversely affected image collection. Biofilm formation was observed in all systems that were subjected to both daily and weekly flushing under neutral pH conditions, with no statistically significant differences apparent between these regimes ($p>0.05$, see Figure 3.10 for an example image). Systems that were flushed less frequently (i.e. monthly or stagnant systems) showed an absence of biofilm-bound cells (Figure 3.9, overleaf), and no statistically significant differences were observed between these regimes

(p>0.05). Any cells that were observed under these conditions appeared to be present as individuals, although this was difficult to confirm given the relatively low magnification available (data not shown), and the generally poor fluorescence of these cells.

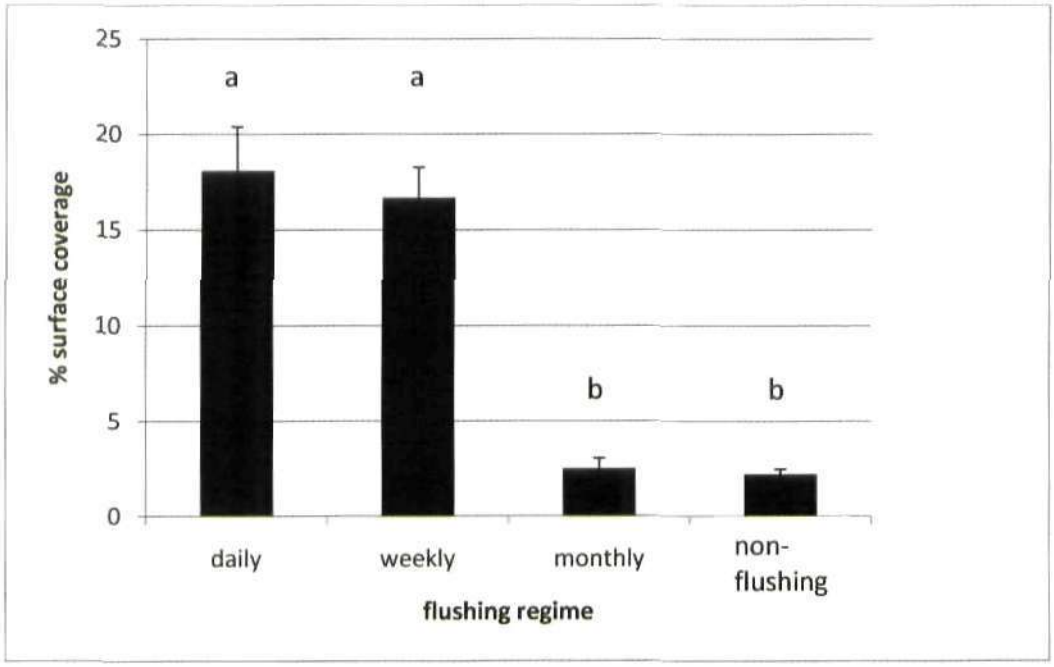


Figure 3.9 Surface coverage of test rig inserts after 84 days of exposure to neutral pH conditions, assessed using the nucleic acid stain Syto9 and confocal microscopy. (^{a,b} - Significant differences between groups are indicated by difference in superscript letters (p<0.05))

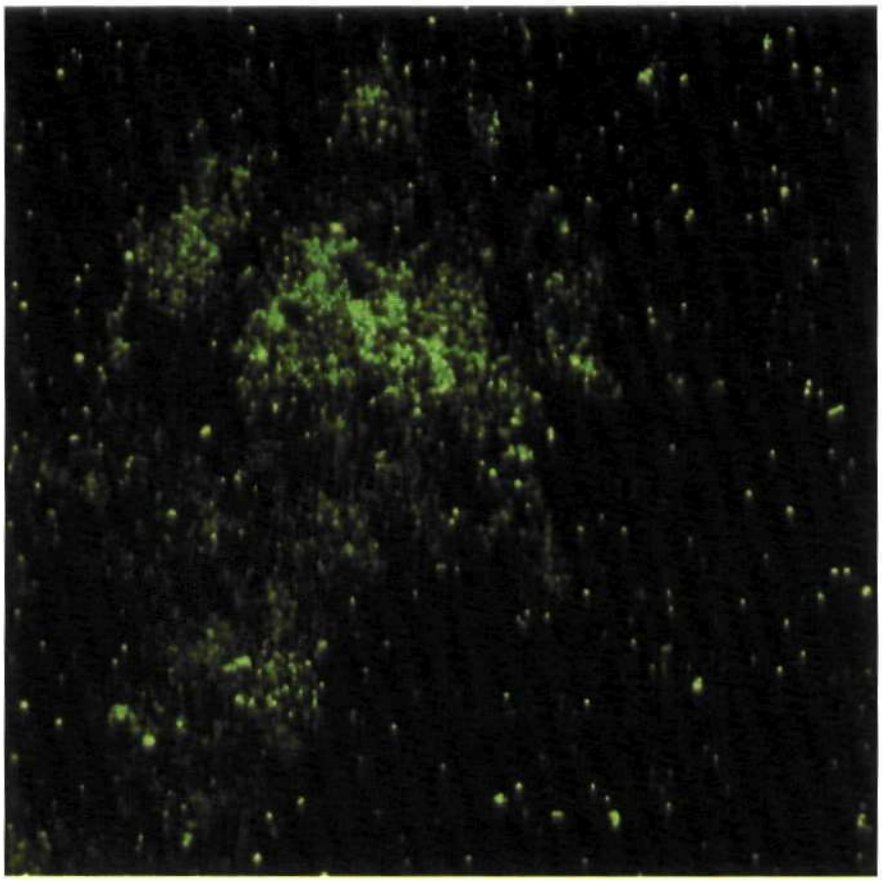


Figure 3.10 Example confocal image of biofilm formed after 84 days of daily flushing under 'neutral' pH conditions and stained using the nucleic acid stain Syto9 (x40 water dipping objective). The variation in fluorescence signals may be due to inter-species differences in, for example, stain uptake and metabolic rate.

3.4 Discussion

Periods of stagnation have been shown to predispose large-scale water distribution systems (WDSs) to elevated bacterial counts within the water column, and porous, loosely adhered biofilm formation (Wadowsky *et al.* 1982; Harper 1988; Halabi *et al.* 2001; Lehtola *et al.* 2007; Manuel *et al.* 2010). This potentially presents a significant health hazard, particularly when sudden changes in shear stress occur, causing biofilm detachment events. Particular interest has been paid to the effect that flow regimes exert on numbers of *Legionella* found in WDSs and, whilst it has been stated that *Legionella* growth (associated with biofilms) is promoted in stagnating warm water (Ciesielski *et al.*

1984), other more recent studies have contradicted this, seeking to elucidate the role of flow conditions in both *Legionella* growth and overall biofilm formation (Lehtola *et al.* 2006; Liu *et al.* 2006). Some modern practices concerned with *Legionella* and biofilm control in WDSs, particularly in healthcare situations, are based on the premise that stagnant conditions are to be avoided. These include regular flushing of taps and showers with chlorinated and/or super heated water.

In everyday parlance, the terms 'stagnant' and 'stagnation' vary contextually. Their use in this study is clearly defined and refers to the *non-flow of water within the test rig between flushes*. Whilst it may be tempting to describe stagnation in the context of much longer periods of non-flow, misinterpretation of such terms in this case may potentially lead to an incorrect assessment of the data obtained. It is clear that even short stagnation periods can have serious implications in terms of the microbiological quality of water, if substantial biofilm is already present, as the yield of planktonic cells and biofilm aggregates is linked to biofilm development (Lehtola *et al.* 2006; Bester *et al.* 2009). During this study, even the most frequently flushed systems (24 hourly) were subjected to a significant stagnation period. Whilst it is recognized that such flushing regimes may not necessarily reflect those employed in, for example, a clinical situation, the decision to flush the test rig every 24 hours was taken in order to preserve the scientific integrity of the study in this respect, given the difficulty in replicating everyday tap usage under laboratory conditions. ATP-, SEM-, confocal-, and culture-based analysis all showed that biofilm formation under neutral pH conditions (pH range 7.15-7.88) was significantly enhanced in the daily- and weekly-flushed systems. At first glance, this observation may initially appear to refute the suggestion that extended stagnation promotes biofilm

formation, given the low numbers associated with monthly-/ non-flushed systems. However, it is possible to explain the results obtained through consideration of factors such as the 'biofilm status' of the copper insert surfaces at the beginning of the study, and also the likely effects within the test rig of performing the flushing procedure. At the beginning of the study, the copper inserts used were newly-manufactured and therefore devoid of biofilm. This study was an investigation into the effects of stagnation on the *formation* of WDS biofilms, as opposed to the effect of stagnation on biofilms which were *already formed*. In this respect, this study differs from those of Lehtola *et al.* (2006) and Manuel *et al.* (2010), which investigated flow effects *post-biofilm formation*, and is similar to the study by Liu *et al.* (2006), who investigated *Legionella* numbers taken from biofilm samples *formed over time* under varying flow conditions.

Whilst the experimental approach employed during this study did not extend to in-depth flow measurements (for example, calculation of Reynold's number etc.), it seems reasonable to assume that the flushing employed during this study was a process dominated by turbulent flow, as water within the pipe is greatly disturbed. This effect would have been exacerbated by the many bends and obstacles which exist within the test rig, although on a micro-scale, areas of eddy formation and stagnation may still exist. Turbulent flow has been shown to lead to increased biofilm formation, in terms of observed surface-associated bacterial numbers (Manuel *et al.* 2010), by increasing deposition of matter to or near the substratum (Melo and Vieira 1999). Higher flushing frequencies were shown to increase biofilm formation (or more specifically, increased culturable aerobic bacterial numbers and surface coverage of the substratum) and metabolic activity (as indicated by increased ATP levels) at the copper insert

surface. This is presumed to be due to the increased availability of nutrients, oxygen and additional microorganisms which may be capable of forming (or being recruited into) a biofilm. Such 'nutrient-delivering events' occurred far less frequently in systems which were subjected to monthly- and non-flushing. It would therefore appear logical that biofilm formation would be greatly reduced under these conditions, and the results of the various analysis methods employed would appear to support this hypothesis. As well as infrequent nutrient and oxygen replenishment, it is likely that the environment in less frequently flushed (i.e. more stagnant) systems is likely to be less conducive to microbial growth for reasons such as increasing concentration of copper ions over time. Other factors which may have contributed to the lack of biofilm formation include decreasing oxygen concentration and a lack of sedimentation of potential nutrients/ populating microorganisms at the pipe surface due to the vertical positioning of the systems.

Whilst a commonly-held belief within the plumbing industry is that stagnation is an active promoter of biofilm formation, this is not wholly accurate. Studies have shown that turbulent flow also promotes biofilm, albeit of a denser, more populous type (Melo and Vieira 1999; Liu *et al.* 2006), and the ability of flow type to dictate the mechanical stability of biofilms is a key principle in their understanding and management in a WDS situation. Stagnation of system components containing pre-formed biofilms potentially increases detachment. Crucially, in a hospital situation it is this scenario of stagnation occurring where a biofilm *is already established* which is likely to be of greater concern, rather than stagnation occurring where a biofilm has yet to be formed. It would therefore seem prudent to adapt WDS management practices accordingly in order to prevent this situation from arising.

Little work has been carried out on the effect of elevated pH on biofilms in water distribution systems in a laboratory setting, and a pH range of 9.53-10.08 was shown in this study to be inhibitory to biofilm formation over 84 days. The findings of this study are partially supported by Nandakumar *et al.* (2002), who investigated the effect of magnesium on *Pseudomonas* sp. adhesion, using magnesium coupons. It was shown that magnesium dissolution caused a rise in the pH of the experimental media used, as well as at the coupon surface, to levels equivalent to or exceeding those observed in the present study. An accompanying drop in the number of adhered viable bacteria was also observed over a period of six days. This drop was considered by the authors to be due to a combination of the rapid increase in pH, coupled with an increase in magnesium concentration. Whilst microorganisms have an intracellular requirement for magnesium due to its many roles within the bacterial cell, exposure to an excessive concentration of magnesium may exceed the tolerance level of many of the microorganisms present (Nandakumar *et al.* 2002). Non-laboratory studies i.e. studies of actual water distribution systems, have suggested the use of high pH as a method of controlling problematic microorganisms (Camper 2000) such as coliforms, as these are of particular interest from the point of view of drinking water provision (Martin *et al.* 1982). It is possible that the use of sacrificial anodes, particularly in soft water locations, may be a way of controlling bacterial growth in small-scale installations, as long as no threat to human health is perceived through this method. Therefore, stringent evaluation of both microbiological and human health aspects would be essential before it could be considered a practical solution. Although the incoming cold feed contained a large number and variety of microorganisms, it is possible that any intolerance to either high pH or elevated magnesium

concentrations was exacerbated due to the stagnation regimes that were employed.

Another observation of interest when considering the role of the sacrificial anode in this study is its apparent role in protecting not only the heating element from corrosion, but also the copper inserts downstream of the heater, as was qualitatively determined by SEM analysis. In comparison to the insert surfaces analyzed following exposure to neutral pH, surface degradation (and consequent roughness) over time appeared reduced, and it would have been of interest to investigate the implications of such changes in terms of bacterial attachment. For example, if surface roughness is enhanced under normal pH conditions, it would be interesting to compare its significance in initiating biofilm formation to other possible biofilm-promoting factors in this situation. Previous studies have demonstrated that surface roughness and topography influences biofilm formation on stainless steel (Percival 1999; George *et al.* 2003), and it would be surprising if it did not exert some influence on biofilm formation in this case. Raising pH has been described as a way of protecting large scale WDSs from corrosion as well as potentially controlling biofilm formation (Wagner and Chamberlain 1997; Camper 2000). However, given the lack of supplementary quantitative data in this case, it is impossible to predict whether the apparent protective effects of sacrificial anode inclusion would be either long-lasting or effective in a larger system, and in turn what the consequences would be in terms of corrosion risk (both chemically and microbially mediated) and biofilm formation. Another important consideration for further studies would be to ascertain the effect of consistent exposure to high pH on non-metallic WDS fittings such as tap outlet fittings

Whilst numerous studies have confirmed the relative toxicity of copper towards microorganisms, and its apparent suitability for use in plumbing systems (Rogers *et al.* 1994; van der Kooij *et al.* 2005; Lehtola *et al.* 2006), culture-based analysis of copper inserts taken from daily and weekly flushed systems suggests that this did not prevent bacterial attachment and biofilm formation. Numerous studies have been conducted into biofilm formation on copper and its associated effects, and all have shown that copper readily supports biofilm formation, albeit to varying degrees in comparison to other materials (Critchley *et al.* 2001; Lehtola *et al.* 2004; Lehtola *et al.* 2005). The rapid initial increase in cell numbers may be due to pioneering microorganisms exploiting the supply of nutrients which is available after the rapid conditioning of a surface with nutrients (Characklis 1990). The initial low numbers observed in the weekly flushed system may be due to the delay in supplementation of this nutrient supply with waterborne nutrients which are added each time the system is flushed.

The confocal microscopy/ image analysis work that was carried out has highlighted the potential for this technology to play an increasingly important role in the context of this work. The data gathered clearly showed the division between daily-/ weekly-flushing and monthly-/ non-flushing, in terms of the biofilm coverage that was observed at the end of the trial. Qualitative analysis of the images of biofilm taken from the daily/ weekly flushed systems demonstrated the existence of substantial mixed-species biofilm, based on morphological observations. It would have been of benefit to have investigated the viability of cells within the biofilm, either by using LIVE/DEAD staining or via the use of a viability stain such as 5-Cyano-2,3-Tolyl-Tetrazolium Chloride (CTC). Other possible future investigations include visualization of the EPS

using fluorescently labelled lectins, as described by Strathmann *et al.* (2002), or employing fluorescence *in situ* hybridization (FISH) to explore various ecological aspects of biofilm formation under these differing conditions. Further work is required to fully exploit the potential of this technology.

This study was conducted over a relatively short period. A study by Lehtola *et al.* (2004) compared biofilm formation in copper and polyethylene (PE) pipes over a one year period. Other studies have run for periods well in excess of the duration of this study (Percival *et al.* 1998), and the benefits of conducting longer studies are obvious, in terms of relatedness to 'real-life' installations, and given that the process of biofilm formation in water distribution systems is slow and subject to a variety of changes over an extended period of maturation (Moritz *et al.* 2010). It is also important to bear in mind that every WDS is unique in terms of construction and environmental conditions, and that a cautionary approach is essential when drawing comparisons with other systems.

In conclusion, daily and weekly flushing were shown to be effective promoters of biofilm formation in this virgin water distribution system, whilst longer stagnation periods were inhibitory. This is believed to be due to the lower frequency of 'nutrient-delivery events' (i.e. flushing) during longer stagnation periods, as well as environmental conditions within the test rig systems subjected to the differing flushing regimes. Future work in this area should involve an assessment of the effectiveness of varying stagnation periods on *pre-formed* biofilm, as this may yield more beneficial knowledge. High pH, induced by the presence of a magnesium sacrificial anode, situated in close proximity to the heater element, was also shown to inhibit biofilm formation and the culturability of microorganisms, an observation which has not previously

been described. Potential may exist for this to be used as a mechanism of biofilm control in small-scale WDSs, but further investigation is required.

Chapter 4. Differential Effects of Thermal Purging on Bacterial Culturability and Viability in Model Warm Water Distribution System Biofilms.

4.1 Introduction

A wide variety of approaches are currently employed in an attempt to control biofilm formation within large-scale water distribution systems (WDS), such as are found in many hospitals and healthcare institutions, as biofilms have been shown to potentially harbour a wide range of pathogens (Momba 2004). All current approaches to pathogen control within WDS have advantages and disadvantages, primarily based on efficacy and cost, and include copper-silver ionisation (Lin *et al.* 1996; Lin *et al.* 1998a; Kusnetsov *et al.* 2001; Kim *et al.* 2008; Silvestry-Rodriguez *et al.* 2008), point-of-use filtration (Ortolano *et al.* 2005; Vonberg *et al.* 2005; Vianelli *et al.* 2006), biocide treatment with chemicals such as chlorine dioxide (Walker *et al.* 1995), ozone (Muraca *et al.* 1987), chlorine (De Beer *et al.* 1994), ultra-violet treatment (Pozos *et al.* 2004) and thermal disinfection (Zacheus and Martikainen 1996; Mouchtouri *et al.* 2007). The mode of action (and cost effectiveness) of these methods varies- some control methods may exhibit a bacteriocidal/ bacteriostatic effect through interference with vital cellular functions (Garcia *et al.* 2009), whilst other methods may rely on physical removal.

Thermal purging (TP) involves the application of water heated in excess of 70 $^{\circ}$C to either the entire WDS, or a section thereof, and is employed mainly as a repeatable, short-term control measure. It was the first documented method of

pathogen control in a hospital situation (Fisherhoch *et al.* 1981; Wright 2000), and its subsequent use has generally been reported as having been employed mainly in response to outbreaks of legionellosis (Fisherhoch *et al.* 1981; Stout *et al.* 1998; Hayes 2006), caused by the generation of infectious aerosols at the water system outlet (Miuetzner *et al.* 1997), rather than as a preventative measure against a broader range of potential pathogens. Many of these potential pathogens may be biofilm-associated, such a *Mycobacterium avium,* which has been shown to be concentrated in poor quality WDSs (Dumoulin *et al.* 1988; Norton *et al.* 2004; Feazel *et al.* 2009). Laboratory studies have shown that the time taken to reduce *Legionella* numbers by one log ranges from 2500 minutes at 45 °C, to less than one minute at 70 °C, with exposure to temperatures above 50 °C bringing about a sharp increase in the rate of loss of viability (Stout *et al.* 1986). Other bacteria commonly associated with *Legionella*, such as *Pseudomonas* spp. have been shown to be less tolerant of high temperature than *Legionella* (Dennis *et al.* 1984).

The exact method of TP application varies according to factors such as system design, the severity and scale of the outbreak, and the infection control policies of the institution involved. The duration of exposure to TP varies greatly, from several minutes (Zacheus and Martikainen 1996) to thirty minutes (Stout *et al.* 1998). The Health and Safety Council currently recommend that, where possible, TP be carried out at a temperature not less than 60 °C for at least one hour (HSC 2008). It is accepted that in order for TP to be effective, then all parts of the system must be exposed equally, although this can be expensive and labour intensive, with potential health and safety risks posed through scalding (Miuetzner *et al.* 1997; Lin *et al.* 1998b), and the even distribution of water at the desired temperature for disinfection may be difficult to achieve in a large

complex system (Stout *et al.* 1986). TP is generally followed by repeated short- and long-term sampling in order to assess its effectiveness, through culturing of sample on appropriate selective media (Ruef 1998).

Previous work on the effectiveness of TP as a control measure has been limited to the reporting of findings within the clinical setting and has focussed primarily on *Legionella*, with little work having been carried out on its effects on the biofilm community as a whole in a laboratory setting.

Given this tendency towards reporting the application of TP in a clinical setting rather than in the laboratory, and in most cases reporting specifically on the control of *Legionella* spp., the aim of this study was to investigate the effects of TP on the culturability and viability of the broader spectrum of bacteria during the early stages of biofilm formation, using a novel model WDS or 'test rig'.

4.2 Materials and methods

4.2.1 Test rig system set-up.

The design and operation of the model warm water system, or 'test rig' used during this experiment is described in detail in chapter 2, section 2.2, page 35.

All eight systems were used, and analyses carried out on both the copper inserts (of which each system was composed) and the attached LLDPE piping which carried effluent from each system. Systems were either thermal-purged (experimental systems), or non-thermal-purged (control systems). The treatment regime relating to the test rig systems is summarized in Table 4.1 (below). This thermal purging regime was undertaken after discussion with DVS regarding current thermal purging practices relating to commercial installations.

Table 4.1 Designation of systems on test rig during current trial (**lhs**- left hand side of system, as viewed from front. **rhs**- right hand side)

System number	*Treatment regime*	*frequency*
1 (lhs)	**Thermally disinfected system**	**Every three days (as per controls on interim days)**
2	Control system (normal flushing)	Every day
3	**Thermally disinfected system**	**Every three days (as per controls on interim days)**
4	Control system (normal flushing)	Every day
5	**Thermally disinfected system**	**Every three days (as per controls on interim days)**
6	Control system (normal flushing)	Every day
7	**Thermally disinfected system**	**Every three days (as per controls on interim days)**
8 (rhs)	Control system (normal flushing)	Every day

4.2.1.1 Description of thermal purging unit and protocol

The unit used to carry out thermal purging is shown in Figure 4.1 (overleaf). The two solenoid plugs shown were attached to the test rig system to be analyzed via the solenoids at the top and bottom of the system, thus bypassing the thermal mixing valve (TMV) and simultaneously allowing flushing to occur.

On day zero of the trial, all systems were manually flushed at a temperature of 41 °C in numerical sequence for thirty seconds using the key switch as described in chapter 2, section 2.2.2, page 36. Flushing was subsequently repeated every 24 hours, with each system remaining stagnant between flushes. On the third day, thermal purge / normal flushing was carried out according to Table 4.1. A thermal purge sequence lasted for a total of one minute, with a maximum temperature of 70 °C being reached after 40 seconds

+/- 2 seconds, and was undertaken in place of the normal flushing sequence. Consequently, purged systems were exposed to 70°C for 20 seconds.

Figure 4.1 Thermal purging unit used during trial, consisting of (A) power supply, (B) solenoid plugs which are simultaneously attached to the solenoids at the top and bottom of each system, (C) key switch unit with digital purge counter, and (D) thermal purge control system.

Effluent water temperature was monitored at the point of exit every 10 seconds using a mercury thermometer. TP was repeated every three days for a total of 27 days. On days when a thermal purge was not scheduled, thermal purged systems were flushed in the same way as control systems.

4.2.2 Culture-based analysis of inserts and LLDPE

Sample collection and culture-based analysis was carried out as described in chapter 2, sections 2.4 and 2.5 (pages 42 and 45). LLDPE samples were taken at the opposite end to that attached to the corresponding test rig system, and

LLDPE sections approximately 20 mm in length were cut using a plastic pipe cutter. The exact length of all sections was recorded in order that the inner surface area could be calculated in each case. Adjustments to the dilution series/ plating range were made as appropriate.

4.2.3 ATP analysis

4.2.3.1 Inserts/ LLDPE

ATP analysis of copper inserts and LLDPE piping was carried out according to the protocol described in chapter 2, section 2.8.1, page 55. LLDPE samples were taken at the point furthest from the test rig system, and LLDPE sections approximately 20 mm in length were cut using a plastic pipe cutter. The exact length of all sections was recorded in order that the inner surface area could be calculated in each case.

4.2.3.2 System effluent

Effluent sample collection and subsequent ATP analysis was carried out according to the protocol described in chapter 2, sections 2.4.3. and 2.8.2 (pages 44 and 56).

4.2.4 SEM analysis

Copper insert and LLDPE piping samples were collected from each system according to the protocol described in chapter 2, section 2.4. LLDPE samples

approximately 5 mm in length were cut using a plastic pipe cutter, at the opposite end to that attached to the corresponding test rig system. Samples were cut longitudinally using a 70% IMS/ flame-sterilized razor blade, in order to reveal the inner surface, and placed into separate 5 ml volumes of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer.

4.2.5 Confocal laser scanning microscopy analysis

4.2.5.1 CTC staining to assess viability of biofilm-associated bacteria

5-cyano-2,3 ditolyl tetrazolium chloride (CTC) was used to assess viability of biofilm-associated bacteria, in association with a nucleic acid counterstain (see Figure 4.2). Biofilm samples were collected from copper inserts and LLDPE sections of known length using cotton wool swabs, moistened in sterile bacteriological (0.85%) saline. The entire inner surface of each section was swabbed thoroughly for 20 seconds. Afterwards each swab was stored at 4 $^{\circ}$C until all swab samples had been collected. Each swab head was removed using scissors which were flame-sterilized using 70% IMS immediately prior to use. The swab head was placed directly into a micro-centrifuge tube containing 500 µl of water which had been taken from the cold feed water supply and filter-sterilized, using a 0.2 µm cellulose nitrate filter, and the sample mixed continuously for one minute using a vortex mixer. Cold feed water was used in an attempt to minimize any negative effects on bacterial metabolism of the removal and disruption procedure. Using metal forceps which were flame-sterilized using 70% IMS immediately prior to use, the swab head was then removed and discarded. CTC solution was prepared by adding 10 µl of a 50

mM stock solution of CTC to the sample, to give a final concentration of 1 mM. The sample was then incubated in the dark at room temperature for four hours, after which time the cells were fixed with 2% v/v formaldehyde, and stored in the dark at 4 °C.

Counterstaining was performed using Syto9, a nucleic acid stain which constitutes part of the BacLight bacterial viability staining kit (Invitrogen, U.K.). A Syto9 stock solution was prepared according to the manufacturer's instructions, to a working concentration of 6 µM, and 100 µl added to each sample, after resuspension of the cells. Tubes were then incubated for 20 minutes in the dark at room temperature. After incubation the samples were filtered onto separate 0.2 µm (pore-size) black nuclepore polycarbonate filters (Whatman, U.K.). A drop of Baclight mounting oil (Invitrogen, U.K.) was applied according to the manufacturer's instructions and a coverslip gently placed on top. Bacteria were imaged using a Zeiss Axioskop LSM510 CLSM (Zeiss, Germany), equipped with a x63 oil immersion objective. Respiring bacteria appeared yellow, due to the formation of red formazan granules within a green cell. Non-respiring bacteria were green (Figure 4.2, below). Monospecies cultures of *Pseudomonas aeruginosa* NCIMB 8626, containing known ratios (1:4, 1:2, 1;1) of live and dead (4% paraformaldehyde-killed) cells were used to provide effective controls in the assessment of staining efficiency, both before and during sample processing (for optimization and checking of staining parameters).

Figure 4.2 Syto9/ CTC stained biofilm bacterial cells. Green cells are non-viable. Yellow cells are viable.

4.2.6 Statistical Analysis

Two sample T-tests (Mann Whitney U test) were performed in order to compare differences within materials due to thermal purging. A *p*-value of <0.05 was used to indicate a significant difference. Microsoft Excel 2007, and SPSS v17.0 were used in data analysis.

4.3 Results

4.3.1 Incoming water parameters

An overview of incoming water parameter data collected at the beginning and end of the study are summarized in table 4.2 (overleaf).

Table 4.2. Overview of incoming water parameters.

Parameter	mains water	cold feed	hot feed
Temperature range, °C	11.5-14.5	15-17.5	69.0-72.0
Water hardness range, dH	2.79-2.81	2.01-2.22	1.98-2.16
pH range	7.44-8.31	7.15-7.88	7.55-7.99
Conductivity range, µS	147-207	177-202	152-224
Total chlorine concentration range, mgl^{-1}	0.6-0.65	0	0
Free chlorine concentration range, mgl^{-1}	0.51-0.58	0	0

4.3.2 Culture-based/ confocal microscopy analysis

A summary of culture-based/ confocal microscopical analysis of bacterial numbers on the two materials in response to frequent thermal purging is presented in Table 4.3. A one minute thermal purge to a maximum temperature of 70 °C on every third day proved to be effective in reducing the numbers of culturable bacteria on both copper and LLDPE. This reduction in numbers was shown to be statistically significant (p-value= 0.001), with a three log reduction observed. The mean count of culturable bacteria on non-thermally purged LLDPE was one log value higher in comparison to copper. Total bacterial counts on LLDPE, assessed using the nucleic acid stain Syto 9, were also

higher. The percentage viability after thermal purging was marginally higher on copper than on LLDPE (21.55% for LLDPE, 25.06% for copper).

Culture based analysis of the test rig effluent after thermal purging revealed a statistically significant (approximately one log value) reduction in total culturable bacteria, in comparison to non thermal purged systems, from 2.21×10^4 cfu ml^{-1} to 1.83×10^3 cfu ml^{-1}. The cold and hot feeds, which supplied the test rig, yielded 4.5×10^4 cfu ml^{-1} and zero culturable bacteria, respectively.

4.3.3 ATP analysis

ATP analysis of the two materials revealed that thermal purging had a statistically significant effect in reducing the level of active biomass within the test rig (Table 4.3). In the case of copper, mean ATP levels were 0.013 pmol cm^{-2} for non-thermal purged copper and 0.0014 pmol cm^{-2} for thermal purged copper ($N=12$, $p<0.05$). ATP levels on LLDPE were 0.0243 pmol cm^{-2} and 0.0003 pmol cm^{-2} for non-thermal purging and thermal purging, respectively, again indicating a highly significant effect ($N=12$, $p<0.05$).

ATP levels in the effluent collected from each system were also reduced in thermally purged systems, from a mean of 0.009 pmol ml^{-1} in effluent from non-thermally purged systems to 0.005 pmol ml^{-1} in effluent from systems subjected to TP. The incoming cold and hot feeds showed ATP levels of 0.0013 pmol ml^{-1}, and 0.0001pmol ml^{-1}, respectively.

Table 4.3 Summary of bacterial counts and ATP levels, in response to TP (* = a statistically significant difference in comparison to control (non-TP) systems).

	Copper		LLDPE	
	control	**thermal purge**	**control**	**thermal purge**
Mean culturable bacteria (cfu cm^{-2}), **C**	7.13×10^{3}	1.36×10^{1} *	6.17×10^{4}	5.81×10^{1}*
SD	**6.47×10^{3}**	**2.59×10^{1}**	**4.28×10^{4}**	**3.61×10^{1}**
% reduction in culturability	**99.81**		**99.91**	
ATP (pmol cm^{-2})	0.013	0.0014*	0.0243	0.0003*
Mean total bacteria (cells cm^{-2})	7.48×10^{6}	7.81×10^{6}	1.55×10^{7}	1.47×10^{7}
SD	**1.71×10^{6}**	**2.04×10^{6}**	**4.28×10^{6}**	**3.40×10^{6}**
Mean viable bacteria, **V** (cells cm^{-2})	**3.09×10^{6}**	**1.96×10^{6}**	**6.33×10^{6}**	**3.18×10^{6}***
SD	7.86×10^{5}	9.64×10^{5}	1.74×10^{6}	1.3×10^{6}
% viability, **T**, (**V/T**)X100	**41.24**	**25.06**	**40.73**	**21.55**
No. of bacteria in VBNC state (**V-C**) (cells cm^{-2})	3.08×10^{6}	1.95×10^{6}	6.33×10^{6}	3.18×10^{6}
% viable bacteria in non-culturable state (VBNC), ((V-C)/V)x100	**99.67**	**99.99**	**99.53**	**99.97**

4.3.4 SEM analysis

SEM analysis of the non-thermally purged materials showed clear microcolony formation (Figures 4.3 and 4.5, pages 104/105). Qualitative assessment of the communities on the two materials revealed differences, in terms of the apparent surface roughness, the microbial community present and the structure of the microcolonies which had formed. In the case of LLDPE, the surface appeared relatively smooth in texture, and the microbial community highly diverse, with both rod-shaped and coccoidal bacteria of various sizes well represented. Bacteria were observed both in close association with each other and individually, and the microcolonies formed appeared to be closely associated with the substratum. Copper, on the other hand, exhibited a high degree of surface roughness. Biofilm microcolonies appeared to have a more organized structure, and were primarily composed of bacteria which were small, coccoidal and uniform in appearance (Figure 4.5), although the presence of rod-shaped bacteria was also noted.

SEM analysis of the thermally purged materials did not suggest any significant changes to the substratum itself in response to this treatment. LLDPE microcolonies appeared to be composed primarily of long rods associated closely with each other and the substratum, being encased in an EPS-like substance (Figure 4.4). Copper biofilm microcolonies of the type observed on non-thermally purged samples were observed less frequently, and there appeared to be a tendency towards a sparser, more varied community, grouped into loose co-associations (Figure 4.6).

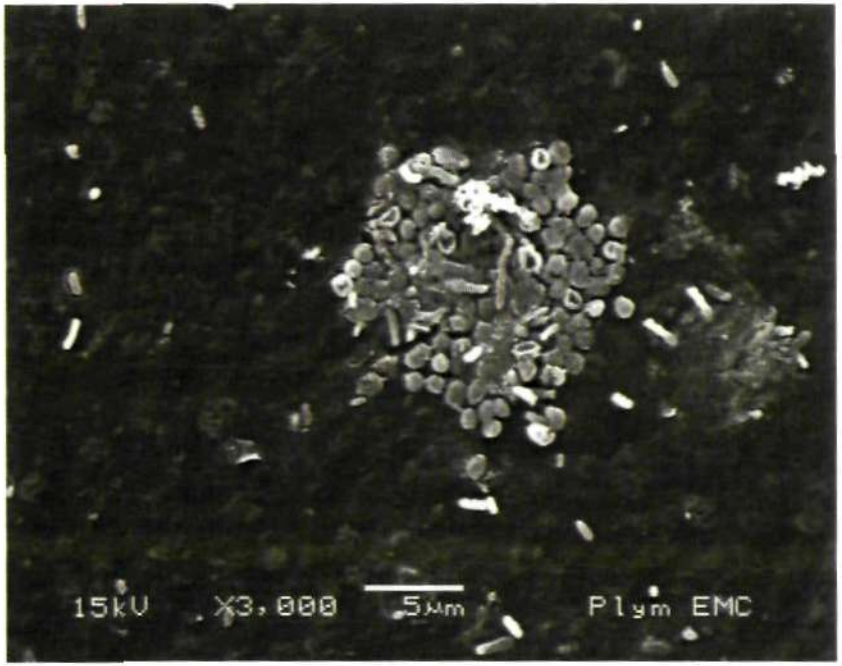


Figure 4.3 Scanning electron micrograph of LLDPE after 28 days non-TP conditions

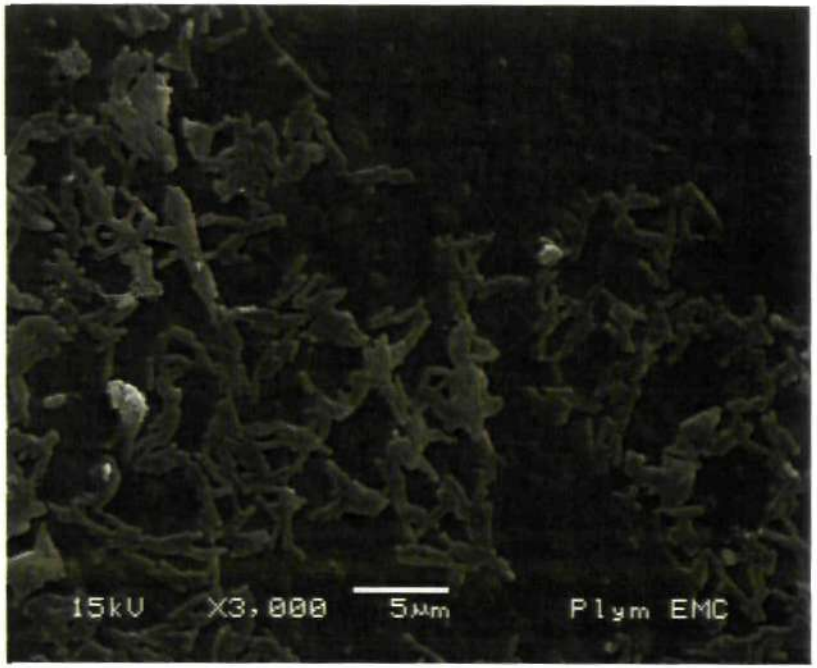


Figure 4.4 Scanning electron micrograph of LLDPE after 28 days TP

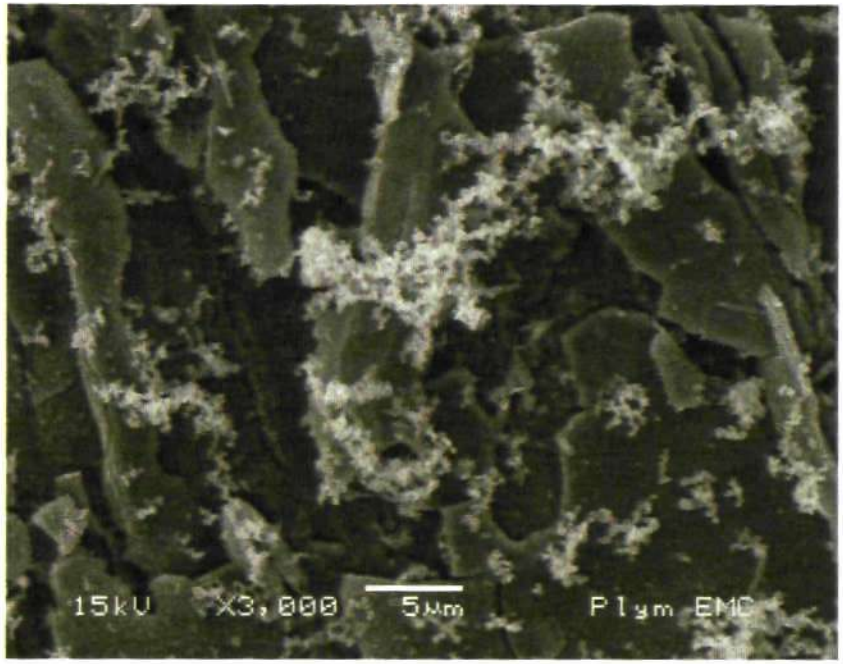


Figure 4.5 Scanning electron micrograph of copper after 28 days non-TP conditions

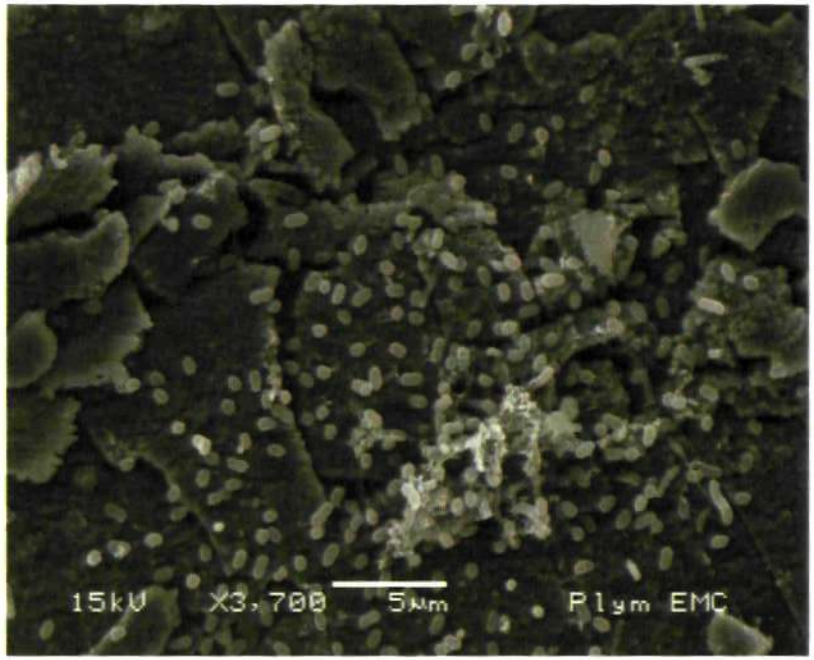


Figure 4.6 Scanning electron micrograph of copper after 28 days TP

4.4 Discussion

The results presented show that thermal purging (TP) significantly reduces the culturability of biofilm-associated bacteria by several orders of magnitude, whilst cell numbers and viability, assessed using the redox dye 5-cyano-2,3 ditolyl tetrazolium chloride (CTC), remain at a relatively high level after frequent exposure to this regime. Under such circumstances, it is suggested that most of the remaining bacteria are in the viable but non-culturable (VBNC) state, and that this state has been induced in the surviving bacterial population (which was previously culturable), through the use of thermal purging. This phenomenon has previously been reported in *Legionella pneumophila* (Chang *et al.* 2006; Garcia *et al.* 2007), and it has been suggested that resistance to thermal purging may be linked to physiological changes associated with the induction of the VBNC state, such as the increased production of protective proteins (Chang *et al.* 2006). This VBNC state has been suggested as the default metabolic state for the majority of biofilm microorganisms, particularly in oligotrophic environments where efficient energy conservation is paramount, with their ability to grow on bacteriological media diminished through either dormancy or cellular damage caused (in this case) by TP.

That biofilm formation is key in providing protection for microorganisms against a range of control measures is well documented, with the EPS providing an efficient and multi-functional 'buffer-zone' in which the bacteria are protected (Flemming *et al.* 2007). Consequently, for TP to be effective, this barrier must be breached i.e. compositional elements (which may vary from one biofilm to another) such as eDNA/ proteins and exopolysaccharides must be at least partially degraded, in order for disinfection to occur. As a considerable number

of bacteria remained viable after TP, it is safe to assume that there is high potential for bacterial regrowth, and that such regrowth would mainly arise from the biofilm which was confirmed by SEM analysis to still be present after the treatment. If the experiment had continued, biofilm levels may even have exceeded those observed when no thermal purging is employed, given the injection of potential nutrients which the test rig would have received, such as corrosion products from the heater, as well as organic matter present from the dead and decaying microorganisms generated during TP but not necessarily removed by flushing. Bacteria such as *L. pneumophila* have been shown to be capable of necrotrophic growth (Temmerman *et al.* 2006), and this is may be an unfortunate side effect of TP, should it be employed in a less than optimal fashion. Incomplete and non-sustained efficacy of TP has been observed following attempts to suppress *Legionella*, due primarily to the presence of biofilm facilitating regrowth (Stout *et al.* 1998; Mouchtouri *et al.* 2007), as well as other factors, such as the presence of protozoa such as *Acanthamoeba*, which may provide extra protection to those bacteria capable of survival within them (Storey *et al.* 2004), and re-colonization via the cold water network (Blanc *et al.* 2005). Another side effect of the use of high temperatures in controlling bacteria in water systems, is the increased demand for, and reduced efficacy of, chlorine (Muraca *et al.* 1987), although in this case the water used during the trial was non-chlorinated, due to the presence of a water storage tank further back in the system allowing chlorine diffusion into the space above the water.

In between thermal purges, the system was flushed every 12 hours, for 30 seconds. Consequently, a lengthy stagnation period was enforced on all systems. Given that stagnation of microbially contaminated water systems is believed to be a known promoter of bacterial growth (Ciesielski *et al.* 1984;

Manuel *et al.* 2010; see also chapters 3 and 5 of this thesis), and that bacterial detachment has been shown to occur soon after sudden increases in flow rate (Lehtola *et al.* 2006) this may have served to enhanced the regrowth of microorganisms after TP, as well as having the additional effect of facilitating biofilm detachment (and subsequent removal from the test rig) before disinfection had occurred. With this in mind, in a clinical situation, the practice of routine regular flushing would seem important in that not only does it avoid the negative effects of stagnation by increasing adherence and reducing biofilm thickness, but it may consequently increase the efficacy of TP.

ATP analysis served to confirm the effectiveness of TP in reducing the activity, if not the number of cells, within experimental systems, and this reduction was seen to be greater on thermal purged LLDPE than copper. This is thought to be because LLDPE possesses greater insulating properties than copper, and so loses heat less rapidly to the environment. This may be enhancing the efficacy of TP by subjecting what appears to be a thinner biofilm to a longer exposure time at high temperature. The culture based/ confocal analysis results would appear to support this. Whilst TP may be marginally more effective on LLDPE, it should be remembered that counts of culturable bacteria on non-thermally purged material were higher than was observed on copper, and that TP may enhance the leaching of potential nutrients into the water column.

Although SEM sample preparation is a highly disruptive process, analysis of the two materials showed clear differences between them in terms of the types of biofilms formed. The closer association of biofilm with the surface which was observed on LLDPE may be a consequence of the higher shear stress to which they are subjected, given the smaller bore of LLDPE piping in comparison to copper. Increasing shear stress has been shown to favour the formation of thin,

firmly attached, and dense biofilms (Melo and Vieira 1999), and it is possible that the variation in biofilm structure/ composition (such has been demonstrated during this study), induced by differences in local environmental conditions, may have consequences for the efficacy of thermal purging. Copper biofilms were seen to contain large numbers of what appear to be small coccoidal cells. These are possibly ultramicrobacteria, as described by Geldreich (1996). Ultramicrobacteria are 'normal' bacteria which are capable of surviving for extremely long periods of time in low nutrient waters (Geldreich 1996). The formation of seemingly more organized microcolony structures on copper may be due to the fact that these bacteria are exposed to a lower shear stress and are therefore seeking to optimize their ability to survive in a harsh oligotrophic environment by adapting the biofilm structure accordingly and incorporating features such as water channels etc.

In this study, TP was carried out every three days, a frequency which was deemed representative of current industry practice, upon installation of new, small-scale systems. Adopting this approach may actually reduce TP efficacy by effectively allowing a 'recovery and reinforcement' period, resulting in an enhanced resistance and regrowth with each round of purging.

Another aspect of the TP regime which requires further optimization is the duration of exposure. During this trial, one minute purges were conducted on each system, with 70 °C- the 'disinfection temperature'- being reached after approximately 40 seconds at the tap outlets, which were the only point at which temperature could be effectively measured. Whilst a longer TP duration would have been preferable, limitations were imposed by the specifications of the heater used, as it was essential that all systems were treated in the same way, and at the same time point. Longer purges were not possible due to the inability

of the heater to maintain the required temperature. Thus, at any point within the test rig the duration of exposure to disinfection temperature was significantly less than the minimum recommendations for disinfection published in a number of studies carried out in hospitals.

Further work is thus required in order to optimize TP efficacy within the test rig. It is essential to bear in mind that the conditions chosen as optimal for this particular installation, may not be directly translatable to another installation, which may differ significantly in a variety of ways, such as layout, size, composition and water quality. Complete eradication of biofilm and biofilm-derived pathogenic bacteria may not actually be essential for the safe operation of water systems (Darelid *et al.* 2002), and is certainly difficult to achieve (Stout *et al.* 1998). Preventing biofilm formation would appear to be a more logical approach than treating it (Simões *et al.* 2010), and in effect this trial has been an attempt at achieving this end. On this basis, given that the results indicate a remaining resident population within thermal purged systems, it may be concluded that this has not been achieved successfully. However, it has provided a useful starting point for further similar investigations and raised some interesting questions with regards to the physiological responses of bacteria to this mode of control, and further studies on the effect of VBNC state induction on the pathogenicity of a variety of clinically relevant microorganisms would be of potential interest in a clinical setting.

Chapter 5 Investigations into the Effect of Material Choice on Biofilm Formation in a Model Warm Water Distribution System

5.1 A comparison into the biofilm formation potential of four commonly used plumbing materials

5.1.1 Introduction

Large scale water distribution systems (WDSs), including those found in healthcare facilities such as hospitals, may be composed of a variety of different materials, with material choice being governed by factors such as cost and durability (Percival 1999). Throughout WDSs, a wide variety of factors, such as design, age of installation, temperature, and flow effects, exert effects on the biofilms within. Materials commonly in use in modern plumbing systems include metals such as copper and stainless steel, rubber-based materials such as ethylene propylene diene monomer (M-class) rubber (EPDM) and plastics such as cross-linked polyethylene (PEX). Various studies have been carried out on these and many other materials, and have shown that type and degree of formation of biofilm on these surfaces may vary significantly, although local environmental conditions also play a significant role (Kerr *et al.* 1999; Kielemoes *et al.* 2000; Zacheus *et al.* 2000; Momba 2004; Lehtola *et al.* 2005; Traczewska and Sitarska 2009).

The majority of biofilm formation in water distribution systems occurs on the pipes, simply because they constitute the greatest surface area available for contamination, and no single material has been developed for use in plumbing

systems which is resistant to biofilm formation (Rogers *et al.* 1994; Camper 2000), even in the presence of high disinfectant concentrations (Momba 2004). Indeed, pipe material characteristics have been shown to have a direct and profound influence on the amount, rate and type of biofilm formation, the subsequent prevalence of potential pathogens in the water, and the effectiveness of disinfection regimes such as UV treatment, ozonation and chlorination in their control (Zacheus *et al.* 2000; Lehtola *et al.* 2005). Consequently, there is a pressing need to continually improve our understanding of bacteria-surface material interactions if the negative effects of biofilm formation in large scale water systems are to be avoided, such as release of potential pathogens into the water and the accelerated corrosion of metal surfaces which is attributed to multispecies biofilms (Rittman 2004).

The influence that pipe materials exert on bacterial adhesion and biofilm formation are rooted in characteristics such as surface structure and chemical composition. For example, surface roughness has been shown to greatly influence bacterial attachment on stainless steel (Arnold and Bailey 2000), and may vary greatly not only between material types, but also between different grades of the same material.

Copper is the most popular modern plumbing material (Wagner and Chamberlain 1997; Percival 1999) for reasons such as its suitability in terms of cost and the ease with which it can be machined into a wide variety of pipes and fittings. Consequently, it is widely used for this purpose. It is also deemed highly suitable due to its perceived antimicrobial properties. However, studies have shown that whilst the antimicrobial properties of copper are employed to good effect in, for example, copper-silver ionization biofilm control systems, they are over time negated at the pipe surface by the formation of biofilms largely

through the chelation of metal ions within the EPS matrix (Starkey *et al.* 2004). Certain biofilm-bound microorganisms may also exhibit some copper-resistance (Kielemoes and Verstraete 2001). The formation of biofilms on copper pipes has also been shown to induce characteristic pitting corrosion known as 'cuprosolvency' which may cause problems such as pipe failure and public health concerns (Critchley *et al.* 2001; Pavissich *et al.* 2010). Stainless steel is an alloy metal of which there are many types and grades (Percival 1999), and is viewed as a possible alternative to copper. In addition to iron, it may contain molybdenum, nickel and chromium, which facilitates the effective resistance to corrosion over long periods, due to the formation of a thin layer of chromium or potentially antimicrobial molybdenum-based oxides at the material surface (Percival 1999). Numerous studies have been carried out with regards to its biofilm-supporting capabilities (Pedersen 1990; Percival *et al.* 1997; Percival *et al.* 1998a; Percival *et al.* 1998b; Percival 1999; Arnold and Bailey 2000; Kielemoes *et al.* 2000; Zacheus *et al.* 2000).

PEX and EPDM are primarily used in flexible plumbing hoses, with the latter also used in fittings such as sealing gaskets. Studies have shown that the tendency of PEX and related materials to leach biodegradable substances such as phosphates into the water leads to the promotion of biofilm formation, particularly in the weeks immediately following installation (Lehtola *et al.* 2004), and therefore debate continues as to the merits of using such materials in plumbing situations.

The aim of this study was to compare the biofilm formation capabilities of copper, stainless steel, PEX, and EPDM, over an 84 day period.

5.1.2 Materials and methods

5.1.2.1 Test rig system set-up

The design and operation of the model warm water system, or 'test rig' used during this experiment is described in detail in chapter 2, section 2.2, page 35. The experiment was carried out as two three month trials (designated 3A and 3B), in order that the materials under investigation-copper, Stainless Steel 316 (SS316) , PEX and EPDM- could be accommodated in such a way as to satisfy the need for sound statistical analysis. All systems were flushed twice daily at a temperature of 41 $^{\circ}$C for 30 seconds, using the auto-flushing/ manual flushing facility as appropriate.

5.1.2.2 Description of test materials

79 mm, 13 mm (ID) copper pipe inserts were used. Lengths of previously unused, Water Regulations Advisory Scheme (WRAS)- approved EPDM pipe were removed from lengths of braided stainless steel hose, cut into identical 79 mm sections using scissors (ID 10 mm), and inserted into six virgin 79 mm copper pipe inserts. These copper/ EPDM assemblies were then fitted into the designated test rig system as described in Table 5.1. This process was repeated for PEX (ID 10 mm).

In the case of SS316, six inserts of identical dimensions to copper inserts were fitted used as shown in Table 5.1. As stainless steel is much harder than copper, the external surface of the middle third of each insert had been filed

down before receipt, in order to facilitate cutting and sampling, whilst maintaining the integrity of the material (Figure 5.1, overleaf).

Table 5.1 Allocation of test materials to test rig systems during trials 3A and 3B.

Trial	System numbers	Test material	Notes
A	1,4	Copper	Flushed twice daily, 41 °C, 30 seconds
	2,5,7	EPDM	Flushed twice daily, 41 °C, 30 seconds
	3,6,8	PEX	Flushed twice daily, 41 °C, 30 seconds
B	1,3,5	Copper	Flushed twice daily, 41 °C, 30 seconds
	2,4,6	SS316	Flushed twice daily, 41 °C, 30 seconds

5.1.2.3 Culture-based analysis

5.1.2.3.1 Inserts

Culture-based analysis was carried out as described in chapter 2, section 2.5.1, page 45.

In the case of EPDM and PEX, the material was carefully removed from the copper insert in which it was housed using forceps which were flame-sterilized using 70% IMS immediately prior to use. The material was then cut using laboratory scissors which were flame-sterilized using 70% IMS immediately

prior to use. In all cases, the exact length of each pipe sample (approximately 15 mm) was recorded.

In the case of SS316, cutting was carried out on the thinnest point of the insert using a rotating pipe cutter, at the point indicated in Figure 5.1 (below)

Figure 5.1 Diagrammatic representation of an SS316 insert. External arrow illustrates cutting point, and internal arrow (length 20 mm) indicates the internal area sampled for culture based analysis. Total insert length is 79 mm.

The tenfold dilution series and plating regime was adjusted for each material according to expected numbers of culturable bacteria. The non-selective agar R2A and the *Pseudomonas*-selective agar CFC were used at each sampling point throughout the trials (i.e. every 28 days), whilst samples were also plated onto *Legionella*-selective BCYE/GVPN agar after 84 days. *P. aeruginosa* NCIMB 8626 and *L.pneumophila* NCIMB 12821 were used as positive quality control organisms.

5.1.2.3.2 Effluent

Culture-based analysis of the effluent was carried out as described in chapter 2, section 2.5.2, page 47. The tenfold dilution series and plating regime was adjusted for each material accordingly, and R2A, CFC and BCYE/GVPN agars used as described previously.

Culture-based analysis of the cold feed, hot feed, and mains water was also carried out at each sampling point, and in the same way as described above.

5.1.2.3.2 Culture of protozoa from test rig

Attempts were made to culture and tentatively identify protozoan species associated with EPDM biofilms. Duplicate 100µl volumes of the 10^{-1} and 10^{-2} dilutions used for culture-based analysis were used to inoculate individual 5 ml volumes of Page's amoeba saline solution. This was then poured onto the surface of individual uninoculated R2A agar plates. These plates were then incubated at 20°C for one week, after which time conventional light microscopy techniques were used to check for the presence of protozoa.

5.1.2.4 ATP analysis

5.1.2.4.1 Inserts

ATP analysis of inserts was carried out according to the protocol described chapter 2, section 2.8.1, page 55. In the case of all materials bar copper, pipe samples were prepared for analysis as described in section 5.1.2.3.1. The exact length of all pipe samples was recorded in order that the inner surface area could be calculated in each case.

5.1.2.4.2. Effluent

ATP analysis of effluent from each system was carried out according to the protocol described in chapter 2, section 2.8.2, page 56.
The ATP content of the cold feed, hot feed, and mains water was also recorded in each case.

5.1.2.5 SEM analysis

Sample preparation and analysis of inserts was carried out as described in chapter 2, section 2.6, page 48.

Pieces of EPDM/ PEX approximately 10 mm in length were cut using scissors which were flame-sterilized using 70% IMS immediately prior to use. A sterile razor blade used to cut the pieces of PEX longitudinally, in order to expose the inner surface, due to the greater resistance of PEX to longitudinal cutting.

SS316 pipe samples approximately 10 mm in length were cut from using a rotating pipe cutter. Metal shears were used to cut the pipe sample longitudinally, whilst it was still partially attached to the main body of the insert. This approach yielded small pieces for analysis, measuring approximately 5 mm x 10 mm. Cut samples were labelled on the reverse (i.e. non-imaged) side using a diamond pen.

5.1.2.6 Confocal laser scanning microscopy analysis

Confocal microscopical analysis of materials was carried out as described in chapter 2, section 2.7, page 49, using the nucleic acid stain Syto9 in the assessment of surface coverage.

The Live/Dead Baclight bacterial viability kit was employed to qualitatively assess bacterial cell viability on the different materials. Application of this combination stain to the sample was carried out as described in chapter 2, section 2.7.1, page 49, and visualization carried out using an LSM 510 confocal microscope (Zeiss, Germany).

Attempts were also made to stain the EPS portion of the biofilm on all materials, using a Cy3-tagged lectin, Concanavalin A (MWG Biotech, Germany). A stock solution of the lectin stain, previously prepared to a concentration of 10 μg ml^{-1} in filter-sterilized water and frozen at -20 °C, was thawed to room temperature and then applied to the pipe surface according to the protocol described for Syto9/ Live/Dead staining in chapter 2, section 2.7, page 49. Samples were stained for one hour in the dark at room temperature, and then washed by gentle sequential transfer through three 3 ml volumes of sterile bacteriological saline. Syto9 was then used to visualize bacterial cells within the EPS matrix, and was applied to the sample as described in chapter 2, section 2.7, page 49.

5.1.2.7 Statistical analysis

Data analysis (paired T-tests, Kruskal- Wallis/ one-way ANOVA and *post hoc* LSD tests as appropriate) was carried out using Microsoft Excel and SPSS V17.0 (SPSS Ltd., Chicago, U.S.). A *p*-value of <0.05 was used to indicate a significant difference.

5.1.3 Results

5.1.3.1 Water parameters

An overview of the results of water parameter analysis is given in Table 5.2 (overleaf).

Table 5.2 Overview of incoming water parameters.

parameter	mains water	cold feed	hot feed
Temperature range, °C	12.5-16.5	15-18.5	68.5-71.5
Water hardness range, dH	1.97-2.03	1.88-2.25	2.01-2.21
pH range	7.44-8.01	7.25-7.65	7.30-7.59
Conductivity range, µS	167-217	178-211	158-199
Total chlorine concentration range, mgl^{-1}	0.6-0.68	0	0
Free chlorine concentration range, mgl^{-1}	0.53-0.58	0	0

5.1.3.2 Culture-based analysis

5.1.3.2.1 Inserts

Figure 5.2 (overleaf) shows the results of culture-based analysis of all materials. After 28 days all materials supported high counts of culturable aerobic bacteria (CAB), indicating rapid initial colonization in all cases. EPDM was shown to support significantly higher numbers than the other three materials (7×10^6 cfu cm^{-2}, $p<0.05$), with numbers of EPDM-associated CAB continuing to increase at a steady rate post-28 days, to a statistically significant level of 9.43×10^7 cfu cm^{-2} after 84 days ($p<0.05$). No statistically significant increases were observed on the other three materials post-28 days. Indeed, copper and stainless steel

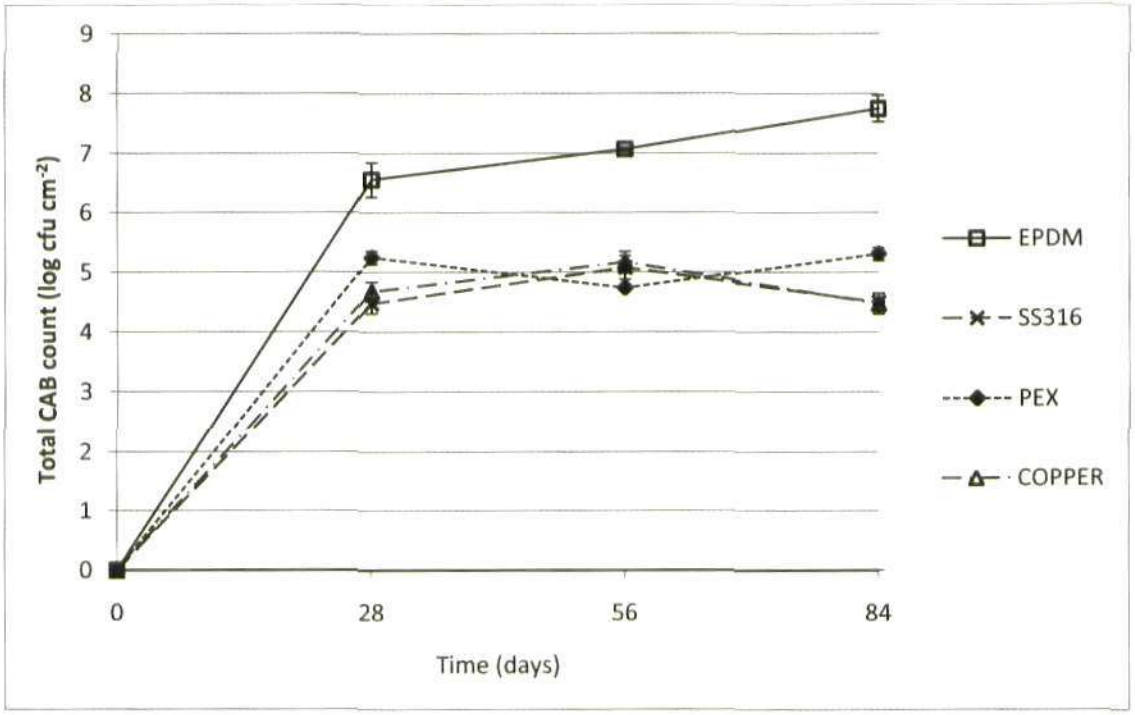


Figure 5.2 Total culturable aerobic bacteria counts on SS316, EPDM, PEX and copper test rig inserts over 84 days. (error bars = SE).

316 showed a marginal drop in CAB numbers after 84 days, to a value of 3.13 x 10^4 cfu cm^{-2}. After 84 days, CAB levels associated with PEX were significantly higher than was observed on both copper and SS316 ($p<0.05$). However, PEX counts were not significantly higher in comparison to numbers seen at day 28 ($p>0.05$).

Counts of culturable *Pseudomonas* spp. indicated a higher level of colonization on EPDM and PEX than was observed on copper and SS316. Indeed, no culturable *Pseudomonas* spp. were isolated from the two metals. In contrast, culturable *Pseudomonas* spp. levels on EPDM were 1.08 x 10^5 cfu cm^{-2} after 28 days, increasing to 2.56 x 10^5 cfu cm^{-2} after 84 days. This indicated that in the case of EPDM, culturable *Pseudomonas* spp. were present in greater numbers than the *total CAB populations* of both copper and SS 316. PEX was also shown to support culturable *Pseudomonas* spp. at a level which increased over time to a maximum of 8.36 x 10^3 cfu cm^{-2} after 84 days.

Attempts to culture *Legionella* spp. from all samples after 84 days were unsuccessful, with no colonies cultured from copper or stainless steel 316. In the case of EPDM and PEX, considerable overgrowth of BCYE plates with non-*Legionella* bacteria was observed. The majority of these microorganisms were confirmed to be *Pseudomonas* spp., *via* culturing of 25 randomly selected colonies onto CFC agar, followed by oxidase testing.

5.1.3.2.2 Effluent

Figure 5.3 (overleaf) shows the results of culture-based analysis of the effluent associated with the test rig.

After 28 days, total CAB numbers associated with effluent samples collected from systems containing EPDM were significantly higher than were observed from systems associated with other materials, reaching a peak level of 3.97 x 10^5 cfu ml^{-1} after 28 days. After 84 days, total CAB numbers associated with EPDM system effluent had dropped (3.38 x 10^5 cfu ml^{-1}), but remained significantly higher than was observed at the start of the experiment ($p<0.05$). Comparison with effluent from systems containing copper, SS316 and PEX after 84 days indicated significantly higher levels of CAB ($p<0.05$). In the case of effluent associated with systems containing PEX, numbers of CAB increased until 56 days, after which time a drop to approximately day zero levels was then observed. CAB numbers associated with both copper and SS 316 effluent did not differ significantly from each other. Culturable *Pseudomonas* spp. were cultured from the effluent associated with all materials throughout the experiment. Colony counts were low relative to the total CAB, with mean values for all effluent samples ranging from 3.87 x 10^1 cfu ml^{-1}, to 1.34 x 10^2 cfu ml^{-1}

during the experiment, following an initial mean peak value of 8.54 x 10^2 cfu ml^{-1} at day zero.

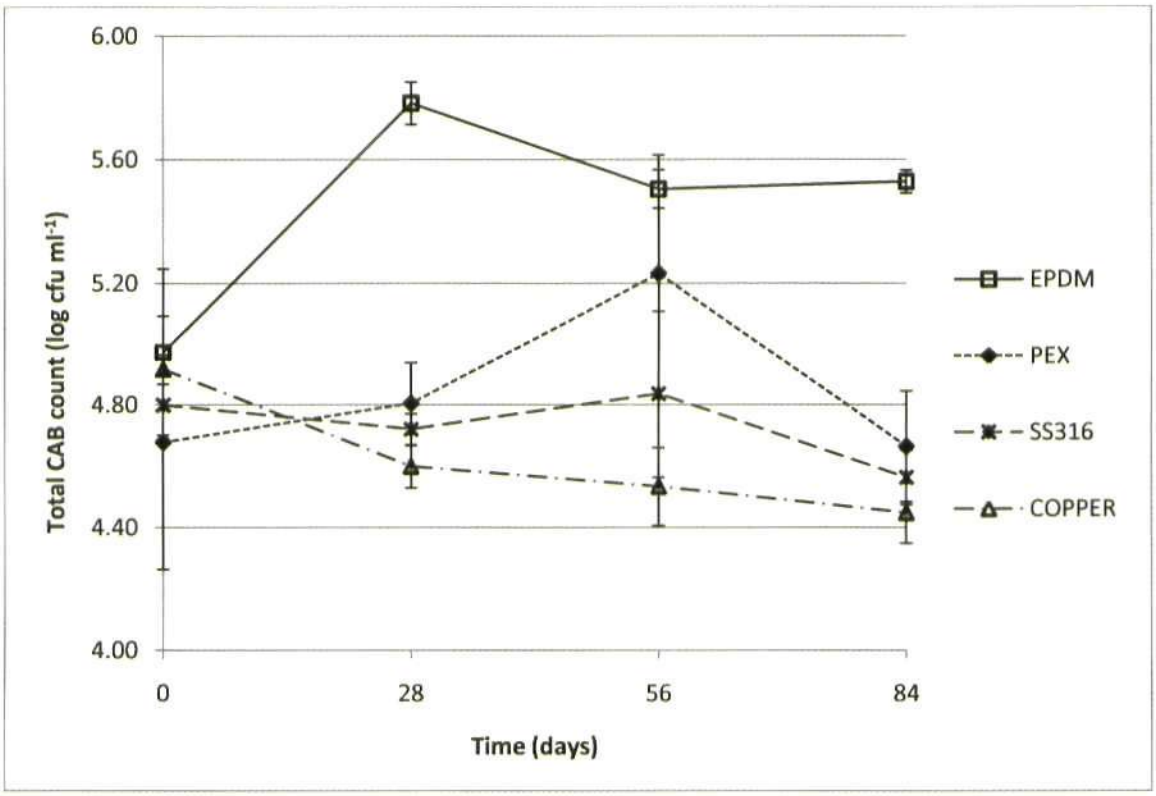


Figure 5.3 Total CAB associated with test rig system effluent over 84 days (Error bars = SE).

No statistically significant changes in effluent-associated culturable *Pseudomonas* spp. numbers were observed during the experiment. No relationship was identified between effluent-associated culturable *Pseudomonas* spp. numbers and material.

Overgrowth of BCYE/GVPN agar plates by non-*Legionella* species was once again observed, and culturing of 25 randomly selected isolates on CFC agar/ oxidase testing indicated that these were primarily culturable *Pseudomonas* spp.

5.1.3.3 ATP analysis

5.1.3.3.1 Inserts

Figure 5.4 (below) shows the results of ATP analysis of materials associated with test rig systems.

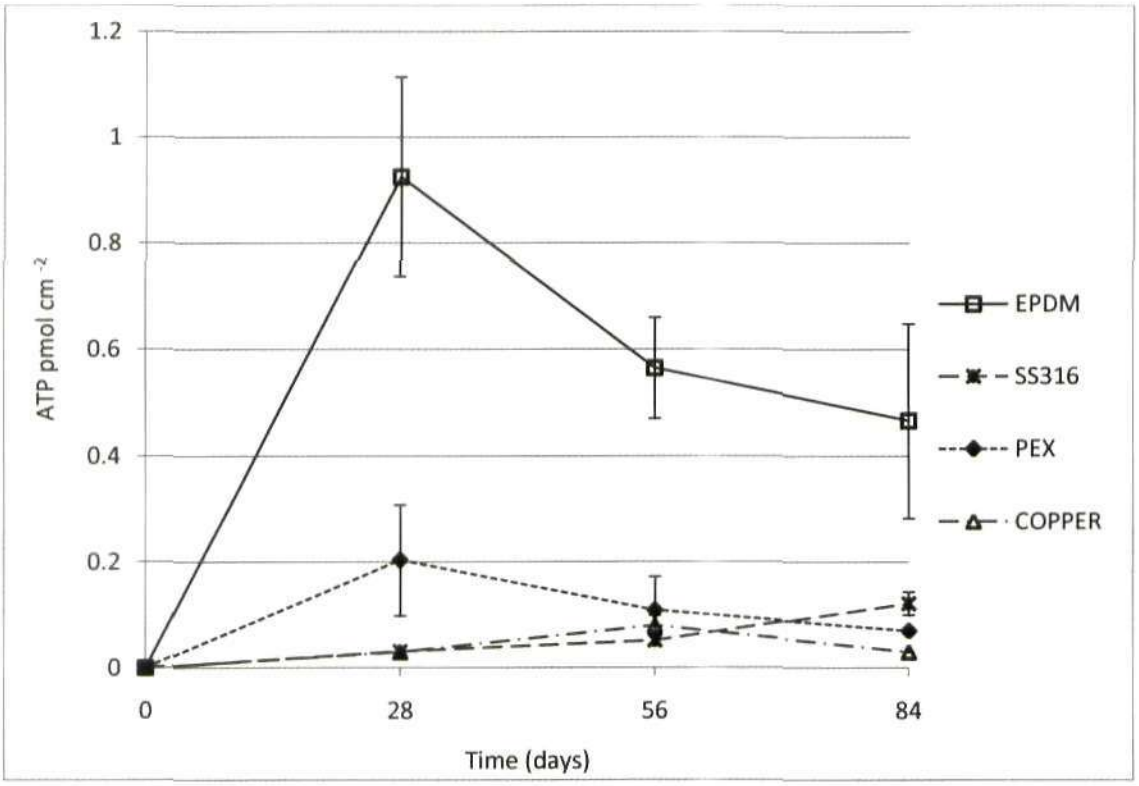


Figure 5.4 ATP concentrations associated with on SS316, EPDM, PEX and copper *test rig inserts over 84 days (Error bars=SE)*

ATP levels associated with EPDM were significantly higher than were observed on other materials at all times post-day zero. A peak mean level of 0.93 pmol cm^{-2} was recorded at 28 days, followed by a statistically significant drop to a mean value of 0.47 pmol cm^{-2} ($p<0.05$) at day 84. Similarly, PEX exhibited a peak in detected ATP at 28 days, and a subsequent gradual drop by the end of

the experiment, although the values observed in this case were significantly less than in the case of EPDM.

SS316 showed a gradual, low-level increase in ATP levels throughout the experiment. At day 84, ATP levels on this material were significantly greater than was observed on both PEX and copper ($p<0.05$), whilst copper itself was shown to support low ATP levels relative to all other materials, with no significant net increase in ATP levels post-day 28, although a marginal steady increase ATP concentration was observed until day 56.

5.1.3.3.2 Effluent

Figure 5.5 (overleaf) shows the results of ATP analysis of effluent associated with test rig systems.

ATP levels associated with EPDM system effluent were significantly higher than were observed on other materials at all times post-day zero, with a peak mean concentration of 0.64 pmol ml^{-1} recorded after 28 days, followed by a gradual drop in concentration to 0.56 pmol ml^{-1} at day 84 ($p<0.05$). In contrast, mean ATP concentrations associated with PEX system effluent exhibited a marginal but statistically insignificant increase over time ($p=0.11$). A marginal net increase was also observed in the case of SS316, with ATP concentrations at 28 and 56 days marginally exceeding those observed in PEX system effluent, before dropping to a concentration which was significantly lower than the final value recorded from PEX system effluent. In contrast, copper system effluent ATP concentrations remained consistently low. Indeed, in this case the ATP concentration recorded after 84 days was not significantly different from the mean value recorded at the start of the experiment.

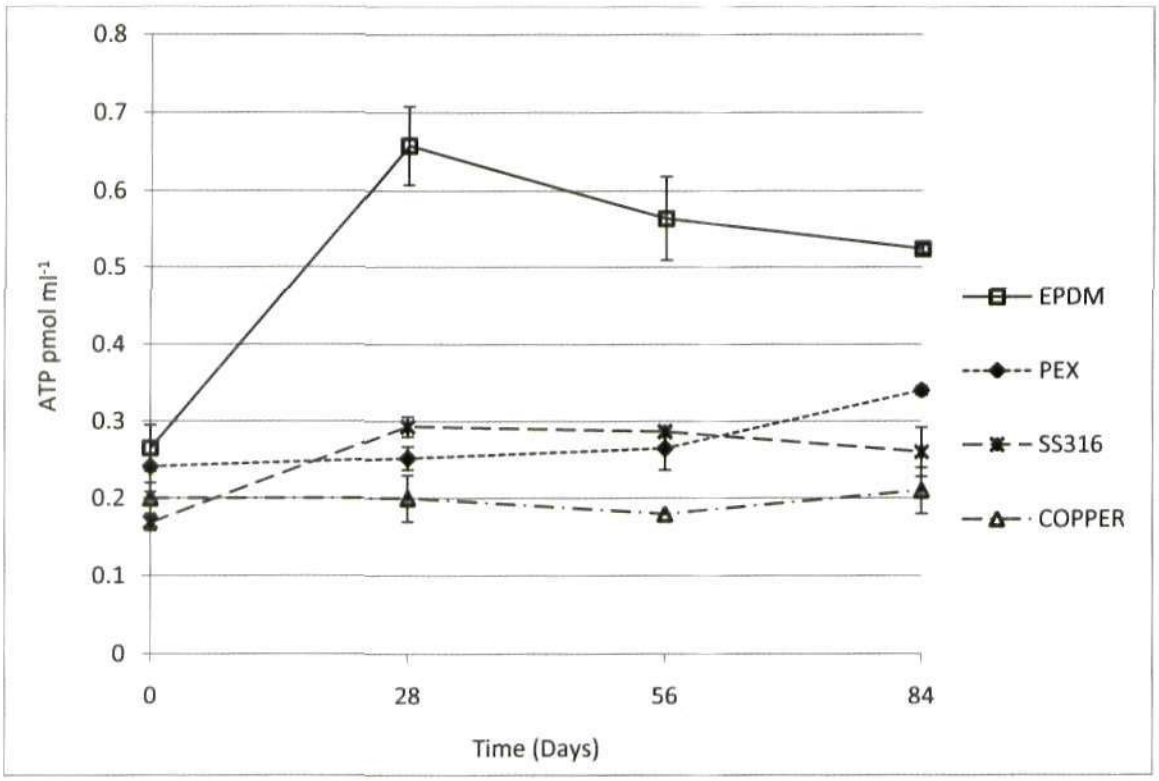


Figure 5.5 ATP concentrations associated with test rig system effluent over 84 days (Error bars=SE)

5.1.3.4 SEM analysis

Qualitative assessment of SEM samples of virgin (i.e. unexposed) material showed clear differences between them, primarily in terms of surface roughness. Copper and SS316 appeared to present a highly irregular, cracked surface. In the case of copper, an amount of seemingly loose material, or 'flaking', was observed. EPDM also demonstrated a high degree of surface roughness and was punctuated at irregular intervals by holes of unknown depth and of irregular size (mean diameter 3.53 µm, SD= 3.33 µm, N=25), an example of which is shown in Figure 5.6 (overleaf). In contrast, PEX did not appear to demonstrate a high degree of roughness and surface irregularities.

There were clear differences between the biofilms which formed on each material, in terms of biomass, overall community diversity, and biofilm structure. In the case of copper, discrete microcolony formation was observed throughout the experiment (Figure 5.7(a) and 5.7(b)). These microcolonies appeared to be composed of small coccoidal cells which were uniformly sized. However, rod-shaped bacteria were also observed, particularly at day 28. Microorganisms appeared to be arranged in a highly structured manner, with a number of pores

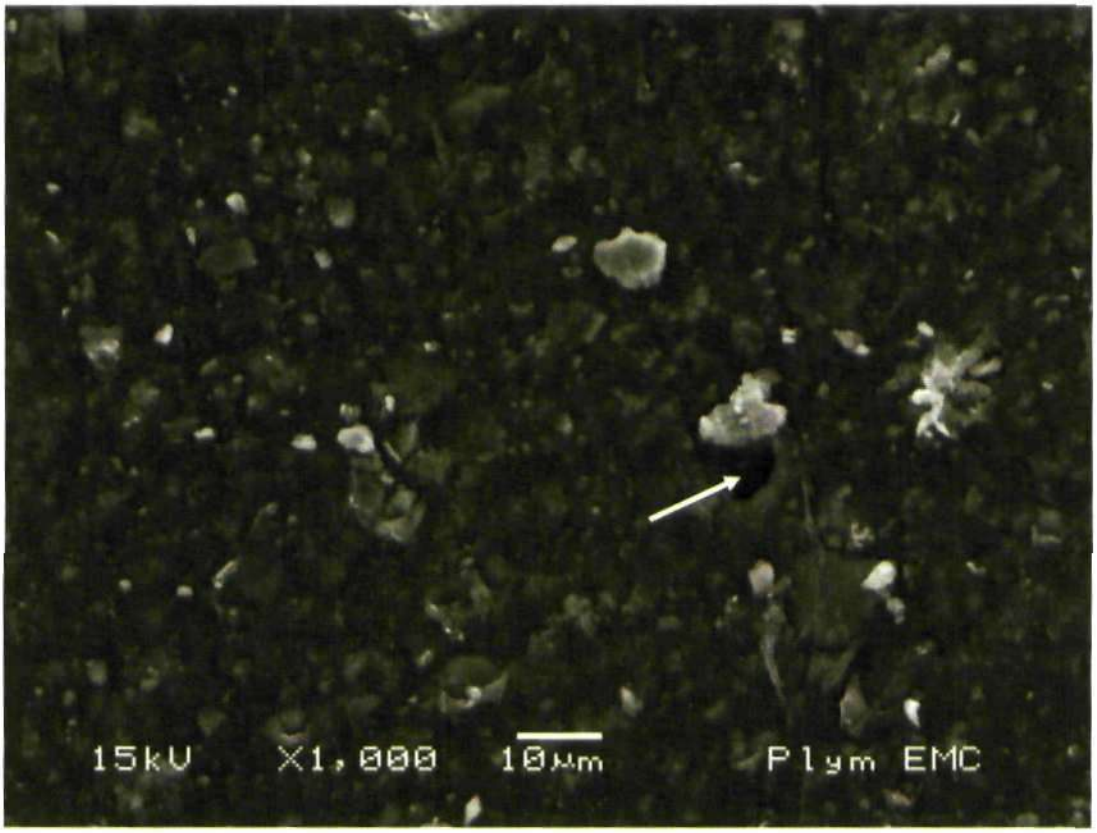


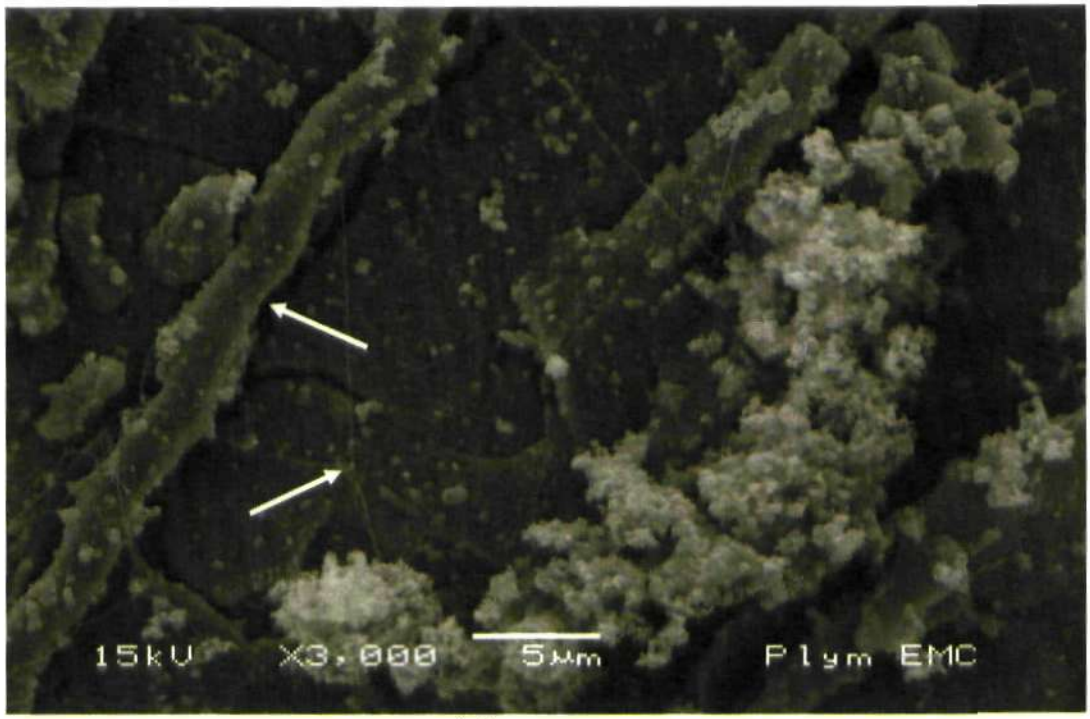
Figure 5.6 Scanning electron micrograph of 'virgin' EPDM inner surface. Particulate matter is spread ubiquitously over the surface, and a general surface roughness is observed. The white arrow indicates a hole in the substratum which was characteristic of this material.

and channels permeating the microcolony structure. SS316 was also shown to support microcolony formation. However, whilst copper microcolonies were at times complex and well-developed structures, on SS316 this was less so. Microcolonies were smaller and less uniform in terms of their degree of

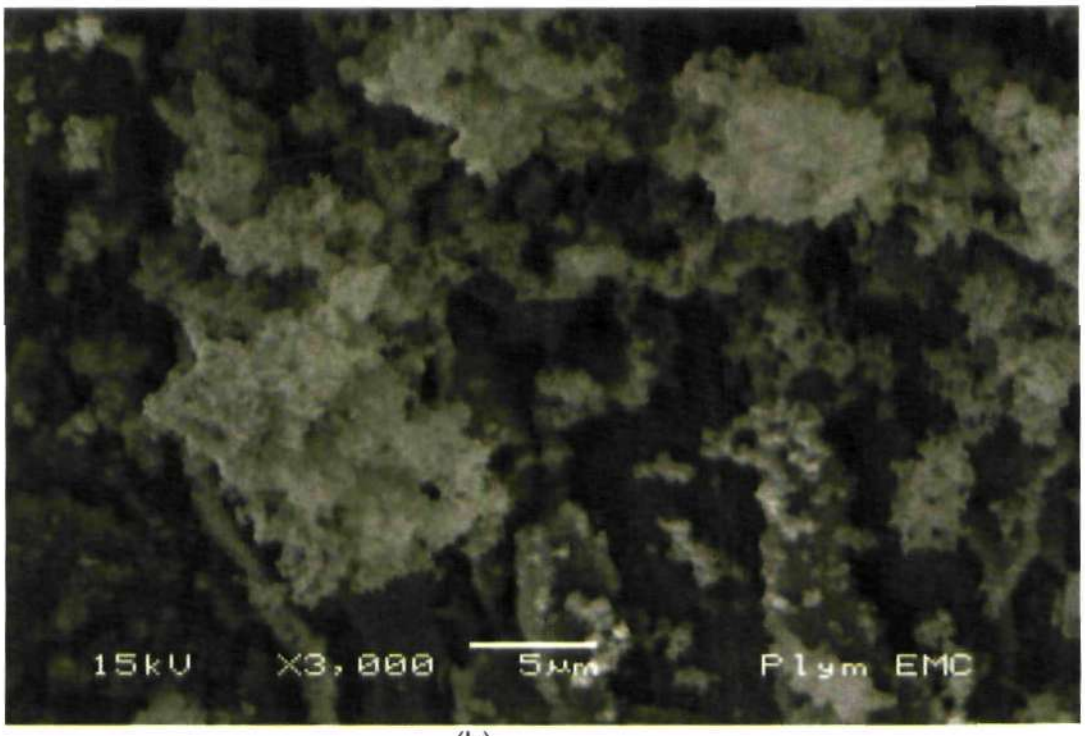
complexity. There was also a greater variety of cell morphologies present and many more individual cells were observed on this material than was observed on copper (Figures 5.8(a) and 5.8(b)). There was no obvious association between microcolony formation and material surface features on either copper or SS316.

PEX biofilms were markedly different from the copper/ SS316 biofilms in that there was no apparent formation of structured microcolonies at any point during the study. Instead, the eventual formation of a thin biofilm layer closely associated with the substratum was observed (Figures 5.9(a) and 5.9(b)). After 28 days the substratum was clearly visible, with bacteria apparently present as individuals, rather than in mutual association. Over time, large amounts of EPS were deposited at the surface, as well as a variety of primarily rod-shaped bacteria, the size and shape of which was suggestive of a diverse community. The type of EPS appeared to also be diverse, although it was difficult to attribute this to the presence of particular microorganisms. By day 84, this EPS layer had developed to such an extent so as to apparently obscure many of the microorganisms present.

SEM analysis of EPDM indicated the rapid formation of copious amounts of diverse biofilm (Figures 5.10(a) and 5.9(b)). After 28 days, there was complete coverage of the substratum and this was the case for the duration of the experiment. A diverse community of microorganisms was observed throughout. At 28 days, testate protozoa were seen in close association with the biofilm, whilst at 56 days, these were no longer observed. Instead larger amoeba-like, non-testate protozoa (Figures 5.11(a) and (b)) predominated. Neither was definitively identified, and attempts to culture them were not successful.

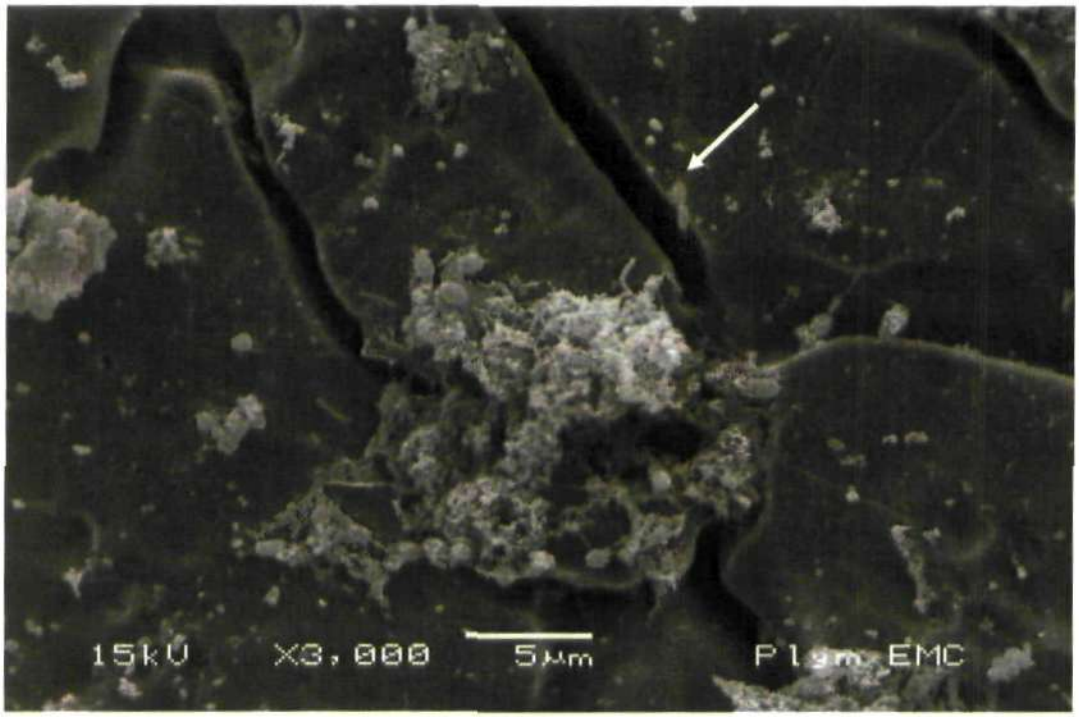


(a)

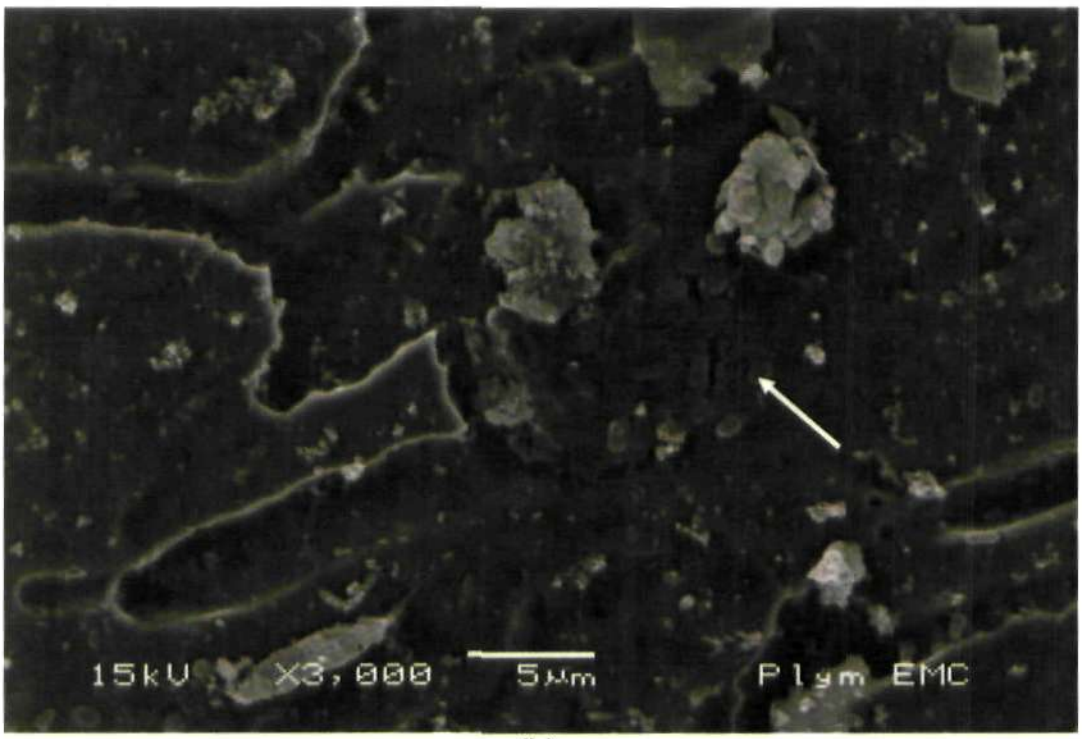


(b)

Figure 5.7 Scanning electron micrographs of copper after (a) 28 days, and (b) 84 days. After 28 days the copper surface is clearly visible, and is characterized by a cracked and fragmented appearance (upper arrow, Figure 5.7 (a)). Also visible are biofilm-associated strands of material (lower arrow, Figure 5.7 (a)) which are spread across the surface, as well as a greater variety of cell morphologies than was observed after days 56 and 84. Both of these features are less apparent after 84 days, after which time surface coverage and microbial biomass have increased.

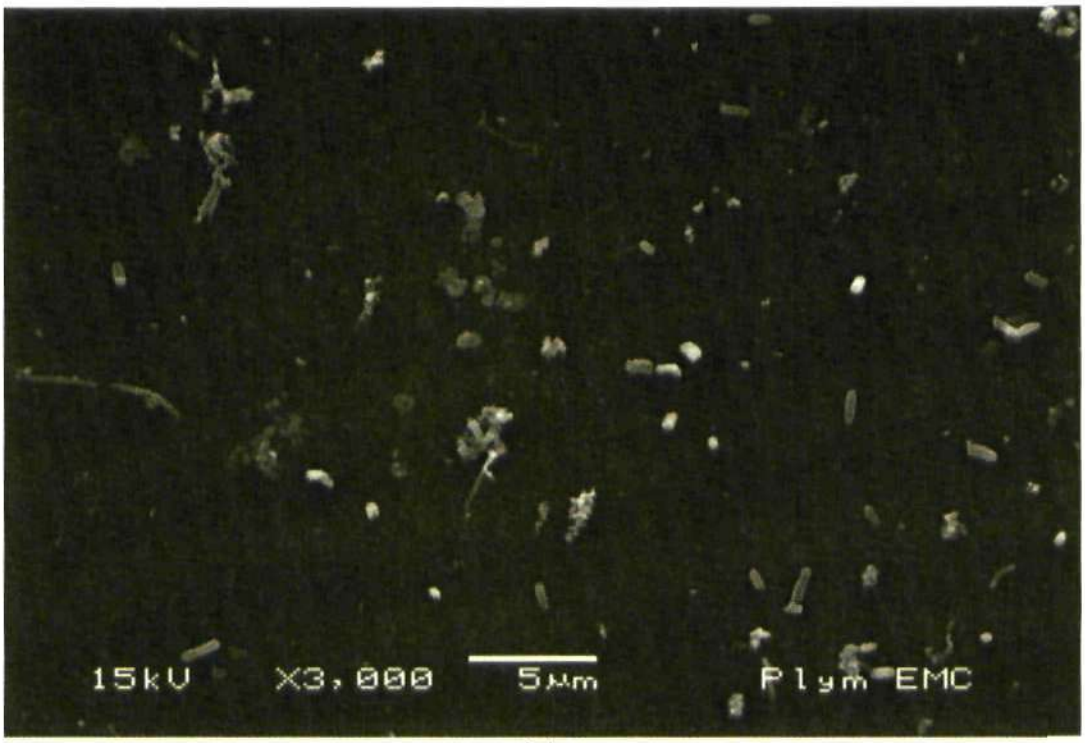


(a)

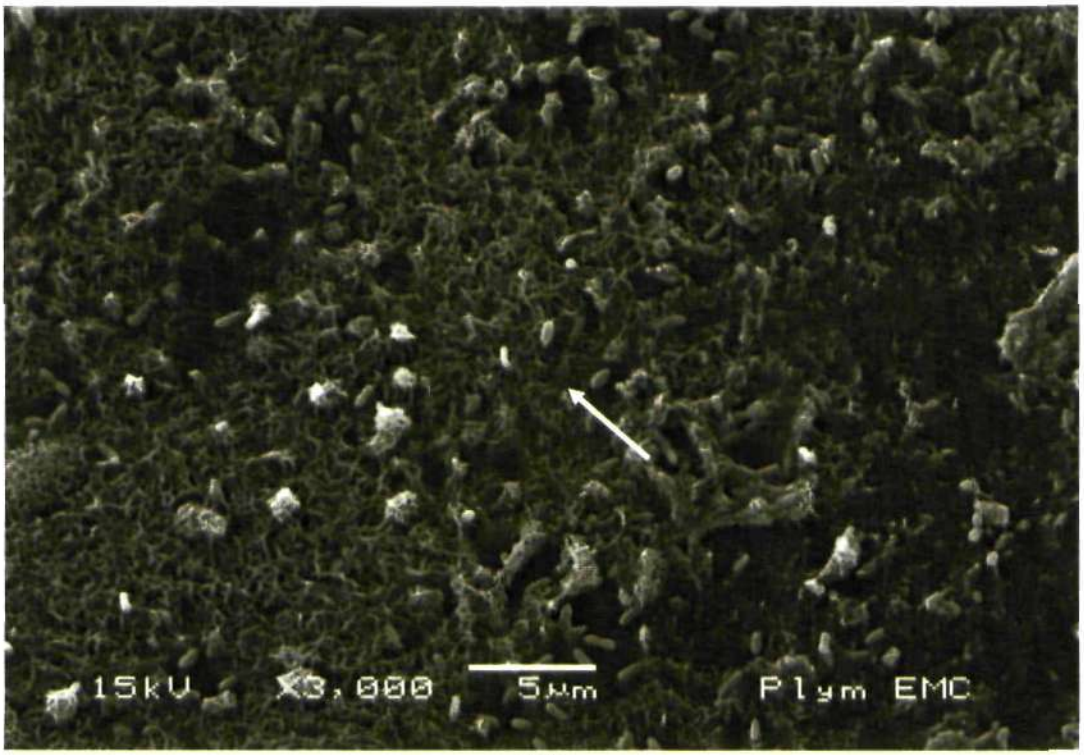


(b)

Figure 5.8 Scanning electron micrographs of SS316 after (a) 28 days, and (b) 84 days. The substratum is clearly visible even after 84 days and is characterized by the presence of surface fissures, with less fragmentation than was observed on copper. A variety of bacterial cell types may be seen in association with the microcolony shown in (a), whilst in (b) the presence of large rod-shaped cells in close association with the substratum (indicated by white arrow) is suggestive of bacterial cells preferentially forming a thin biofilm layer.

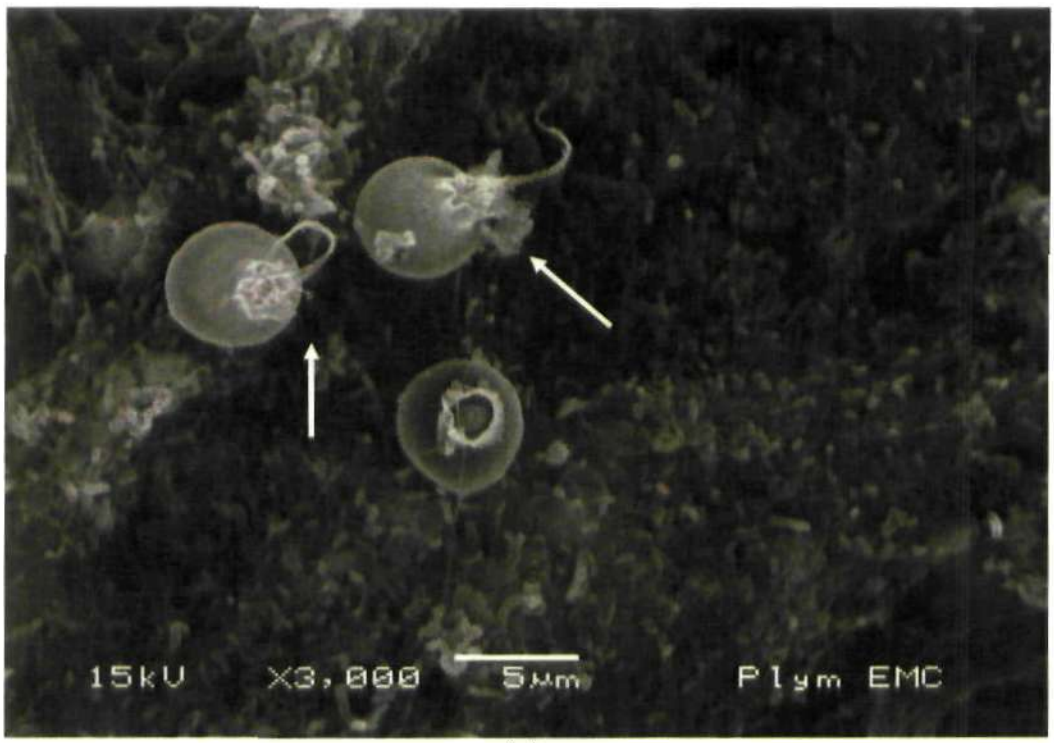


(a)

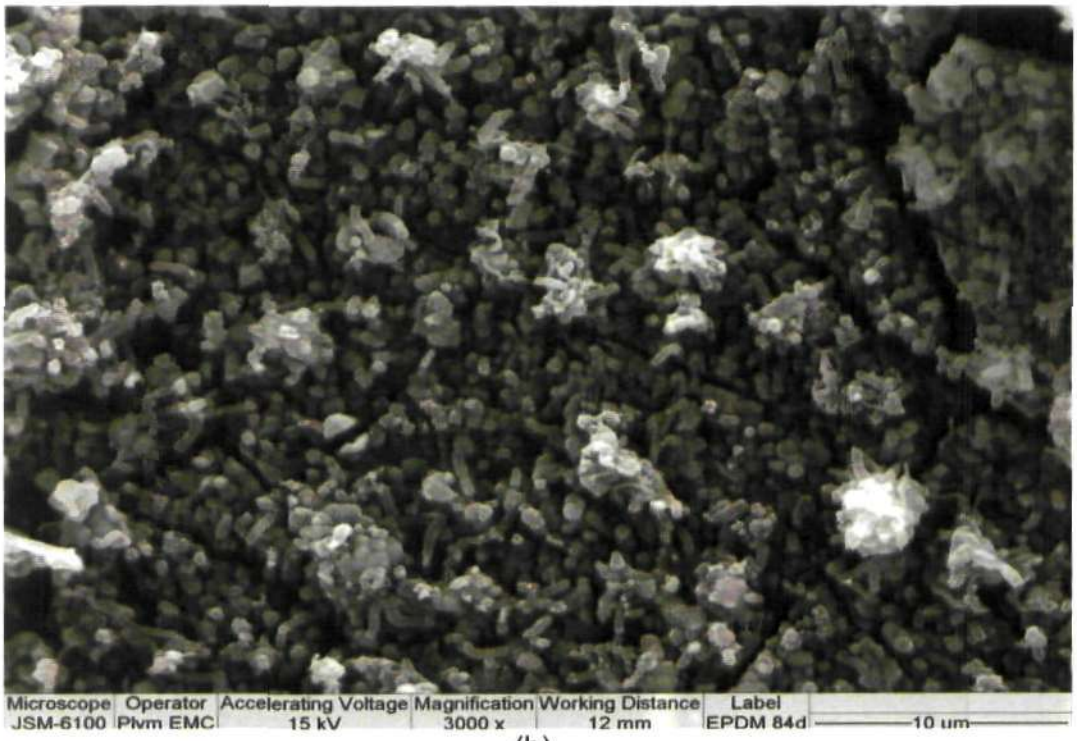


(b)

Figure 5.9 Scanning electron micrographs of PEX after (a) 28 days, and (b) 84 days. After 28 days the substratum, plus a diverse community of associated individual bacteria, are clearly visible. After 84 days, the substratum is covered by a layer of EPS (indicated by white arrow) and associated bacteria, which are primarily rod-shaped. Note the absence of microcolonies as were observed on copper and SS316.

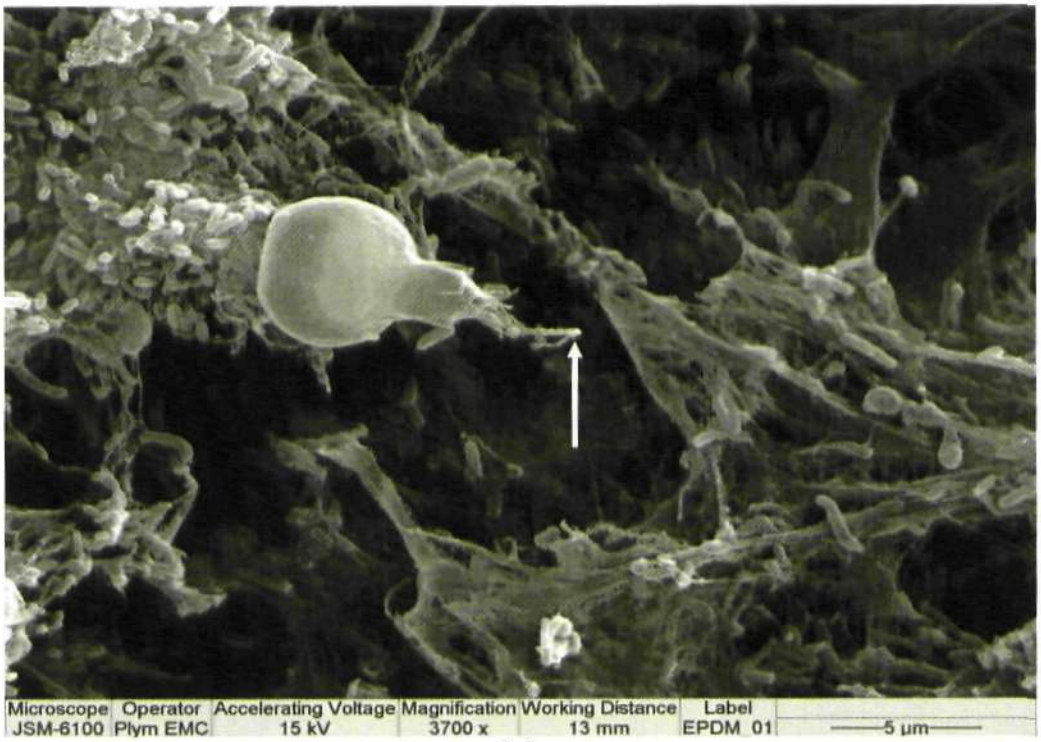


(a)

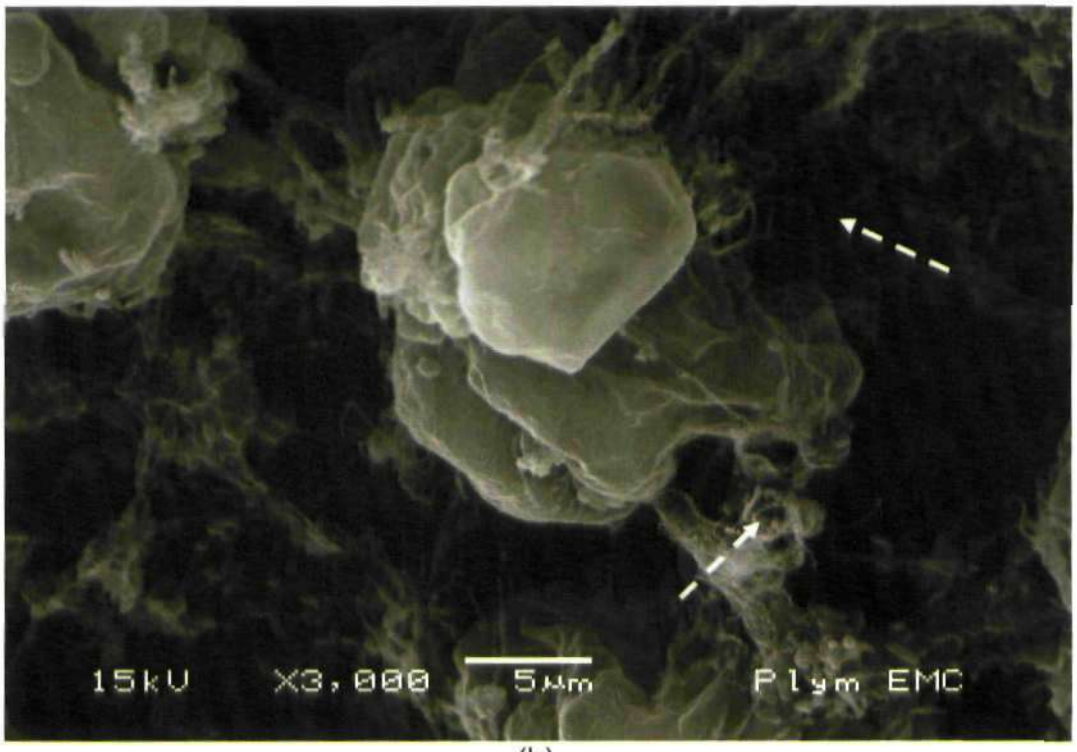


(b)

Figure 5.10 Scanning electron micrographs of EPDM after (a) 28 days, and (b) 84 days. Note complete coverage of the substratum, with copious amounts of EPS housing large numbers of primarily rod-shaped bacteria. Testate protozoa, indicated by white arrows, were also observed in close association with the biofilm. The cracks seen in (b) are artefacts caused by the drying process employed during sample preparation.



(a)



(b)

Figure 5.11 Scanning electron micrographs of protozoa associated with EPDM biofilm after (a) 28 days, and (b) 56 days. In (a), the protozoan is enclosed in a protective test and an emerging flagellum, indicated by a white arrow, is clearly visible. The protozoan in (b) is presumed to be a species of *Amoeba*. Large numbers of *Gallionella*-like bacteria (possibly *Leptospira* spp.) are also present (broken white arrows).

5.1.3.5 Confocal microscopy

5.1.3.5.1 Surface coverage estimation

Figure 5.12 (below) shows the results of surface coverage assessment, using the nucleic acid stain Syto9.

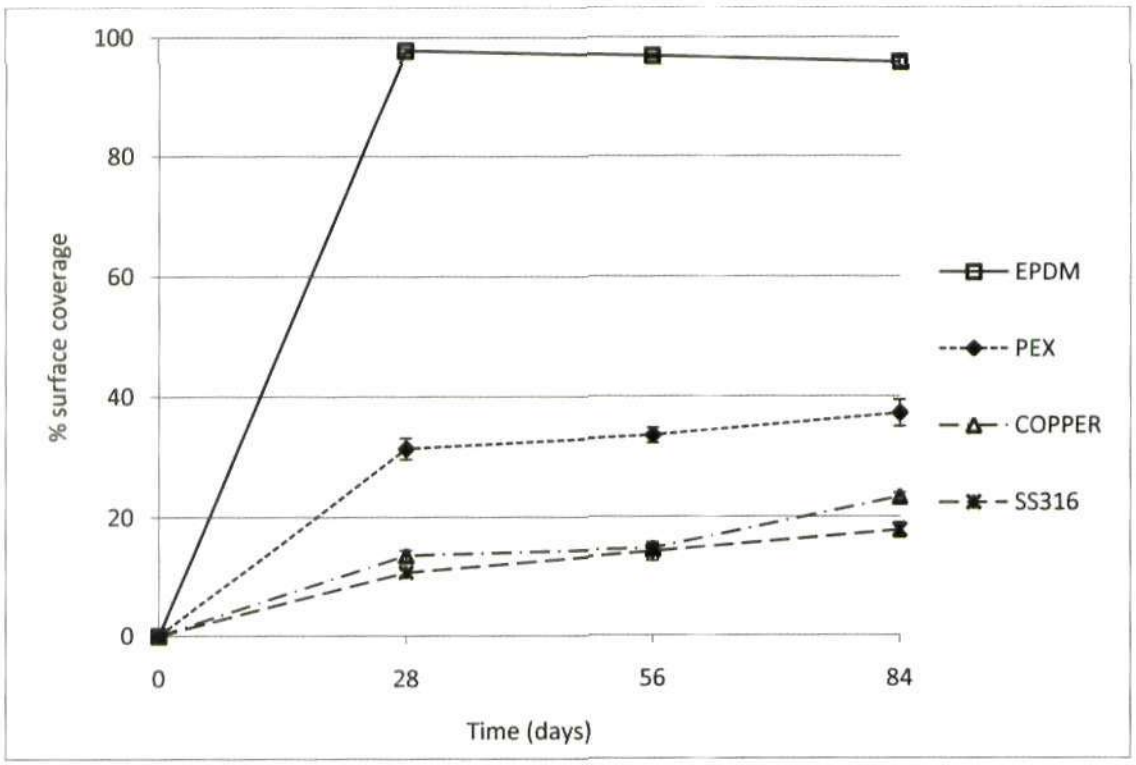


Figure 5.12 Surface coverage of test rig inserts over 84 days, assessed using nucleic acid staining and confocal microscopy.

After 28 days, all materials showed evidence of increasing biofilm coverage, and continued to do so for the duration of the experiment. EPDM demonstrated almost complete surface coverage from 28 days onwards, indicating particularly rapid and complete colonization, whilst biofilm coverage of PEX was shown to be significantly greater than was observed on either copper or SS316 throughout. However, the degree of coverage after 84 days was significantly

less than was observed on EPDM ($p<0.05$). Copper and SS316 showed similar levels of coverage. Indeed, no statistically significant differences were observed until day 84, when SS316 demonstrated marginally greater coverage than copper ($p<0.05$).

5.1.3.5.2 Qualitative assessment of bacterial viability

Qualitative assessment of bacterial viability revealed clear differences between materials stained after 84 days. Live/Dead staining of copper appeared to stain both the cells and the EPS (Figure 5.13, below), demonstrating the co-aggregation of groups of live (green) cells, as well as the existence of large numbers of both viable and non-viable individuals over much of the surface.

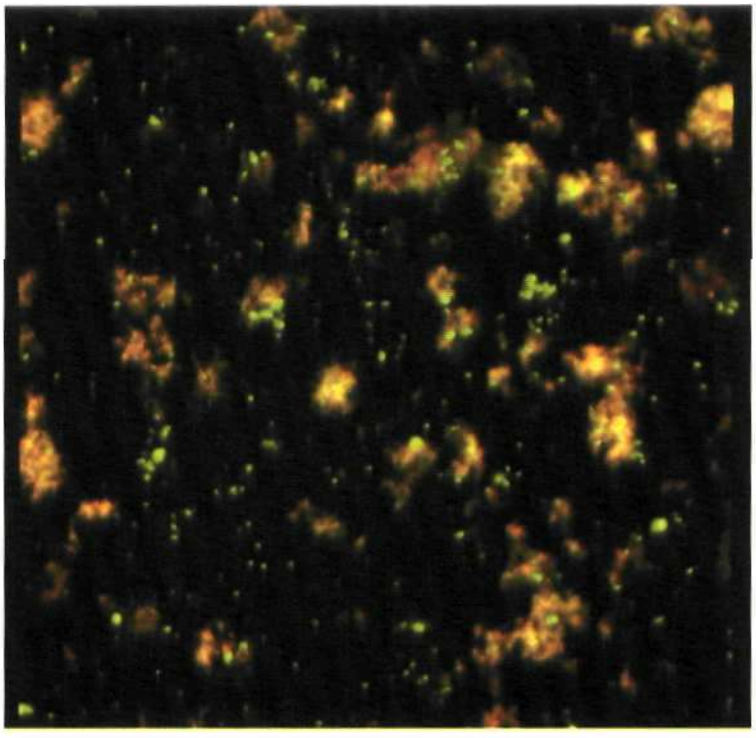


Figure 5.13 Confocal microscopy image of copper biofilm stained after 84 days with Live/Dead BacLight bacterial cell viability stain. Viable cells are green, non-viable cells are red. The EPS appears to have picked up a substantial amount of the stain.

In contrast, Live/ Dead staining of EPDM (Figure 5.14, overleaf) appeared to confirm previous SEM/ coverage observations in that the surface was

completely covered with biofilm material. Microcolonies consisting of viable cells were visible as highly fluorescent greenish-yellow patches. Whilst a large number of non-viable cells were clearly visible, these were not as obviously microcolony-associated. However, the numbers of bacteria present were so great it was difficult to make accurate judgements as to the spatial arrangement of viable/ non-viable cells.

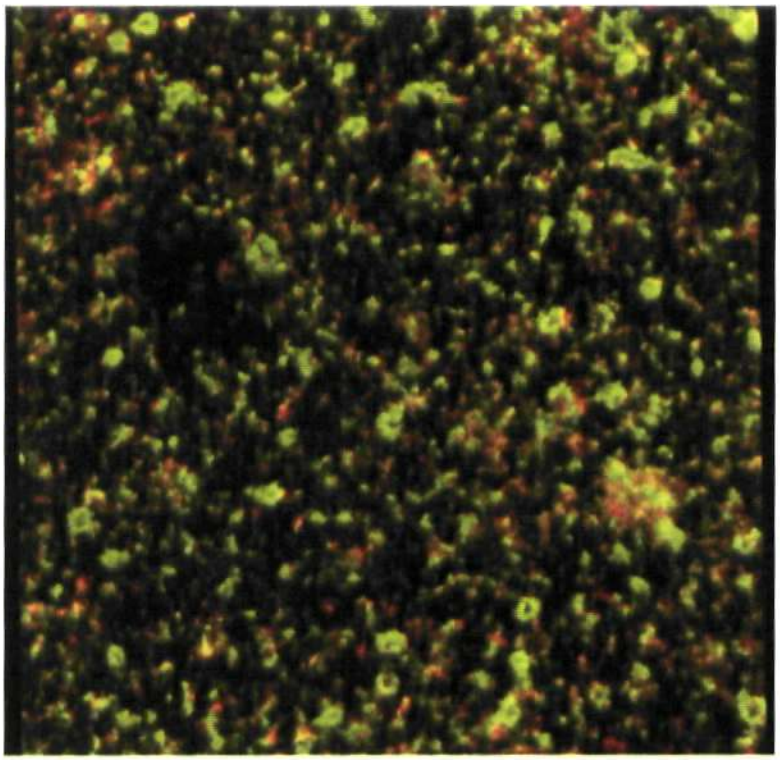


Figure 5.14 Confocal microscopy image of EPDM biofilm stained after 84 days with Live/Dead BacLight bacterial cell viability stain. The majority of viable bacteria appear to be associated with discrete microcolonies.

Microcolonies consisting primarily of viable cells were observed in biofilms formed on PEX after 84 days (Figure 5.15, overleaf). Investigation of these microcolonies through manipulation of the projected (3D) image suggested that these were closely adhered to the substratum and lacking the structure observed in microcolonies observed on other materials, thus supporting SEM observations which presented little evidence of any microcolony formation

perpendicular to the surface. The majority of cells existing as individuals on PEX appeared to be non-viable.

In the case of SS316, the majority of viable bacteria were once again shown to be contained within microcolonies, with non-viable bacteria mainly distributed as individuals over the surface (Figure 5.16, overleaf). Many of these microcolonies were small, generally consisting of fewer cells than was observed on copper. The occasional single viable individual cell was observed. This distribution of small microcolonies supports the SEM analysis, where the similar observations were made.

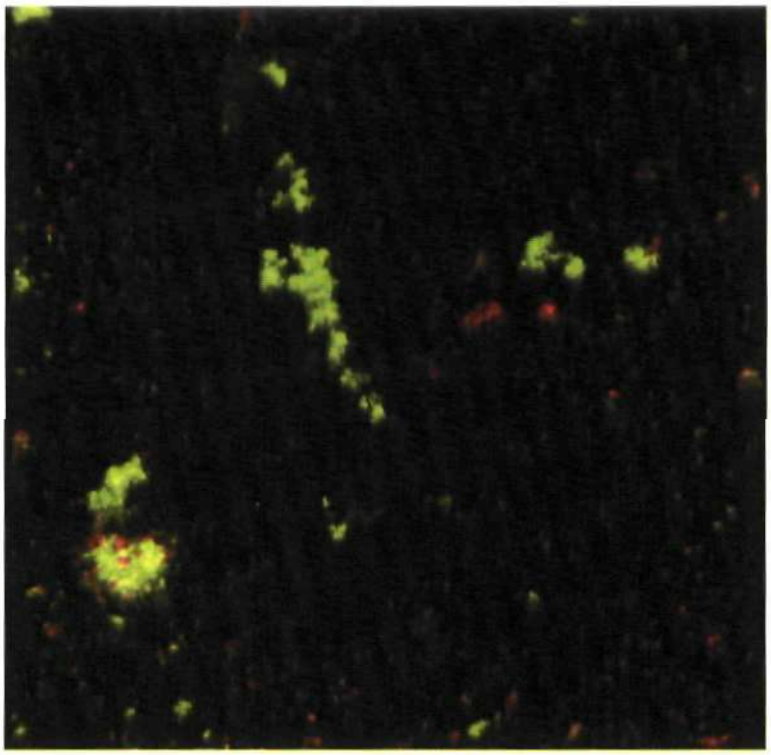


Figure 5.15 Confocal microscopy image of PEX biofilm stained after 84 days with Live/Dead BacLight bacterial cell viability stain. Viable cells are once again associated with microcolonies and the majority of individual cells non-viable.

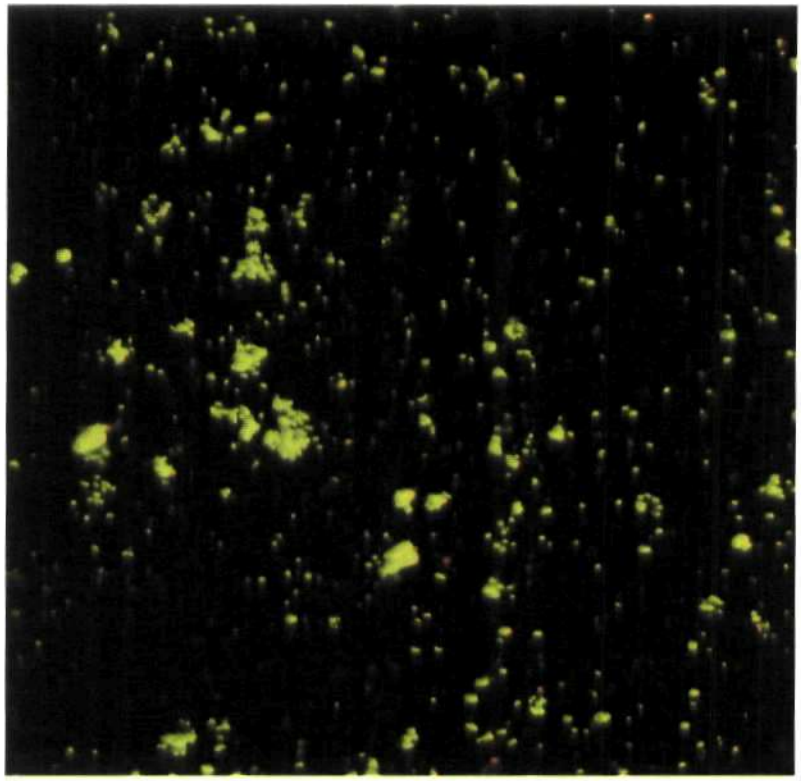


Figure 5.16 Confocal microscopy image of SS316 biofilm stained after 84 days with Live/Dead BacLight bacterial cell viability stain. Small microcolonies are ubiquitously spread over the surface, with non-viable (red) individuals also clearly visible.

5.1.3.5.3 Qualitative assessment of EPS using lectin staining

Figure 5.17 (overleaf) shows a copper biofilm which has been stained with Cy3-labelled concanavalin A lectin stain and the nucleic acid stain Syto 9, after 84 days. Both the EPS and the cellular component of the biofilm can be seen, with the majority of cells located in close association with the EPS. Attempts to visualize the EPS on biofilms formed on the other test materials were not successful, in that it was not possible to differentiate clearly between the cellular and EPS component (data not shown).

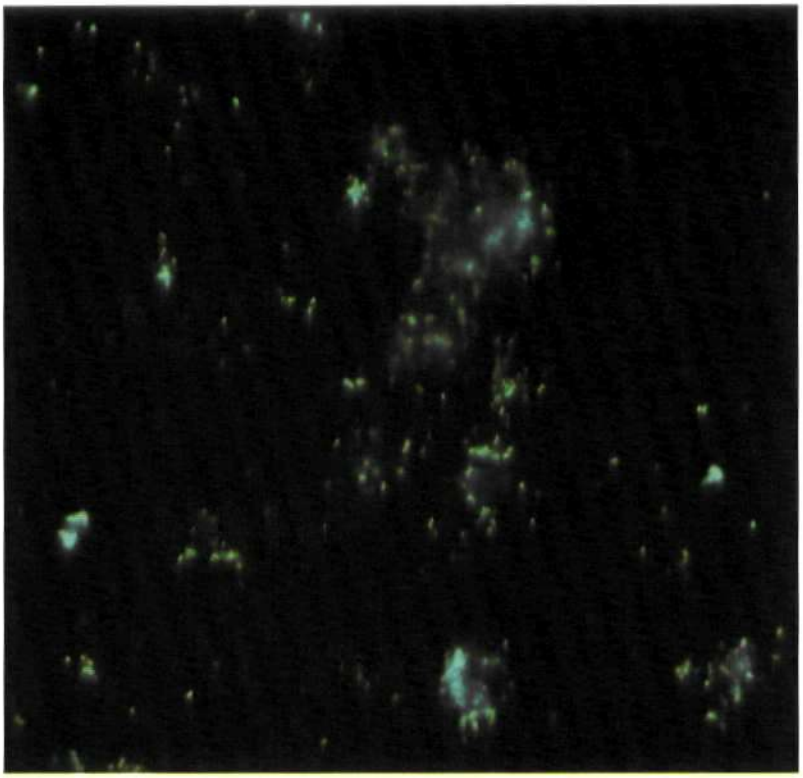


Figure 5.17 Confocal microscopy image of copper biofilm stained with Cy3-labelled concanavalin A lectin stain and the nucleic acid stain Syto9, after 84 days. The majority of bacterial cells (green) are clearly seen in close association with the EPS (blue).

5.1.4 Discussion

Material choice in the context of a water distribution systems (WDSs), such as are found in clinical situations, has a major influence on the type and extent of biofilm formation therein, and therefore also on the potential for pathogen dissemination into the immediate environment. This, coupled with the fact that progress toward the development of commercially-viable, biofilm-proof materials appears to be slow, is a testament to the adaptive abilities of the vast majority of microorganisms to form biofilms. The results presented would appear to support this summation in that the materials used during this study all supported biofilm growth to some degree, yet these biofilms exhibited some obvious differences.

There have been many studies conducted on the effect of material choice on biofilm formation, investigating a wide range of commonly-used materials. The approaches employed in these studies are varied, from *in situ* studies in a clinical setting (Percival 1999), to laboratory studies using reactors (Bressler *et al.* 2009; Moritz *et al.* 2010) and model distribution systems (Zacheus *et al.* 2000; Lehtola *et al.* 2005). This study would appear to represent the first time that copper, SS316, PEX and EPDM have been investigated concurrently, and the test rig employed is unique in its design. Whilst it is important to keep in mind that biofilm formation may differ significantly between water systems, due to differing conditions therein, the results obtained are generally reflective of previous studies involving these materials and allow several conclusions to be drawn.

Perhaps the most striking observation was that EPDM supported biofilm formation at a consistently higher level than the other materials studied. This is in agreement with recent studies by Bressler *et al.* (2009) and Moritz *et al.* (2010), with the former reporting CAB counts of 10^8 cfu cm^{-2} after 14 days, whilst the latter reported approximately 10^7 cfu cm^{-2} over the same period, with a marginal net drop in numbers of CAB over the remaining 29 days of the study. *Pseudomonas* spp. were cultured at levels higher than the numbers of total CAB seen on any other material, and the inclusion of *P. aeruginosa* into WDS biofilms has previously been reported (Rogers *et al.* 1994; Lee and Kim 2003; Bressler *et al.* 2009; Moritz *et al.* 2010). Given that culture-based analysis severely underestimates bacterial numbers in a sample, this observation alone gives cause for concern, given the high profile occupied by members of this genus as nosocomial pathogens, in particular *P. aeruginosa* (Anaissie *et al.* 2002; Ortolano *et al.* 2005; Bressler *et al.* 2009). It is also noteworthy that whilst

no *Legionella* were cultured after 84 days, the possibility of the presence of non-culturable *Legionella* bacteria (plus other potential pathogens such as *M. avium*) at any point during the study cannot be discounted, and investigations into the presence and physiological status of such non-culturable bacteria could form the basis of future work which could prove to be of great potential benefit from a clinical perspective.

In recent years, the suitability of EPDM for use as a plumbing material has been subject to some debate, with the non-WRAS-approved equivalent having previously raised some safety concerns from a microbiological viewpoint (Anon. 2006). The tendency for elastomeric materials such as EPDM, and plastics such as PEX, to leach biodegradable components such as phosphorus-based compounds into the water is believed to be a major contributory factor towards the formation of biofilm thereupon, and EPDM has previously been shown to support high numbers of autochthonous drinking water bacteria, as well as *P. aeruginosa* and *L. pneumophila,* in comparison to commonly employed metals and plastics (Rogers *et al.* 1994; Kilb *et al.* 2003; Lehtola *et al.* 2004; Bressler *et al.* 2009). It is possible that initial careful flushing of such 'leachable' materials, after manufacture and before installation, may be a way of alleviating such rapid and profuse biofilm growth such as was apparent in this study. However, it must be borne in mind that other factors, such as surface roughness and chemistry also play a major role in biofilm formation. Therefore removal of leachable biodegradable material should not be expected to provide the sole solution. Based upon the evidence presented in this study, coupled with that of other EPDM-based studies, EPDM's suitability for use as a material in plumbing systems is questionable.

The duration of any study of this kind will undoubtedly have a major bearing on the results obtained, given the effect of time on dynamic aspects of biofilm formation, such as biomass development and community changes. As with any study of WDS biofilms, it may be argued that gathering data over a period longer than the 84 days of this study would be of greater practical benefit, given that most systems are intended for long-term use post-installation, and the slow-growth of bacteria often associated with such a challenging environment. Over extended periods (>200 days) it has been shown that copper and PEX support similar levels of biofilm, even if plastic materials such as PEX initially form biofilms more rapidly (Zacheus *et al.* 2000; van der Kooij *et al.* 2005). This is thought be due to the initial leaching of biodegradable substances from PEX (Lehtola *et al.* 2004), although copper may also be exerting an initial antimicrobial effect in this case. These effects are eventually negated and hence the two materials begin to show similar levels of biofilm.

CLSM analysis of Live/Dead-stained biofilms after 84 days yielded fascinating insights into how their physiological state differed according to the material on which they were grown. In all cases, it was clear that co-aggregation of microorganisms was closely linked to bacterial viability, with individual cells predominantly exhibiting non-viability. This would appear to support the accepted theory that biofilm existence confers clear advantages in terms of survivability, over existence as an individual, particularly in challenging, oligotrophic environments. The inclusion of CLSM analysis where possible may be considered fundamental to modern biofilm study as it provides insights which are not possible using other microscopical techniques such as SEM. Less versatile than CLSM, SEM involves highly destructive preparation processes. However, this study shows that the two techniques employed in tandem are

highly complementary. Microscopical analysis of EPDM biofilm indicated the presence of microcolonies of viable cells under CLSM, a detail which was missed under SEM. Conversely, SEM revealed a wide variety of microorganism morphology, including protozoa, which were difficult to see clearly under CLSM due to the lower available magnification. SEM also provided a qualitative, if crude, evaluation of community diversity. The benefits of using SEM and CLSM together where possible are therefore clearly illustrated, and the hidden complexity of biofilms on EPDM and other materials is revealed to a greater extent than would have been the case should only one technique have been used. The association of protozoa with mature WDS biofilms has been well documented, particularly with reference to the exploitation of species such as *Acanthamoeba* and *Hartmanella* by *Legionella* spp. (Rowbotham 1984; Thomas *et al.* 2004; Garcia *et al.* 2007), and has also been reported on both stainless steel and PVC (Pedersen 1990). The fact that no protozoa were observed on any surface apart from EPDM during this study may be due to the comparative short term nature of this study, and the consequent relative immaturity of non-EPDM biofilms. Although culturing of the protozoa species present was attempted but unsuccessful, further work in establishing their relationship with EPDM biofilm-associated bacteria, in particular *Legionella* spp., would enhance understanding of biofilm ecology and pathogen dissemination associated with this material.

CLSM/ SEM investigation of copper revealed the presence of microcolonies. The apparent small size of the bacteria therein suggests that they may have been ultramicrobacteria, as described by Geldreich (1996) and also referred to in chapter 4 of this thesis. However, CLSM investigation potentially contradicted this conclusion, in that these 'cells' did not appear to be readily Live/Dead

stained. Instead, Live/ Dead staining appeared to preferentially stain the EPS component, and whilst the presence of EPS-associated cells was confirmed, many viable cells were apparently not closely associated with this EPS. This suggests that the perceived presence of such ultramicrobacteria may in fact be artefactual, although the induction of the VBNC state on copper, of which the presence of ultramicrobacteria may be an indicator, has been presented as a possible explanation for the reduced culturability (in comparison to total bacterial numbers) which was observed by Moritz *et al.* (2010).

The heavy staining of the EPS component may be due to the presence of high levels of extracellular (e)DNA, given that the Live/Dead staining kit is composed of two nucleic acid stains. It is possible that preferential binding of the stain to eDNA in the EPS may have occurred, thus reducing the efficiency with which the stain bound to the target bacteria, particularly those exhibiting low levels of RNA, a possible indicator of low metabolic activity and a target molecule for the nucleic acid stains used. ATP analysis suggested that overall metabolic activity of copper biofilms was indeed lower at 84 days than on any other material, thus partially supporting this theory. Extracellular DNA has been described as a key structural component in many biofilms under certain environmental conditions, such as those formed by *P. aeruginosa* (Allesen-Holm *et al.* 2006) and *Neisseria meningitidis* (Lappann *et al.* 2010). However, its employment as a structural component in mixed environmental biofilms has not been widely studied. Interestingly, copper was the only material on which possible profuse eDNA production, and its subsequent Live/ Dead detection, was observed, and even between copper samples its presence was not always obvious. It would of course have been prudent to confirm its presence/absence by other means, in order to rule out the possibility of non-specific binding to other EPS

components, for example by following the protocol used by Lappan *et al.*(2010). Unfortunately, time and technical constraints did not permit this but it is certainly an area worthy of further investigation. It is not clear whether eDNA was present on EPDM, although it is considered likely, giving the confirmed presence of large numbers of *Pseudomonas* spp.

Microscopic investigation of SS316 and PEX again provided useful insights into what were revealed to be structurally very different biofilms. As was the case with copper, SS316 biofilms were characterized by microcolonies in which viable bacteria predominated, whilst PEX was unique in that a large number of individual (i.e. non-aggregated), surface-associated cells were observed in comparison with other materials. However, as with other materials, aggregates of viable bacteria were again observed, thus once again supportive of the theory that biofilm formation is a preferable mode of existence.

That PEX failed to support the obvious development of microcolonies extending away from the pipe surface may be explained by examining the hydraulic forces at work within the inserts composed of this material. Because PEX had a smaller inner diameter in comparison to the two metals, the shear stresses acting on the attached bacteria would have been greater, therefore favouring the formation of thinner biofilms. Whilst EPDM had the same dimensions as PEX, it is possible that other factors, such as greater surface roughness, which increases the available colonisable surface area whilst interrupting normal water flow near a surface, and available biodegradable materials, may have negated such hydraulic effects in this case.

A more in-depth analysis of the biofilm EPS on each material would have been of value, given that this component forms a considerable proportion of many biofilms and plays a crucial role in biofilm form and function, playing a variety of

constructive, protective and even nutritive roles (Flemming *et al.* 2007). On this basis, it would be wise to factor EPS analysis into any future biofilm study. Confocal analysis during this study was largely confined to analysis of the bacterial cell component alone. A popular method of EPS analysis currently employed utilizes fluorescently-labelled lectins, such as concanavalin A and wheat germ agglutinin. Several attempts were made to label the EPS with the former and were met with partial success (section 5.1.3.4.3). This is presumed to be due to the fact that concanavalin A binds specifically to alpha-mannosyl groups found in various sugars and glycoproteins, and that the success of this particular method is therefore dependent on the presence of such target groups within the biofilm at a detectable level. It was apparent that for the lectin labelling approach to be successful, some preliminary chemical characterization of the EPS was necessary, an approach suggested by Neu *et al.* (2001). Analysis of surface coverage using nucleic acid staining and CLSM clearly supported observations using other techniques, in that rapid and complete coverage of the EPDM surface was seen after 28 days, whereas in the case of the other materials coverage was seen to increase gradually over time following an initial rapid development of a non-confluent biofilm. It provided perhaps the clearest indication that the non-metallic materials were promoting greater initial biofilm formation, and that SS316 and copper demonstrated similar coverage levels over time. It is important to recognize the possible limitations in analyzing the material surface in this way, and thus once again be reminded of the importance of adopting a range of approaches in biofilm analysis. Firstly, application of a nucleic acid stain means that the majority of what was analyzed was likely to be cellular in nature, although as mentioned the possibility of binding to structural (and non-structural), EPS-bound eDNA is a possibility.

Therefore, the EPS component is largely ignored. Secondly, analyzing a two-dimensional or a 'plan' view of a curved pipe surface largely ignores the fact that biofilm growth may not only be occurring over the pipe surface but also approximately perpendicular to the surface, depending on the overriding local flow conditions. Therefore, a potentially significant proportion of the total biomass may be overlooked, particularly in situations when laminar flow or stagnation predominate, as this has been shown to promote the growth of thicker, less cell-dense biofilms (Melo and Vieira 1999). These limitations are recognized. However, adopting this approach was deemed acceptable, and it is believed that the results are in the main reflective of the overall biofilm development process, in that copper and SS316 biofilm coverage was similar throughout the study, with PEX biofilm coverage levels slightly greater, and EPDM exhibiting gross contamination.

The mode of operation of the test rig undoubtedly played a significant role in determining the way in which biofilms developed therein. In any WDS, a myriad of factors influence biofilm development, and it is difficult to relate colonization to a single determining factor (Kielemoes *et al.* 2000). Consequently, every system may be considered unique in that the likelihood of any two operating systems exhibiting identical biofilm-influencing conditions is low. Therefore, it is necessary to exercise caution when drawing specific comparisons between systems in terms of biofilm development. The selected flushing regime, whilst not necessarily reflective of normal WDS use, enabled this study to be carried out in a controlled manner, whilst ensuring regular delivery of water to each system and measurable biofilm formation.

As it was, a lengthy stagnation period was enforced on the forming biofilms for the majority of the time, and this can lead to increased detachment both during

the stagnation period and at the point of reintroduction of turbulent flow (Lehtola *et al.* 2006). This may go some way in accounting for the high numbers of CAB (and ATP) observed in the EPDM system effluent at day 28, after which time the biofilm is stabilised as it reaches a stationary state and adapts to the flow regime employed, a situation which manifested itself as a reduction in CAB and ATP levels. However, the limitations of these techniques should be borne in mind and that the possibility of the presence in the effluent of bacteria in the VBNC state (and exhibiting low activity) remains.

Bacterial attachment on stainless steel has been extensively researched due to its use in a wide range of industrial processes which expose it to potential microbial contamination, with the two main focal points of this research being the effects of surface finish (Arnold and Bailey 2000) and stainless steel grade (Percival *et al.* 1998b; Percival 1999) on microbial colonization. Several authors have reported a tendency for bacteria to preferentially colonize grain boundaries, scratches and surface imperfections, with highly polished surfaces showing a lesser degree of colonization (Kielemoes *et al.* 2000; Medilanski *et al.* 2002). However, during this study there was no apparent evidence of this, with surface distribution of bacteria (as either individuals and in microcolonies) seemingly quite homogenous. Further in-depth analysis may have revealed possible preferential localization (for example, by employing quantitative SEM/ image analysis) but this was beyond the scope of this study.

Other studies involving stainless steel have also included other materials, in various combinations, such as copper, PVC, and PE (Niquette *et al.* 2000; Zacheus *et al.* 2000; van der Kooij *et al.* 2002). Generally speaking, these studies have shown that there is little difference over extended periods of time (up to two years) between these materials, as effects both conducive or

inhibitive to biofilm formation are gradually negated. Therefore, if a new WDS is to be installed, budgetary considerations and resistance to microbially induced corrosion, a phenomenon to which copper is particularly susceptible (Walker *et al.* 1991; Percival *et al.* 1997; Critchley *et al.* 2001), may be factors which ultimately govern material choice.

In conclusion, results would suggest that copper and SS316 show no significant overall differences in terms of biofilm formation, whilst ATP and culture-based analysis of inserts suggests that PEX may also performing at a very similar level. These observations are generally in agreement with the results of other, longer-term studies (Zacheus *et al.* 2000; van der Kooij *et al.* 2002). On this basis, it may be concluded that the main benefit of this study has been to unequivocally identify EPDM as an unsuitable material for use in WDS, particularly in a clinical situation.

5.2 A preliminary study of biofilm formation on materials and tap outlet fittings downstream of test rig systems

5.2.1 Introduction

Tap and shower usage provides a source for repeated exposure to microbes through either aerosolization or direct contact with water which potentially contains biofilm-derived opportunistic pathogens, and the inside of such outlets provides a niche which is moist and frequently replenished with seed organisms derived from biofilms associated with the water distribution system (WDS) upstream (Feazel *et al.* 2009). A variety of tap outlet fittings have been developed and incorporated into tap design, in order to (a) reduce water wastage, (b) reduce aerosolization, and (c) produce a water flow effect which is aesthetically pleasing to the customer. Examples of such tap outlet fittings include aerators, laminar flow outlets (also known as laminar flow regulators) and flow straighteners, with a wide variety of designs currently commercially available. The development of antimicrobial technologies and their incorporation into tap outlet fitting design is an area of increasing commercial interest, following widespread recognition of the role of the WDS in biofilm production and biofilm-derived pathogen dissemination.

Previous work has shown tap outlet fittings, in particular aerators, to be potential areas of microbial contamination with the ability to disseminate potential pathogens into the healthcare environment (Ciesielski *et al.* 1984; Weber *et al.* 1999; Halabi *et al.* 2001; Inglis *et al.* 2010). However, there is a paucity of available literature on the nature and degree of biofilm formation on more modern designs of tap outlet fittings. This suggests that it is an area in need of

further investigation, in order that the public health risk may be accurately assessed, and ideas for effectively designing and testing future products proposed.

The aims of this preliminary study were to investigate the formation of biofilm on (a) a variety of tap outlet fittings, and (b) linear low density polyethylene (LLDPE) piping, a flexible material used in many modern plumbing installations and which, in this case, connected the test rig systems to the no-touch taps used during this research. These materials are commonly used in a variety of commercial installations, including the healthcare sector.

5.2.2 Materials and methods

5.2.2.1 Test rig system set-up and operation

The design and operation of the model warm water distribution system, or 'test rig' used during this experiment is described in detail in chapter 2, section 2.2, page 35.

The taps (and associated fittings) were attached to test rig systems one to six via identical lengths of LLDPE piping. Flushing of each system was carried out every 12 hours, at a temperature of 41 °C, and for 30 seconds duration, using the auto-flushing facility. The study was carried out over two 84 day periods, with sampling carried out every 28 days. Table 5.2 (overleaf) details distribution of tap outlet fittings among the test rig systems.

Table 5.2 Distribution of tap outlet fittings amongst test rig systems/ tap outlets (LFO= Laminar Flow Outlet, SG= Sealing Gasket, ALFO= Antimicrobial Laminar Flow Outlet, STR= Flow Straightener)

System/ tap outlet number	Tap outlet fitting	Time (days,d) at which sampled
1,3,5	LFO + SG	1=28d 3=56d 5=84d
2,4,6	ALFO + SG	2=28d 4=56d 6=84d
1,3,5	STR + SG	1=28d 2=56d 3=84d
2,4,6	ALFO + SG	2=28d 4=56d 6=84d

5.2.2.2 Description of tap outlet fittings

The tap outlets under investigation were housed in no-touch taps which are described in chapter 2, section 2.2, page 35. Four types of tap outlet fitting were investigated: laminar flow outlets, antimicrobial laminar flow outlets, flow straighteners, and EPDM sealing gaskets (Figure 5.18, overleaf). All were supplied by DVS Ltd. Laminar flow outlets and flow straighteners are designed to reduce water wastage, eliminate aerosol production, and provide an aesthetically pleasing columnar flow of water. Antimicrobial laminar flow outlets possess the same multi-functionality, whilst their chemical composition included an antimicrobial element, the exact nature of which was not disclosed by the manufacturer.

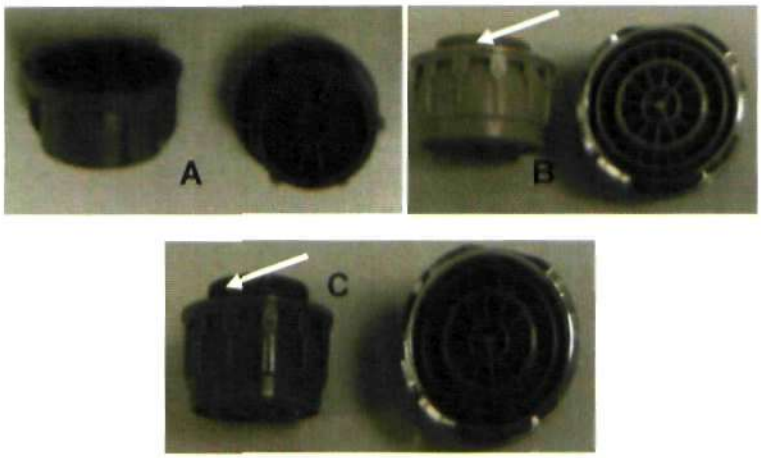


Figure 5.18 Flow straightener (A), antimicrobial laminar flow outlet (B), and laminar flow outlet (C) used during current study. The antimicrobial laminar flow outlet is identical to the laminar flow outlet, with the incorporation of an antimicrobial ingredient. The white arrows indicate a 'nest' - a fine plastic mesh which serves to filter out large particles, as well as acting as the initial point of flow control

5.2.2.3 Removal of materials for analysis

5.2.2.3.1 LLDPE

At each sampling, LLDPE sections were cut using a 70% IMS/ flame sterilized plastic pipe cutter, and exact lengths recorded where appropriate, for SEM-, culture based-, ATP-, and CLSM analysis. Sections were taken from piping associated with each no-touch tap. The point of entry of the LLDPE piping into the isolating chrome ball valve associated with each no-touch tap was selected as the sampling point, as this represented the easiest point at which sections could be taken. The approximate length of these sections was 10 mm, and cut sections were handled and treated as described for PEX in section 5.1.2, given the similarity in the mechanical properties of the two materials.

5.2.2.3.2 Tap outlet fittings

At each sampling, tap outlet fittings were removed individually from the test rig, and subsequently handled using fine laboratory forceps, which had been flame-sterilized using 70% IMS. They were then placed onto separate sterile 90 mm glass Petri dishes, in order that samples could be collected for SEM analysis (see section 5.2.2.6, page 156).

5.2.2.4 Culture-based analysis

5.2.2.4.1 LLDPE

Culture-based analysis was carried out on each LLDPE section as described in chapter 2, section 2.5.1, page 45.

The non-selective agar R2A and the *Pseudomonas*-selective agar CFC were used at each sampling (i.e. every 28 days), whilst samples were also plated onto *Legionella*-selective BCYE/GVPN agar after 84 days.

5.2.2.4.2 Tap outlet fittings

After collection of samples for SEM analysis, tap outlet fittings were placed into individual 50 ml polypropylene centrifuge tubes, each containing 10 ml sterile bacteriological saline. Tubes were then secured onto a vortex mixer and vortexed for five minutes.

After vortexing, 100 µl samples were used for culture-based analysis. The tenfold dilution series and plating regime was adjusted for each outlet fitting

according to expected numbers of culturable bacteria. The non-selective agar R2A and the *Pseudomonas*-selective agar CFC were used at each sampling point, whilst samples were also plated onto *Legionella*-selective BCYE/GVPN agar after 84 days.

5.2.2.5 ATP analysis

5.2.2.5.1 LLDPE

ATP analysis was carried out every 28 days according to the protocol described chapter 2, section 2.8.1 (page 55). The exact length of all pipe samples was recorded in order that the inner surface area could be calculated in each case.

5.2.2.5.2 Tap outlet fittings

ATP analysis of tap outlet fittings was carried out using the samples which had been prepared for culture based analysis. After culture-based analysis had been performed, fittings were removed and discarded, and the remaining suspension was centrifuged at 30000g for 10 minutes. The supernatant was removed using a pipettor until a volume of less than 1ml remained. This was pipetted into a sterile 1.5 ml microcentrifuge tube and centrifuged at 10000g for 10 minutes. The remaining pellet was resuspended in 200 µl of sterile bacteriological saline and a 100 µl aliquot removed and placed into a sterile 1.5 ml microcentrifuge tube for 24 hour storage at -20 °C, before transfer to -80 °C until analysis could be performed. The remaining aliquot was used for PCR-DGGE analysis.

5.2.2.6 SEM analysis

5.2.2.6.1 LLDPE

Sections of LLDPE were prepared for SEM analysis in the same way as described for PEX in section 5.1.2.5, page 118.

5.2.2.6.2 Tap outlet fittings

In the case of all tap outlet fittings except sealing gaskets, metal shears, which had been flame-sterilized using 70% IMS, were used to cut two small samples from the radiating fins of each outlet. Absolute uniformity in the dimensions of these small samples was hard to achieve, given the hardness and brittle nature of the material. These samples measured approximately 5 mm x 5 mm. In the case of both the laminar flow outlet and the antimicrobial laminar flow outlet an additional sample, measuring approximately 3 mm x 3 mm, was cut from the 'nest' situated on top of the outlet as this was considered an area of particular interest from a microbial contamination viewpoint, due to its mesh-like structure and consequently large surface area. In the case of the sealing gasket, laboratory scissors, which had been flame-sterilized with 70% IMS, were used to cut single samples approximately 3 mm x 3 mm.

Subsequent preparation and analysis of outlet samples was carried out as described in chapter 2, section 2.6, page .48

5.2.2.7 Confocal laser scanning microscopy analysis of LLDPE

After 84 days, confocal microscopical analysis of LLDPE was carried out as described in chapter 2, section 2.7, page 49, using the nucleic acid stain Syto9 in the assessment of surface coverage.

The Live/Dead Baclight bacterial viability kit was also employed after 84 days to qualitatively assess bacterial cell viability. Application of this combination stain to the sample was carried out as described in chapter 2, section 2.7.1, page 49.

5.2.2.8 Statistical analysis

Statistical analysis was carried out as described in section 5.1.2.7, page 119.

5.2.3 Results

5.2.3.1 Culture based analysis

5.2.3.1.1 LLDPE

After 28 days, large numbers of culturable aerobic bacteria (CAB) were cultured from the surface of LLDPE piping (mean value 1.08×10^5 cfu cm^{-2}, SD= 3.48×10^4, N=6). After 84 day a significant increase in mean CAB numbers had occurred (9.14×10^5 cfu cm^{-2}, SD= 3.55×10^5, N=6, $p<0.05$).

After 84 days, LLDPE was also shown to support culturable *Pseudomonas* spp. at a level of 9.89×10^2 cfu cm^{-2} (SD= 5.1×10^1, N=6). Attempts to culture

Legionella were unsuccessful, with BCYE/GVPN plates exhibiting overgrowth with non-*Legionella* spp.

5.2.3.1.2 Tap outlet fittings

Large numbers of CAB were cultured from all fittings, and at very similar levels (Figure 5.19, below).

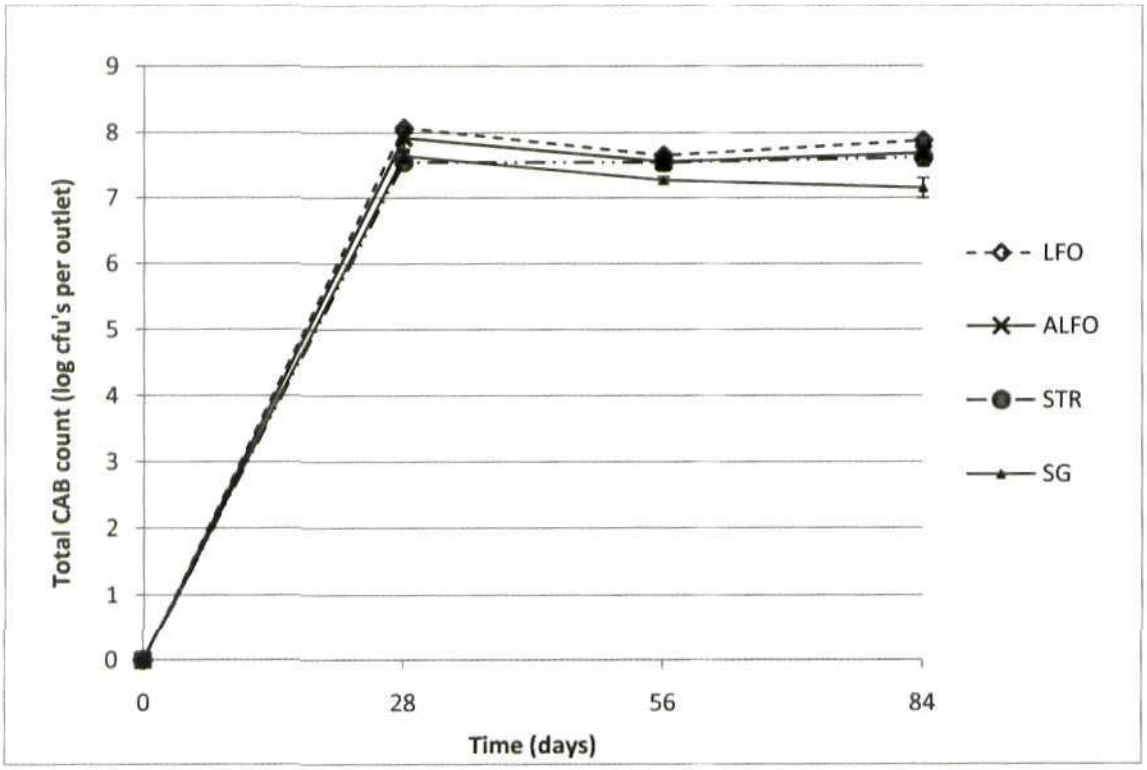


Figure 5.19 Total CAB associated with laminar flow outlets (LFO), antimicrobial laminar flow outlets (ALFO), flow straighteners (STR) and sealing gaskets (SG) over 84 days (Error bars= SE).

There was clear evidence of a rapid increase in numbers of CAB in all outlets after 28 days, with statistical analysis revealing no significant differences between any of the fittings, including the laminar flow outlet and its antimicrobial equivalent ($p>0.05$). During the remainder of the study there were no statistically significant differences observed between the flow straightener, the laminar flow outlet and its antimicrobial equivalent, whilst all of these fittings

demonstrated significantly more CAB than were observed on sealing gaskets at all times post-day 28 ($p<0.05$). Numbers of CAB at the end of the study showed no statistically significant differences when compared with numbers observed at day 28, regardless of fitting type ($p<0.05$ in all cases).

High numbers of culturable *Pseudomonas* spp. were associated with all fittings after 28 days, after which time no significant net increase in culturable *Pseudomonas* numbers was observed. At the end of the study, mean culturable *Pseudomonas* spp. numbers associated with the flow straightener, laminar flow outlet, antimicrobial laminar flow outlet, and sealing gasket were 2.48×10^5 (SD=1.05×10^4, N=5) 2.30×10^6 (SD=7.18×10^5, N=5), 5.18×10^6 (SD= 1.16×10^5, N=5), and 9.50×10^5 (SD=7.55×10^4, N=5) cfu per outlet, respectively.

No *Legionella* were cultured at the end of the study. Overgrowth of BCYE/GVPN agar plates by non-*Legionella* species was once again observed, and sub-culturing of 25 randomly selected isolates onto CFC agar/ oxidase testing indicated that these were primarily culturable *Pseudomonas* spp.(17/25, 68%).

5.2.3.2 ATP analysis

5.2.3.2.1 LLDPE

ATP levels associated with LLDPE exhibited a steady increase throughout the analysis period, rising from 0.012 pmol cm^{-2} after 28 days (SD=0.004, N=3), to 0.052 pmol cm^{-2} after 84 days (SD= 0.005, N=3).

5.2.3.2.2 Tap outlet fittings

Figure 5.20 (overleaf) shows the results of analysis of ATP levels during the study. No statistically significant differences were observed between fittings after 28 days ($p>0.05$). After 56 days, a statistically significant drop in ATP concentration was observed in sealing gasket samples ($p<0.05$), and subsequent analysis revealed low levels of ATP after 84 days. In contrast, the other fittings exhibited significant net increases between 28 and 84 days, with a sharp rise in ATP levels being evident in all cases after 56 days. After 84 days, significantly different ATP levels were observed on all fittings, with the antimicrobial laminar flow outlet demonstrating the highest values.

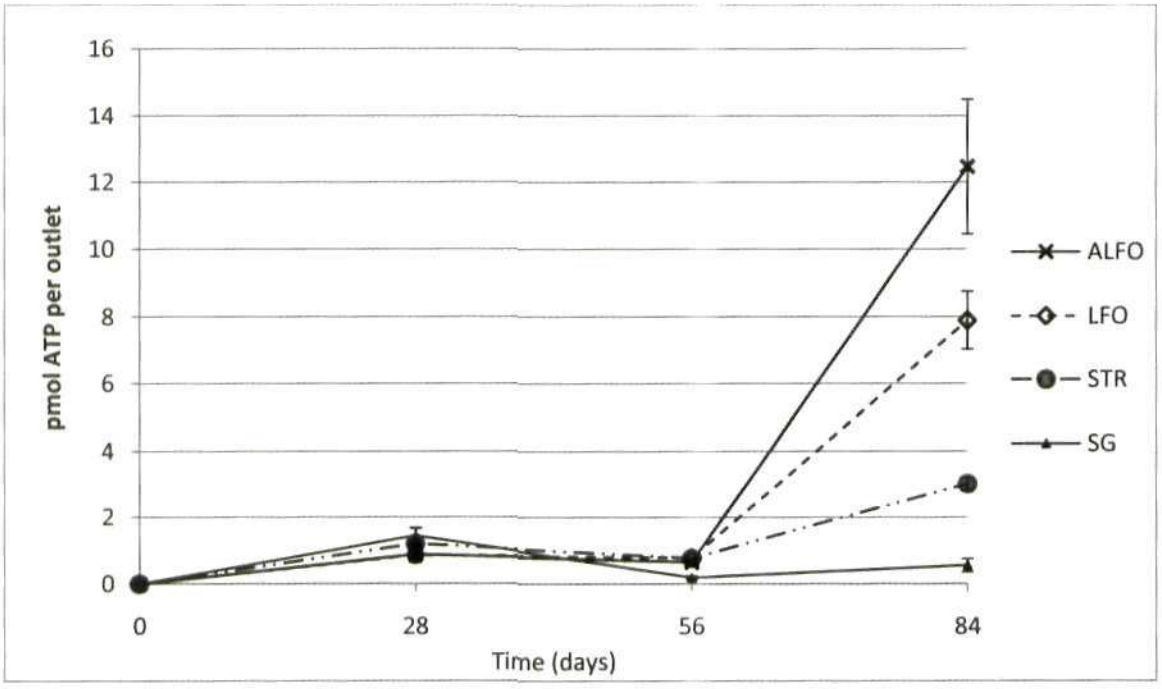


Figure 5.20 ATP levels associated with laminar flow outlets (LFO), antimicrobial laminar flow outlets (ALFO), flow straighteners and sealing gaskets (SG) over 84 days (Error Bars=SE)

5.2.3.3 SEM analysis

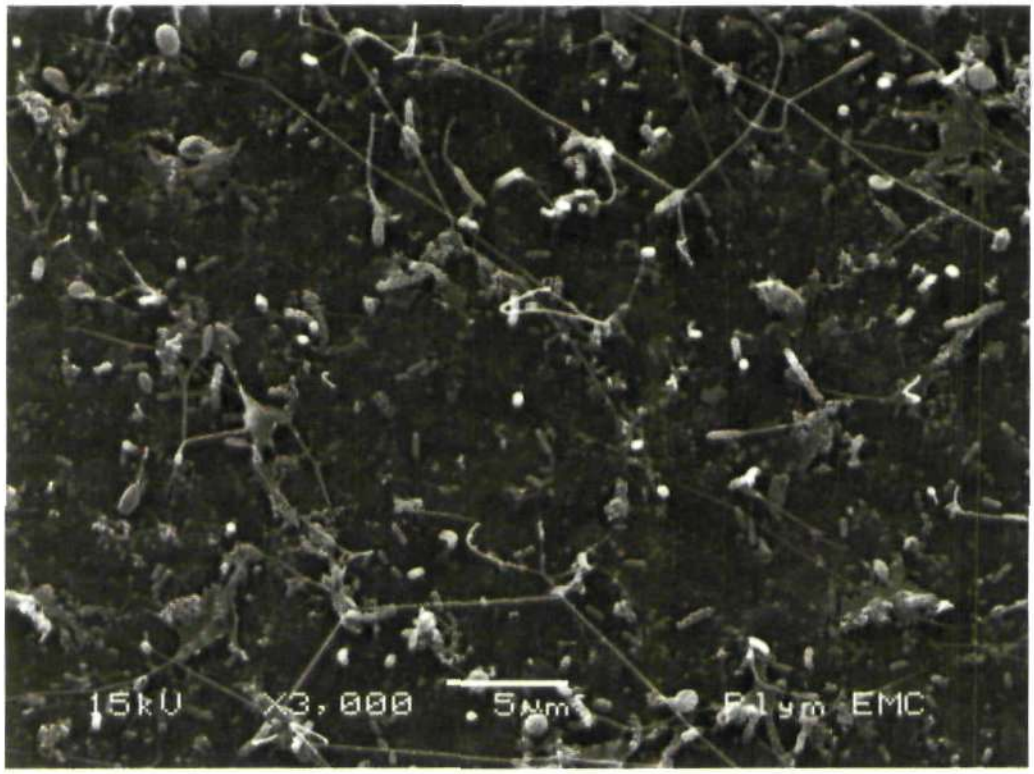
5.2.3.3.1 LLDPE

SEM analysis of LLDPE after 84 days provided evidence of the formation of a highly diverse multispecies biofilm (Figure 5.21, overleaf). The observed biofilm was notable for the presence of distinctively-shaped spore-like structures, possibly indicative of the actinomycete genus *Micromonospora*, which were not observed at any other point in the test rig. Also present were large numbers of prosthecae-bearing bacteria, possibly of the genera *Caulobacter* and/or *Hyphomicrobium*, at a level which appeared proportionally much greater than was observed on any other materials.

5.2.3.3.2 Tap outlet fittings

SEM analysis of tap outlet fittings provided clear evidence of copious biofilm formation on all the fittings tested, from day 28 onwards. In all cases, biofilms were characterized by a highly diverse microbial community associated with large amounts of EPS.

Figures 5.22 (page 165) and 5.23 (page 166) show biofilms formed on the radiating fins of laminar flow outlets and antimicrobial laminar flow outlets, respectively, during the course of the study. Artefactual damage to the surfaces, caused by exposure to the electron beam, can be observed on some of the images as cracking. Also, it should be noted that severe dehydration of the biofilms has occurred, and is caused by the harsh processes involved in sample preparation. In general, SEM analysis of virgin surfaces indicated an apparently



(a)

(b)

Figure 5.21 Scanning electron micrograph of LLDPE piping after 84 days, at (a) 3000x magnification, and (b) 10000x magnification. In (b), Prosthecae-bearing bacteria, *Spirillum*-like bacteria (next to scale bar) and distinctively-shaped spore-like structures (centre of image) are clearly shown.

low degree of surface roughness on 'flat' surfaces (not shown). However, this was difficult to accurately assess using SEM alone, partly because the surface was prone to rapid electron beam damage during analysis.

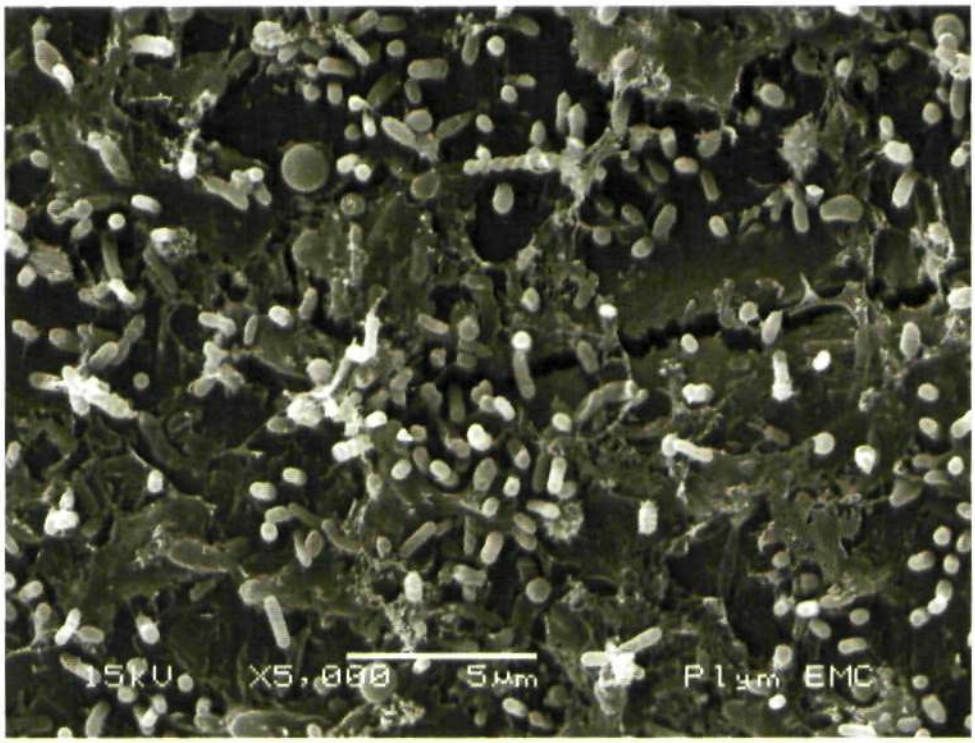
Large numbers of EPS-encased microorganisms were present in all cases after 28 days, with these microorganisms highly diverse in terms of size and morphology. Complex multispecies biofilms were highly evident, and contained bacteria, fungal hyphae, and yeasts. After 84 days, there appeared to be almost complete surface coverage on all materials. Whilst rod-shaped bacteria predominated, all major bacterial morphotypes were represented in some measure. No discernible differences in terms of attached biomass levels could be identified between fitting types, based upon the images analyzed.

Analysis of the 'nest' atop the both the laminar flow outlets and the antimicrobial laminar flow outlets was also shown to be prone to heavy microbial contamination (Figure 5.24, page 167), with the majority of bacteria attached to the inner walls of the nests.

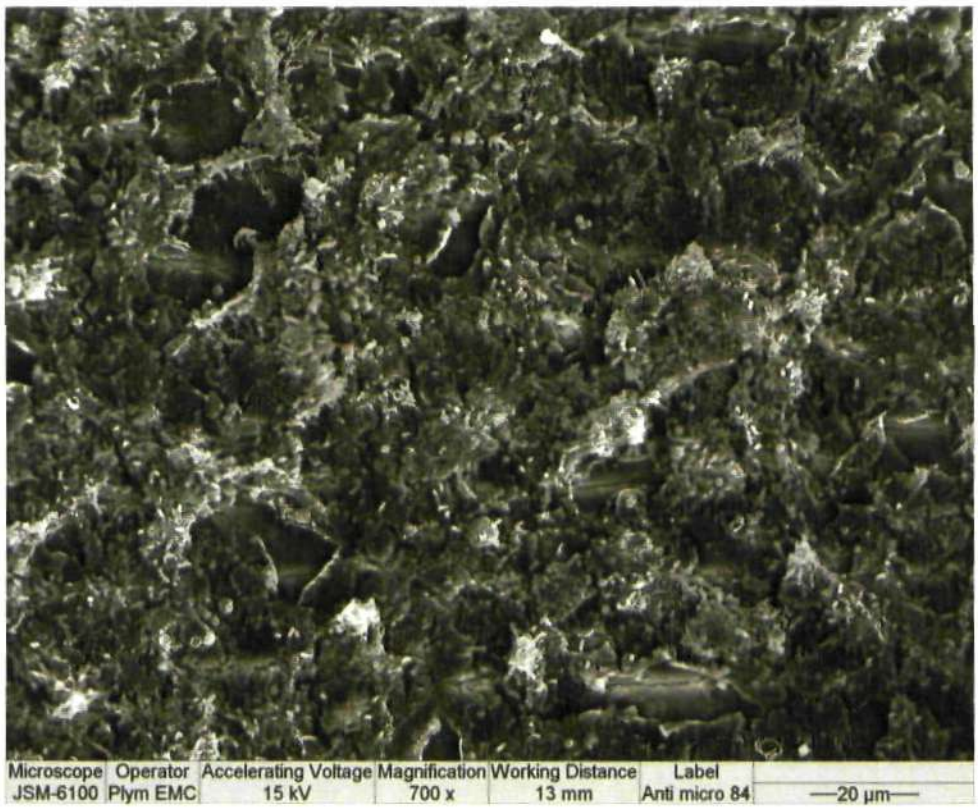
Figure 5.25 (page 168) show bacteria and associated biofilm material adhered to the radiating fins of flow straighteners during the course of the study. As was the case with the laminar flow outlets, image acquisition caused a degree of substratum damage. After 28 days, a variety of cell types are clearly visible (Figure 5.25 (a)), and EPS production appears reduced. However, after 84 days biofilm is clearly present, with rod-shaped cells predominating and encased within a profusion of EPS (Figure 5.25 (b)).

The surface of virgin EPDM sealing gaskets was very different in appearance to that of the other fittings analyzed, exhibiting a degree of roughness much greater than was observed on EPDM piping. Figure 5.26 (page 169) shows biofilm formation on sealing gaskets after 28 and 84 days. After 28 days there

was evidence of profuse biofilm formation, with which protozoa were closely associated (Figure 5.26 (a)). These protozoa exhibited the same morphology as those observed on EPDM piping (Figure 5.10 (a), also after 28 days). After 84 days, patches of thick biofilm predominated on the surface, although these were interspersed with partially covered areas demonstrating the presence of yeast-like cells, fungal hyphae, large numbers of *Spirillum*-like bacteria, and bacteria exhibiting prominent prosthecae.

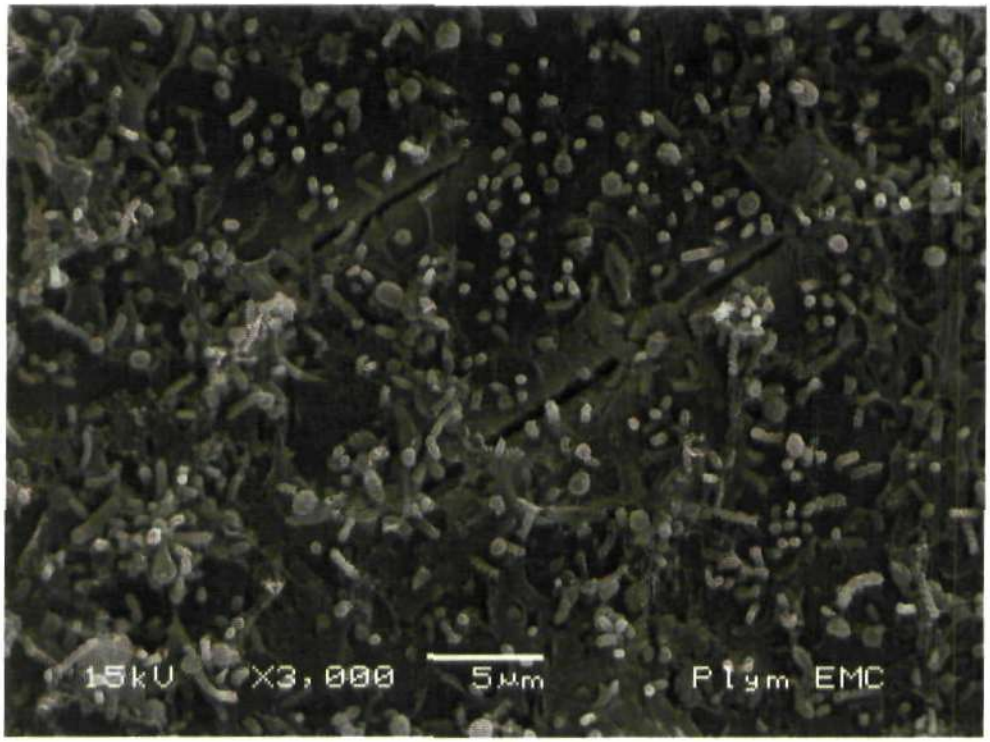


(a)

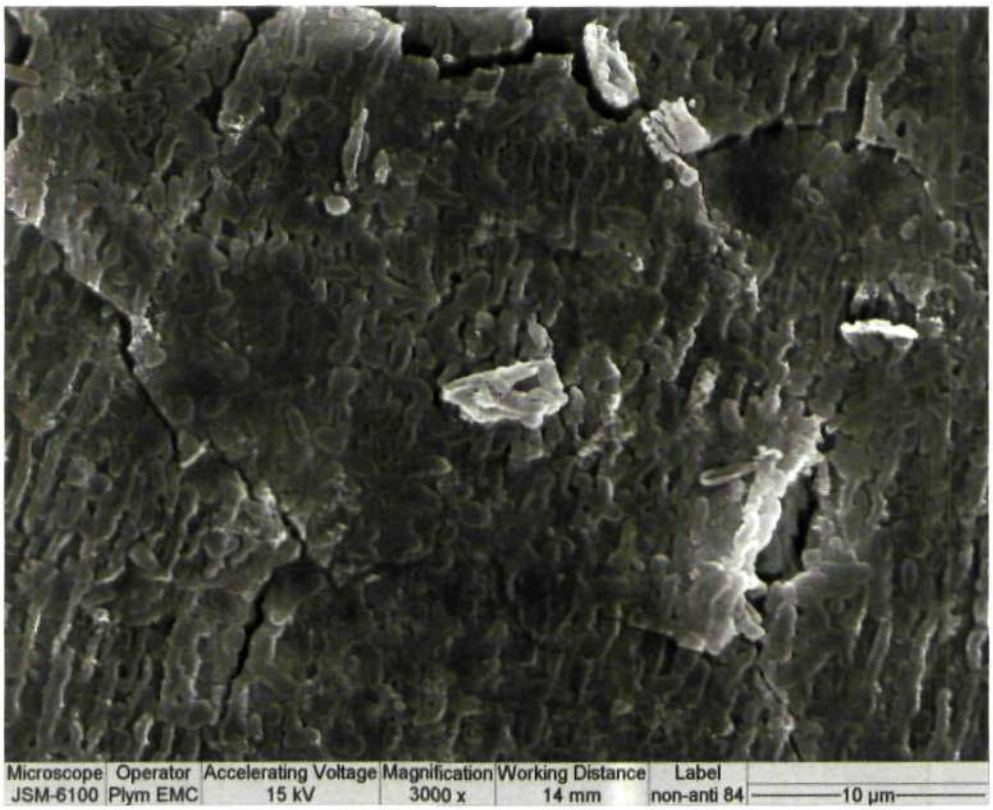


(b)

Figure 5.22 Scanning electron micrograph of laminar flow outlet surface after (a) 28 days, and (b) 84 days, showing copious, diverse biofilm. (b) presents a low-magnification image in order to emphasize the degree of biofilm growth over the surface. (Note: Micrograph (b) was taken using JEOL 6100 SEM)

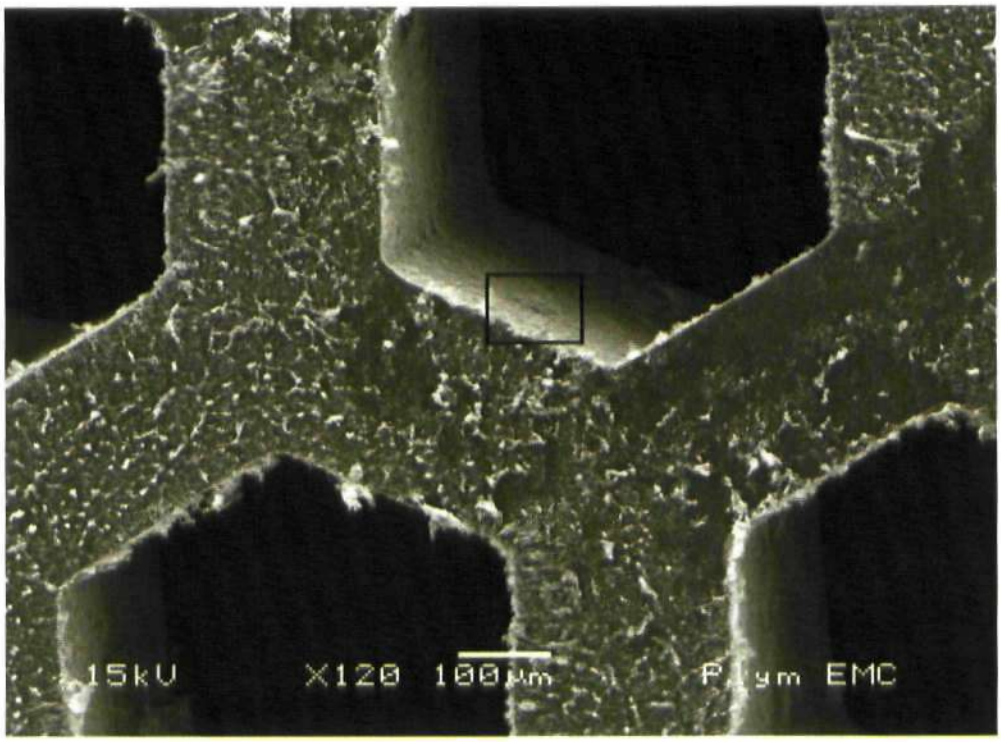


(a)

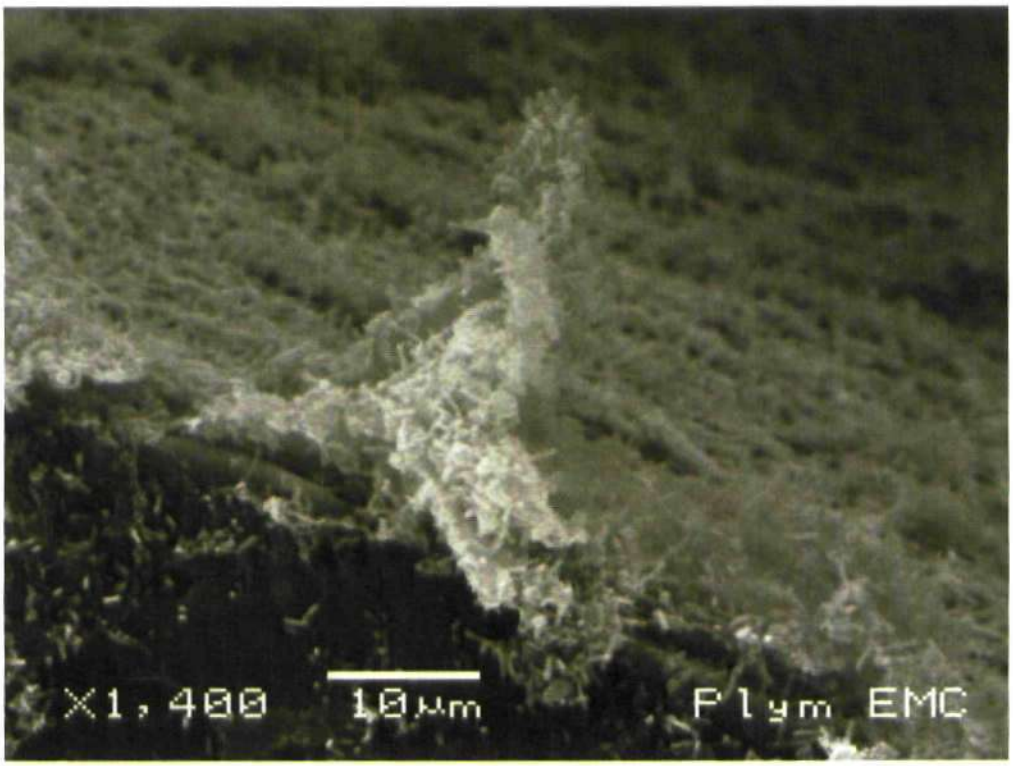


(b)

Figure 5.23 Scanning electron micrograph of antimicrobial laminar flow outlet *surface after (a) 28 days, and (b) 84 days. Complete surface coverage was* observed after 84 days.

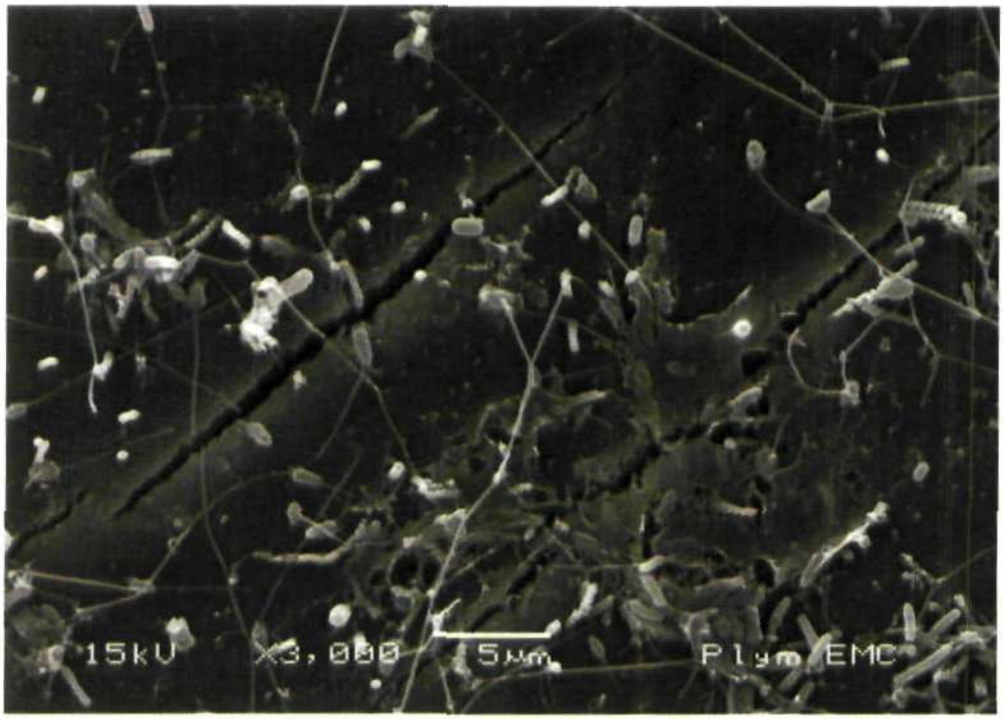


(a)

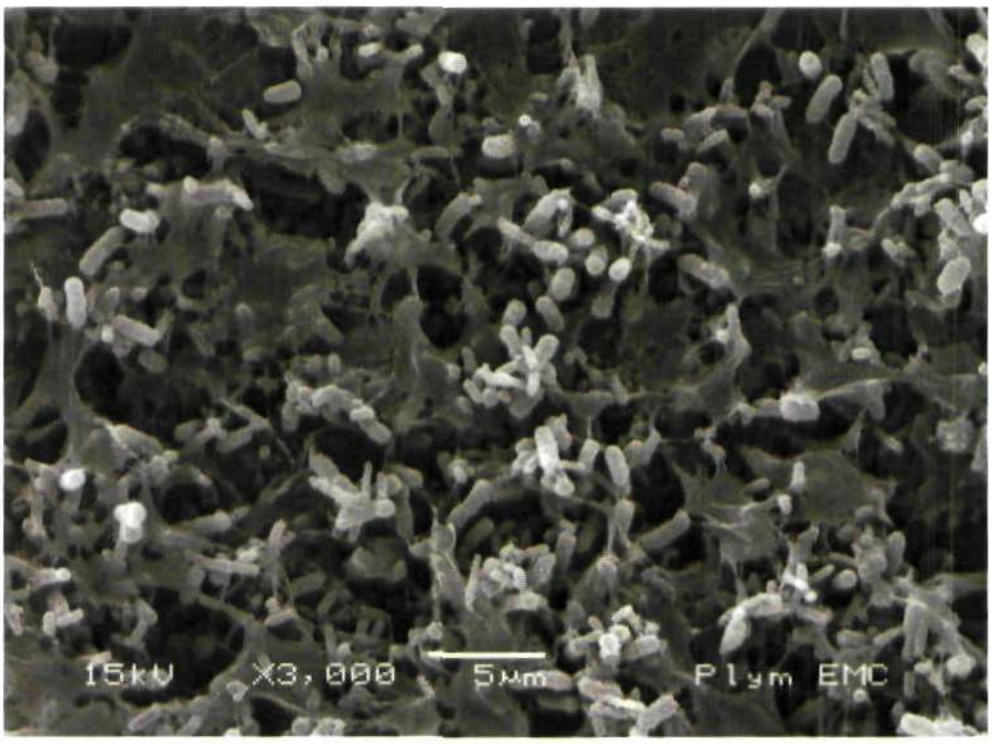


(b)

Figure 5.24 Scanning electron micrograph of laminar flow outlet 'nest' after 28 days. The small boxed area in (a) has been magnified in (b), and the difference in attached biomass between the inner wall and the top of the nest clear shown. Micrographs of antimicrobial flow laminar 'nests' revealed very similar levels of contamination (not shown).

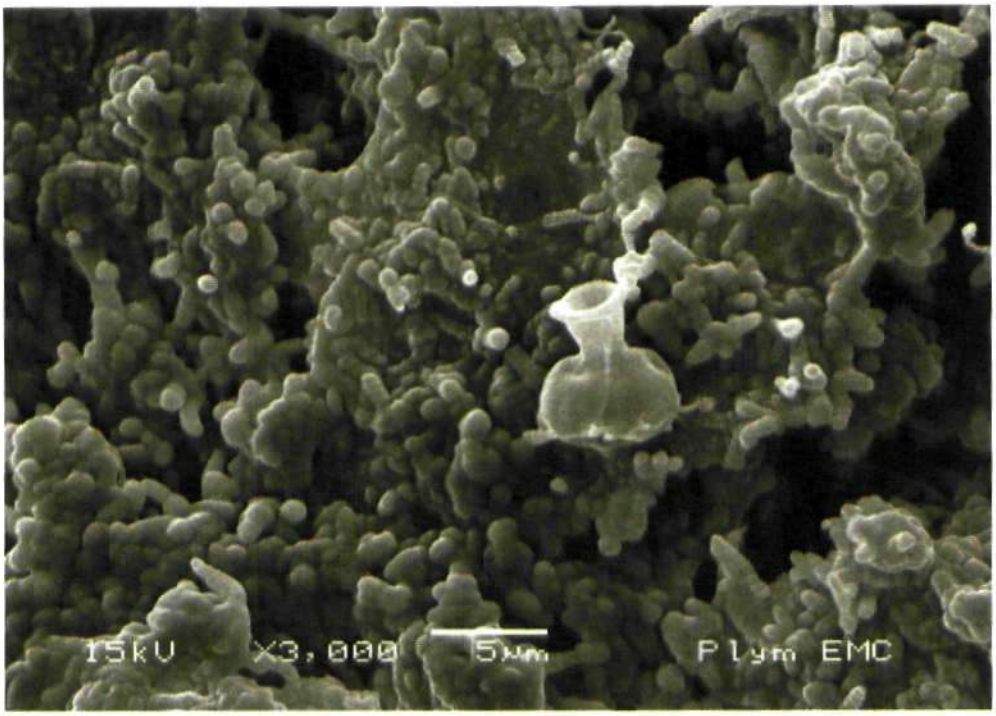


(a)

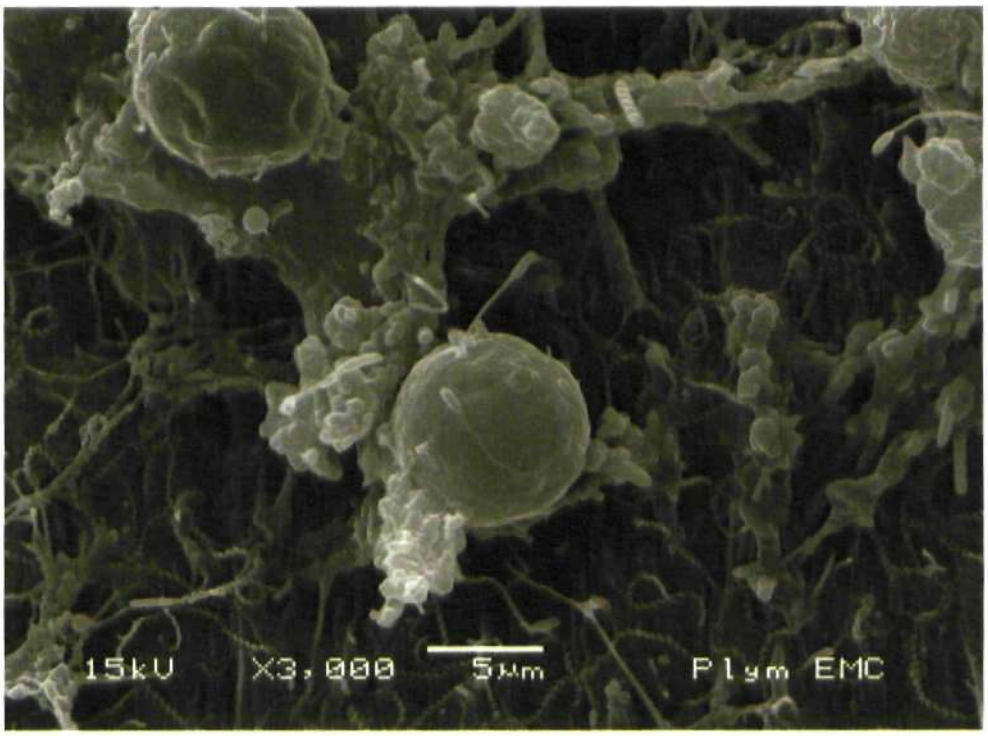


(b)

Figure 5.25 Scanning electron micrograph of flow straightener after (a) 28, and (b) 84 days. Prosthecae-bearing bacteria (possibly *Caulobacter* spp. or *Hyphomicrobium* spp.) are clearly visible after 28 days, whilst after 84 days, copious biofilm and *associated rod-shaped bacteria predominate. The surface cracking observed in (a) is* artefactual damage caused during image acquisition.



(a)



(b)

Figure 5.26 Scanning electron micrograph of sealing gasket after (a) 28 days, and (b) 84 days. Protozoa, large, yeast-like cells, and *Spirillum*-like bacteria- possibly *Leptospira* spp.- are clearly shown.

5.2.3.4 Confocal microscopical analysis of LLDPE

After 84 days, mean coverage of LLDPE was 29.63% (SD= 6.04). An example image of a Live/Dead stained LLDPE biofilm is shown in Figure 5.27 (below).

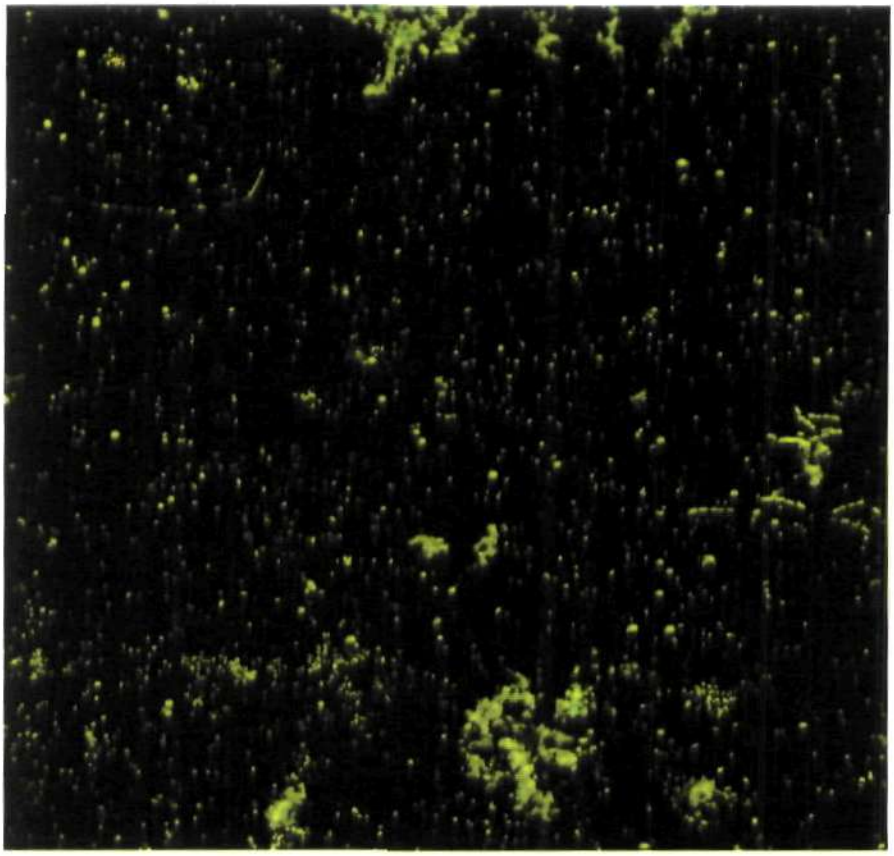


Figure 5.27 Confocal microscopy image of LLDPE biofilm stained after 84 days with Live/Dead BacLight bacterial cell viability stain. Viable cells are green/ yellow, non-viable cells are red.

Live/ Dead staining revealed microcolonies which primarily consisted of viable cells, as well as large numbers of viable individual bacteria. Very few non-viable bacteria were observed. Investigation of microcolonies through manipulation of the projected (3D) images suggested that these were generally thin and closely adhered to the substratum, thus supporting SEM images which also suggested *little microcolony development away from the pipe surface.*

5.2.4 Discussion

Water distribution systems (WDS) present microorganisms with a wide variety of habitats in which to settle and become established in biofilms, the development of which is influenced to a great extent by local environmental conditions. The aim of this preliminary study was to assess biofilm formation on elements of the test rig downstream of the systems in which prior studies into stagnation, thermal purging and material choice have been conducted and reported elsewhere. Studies of this kind may be considered to be of potential benefit to both the manufacturer and the consumer alike, given the widespread use of LLDPE and tap outlet fittings at the distal points in many commercial installations, and hence their proximity to the consumer.

Biofilm formation on PEX has been clearly demonstrated and described in section 5.1, and has also been previously reported on other types of PE piping (Cunliffe *et al.* 1999; Niquette *et al.* 2000; Zacheus *et al.* 2000; Lehtola *et al.* 2005; Moritz *et al.* 2010). However, the ability of LLDPE piping to support biofilm has not been specifically investigated. Instead, the reporting of biofilm formation/ bacterial adherence to LLDPE is largely limited to research into the effectiveness of antimicrobial additives such as polymeric biocides (Seyfriedsberger *et al.* 2006), and the bulk of this work is not concerned with biofilm formation in water system pipework. Given prior knowledge of biofilm formation on other types of PE, it is perhaps unsurprising that biofilm formation was shown to occur on LLDPE piping. Nevertheless this investigation, although preliminary in nature, serves to further elucidate the nature of biofilm within the test rig, and would appear to be the first time that biofilm has been shown on water system LLDPE. Interestingly, SEM analysis would appear to suggest that

the LLDPE piping represents a particularly suitable area for colonization by large numbers of bacteria which exhibit phenotypic characteristics which were infrequently observed upstream i.e. in the test rig systems, such as prosthecae, and it is possible that their presence on LLDPE may to some degree have facilitated the colonization of downstream tap outlet fittings with similar bacteria. Such appendages may be indicators of the presence of *Caulobacter* spp. and *Hyphomicrobium* spp., both of which have been described previously in association with drinking water biofilms (Manz *et al.* 1993).

The majority of studies dealing with biofilm formation on tap outlet fittings report on circumstantial findings in clinical settings, dating as far back as 1966 (Cross *et al.* 1966). Consequently, there would appear to have long been a degree of awareness of the potential threat to health posed by tap outlet fittings, from a microbial contamination standpoint. It is therefore surprising that few in-depth laboratory-based studies have been published in this area. The outlet fittings used in this study were not aerators, but rather fittings which are designed to reduce aerosolization, which has long been recognized as a mode of dissemination of water system-derived bacteria such as *Legionella*, into the environment (Bollin *et al.* 1985). Consequently, from a clinical viewpoint, such fittings would appear to be a more sensible choice for installation in hospital situations.

The results of this short-term study clearly indicate that the antimicrobial element which was incorporated into one of the laminar flow outlets during manufacture is ineffective at controlling biofilm formation in this case, although adequate replication is required to confirm this. It is unfortunate that more information was not available with regards to the exact nature of the antimicrobial ingredient, as well as its concentration within the tap outlet fitting,

as this knowledge would potentially enable the identification of either a more appropriate usage level, or of a more effective antimicrobial ingredient than the one which was used in this case. The high levels of contamination on both the laminar flow outlet and its antimicrobial equivalent would presumably constitute a major cause for concern, were these findings to be observed in a clinical setting, given the large numbers of CAB (including *Pseudomonas* spp.) which were cultured from 28 days onwards. Although beyond the scope of this study, it would have been of interest to have established an approximate maximum level of microbial contamination required for any antimicrobial effect to have been observed using the antimicrobial laminar flow outlet, and also the duration for which this effect could have been maintained, as this type of information may be useful in defining situations under which outlet fittings of this type could be successfully utilized and also when they are perhaps likely to be ineffective. The high levels of contamination observed on the EPDM sealing gaskets was expected, given the poor performance of this material as reported in section 5.1. The intricate design of tap outlet fittings represents a variety of micro-environments in which bacteria may settle and proliferate. The total surface area available for bacterial colonization is large and virtually incalculable, particularly in the case of laminar flow outlet fittings, and it is presumed that the reduction in flow for which such fittings are designed, coupled with the prolonged periods of stagnation that were induced by the twice daily flushing regime employed during this study, would be conducive to the formation of biofilm. It was interesting to observe the preferential formation of a biofilm 'carpet' on the inner surfaces of the 'nest' portion of the laminar flow outlet, which constitutes a considerable area available for colonization by both individual bacteria and detached bacterial aggregates originating from further

upstream. This inner surface colonization may be in response to the uni-directional flow of water through the mesh of which the nest is composed, resulting in conditions which are preferable to those encountered on the upper surface of the nest in terms of shear force consistency and nutrient delivery. The fact that tap outlet fittings are situated at the most distal point in the water system means that biofilm microorganisms associated with such fittings are possibly in a position to receive elevated levels of oxygen and nutrients, in comparison with those microorganisms further upstream. It is also likely that events such as detachment of upstream biofilm material are likely to provide significant 'seeding populations' for the rapid commencement of biofilm formation on tap outlet fittings. Consequently, material choices and system design further upstream may influence biofilm formation at this point.

It was noted that both types of laminar flow outlet fitting retained water between flushes, a situation which would have provided stagnant conditions under which biofilms, including those containing potential pathogens, have been shown to proliferate in a way which renders them vulnerable to detachment when changes in shear force occur (Ciesielski *et al.* 1984; Manuel *et al.* 2010). Minimizing this water retention may be an important consideration in improving future designs. Theoretical examples of how this could be achieved include the use of materials which exhibit a higher degree of hydrophobicity, or through the integration of a mechanism which mechanically removes any residual water between flushes. In this study, all the tap outlet fittings investigated were shown to be effectively amplifying microbial contamination levels at arguably the least acceptable point in the water system.

Forming a seal between the inside of the tap and the top of the laminar flow outlet fittings, sealing gaskets prevent undesirable water leakage during tap

operation. Given their position within the tap/ fitting assembly, sealing gasket surfaces represent another unique micro-environment, which differs from those found on laminar flow outlets. Previous findings, which describe the potential non-suitability of EPDM for use as a plumbing material within the test rig (section 5.1), coupled with SEM observations of the gasket surface, were suggestive of a material which is highly suitable for microbial colonization. This was indeed shown to be the case, with biofilm formation possibly affected by upstream detachment events, coupled with localized influences such as nutrient leaching and contamination with laminar flow outlet-derived microorganisms, with interactions between microorganisms derived from the gaskets and the outlet fittings presumably occurring. Stagnation and reduced oxygen availability may also have played a role in modulating aspects of microbial physiology and ecology, for example by favouring the proliferation of micro-aerophilic species. Whilst culture-based analysis continued to yield high CAB counts from the sealing gaskets, it seems feasible that metabolic activity of gasket biofilm-bound bacteria *in situ* would be reduced in such a micro-aerophilic (possibly anaerobic) environment, and that this manifested itself as significantly reduced ATP levels in comparison to those observed on other fittings. In this scenario, it is possible that processing samples for culture-based analysis may have facilitated a revival of these facultative microorganisms, as well as those which, before sampling, may have been in an *in situ* VBNC state.

Culture-based analysis revealed high levels of CAB on all fittings after 28 days, with little change in the number of CAB thereafter, and it is certain that total cell numbers would have been significantly greater. This was also observed during analysis of pipe materials, suggesting once again that the biofilms had reached a quasi-stationary state before the first sampling was undertaken. The presence

of such high numbers of *Pseudomonas* spp. also gives cause for concern, and although no *Legionella* were detected, their presence in the VBNC state must not be discounted. ATP analysis of laminar flow outlets suggested a continuing increase in metabolic activity throughout the study, in sharp contrast to the drop in activity observed on the sealing gaskets after 28 days. This is presumably due to the continuing colonization of the many available micro-niches that such outlets contain, possibly with bacteria which are non-culturable and which were therefore overlooked during concurrent culture-based analysis.

Whilst ATP- and culture-based analysis have yielded insights into biofilm formation on these fittings, it is important to recognize the limitations of these experimental approaches. Culture-based analysis relies on the fulfilment of several conditions in order to be considered an accurate reflection of bacterial numbers, with factors such as bacterial culturability and sampling efficiency being important determinants of success in this regard. Therefore, it is often used in conjunction with other techniques such as ATP analysis and quantitative fluorescence microscopy. Due to the time available and technical constraints, the latter was not employed, but would have provided a useful insight into, for example, the total numbers of bacteria present, as well as various physiological and ecological aspects.

A significant proportion of environmental bacteria are non-culturable. This is due to factors such as fastidious nutritional requirements, physiological status (such as existence in a damaged or VBNC state), or a combination of the two. The sampling of the laminar flow outlets was made challenging by their intricate design, as this prevented both accurate surface area calculation, and (presumably) effective biofilm removal, therefore any downstream analyses of biomass levels (in this case ATP- and culture-based analyses) are likely to be

affected. The fact that sealing gaskets are of a completely different design to the laminar flow outlets would suggest that assessment of their biofilm forming capabilities independent of those of the laminar flow outlets would constitute, from a scientific viewpoint, a wiser approach than making direct quantitative comparisons.

Possible points to consider in the design of future outlet fittings include reduction of total surface area (including minimization of surface roughness) and complexity, elimination of biodegradable leachates, elimination of retained water between flushes (and therefore stagnation), and the inclusion of technologies which would facilitate localized disinfection of tap outlets such as thermal purging and ozonation. Effective microbiological testing of such novel tap outlet fitting designs/ technologies, particularly those which are intended for use in clinical situations, should be considered essential before any commitment to manufacture is undertaken. Reliance on quality control protocols which centre around short-term exposure of materials and fittings to laboratory strains may be inadequate in effectively assessing risk, and it would seem prudent to carry out periodic 'post-installation' studies such as has been described. Such studies could perhaps involve the use of a similar, simplified test rig, seeded with an inoculum which, whilst reflective of commonly-occurring WDS microflora, is produced in such a way as to be consistent in its composition on a test-by-test basis. Sample analysis should also extend beyond the conventional culture-based analysis which, whilst cost-effective, is limited in its ability to accurately assess bacterial diversity, numbers and metabolic activity. This may have prohibitive cost-implications but would significantly enhance the value of the results obtained in comparison to a purely monospecies, culture-based approach. The way in which this testing is

conducted ultimately dictates its effectiveness in determining the 'anti-biofilm' capabilities of the test material. Incorporation of sample fittings into a model distribution system, such as the test rig used in this study, and subsequent analysis over as long a period as is practicable, would appear to be a useful way of assessing biofilm contamination, and is certainly preferable to unrealistic laboratory reactor-based studies. However, it must always be borne in mind that all water distribution systems differ in terms of the parameters under which they operate, with every outlet and fitting providing a unique micro-niche. During this study, only one outlet fitting was sampled every 28 days, and this lack of replication is recognized as something to be addressed in subsequent studies, given the likelihood of variability in the type and extent of biofilm formation. A preferable approach would have been to assess all outlets at the same time.

In conclusion, previous studies have identified tap outlet fittings as sources of potentially hazardous microbial contamination, and little work has been carried out to assess biofilm formation on LLDPE. This study, whilst preliminary in nature, has clearly demonstrated that LLDPE, flow straighteners, laminar flow outlets and EPDM sealing gaskets are all prone to microbial contamination. In this case, the incorporation of an antimicrobial component into the LFO proved ineffective, and if further in-depth studies were to support these preliminary findings, then this may have serious financial and logistical implications for healthcare professionals and those involved in implementing infection control strategies. It appears that the microbial communities observed on the outlet fittings differ significantly both between fittings and also from those observed within the test rig systems. This study has further elucidated the nature of biofilm formation within the different areas of the test rig, and opens the door to

further in-depth investigations which may shed some light on the processes involved, and the risks posed by, biofilm formation on such materials.

Chapter 6 Molecular Microbial Ecology Investigations of Test Rig Biofilms

6.1 Introduction

The development of molecular (nucleic acid-based) approaches has enabled the study of microbial groups which had previously remained undetected due to the limitations of classical microbiological methods, the most significant of which being the difficulties often encountered in culturing many environmental bacteria. Such limitations may be due to species-species interdependence in certain situations, and is certainly due to a lack of knowledge with respect to actual nutritional requirements of these non-culturable microbes (Muyzer and Smalla 1998). Another limiting factor is likely to be the existence of microbes in the VBNC state. Investigations of oligotrophic environments such as water systems have frequently revealed total cell counts, estimated by methods such as fluorescent microscopy and CTC (redox) staining, to be several orders of magnitude higher than those observed through culturing (e.g. Boulos *et al.* 1999; Wingender and Flemming 2003; Moritz *et al.* 2010). Therefore, adopting molecular microbial ecology techniques dramatically improves the chances of a successful analysis of the microbial community in its entirety.

The foundation of nucleic-acid based approaches lies in the development of techniques concerned with their direct extraction from the environment and the subsequent application of PCR techniques to amplify gene sequences, thus facilitating diversity studies by fingerprinting and sequencing. Methodologies based around these principles have been applied to microbial populations residing in a diverse range of environments, and among the most popular

current techniques is denaturing gradient gel electrophoresis (DGGE), a technique first described (in a microbiological context) by Muyzer *et al.* (1993). In the context of the investigation of man-made water systems, DGGE has been successfully employed in a range of studies involving, amongst others, corroding heating systems (Kjellerup *et al.* 2005), iron piping corrosion (Teng *et al.* 2008), elastomeric materials (Bressler *et al.* 2009), the development of *in situ* water distribution system sampling devices (Deines *et al.* 2010), and various aspects of wastewater treatment (Fernández *et al.* 2008; Zhang *et al.* 2008).

The purpose of the following molecular microbial ecology investigations was to attempt to identify changes in the bacterial community as influenced by factors such as time, material type and thermal purging.

6.2 General materials and methods

6.2.1 DNA extraction

6.2.1.1 Collection of insert-derived biofilm samples for DNA extraction.

Biofilm samples for DNA extraction from test rig inserts were collected as previously described in chapter 2, section 2.4, page 42. Swabs were retained and separate extractions carried out on both the swabs and the vortexed samples. After DNA extraction these samples were recombined in a single volume of Tris-EDTA (TE) buffer for downstream processing.

6.2.1.2 Collection of tap outlet fitting-derived biofilm samples for DNA extraction

Biofilm samples for DNA extraction from tap outlet fittings consisted of sub-samples derived from the collection of samples for culture-based analysis (chapter 5, section 5.2.2.4.2, page 155). After preparation of serial tenfold dilutions for culture-based analysis, the remaining undiluted suspension was centrifuged at 30000g for 15 minutes. The supernatant was carefully poured away and the pellet frozen at -20 °C for 24 hours, before transfer to -80 °C.

6.2.1.3 DNA extraction procedure

Compositions of all buffers used in the following procedure are documented in Appendix I. Chemicals were purchased from Sigma Aldrich (Poole, U.K.) unless otherwise stated.

50 µl of lysozyme solution was added to each sample at a concentration of 50 mg ml^{-1} in TE buffer, before incubation for 30 minutes at 37 °C. 35 µl of lysis solution was then added, before addition of 200 ml of extraction solution, pre-warmed to 60 °C. The total volume was then transferred to a 15 ml falcon tube in a fume hood. An equal volume of TE-equilibrated, ice-cold phenol solution was then added. After gentle mixing, each sample was left on ice for 10 minutes, before addition of 1 ml chloroform, followed by a further gentle mix. Each sample was then centrifuged for 15 minutes at 3000g. The resulting upper aqueous layer was carefully removed using a 200 µl micropipette and transferred to a sterile 1.5 ml micro-centrifuge tube.

DNA was precipitated by the addition of approximately 0.5 volumes of ice-cold isopropanol, and centrifuged at 10000g for 30 minutes in a bench top micro-centrifuge. The DNA pellet was washed twice using 1 ml 70% molecular-grade ethanol, each time being re-pelleted as described, before gentle drying using a drying chamber / vacuum pump assembly, and resuspension overnight at 4 °C in 20 µl sterile molecular-grade water.

The DNA concentration of samples was determined at 260 nm using a NanoDrop™1000 spectrophotometer (Thermo Scientific Ltd., DE, USA). Duplicate measurements were taken for each sample.

6.2.2 Polymerase chain reaction

PCR amplification of the variable V3 region of 16S rRNA genes was carried out using the reverse primer P2 (5'- ATT ACC GCG GCT GCT GG -3') and the forward primer P3 (5'- CC TAC GGG AGG CAG CAG -3'), which had a GC clamp attached at the 5' end (5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G -3'). Both primers were synthesized by Eurofins MWG Biotech Ltd., Germany. The following reagents were added to each PCR tube: 1 µl of primer P2 and 1 µl of P3 (50 pmol litre^{-1}), 3 µl DNA template, 25 µl RedTaq™ PCR Reaction Mix and 20 µl molecular grade water. This gave a final concentration of 1.5 units Taq DNA polymerase, 10 mM TrisHCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, and 0.2 mM dNTPs. PCR conditions employed were as follows: 95 °C for 5 min, followed by 2 cycles at 95 °C for 1 min, 65 °C for 2 min, 72 °C for 3 min. This cycling regime was repeated with a drop in annealing temperature of 1 °C after every second cycle until a final annealing temperature of 55 °C was reached, whereupon a further 10 cycles were run.

This PCR program was intended to counter the formation of any spurious amplicons during the amplification process, having first been employed by Muyzer *et al.* (1993). Thermal cycling was conducted using a Techne TC312 thermal cycler (MIDSCI, MO, USA), and all reactions prepared in a Labcaire PCR hood, after UV treatment of all equipment.

6.2.3 Agarose gel electrophoresis

DNA extracts and PCR products were run on agarose gels to assess quality and PCR success, before any further work was attempted. All gel electrophoresis was conducted using a 1.5% agarose gel (Fisher Scientific, Loughborough, U.K.), made using 60 mls 1x Tris-acetate EDTA (TAE) buffer and run in either a Pharmacia Biotech (Amersham, UK) or a Fisher Scientific electrophoresis tank containing an appropriate volume of the same buffer. All gels were pre-stained with 6 µl of a 10000x stock solution of SybrSafe nucleic acid gel stain (Invitrogen, U.K.). 8 µl of sample (plus 3 µl 6x loading buffer) was loaded into each well, with 5 µl of 1 Kb ladder (Thistle Scientific, U.K.) loaded at either end of the well series, in order to confirm the size of the PCR product. Visualization of agarose gels was achieved with a BioRad universal hood II (BioRad laboratories, Italy).

6.2.4 Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed using a DGGE-2001 system (C.B.S. scientific, CA, USA). 20 µl of PCR products were mixed with 8 µl of loading buffer and run on 8% polyacrylamide gels (160 mm x 160 mm x 1 mm) containing a denaturing

gradient of 40%- 60% (where 100% denaturant is 7 M urea and 40% formamide). Gels were poured using a BioRad gradient delivery system (BioRad Laboratories, Italy). 200 µl of loading buffer was included in the high denaturant acrylamide mixture in order to assess the quality of the gradient achieved. All gels were run at 65V for 17 hr at 60 °C in 20 L of 1 x TAE buffer. In order to avoid issues relating to reproducibility, all samples relating to the same study were loaded in triplicate on the same gel, as shown in Figure 6.1 (page 188). The outer lanes were not used as these were subject to an undesirable 'smiling' effect. Staining of DGGE gels was achieved by placing each gel in a shallow staining box containing a staining solution, consisting of 10 µl of a 10000x stock solution of SYBR Gold nucleic acid gel stain (Molecular Probes, U.K.) in 100 mls 1 x TAE buffer. The staining box was covered in aluminium foil, placed on a shaking platform, and gently agitated for 20 minutes. Destaining was carried out by draining off the staining solution, and replacing it with an equal volume of 1 x TAE buffer. Gentle agitation was carried out as previously described, for five minutes, before visualization under ultraviolet light in a BioRad 1387 universal hood II (BioRad laboratories, Italy).

6.2.5 Band excision and sequence analysis

After qualitative assessment of each gel in terms of staining effectiveness, band clarity etc., and image gathering, samples of individual DGGE bands were excised for DNA sequencing. Efforts were made to excise as many unique bands as was practically possible from each gel. Band excision was carried out using a flame-sterilized razor blade. Excised bands were added to pre-labelled, sterile 1.5 ml microcentrifuge tubes containing 50 µl of sterile double-distilled

water, and the DNA eluted overnight at 4 °C. 20 bands were then selected for sequencing, and reamplified according to the PCR protocol described in section 6.2.2, the only difference being that primer P3 in this case did not have a GC clamp attached.

The PCR products were cleaned using a Sureclean PCR purification kit (Bioline, U.K.), according to the manufacturer's instructions. The DNA concentration was determined spectrophotometrically as described in section 6.2.1.3. Sequencing was carried out by GATC biotech laboratories (Germany), and the forward primer P3 (minus GC clamp) used as the sequencing primer. A BLAST search in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was carried out to identify the taxonomic groups for which the sequences showed the highest alignment identities.

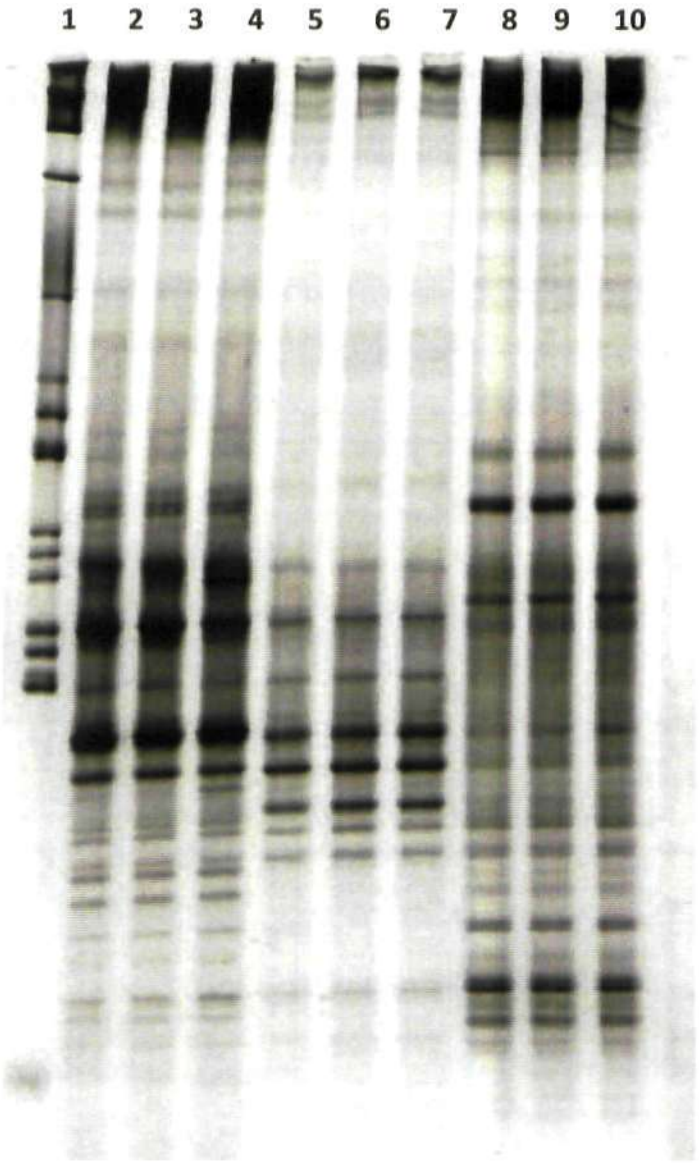


Figure 6.1 Example image of DGGE fingerprints. The first lane on the left (lane 1) is a 1Kb molecular weight marker. The next three lanes (lanes 2-4) are derived from an Antimicrobial Laminar Flow Outlet (ALFO) biofilm. The next three lanes (lanes 5-7) are derived from a sealing gasket (SG) biofilm, whilst the three lanes on the right (lanes 8-10) are derived from a Non-Antimicrobial Laminar Flow Outlet biofilm. All three biofilm samples were 84 days old.

6.2.6 Analysis of band patterns acquired using DGGE

The step-wise protocol employed for analysis of DNA profiles acquired using DGGE is described below:

a) Gel images (saved as '.tif' files) were opened using the image analysis software package 'Image J' (http://rsbweb.nih.gov/ij/).

b) Images were then manipulated using the 'crop', 'subtract background' and 'threshold' functions, in order to ensure optimal band visualization, before printing at the highest available resolution and quality.

c) Bands were numerically labelled according to their migration distance, accounting for the absence of bands in some lanes, and each lane was outlined using the 'Analyze>Gels>Outline First/ Other Lane' function.

d) Using the 'Analyze>Gels>Plot lanes' function, an arbitrary graphical representation of band intensity in each lane was generated and printed. Successive peaks represented the bands present, and printing facilitated the correct labelling of these peaks according to their corresponding bands.

e) Peak area values (expressed as pixel number) were generated using the 'wand (tracing)' tool, and these values transferred to MS Excel.

f) Statistical analysis of this data was carried out using the community ecology analysis software Primer 6 (University of Plymouth, U.K.). For each gel, two- dimensional non-metric multidimensional scaling (nMDS) analysis was used to represent the relative similarities between the different conditions represented on each gel. Cluster analysis was used to check the observed groupings, and an analysis of similarity (ANOSIM) analysis was also performed as a measure of the similarity of replicates both within and between groups.

6.2.7 Description of DGGE analyses undertaken

Six separate DGGE analyses were carried out, and are listed below:

- Effect of thermal purging on copper- / LLDPE- associated bacterial communities.
- Effect of material selection on residential bacterial communities
- Time course analysis of bacterial community changes within copper biofilms over 84 days.
- Time course analysis of bacterial community changes within PEX biofilms over 84 days.
- Time course analysis of bacterial community changes within EPDM biofilms over 84 days.
- Comparison of bacterial communities associated with a variety of tap outlet fittings- non-antimicrobial laminar flow outlet (NALFO), antimicrobial laminar flow outlet (ALFO) and sealing gasket (SG). *These tap outlet fittings are described in more detail in chapter 5,* section 5.2.2.2, page 152.

Sample collection in each case was carried out as described in chapters 4 and 5.

6.3 Results

6.3.1 Effect of thermal purging on copper- / LLDPE- associated bacterial communities

Non-metric multidimensional scaling (nMDS) analysis of DGGE fingerprints is shown in Figure 6.2, and cluster analysis of the same fingerprints is shown in Figure 6.3. Both analyses clearly indicate that bacterial populations associated with LLDPE (both thermal-purged (TP) and non-thermal-purged (nTP)) and the nTP copper are more similar than the TP copper. Average similarities between replicates were high in nTP copper and TP/ nTP LLDPE, ranging from 86.62% to 91.70%. Average similarities between these sample types were also high between TP and nTP LLDPE (76.03%) and between nTP copper and both LLDPE samples (70.03%). However, the average similarity between TP copper and all other replicates was much lower (43.05%). Thermal purging was shown to have had a negative impact on the species richness, indicated by the number of bands present (Table 6.1), of both copper and LLDPE, with reductions of 25.8% and 16.2% observed, respectively. The similarity half matrix of the DGGE fingerprints is shown in Table 6.1. Similarity between the TP and the nTP copper was less than 45% (mean 42.71%, SD=1.10%), whilst an equivalent assessment of LLDPE under the two purging regimes indicated a mean similarity of 76.90% (SD= 2.16%). An ANOSIM R statistic of 1 was calculated, suggesting that replicates within groups were more similar than those from other groups.

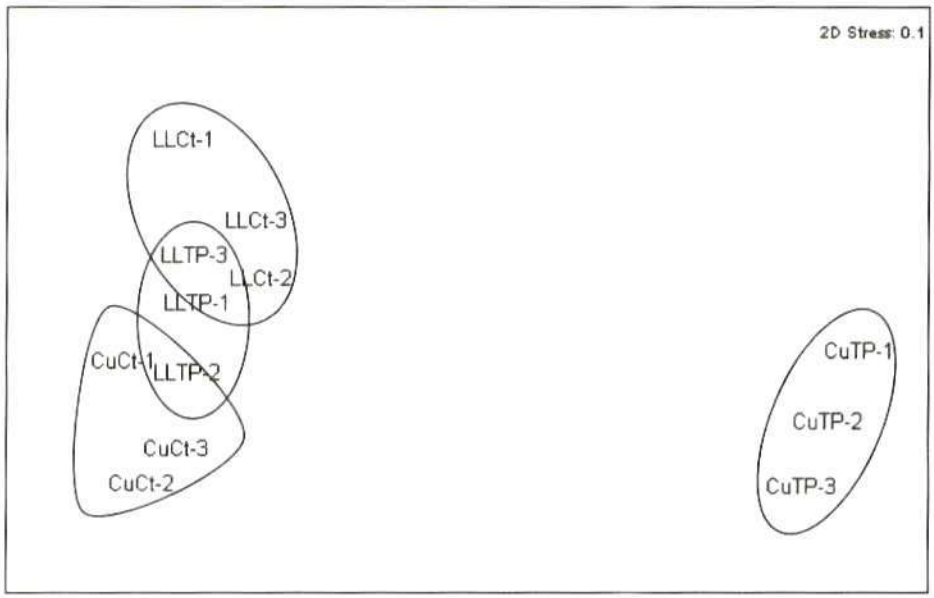


Figure 6.2 Non-metric multidimensional scaling analysis plot of DGGE fingerprints showing similarities between thermal purged (TP) and non-thermal purged (Ct) copper and LLDPE biofilm communities (CuTP= thermal purged copper, CuCt= 'control' copper i.e. non-thermal purged, LLTP= thermal purged LLDPE, LLCt= 'control' LLDPE i.e. non-thermal purged. 1-3 denotes replicate number in each case).

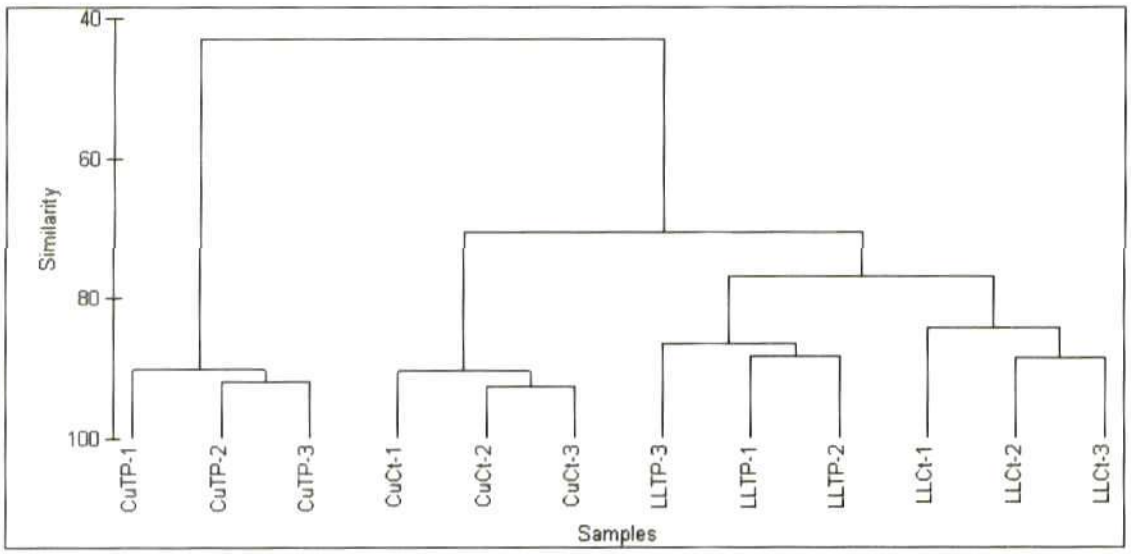


Figure 6.3 Cluster analysis of DGGE fingerprint indicating similarity relationships between thermal purged (TP)/ non-thermal purged (Ct) copper and LLDPE biofilm communities. Samples are denoted as described in Figure 6.2.

Table 6.1 Similarity half matrix between presence/absence of DGGE bands of thermal purged (TP)/ non-thermal purged (nTP) copper and LLDPE biofilm communities. (**Sp. r/ness**= species richness expressed as mean numbers of bands observed in three replicates).

			Copper (TP)			Copper (Ct)			LLDPE (TP)			LLDPE (Ct)		
	Replicate	**Sp. r/ness**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Copper (TP)	**1**	19.3	100.00											
	2		91.82	100.00										
	3		88.89	91.97	100.00									
Copper (Ct)	**1**	26	43.05	42.30	40.71	100.00								
	2		42.47	42.23	41.78	90.59	100.00							
	3		44.65	43.83	43.38	90.50	92.77	100.00						
LLDPE (TP)	**1**	26	41.93	41.53	42.14	72.83	67.15	69.06	100.00					
	2		40.91	40.42	40.98	75.64	70.62	70.60	88.43	100.00				
	3		40.00	38.90	39.74	77.36	72.59	76.29	85.37	87.73	100.00			
LLDPE (Ct)	**1**	31	48.27	46.78	45.38	67.90	63.70	65.38	76.12	76.36	76.53	100.00		
	2		47.53	46.29	44.38	73.78	70.08	72.04	77.94	78.90	79.37	85.21	100.00	
	3		45.40	43.56	43.79	68.54	66.86	70.68	73.36	74.26	79.29	83.72	88.63	100.00

6.3.2 Effect of material selection on residential bacterial communities

Non-metric multidimensional scaling (nMDS) analysis of DGGE fingerprints is shown in Figure 6.4, and cluster analysis of the same fingerprints is shown in Figure 6.5. Both analyses clearly indicate the existence of distinct bacterial communities associated with each of the four materials. Average similarities between replicates of each material were high in all cases, ranging from 77.80% to 88.92%. The average similarities of DGGE fingerprints between material types were lower e.g. 53.10% between copper and PEX, and 38.38% between SS and all other materials. These relationships are clearly represented in Figure 6.4. Species richness, indicated by the mean number of bands present (Table 6.2), was highest on EPDM (18). The lowest species richness was observed on SS and PEX (15). The similarity half matrix of the DGGE fingerprints is shown in Table 6.2. The highest similarity was observed between copper and PEX (mean 53.10%, SD=3.82%) whilst copper and SS exhibited the greatest dissimilarity (mean 30.03%, SD= 2.76%). An ANOSIM R statistic of 1 was calculated, suggesting that replicates within groups were more similar than those from other groups.

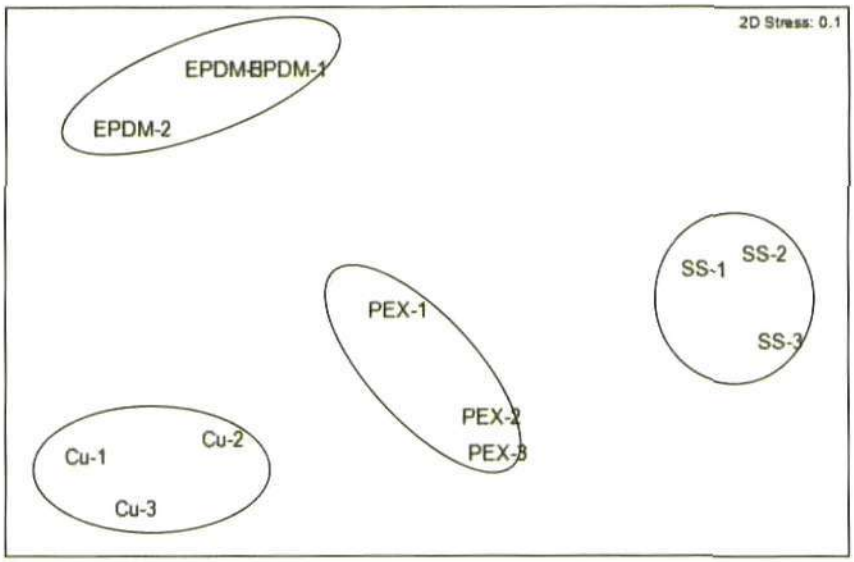


Figure 6.4 Non-metric multidimensional scaling analysis plot of DGGE fingerprints showing relative similarities between copper (Cu), stainless steel 316 (SS), EPDM and PEX biofilm communities after 84 days (1-3 denotes replicate number in each case).

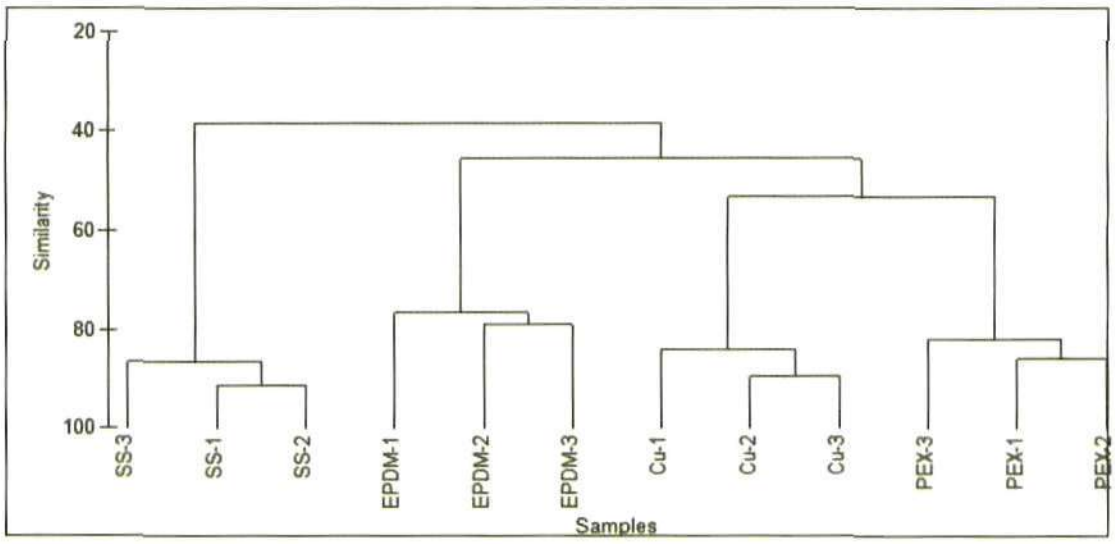


Figure 6.5 Cluster analysis of DGGE fingerprint indicating levels of similarity between copper (Cu), stainless steel 316 (SS), EPDM and PEX biofilm communities after 84 days (1-3 denotes replicate number in each case).

Table 6.2 Similarity half matrix between presence/absence of DGGE bands of EPDM, PEX, stainless steel 316 (SS) and copper (Cu) biofilm communities after 84 days 12 hourly, 30 second flushing at 41 °C. (**Sp. r/ness**= species richness expressed as mean number of bands observed in three replicates).

			EPDM			PEX			SS			Cu		
	Replicate	**Sp. r/ness**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
EPDM	**1**	18	100.00											
	2		74.98	100.00										
	3		78.16	79.02	100.00									
PEX	**1**	15	55.00	49.63	49.25	100.00								
	2		49.10	42.39	42.65	85.94	100.00							
	3		51.67	44.87	48.03	78.78	84.93	100.00						
SS	**1**	15	40.45	32.57	35.89	49.77	51.75	52.68	100.00					
	2		40.45	32.06	37.62	49.05	49.37	50.67	91.32	100.00				
	3		36.22	29.26	31.59	47.80	50.97	47.73	86.79	86.25	100.00			
Cu	**1**	16	46.69	45.32	49.91	53.61	47.09	48.06	27.41	27.28	25.64	100.00		
	2		43.29	38.89	44.29	58.72	55.78	55.25	33.35	33.66	31.51	83.46	100.00	
	3		39.47	37.16	41.10	55.50	53.25	50.61	30.97	30.40	30.04	84.55	89.55	100.00

6.3.3 Time course analysis of bacterial community changes within copper biofilms over 84 days.

Non-metric multidimensional scaling (nMDS) analysis of DGGE fingerprints is shown in Figure 6.6, and cluster analysis of the same fingerprints is shown in Figure 6.7. Both analyses clearly indicate the existence of distinct bacterial communities associated with copper at 28 and 84 days. Average similarities between replicates within each time point were high in both cases (91.15% after 28 days, and 83.72% after 84 days). The average similarity of the DGGE fingerprints between time points was 51.04%, and species richness, indicated by the mean number of bands present (Table 6.3), was higher after 28 days (20) than after 84 days (17). The similarity half matrix of the DGGE fingerprints is shown in Table 6.3, and a mean similarity of 52.01% (SD= 2.36%) was observed. An ANOSIM R statistic of 1 was calculated, suggesting that replicates within groups were more similar than those from other groups.

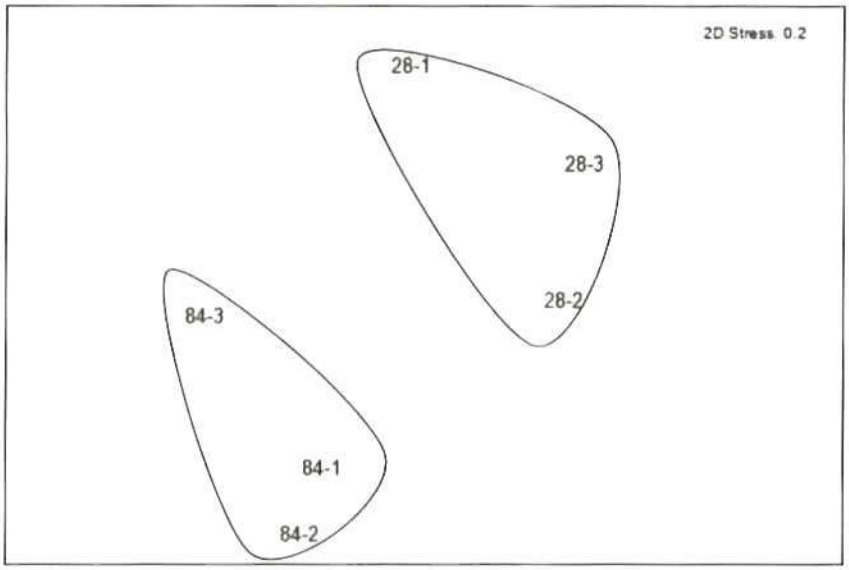


Figure 6.6 Non-metric multidimensional scaling analysis plot of DGGE fingerprints showing relative similarities of 28 day- and 84 day copper biofilm communities over 84 days of 12 hourly, 30 second flushing at 41 °C (1-3 denotes replicate number in each case).

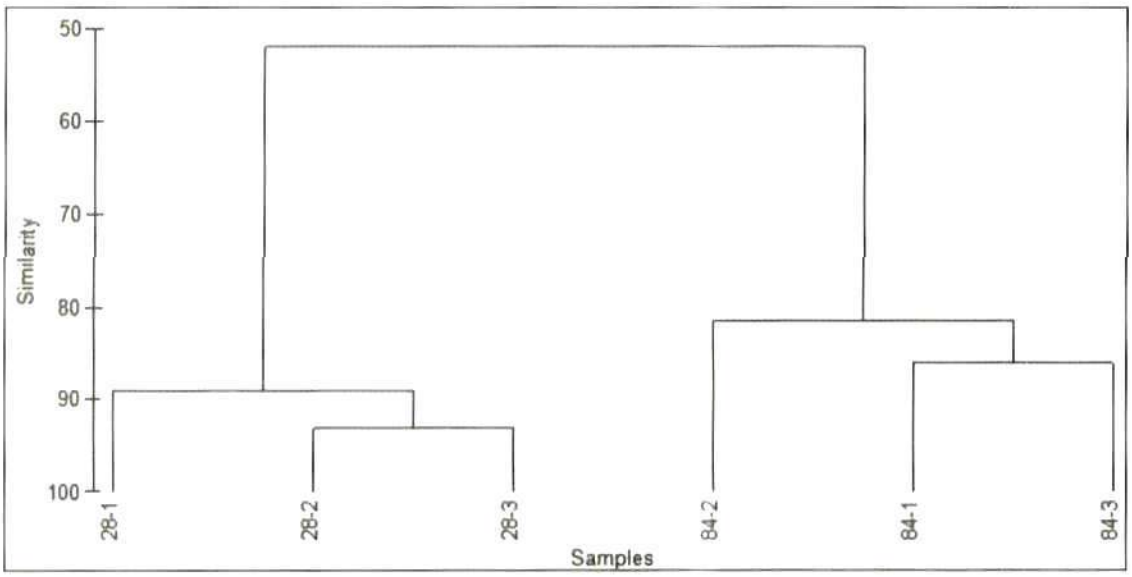


Figure 6.7 Cluster analysis of DGGE fingerprint indicating levels of similarity between 28 day and 84 day copper biofilm communities over 84 days of 12 hourly, 30 second flushing at 41 °C (1-3 denotes replicate number in each case).

Table 6.3 Similarity half matrix between presence/absence of DGGE bands of copper biofilm communities, after 28 and 84 days of 12 hourly, 30 second flushing at 41 °C. (**Sp. r/ness**= species richness expressed as mean numbers of bands observed in three replicates).

			Copper (28 days)			Copper (84 days)		
	Replicate	**Sp. r/ness**	**1**	**2**	**3**	**1**	**2**	**3**
Copper (28 days)	**1**	20	100.00					
	2		91.32	100.00				
	3		86.79	86.25	100.00			
Copper (84 days)	**1**	17	50.91	53.83	54.67	100.00		
	2		47.38	50.29	50.52	83.46	100.00	
	3		51.68	53.92	54.86	84.55	89.55	100.00

6.3.4 Time course analysis of bacterial community changes within PEX biofilms over 84 days.

Non-metric multidimensional scaling (nMDS) analysis of DGGE fingerprints is shown in Figure 6.8, and cluster analysis of the same fingerprints is shown in Figure 6.9. Both analyses clearly indicate the existence of distinct bacterial communities associated with PEX at 28, 56 and 84 days. Average similarities between replicates within each time point exhibited a successive decrease from 87.62% after 28 days to 74.95% after 84 days. In comparison, the average similarities between time points were considerably lower (37.54% between 56 and 84 days, and 29.13% between 28 and 56/ 84 days). Species richness, indicated by the mean number of bands present (Table 6.4), was highest after 56 days (27) and lowest after 28 days (13). The similarity half matrix of the DGGE fingerprints is shown in Table 6.4. The highest similarity was observed between 56 and 84 days (mean 37.54%, SD= 5.89%), and the lowest between 28 and 84 days (mean 27.25%, SD= 1.27%). An ANOSIM R statistic of 1 was calculated, suggesting that replicates within groups were more similar than those from other groups.

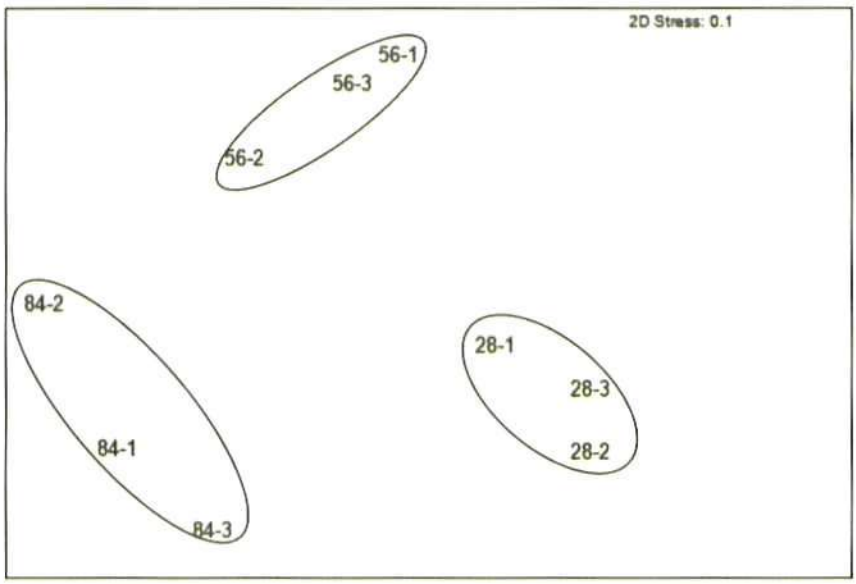


Figure 6.8 Non-metric multidimensional scaling analysis plot of DGGE fingerprints showing relative similarities between 28 day, 56 day and 84 day PEX biofilm communities over 84 days of 12 hourly, 30 second flushing at 41 °C (1-3 denotes replicate number in each case).

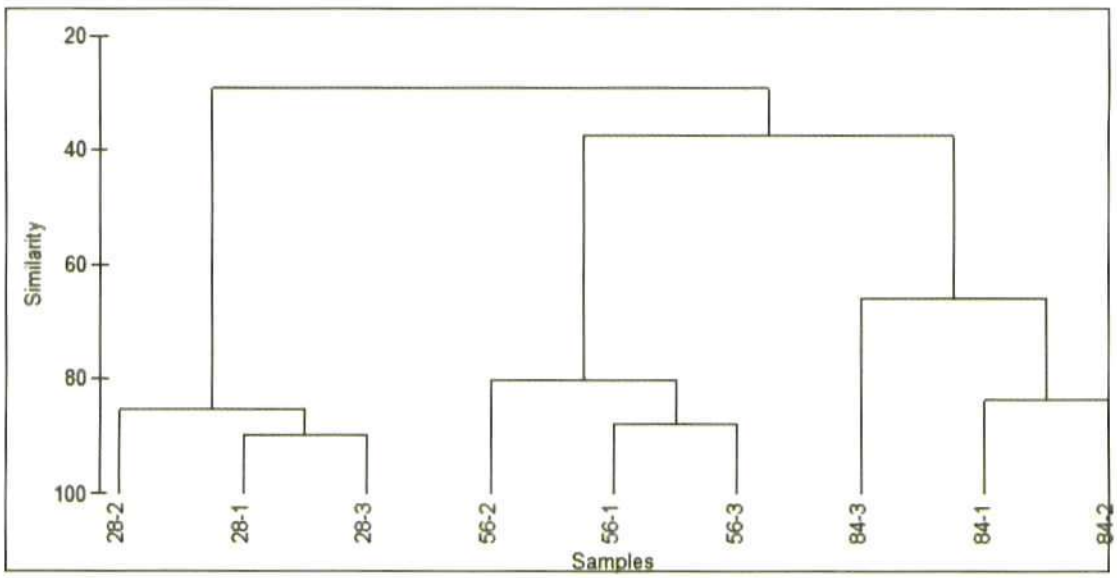


Figure 6.9 Cluster analysis of DGGE fingerprints indicating levels of similarity between 28 day, 56 day and 84 day PEX biofilm communities over 84 days of 12 hourly, 30 second flushing at 41 °C (1-3 denotes replicate number in each case).

Table 6.4 Similarity half matrix between presence/absence of DGGE bands PEX biofilm communities after 28, 56 and 84 days of 12 hourly, 30 second flushing at 41 °C. (**Sp. r/ness**= species richness expressed as mean numbers of bands observed in three replicates).

			PEX (28 days)			PEX (56 days)			PEX (84 days)		
	Replicate	**Sp. r/ness**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
PEX (28 days)	**1**	13	100.00								
	2		83.48	100.00							
	3		89.81	87.36	100.00						
PEX (56 days)	**1**	27	28.17	25.73	27.10	100.00					
	2		40.00	34.96	38.81	75.46	100.00				
	3		32.59	29.46	31.31	87.93	85.15	100.00			
PEX (84 days)	**1**	15	27.81	26.39	27.43	31.59	46.83	36.76	100.00		
	2		25.42	25.16	24.44	30.41	45.11	35.07	83.80	100.00	
	3		27.94	25.28	26.40	34.13	42.45	35.50	62.93	69.27	100.00

6.3.5 *Time course analysis of bacterial community changes within EPDM biofilms over 84 days.*

Non-metric multidimensional scaling (nMDS) analysis of DGGE fingerprints is shown in Figure 6.10, and cluster analysis of the same fingerprints is shown in Figure 6.11. Both analyses clearly indicate the existence of distinct bacterial communities associated with EPDM after 28 and 84 days. Average similarities between replicates within each time point were high in both cases with a marginal decrease observed over time (82.79% after 28 days, and 77.93% after 84 days). The SIMPER average similarity of the DGGE fingerprints between 28 and 84 days was 41.24%, and species richness, indicated by the mean number of bands present (Table 6.3), was higher after 84 days (22) than after 28 days (17). The similarity half matrix of the DGGE fingerprints is shown in Table 6.3, and a mean similarity of 41.24% (SD= 3.00%) was observed. An ANOSIM R statistic of 1 was calculated, suggesting that replicates within groups were more similar than those from other groups.

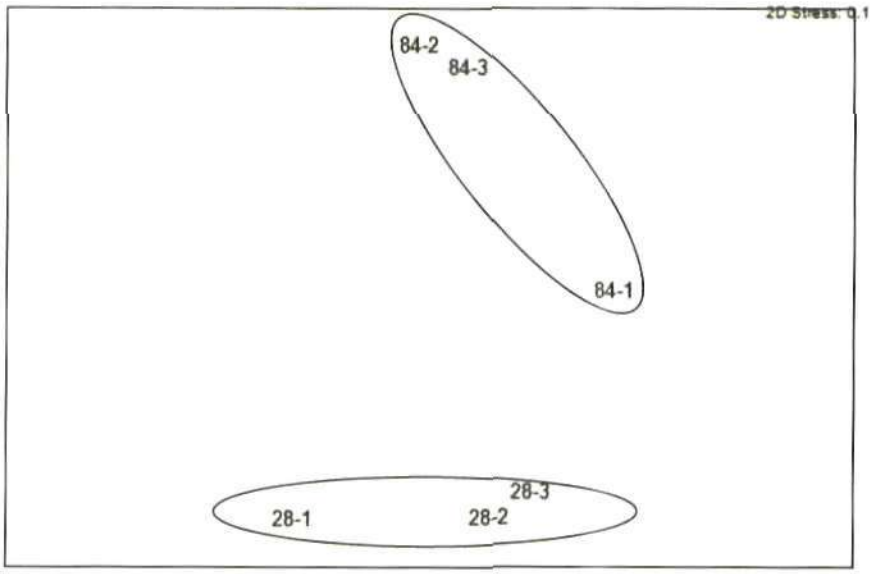


Figure 6.10 Non-metric multidimensional scaling analysis plot of DGGE fingerprints showing similarities between 28 day and 84 day EPDM biofilm communities over 84 days of 12 hourly, 30 second flushing at 41 °C (1-3 denotes replicate number in each case).

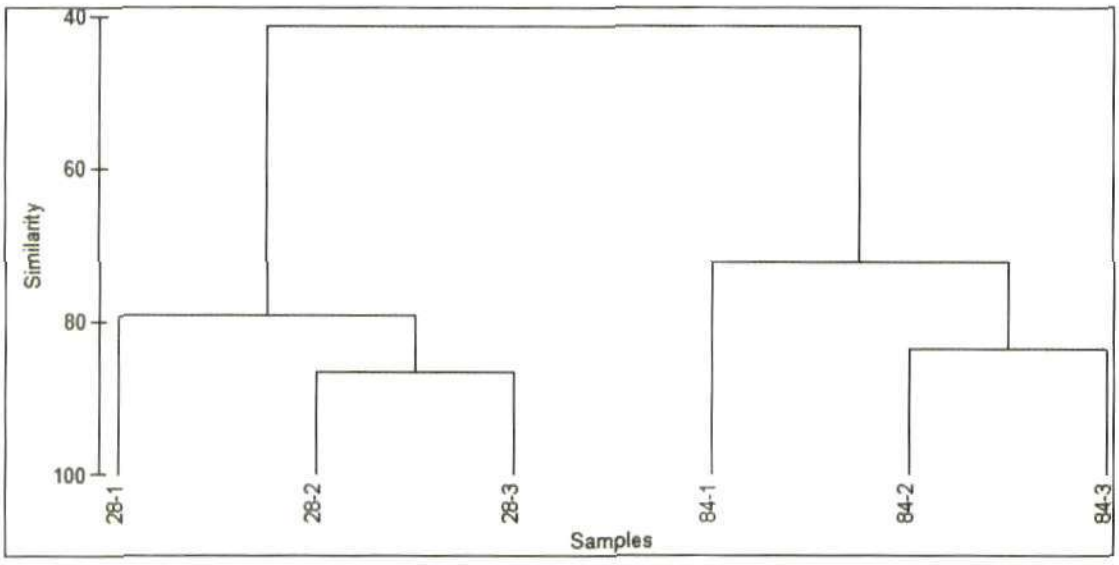


Figure 6.11 Cluster analysis of DGGE fingerprints showing similarities between 28 day and 84 day EPDM biofilm communities over 84 days of 12 hourly, 30 second flushing at 41 °C (1-3 denotes replicate number in each case).

Table 6.5 Similarity half matrix between presence/absence of DGGE bands of EPDM biofilm communities, after 28 and 84 days of 12 hourly, 30 second flushing at 41 °C. (**Sp. r/ness**= species richness expressed as mean numbers of bands observed in three replicates).

			28 days			84 days		
	Replicate	**Sp. r/ness**	**1**	**2**	**3**	**1**	**2**	**3**
28 days	**1**	17	100.00					
	2		82.18	100.00				
	3		76.12	86.43	100.00			
84 days	**1**	22	42.45	45.44	44.28	100.00		
	2		36.59	37.92	39.37	68.95	100.00	
	3		39.91	41.57	43.64	75.50	86.96	100.00

6.3.6 Comparison of bacterial communities associated with a variety of tap outlet fittings

Non-metric multidimensional scaling (nMDS) analysis of DGGE fingerprints is shown in Figure 6.12, and cluster analysis of the same fingerprints is shown in Figure 6.13. Both analyses clearly indicate the existence of distinct bacterial communities associated with the different tap outlet fittings after 84 days. Average similarities between replicates associated with the SG, AFL and NAFL after 84 days were 74.92%, 83.89% and 81.65%, respectively. In comparison, the average similarities between the different fittings were considerably lower (39.89% between the AFLO and the NAFLO and 39.01% between the SG and the two laminar flow outlet fittings. Species richness, indicated by the mean number of bands present (Table 6.4), was highest on the NAFL (31) and lowest on the SG (22). The similarity half matrix of the DGGE fingerprints is shown in Table 6.5. The highest similarity was observed between the AFL and the NAFL (mean 39.89%, SD= 1.67%), and the lowest between the SG and the AFL (mean 38.80%, SD= 3.43%). Given the marginal differences between these values, they are not considered to be significant. An ANOSIM R statistic of 1 was calculated, suggesting that replicates within groups were more similar than those from other groups.

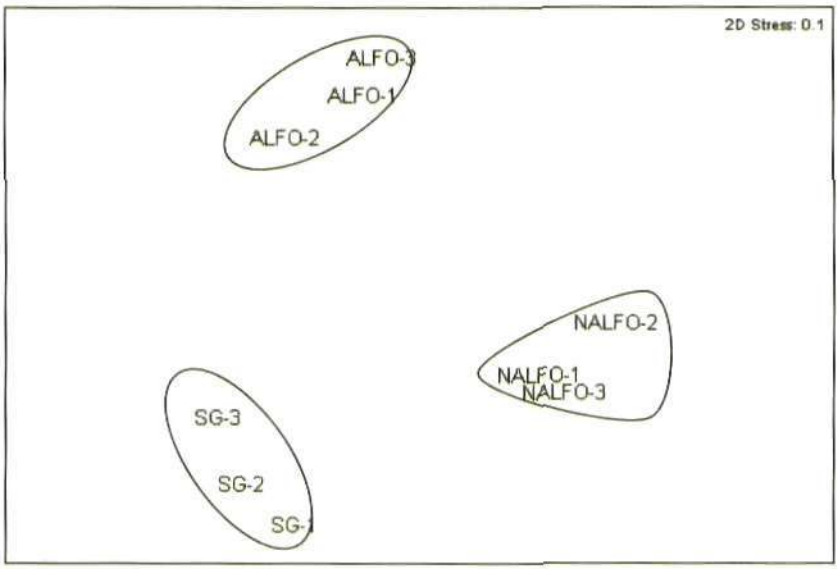


Figure 6.12 Non-metric multidimensional scaling analysis plot of DGGE fingerprints showing similarities between biofilm communities associated with sealing gaskets (SG), antimicrobial laminar outlet (ALFO) and non-antimicrobial laminar flow outlet (NALFO) after 84 days of 12 hourly, 30 second flushing at 41 °C (1-3 denotes replicate number in each case). .

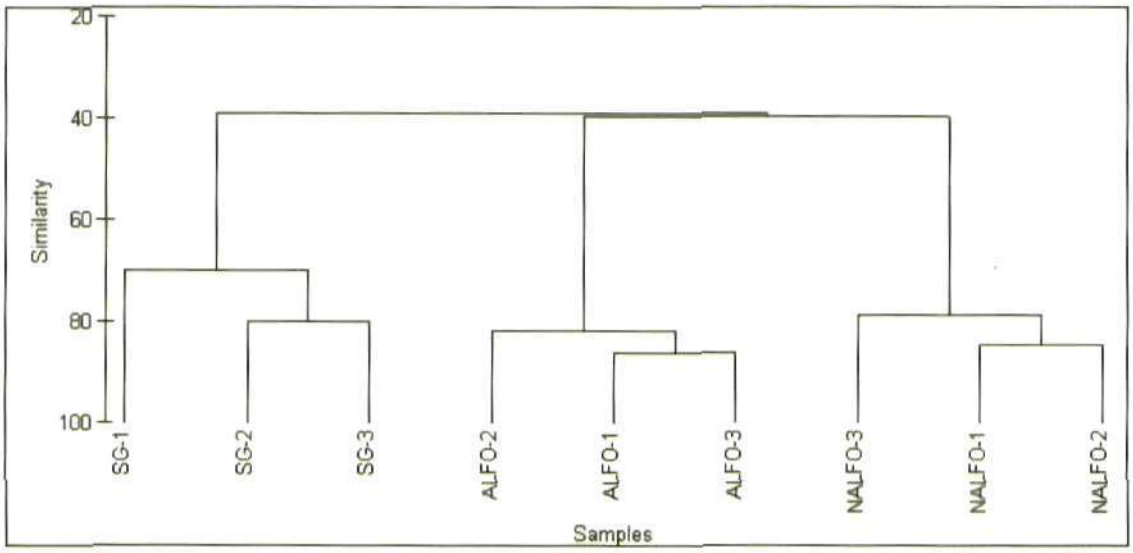


Figure 6.13 Cluster analysis of DGGE fingerprints showing similarities between biofilm communities associated with sealing gaskets (SG), antimicrobial laminar outlet (ALFO) and non-antimicrobial laminar flow outlet (NALFO) after 84 days of 12 hourly, 30 second flushing at 41 °C (1-3 denotes replicate number in each case).

Table 6.6 Similarity half matrix between presence/absence of DGGE bands of biofilm communities associated with sealing gaskets (SG), antimicrobial laminar flow outlet (ALFO) and non-antimicrobial laminar flow outlet (NALFO) after 84 days of 12 hourly, 30 second flushing at 41 °C. (**Sp. r/ness**= species richness expressed as mean numbers of bands observed in three replicates).

			SG			ALFO			NALFO		
	Replicate	**Sp. r/ness**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
SG	**1**	**22**	100.00								
	2		83.48	100.00							
	3		89.81	87.36	100.00						
ALFO	**1**	**29**	28.17	25.73	27.10	100.00					
	2		40.00	34.96	38.81	75.46	100.00				
	3		32.59	29.46	31.31	87.93	85.15	100.00			
NALFO	**1**	**31**	27.81	26.39	27.43	31.59	46.83	36.76	100.00		
	2		25.42	25.16	24.44	30.41	45.11	35.07	83.80	100.00	
	3		27.94	25.28	26.40	34.13	42.45	35.50	62.93	69.27	100.00

6.3.7 Sequence analysis

Successful sequencing was achieved for only 11 out of the 20 PCR fragments which were sent for analysis. The results of sequence analysis are shown in Table 6.7 (overleaf). In other cases, sequence quality was deemed to be below the required standard and no sequencing data was returned. Recovered sequence lengths ranged from 45 to 119 base pairs (bp). Maximum identification percentages ('Max Ident') ranged from 92% to 100%, and the order Sphingomonadales, including *Novosphingobium* and *Sphingomonas,* featured heavily in the resulting BLAST searches. One isolate of the order Comamonodaceae was shown to be present on non-thermal purged LLDPE, and several unidentified, uncultured bacteria were also in evidence.

Table 6.7 Summary of sequence analysis undertaken on excised bands. (ALFO- antimicrobial laminar flow outlet).

Recovered sequence length (bp)	Source	BLAST identification/ Max ident percentage
58	ALFO biofilm 84 days	***Sphingobium* sp./ 97%**
119	Non-thermal purged LLDPE	**Order Comamonodaceae/ 100%**
104	Thermal-purged copper	**Uncultured bacterium isolate/ 92%**
45	Thermal-purged copper	**Uncultured bacterium clone/ 93%**
112	Copper biofilm- 84 days	***Sphingomonas* sp./ 94%**
93	Copper biofilm 84 days	**Uncultured bacterium clone/ 97%**
109	EPDM biofilm 84 days	***Novosphingobium* sp./97%**
114	EPDM biofilm 84 days	**Uncultured bacterium clone/ 92%**
108	EPDM biofilm 84 days	**Sphingomonadales/ 97%**
79	PEX biofilm 84 days	**Uncultured bacterium partial 16S rRNA gene/ 100%**
109	PEX biofilm- 84 days	**Uncultured Sphingomonadaceae bacterium/ 100%**

6.4 Discussion

Six PCR/DGGE analyses were undertaken in order to investigate the effect of factors such as thermal purging, temporal influence and material choice on the bacterial communities within the test rig. Without exception, each investigation produced clear evidence of a large degree of variation within the bacterial communities investigated. This is perhaps unsurprising given that the test rig represents a huge diversity of micro-environments, and within any one study, individual biofilms (or, more specifically, the biofilm samples which have provided the genetic material for study) were either temporally or geographically well separated.

The route from sample collection to final community analysis using DGGE (and equivalents such as temperature gradient gel electrophoresis) is multistage and complex. Consequently, the potential for the introduction of undesired bias at every point in the process is considerable and has been frequently reported (Zhang and Fang 2000; Sekiguchi *et al.* 2001; Petersen and Dahllöf 2005). Therefore, critical analysis of the methods employed during these studies is essential in order to identify areas for future improvement. The complex nature of the processes employed during these studies means that as much attention as is practicable must be paid to each step in order to optimize the process in question and maximize the chances of ultimately gaining data which accurately reflects the bacterial community as it occurs in the environment. Nowadays, a multitude of bacterial cell recovery and DNA extraction strategies are available. In many cases these protocols are specifically tailored towards isolating DNA from particular environments and, whilst cost and time may be limiting factors, any preliminary efforts which can be made to optimize the recovery of bacterial

DNA from a specific environment would of course be beneficial in improving the quality of data gathering during downstream processes. In the context of the studies under discussion, conventional phenol: chloroform extraction was used. This was primarily due to its low cost and general reliability when employed to extract DNA from a variety of sample types. However, on occasion it proved challenging to recover sufficient DNA for downstream processing and this was possibly due to high levels of inhibitory substances such as humic acids and/ or metals. Consequently, it is felt that further optimization of the method to counter this may have been useful in overcoming such issues.

Another aspect of the DNA extraction procedure which undoubtedly influenced downstream processing is the measurement of DNA concentration post-extraction, for the simple reason that downstream processes were in this case specifically tailored towards targeting bacterial DNA, whereas the initial extraction procedure is presumed to have resulted in a complex mixture of both bacterial and non-bacterial DNA, the proportions of which may have varied between samples and are unknown. As a consequence, estimation of exact yields of bacterial DNA with a view to standardisation of template (target) DNA was impossible, although DNA measurement was deemed essential as a useful indicator of the success of the extraction procedure.

Further optimization of the PCR procedure may have increased the robustness of the data gathered. For example, identification and trialling of different primer sets, targeting different areas of the variable V3 region of the 16S rRNA gene, may have yielded more useful sequence data. The primer set used during these studies yields a relatively small fragment (193bp) and it is presumed that a larger fragment would have led to greater success at species-level identification. It is also possible that different primer sets and PCR conditions

may affect subsequent band separation, and consequently influence downstream processes in this way. Any trialling of new primer sets, experimentation with changes in PCR conditions, or re-positioning of the GC clamp (onto the reverse primer instead of the forward primer, as was used in this case), entails a great deal of optimization, and temporal/ financial constraints imposed strict limits on the amount of optimization which was undertaken.

Selection of bands for excision and subsequent sequencing was largely dictated by what was observable directly under UV illumination, rather than selecting bands observed on the computer-generated image. This is because in practice the two were often dissimilar, with the numbers of directly observable bands (under UV illumination) being the limiting factor in this regard. It is therefore unfortunate that what were perhaps perceived as bands of potential importance were not always selected for sequencing analysis.

Another limiting factor regarding the excision of bands for sequencing was the close proximity of bands. Such situations of co-migration of PCR products with similar melting characteristics were observed many times and necessitated a cautious approach to band excision. Whilst it may be tempting to assume that single bands refer to single species, the terms 'co-migrating taxonomic unit' (CTU) and 'operative taxonomic unit' (OTU) have previously been proposed as terms suitable for use in recognition of the fact that this may not be the case (Gafan and Spratt 2005). Indeed, Gafan and Spratt (2005) and Sekiguchi *et al.* (2001) demonstrated that single bands may, under tighter denaturing gradient conditions, yield 'sub-bands' (representing in some cases both Gram-negative and Gram-positive bacteria) and as a consequence suggest a cautionary approach to analysis. Analysis of the gels used in this study suggested that

some re-running of gels and manipulation of the gradients subsequently utilized may have improved band isolation for downstream processing, as many bands were in close proximity to each other. However constraints on resources prevented this.

The image/ statistical analysis methodology employed here is one of many currently available, and required a degree of subjective assessment of band identification. Similar approaches to DGGE analysis have been reported by Powell *et al.* (2003) and Dimitroglou *et al.* (2009), and the Primer software has also recently been used in conjunction with alternative molecular methods to investigate microbial communities associated with copper corrosion (Pavissich *et al.* 2010). Whilst time consuming, it allowed a great deal of control over the way in which bands were assigned and enabled analysis of the data in a variety of ways.

It is acknowledged that greater sample replication at the PCR stage would undoubtedly have resulted in more robust data, and also that carrying out PCR on templates derived from multiple DNA extractions (as opposed to conducting multiple PCR reactions from a single DNA extraction) may have better reflected the original bacterial community. However, the fact that sample sizes for DNA extraction were deemed to be too small to guarantee the success of triplicated DNA extractions necessitated a 'single extraction/ multiple-PCR' approach.

Many experimental approaches employ standardization techniques in an effort to overcome the inherent bias associated with the analysis of environmental samples. For example, ATP analysis as described elsewhere employs an ATP standard of a known concentration. During the course of analyses of this kind the use of internal standards in DNA extraction or PCR may be useful in ensuring that the final results may be reliably related back to the original

sample. Such an approach may involve sample 'spiking' before DNA extraction and has been proposed previously (Petersen and Dahllöf 2005). However, it is presumed that inherent biases such as primer mismatch during PCR, diversity of template DNA etc. still remain.

Analysis of bacterial communities associated with thermal-purged and non-thermal purged copper/ LLDPE show that, whilst some differences were observed between all four groups, thermal purging of copper gave rise to a community profile which differed significantly from that observed on non-thermal purged copper. Whilst differences in the LLDPE community under the two regimes were also observed, these differences were less obvious and this may be due to factors such as the differing nature of the two materials, and their influence on the efficiency of the thermal purging procedure. Given that plasticized materials such as LLDPE are known to leach substances of potential nutritional value to microbes, and that copper is potentially toxic to many microorganisms, it is perhaps unsurprising that species richness was greater on the former. It is not known whether thermal treatment of copper induces an increased toxic effect upon residential bacteria. It is possible that this may account, in part, for the decrease in species richness which was observed and could potentially form the basis of further future investigations which could, for example, investigate the occurrence of genetically-controlled copper resistance mechanisms within the bacterial population.

Primer analysis of bacterial communities associated with different materials after 84 days showed that, whilst species richness did not vary greatly between the four materials, the communities were clearly very different. EPDM demonstrated the greatest species richness. Whilst this is perhaps unsurprising given the high incidence of biofilm formation associated with this material (see

chapter 5), Bressler *et al.* (2009) reported up to 68 DGGE bands derived from drinking water biofilms which had been grown on EPDM coupons immersed in a laboratory flow-through reactor system for 14 days. However, it is important to recognise that any direct comparisons would be unwise, given the differing experimental procedures employed. It is presumed that the growth of copious biofilm such as was observed (see chapter 5) would offer greater opportunities for recruitment of new bacterial species into the existing biofilm, resulting in the increased species richness (in comparison to other materials) which was indeed observed, albeit at what is possibly a lower-than-expected level. Few studies have specifically employed DGGE to investigate bacterial communities on these materials, preferring instead to employ techniques such as fluorescence *in situ* hybridization (FISH) and culturing to target specific microorganisms (for example, Moritz *et al.* (2010)). Pavissich *et al.* (2010) used terminal restriction length polymorphism (T-RFLP) to analyze communities associated with copper corrosion. Other studies have used alternative methods for community analysis which involve neither culturing nor analysis of genetic material. For example Lehtola *et al.* (2004) used two lipid biomarkers to study changes in the bacterial community on copper and polyethylene pipes over 308 days, and, in common with the current, albeit shorter, study, found distinctly different microbial communities associated with the two materials after this period.

Analysis of three types of tap outlet fitting again revealed three distinct bacterial communities, although it is recognised that the lack of replication in terms of number of outlets used necessitates caution in drawing any generalized conclusions with regards to the types of communities which are likely to form on these fittings. However, the data would appear to suggest that the incorporation of an antimicrobial substance into the laminar flow outlet fitting exerts an

influence on the associated bacterial community, when compared to the non-antimicrobial outlet fitting. It is perhaps unsurprising that the bacterial community associated with the sealing gasket showed the greatest level of dissimilarity, as it is hugely different in terms of both design and functionality. As a consequence, the resulting micro-environment is likely to be vastly different from that found on the two types of laminar flow outlet fitting. The communities observed on these fittings are likely to be influenced by upstream events, as these fittings represent a considerable barrier to waterborne microbes before they exit the water system.

Several analyses were carried out into temporal effects on bacterial communities observed on copper, PEX and EPDM, and it is unfortunate that day 56 data was not obtained in all cases. This was due to difficulties encountered during pre-DGGE procedures which would suggest that further refinement of the method to avoid possibly co-extraction of inhibitory substances was required. Nevertheless, some interesting results were obtained, especially in the case of PEX, when a sharp peak in species richness was observed after 56 days. A possible reason for this may be the existence of a correlation between species richness and the degree of leaching of potential nutrients from the material itself. Analysis of copper biofilms revealed a slight decrease in species richness over time. This may be due to the maturation of the biofilm and the establishment of a less diverse community which is dominated by species which are better suited to living on a copper surface. A potential future line of research would be to investigate whether there is an increase in the incidence of genes actively involved in reducing the toxic effects of copper over time, in an effort to confirm this.

The dynamic, adaptive nature of biofilms means that changes in the local micro-environment in which they are found are likely to influence their development in a number of ways, and this may extend to manipulating the bacterial communities present therein. For example, if any change in the microbiological profile of the water system upstream of the developing biofilm under investigation was to occur, then it is possible that subsequent analyses may reveal changes in the community which are due to these 'extra-biofilm events' rather than changes occurring solely as a result of the maturation of the biofilm under investigation ('intra-biofilm' events). However, it may be argued that in experimental set ups such as the test rig employed in this case, and indeed in 'real-life' installations, the existence of external influences is unavoidable. Therefore, any reasonable steps in understanding of the surrounding environment should be taken, and in this case it may have been beneficial to obtain DGGE profiles of the water itself, as it seems reasonable to assume that the water supply is the main provider of seed microbes for biofilm formation. This would also have represented a suitable experimental control.

Sequencing analysis yielded little in the way of definitive identification of bacterial species, and this is believed to be closely linked to both primer set used and the recovered sequence length. Consequently, it may be worthwhile to focus future efforts on investigating the use of alternative primer sets which will results in longer PCR products and consequently more informative sequencing data. It may be possible that multiple bands were co-excised, and that this may have led to problems in generating complete and reliable sequence information. It is not known whether prolonged exposure to UV at the band excision stage would have adversely affected downstream processing procedures. However, the possibility of this should not be discounted and any

future improvements in this particular aspect of post-DGGE processing should be investigated and implemented.

Of the sequences which were retrieved successfully, bacteria of the order Sphingomonadales appear to have been well represented. That this should have been the case across a range of source material may be indicative of their dominance within the test rig. However, it is acknowledged that the paucity of sequencing information available means that it is difficult to make such an assumption with any degree of certainty. The presence of bacteria of this type is not unexpected, given that these Gram-negative, yellow pigmented, chemo-heterotrophic, non-sporulating, pleiomorphic cells are commonly associated with water (Brenner *et al.* 2005) and have been implicated in water-related nosocomial infections (Perola *et al.* 2002). A review by White *et al.* (1996) describes a unique ability to produce highly viscous gellan polysaccharides. Such a feature, coupled with their chemotrophic abilities, may be instrumental in their apparently highly successful existence within water system biofilms. It would be of interest to investigate further the extent to which bacteria such as those of the order Sphingomonadales dominate the microbial communities within the test rig, as well as whether their chemotrophic abilities extend to degradation of leached compounds from materials such as EPDM, PEX and LLDPE.

The recent development of advanced sequencing technologies is likely to lead to their increasing employment in WDS settings, and would bypass many of the challenges encountered during this study during the PCR/DGGE/sequencing procedure. For example, 454 pyrosequencing allows for high-throughput sequencing in a single sequencing run, and has recently been employed by Hong *et al.* (2010) to investigate bacterial communities in water meters. The

advantages of such a technique in defining the microbial ecology in a variety of WDS situations would appear to be manifold.

Bacteria are highly likely to be a constituent, albeit a major one, of a larger and more taxonomically complex microbial biofilm community incorporating, for example, many species of fungi, protozoa and bacteriophages, which may otherwise be undetected. With this in mind, expanding future studies, by employing a range of specific primer sets and refined DNA extraction techniques, would be beneficial in furthering current understanding of overall diversity of the microbial community. The identification of patterns of community diversity in response to varying environmental conditions may open the door to biofilm management methods based on the identification and exploitation of inter-dependency of certain microbial groups. It is postulated that furthering understanding of the relationship between host-specific biofilm-bound bacteriophages using molecular methods of identification and monitoring may lead to novel control methods in water system settings, in a similar way that bacteriophage therapy is employed in clinical settings (see Donlan (2009) for a review of this subject).

In conclusion, whilst the limitations of the methods employed and the existence of potential bias at various stages of what is a complex procedure are recognized, these studies have provided an insight into how a variety of factors are capable of inducing bacterial community changes within the test rig. Future molecular studies of interest may include the use of methods such as FISH to further elucidate the complex relationships which exist within WDS biofilm communities.

Chapter 7 General discussion, conclusions, and suggestions for further work

A range of techniques has been employed during this research in order to assess biofilm formation, under a variety of conditions, within the test rig. The use of model water distribution systems (WDSs) as tools for biofilm study have been described previously, with such systems varying greatly in terms of design, scale, and functionality. The test rig used during this research was designed specifically for this project and is highly complex in its construction. It has demonstrated its versatility in terms of the types of studies which it is capable of supporting, and may be viewed as a valuable resource for future research projects. However, any novel technology should be subjected to a critical appraisal of its performance post-use and it is felt that some minor technical modifications would improve the test rig's performance as a research tool, from both an ergonomic and a scientific perspective. Generally speaking, from an engineering and manufacture point of view, the test rig has performed extremely well and little remedial work has been required post-installation. This has allowed research to be carried out largely uninterrupted by technical issues, and is a testament to the high degree of engineering expertise involved in its manufacture. Examples of possible modifications are given in Table 7.1 (page 223).

Throughout the course of this research, numbers of culturable aerobic bacteria isolated from the cold feed water entering the test rig remained consistently high (in the order of 10^4 cfu ml^{-1}). This is believed to be due to the fact that the cold water storage tank was acting as a reservoir for microbial growth. It was also apparent that the residence time of water within this tank i.e. the period of

stagnation between flushes, was sufficient for the total removal of mains water-derived chlorine from the water at this point (confirmed *via* measurement of mains water/ cold feed chlorine levels at time of sampling), thus bacterial growth and biofilm formation within the test rig was not subject to any form of disinfection pressure. This water storage tank is presumed to be of a type commonly used in the plumbing industry. However, the inclusion of a downstream dosing system for the accurate administration of a desired disinfecting chemical may be useful in conducting research which bears greater comparison with 'real-life' installations.

There are a number of parameters which it would have been potentially useful to measure, in order to enhance our understanding of the conditions under which the observed biofilms were being formed. For example, in chapter 3 reference is made to the potential drop in oxygen levels within test rig systems under stagnant conditions, and the negative effect that this may have had on the growth of aerobic bacteria. The incorporation of a means of accurately measuring oxygen concentrations within the test rig systems would have been useful in providing evidence of this. Another parameter which it may have been useful to measure within the test rig systems was temperature, in order that fluctuations within the test rig systems (i.e. at the point of biofilm formation) could be accurately recorded.

The incorporation of a data logging system would be useful in maintaining a record of automatic flushing times out of hours, if only to allow the researcher to address any potential issues regarding failure of equipment out of hours, a scenario which may have serious repercussions for studies of this type.

Table 7.1 Potential modifications to optimize test rig utilization from an ergonomic and scientific viewpoint

Test rig adjustment	Benefit to research
Manufacture/ installation of an extra system	A total of nine systems would provide an extra level of statistical robustness to investigations involving cross-system comparisons
Increased capacity of heater or replacement with an in-line heating system and accurate temperature monitoring at outlets.	Greater flexibility with regards to optimization of thermal purging regimes
Inclusion of two extra auto-flushing taps	Greater flexibility with regards to investigation of tap outlet fittings
Inclusion of flow rate/ oxygen meters	Increased understanding of hydrodynamic aspects/ oxygen levels within the test rig
Inclusion of appropriate disinfectant dosing system and/ or copper/ silver ionization system	This would facilitate the investigation of different biofilm disinfection technologies
Make adjustments which would increase flexibility with regards to the incorporation/ testing of alternative water system fittings	This would facilitate testing of a range of water system fitting designs, such as TMVs, taps and tap outlets
Make adjustments which would improve the performance of auto-flushing technology	This would allow a greater range of flushing frequencies and/ or durations to be explored

The test rig's complex construction, coupled with the range of valuable research opportunities which it provides, necessitates a cautionary approach by researchers when designing future experiments in order to ensure that the scope of such work is of sufficient breadth to facilitate the gathering of as much useful data as possible, whilst remaining unencumbered by external influences and therefore focussed on the core objectives of the research in question.

When the dynamic nature of biofilm development and the unique design of the test rig are considered together, the advantages of adopting a multi-faceted, rather than a singular, approach are clear. The application of a variety of microscopy, molecular biology and classic microbiological techniques in tandem ensured that biofilm studies were undertaken from a much broader perspective than the employment of a single technique would have allowed.

Within the water supply industry, stagnation has long been associated with microbial contamination problems, in particular the biofilm-associated proliferation of potentially pathogenic bacteria such as *Legionella*, *Pseudomonas aeruginosa* and *Mycobacterium avium*, and has been shown to adversely affect water quality from a microbiological viewpoint in WDS by many authors. Chapter 3 comprised an investigation into the effects of varying stagnation period on biofilm formation within the test rig, and at the end of the trial it was clear that more frequent flushing (i.e. shorter stagnation) was more conducive to biofilm formation than either monthly flushing or complete stagnation. These observations were explained through consideration of the potential benefits which performing a 30 second flush is likely to impart to a biofilm-forming population. Each flushing 'event' replenishes nutrients, raises oxygen levels, and also transports additional potential biofilm-forming microorganisms into the test rig, whilst at the same time removing potentially

toxic substances, such as copper ions, which may be leached into the bulk fluid from the substratum during lengthier periods of stagnation. It would appear logical to conclude that the greater the number of flushing events, the greater the level of biofilm formation, and this was indeed observed. It is also likely that the turbulence created by flushing plays a significant role in dictating the way in which biofilms form within the test rig, having previously been shown to be a promoter of denser, more populous biofilms than are seen during extended stagnation, when biofilms are looser and more prone to detachment. Therefore, the commonly-held industry view that stagnation is an active promoter of biofilm is not strictly accurate, and the potential hazards to health which are associated with stagnation exist primarily because of the *type* of biofilm which is formed (i.e. porous and 'loose') and the loosening effect that extended stagnation, followed by sudden changes in shear stress, has on pre-formed biofilms.

The effect of pH on biofilm formation under these varying stagnation conditions was also investigated. The results of this study suggest that high pH conditions within the test rig, induced by the inclusion of a magnesium sacrificial anode, represent an effective method of controlling, or at least retarding, biofilm formation. The applicability of this phenomenon as a biofilm control method is difficult to ascertain based on the results presented. However, there is certainly scope for future investigations into such aspects as the effect of pH on a variety of ecological, biochemical and physical aspects associated with any biofilms which do manage to form under such conditions, as well as the effect of high pH on the various materials of which the test rig is composed with a view to identifying any advantageous/ deleterious effects.

The use of thermal purging (TP) as a method of controlling biofilm and, in particular, *Legionella*, has been documented in a range of clinical settings

(Fisherhoch *et al.* 1981; Stout *et al.* 1998; Hayes 2006). However, few studies have been carried out in a controlled experimental setting. Chapter 4 examined the effect of TP on culturability and viability. A significant decline in culturability was observed as a result of TP, whilst CLSM studies indicated that the total number of viable bacteria remained relatively high and was suggestive of the existence of a large population of bacteria existing in the viable but non-culturable (VBNC) state. Consequently, it was concluded that the potential for bacterial regrowth, possibly to levels exceeding those observed without TP, was high due to the possible injection of nutrients and the tendency of some bacteria, including *Legionella*, to demonstrate necrotrophic growth (Temmerman *et al.* 2006). The increased resistance of *Legionella* in response to TP, and the incomplete destruction of biofilm, even after 30 minutes TP, has recently been demonstrated by Farhat *et al.* (2010). Webb *et al.* (2003) proposed bacteriophage-mediated cell death as a mechanism of biofilm cluster dispersal and subsequent biofilm development. Whilst this type of cell death comprises a different mechanism to the TP regime utilized during this study, it seems reasonable to suggest that as cell death is a normal component of multicellular development (Webb *et al.* 2003), and that the existence of a large sub-population of potentially detached biofilm cells after TP may facilitate downstream biofilm development. The detection of bacteriophages and their role in cell lysis and dispersal may offer clues as to a novel 'phage-therapy' approach to biofilm control in small scale clinical WDS situations, particular given that phage therapy is currently used as a mode of biofilm control on in-dwelling medical devices (Curtin and Donlan 2006; reviewed by Donlan 2009)). Other authors, for example Sutherland *et al.* (2004) are somewhat cautious with regards to the potential use of phages against biofilm bacteria. Nonetheless,

given the paucity of literature concerning their presence in WDS biofilms, it is felt that this is an area worthy of future research. The use of the particular TP regime employed during this study may not constitute an appropriate biofilm control measure. Reports into the use of TP in clinical settings consistently adhere to a minimum 60 °C (or above) exposure duration of at least 30 minutes (Snyder *et al.* 1990; Steinert *et al.* 1998), and are primarily concerned with the control of *Legionella*, rather than the wider biofilm population. As the majority of biofilm bacteria appear to be non-pathogenic, the theory that complete eradication of biofilm and biofilm-derived pathogenic bacteria may not actually be essential for the safe operation of water systems (Darelid *et al.* 2002) is worth considering in terms of future control strategies. Indeed, it may be that under certain circumstances, manipulation of the regulating ('probiotic') effect of such bacteria could prove a useful tool in the control of *Legionella*. However, the potential presence of opportunistic pathogens is a factor which should not be overlooked, particularly in a healthcare environment. Further work in this area is required, and should encompass optimization of all relevant parameters including TP frequency, duration, and TP apparatus design. Concurrent physiological/ ecological investigations into the response of biofilms to such a control measure would be essential in order that accurate assessments of TP effects can be made. One point which it is essential to bear in mind when considering biofilm management strategies is that, as is so often the case, prevention is better than cure.

As material choice has a major influence upon biofilm formation within WDS, comparative test rig studies were carried out with copper, stainless steel 316 (SS), PEX and EPDM. It was demonstrated that EPDM supported significantly greater levels of copious biofilm than were observed on the other materials.

This is in agreement with a number of other studies, which have also suggested that rubber materials support high levels of biofilm (Bourion and Cerf 1996; Kilb *et al.* 2003; Moritz *et al.* 2010), and should be of great interest to those employed within the water industry. Chapter 5 also provided a preliminary glimpse into the biofilm-supporting abilities of a variety of tap outlet fittings, the manufactured complexity of which was believed to be a major influencing factor in terms of biofilm formation. Their position at the most distal points of any WDS also means that they constitute important potential 'vectors' of biofilm dissemination, and the implications of this with regards to patient health in a hospital environment should be considered. Rather surprisingly, relatively few studies appear to have been carried out on tap outlet fittings even though observations relating to colonization of tap aerators by *Pseudomonas* were made over 40 years ago (Cross *et al.* 1966). Results indicated that contamination levels of supposed antimicrobial laminar flow outlet fittings showed no detectable decrease when compared to a non-antimicrobial equivalent, whilst PCR-DGGE analysis, described in chapter 6, revealed that the associated bacterial communities exhibited less than 40% similarity. The origin of such low similarity may simply be a consequence of the spatial separation of the two outlet fittings, rather than as a consequence of the inclusion of an antimicrobial ingredient. Only further rigorous scientific investigations, with greater replication, will clarify this point. Clear differences were observed between the four pipe materials tested in terms of their respective associated bacterial communities, and it is hoped that future investigations employing commonly-used molecular microbial ecology techniques such as FISH will provide further insight into the composition of these communities and the influence of material choice thereupon.

Consequently, there is considerable scope for the integration of other scientific disciplines in further studies of this type, in order that the influence of factors such as surface topography and chemical composition may be better understood, as these have been widely cited as exerting a considerable influence upon biofilm formation.

The flexibility which the test rig offers in terms of its design and operation means that there is huge potential for future work using this system. However, it is important to bear in mind that the inherent uniqueness of the test rig (and arguably all WDSs), coupled with the incredible adaptability and diversity associated with biofilms and their development, means that every opportunity must be taken to carry out investigations in actual clinical/ commercial installations. It is hoped that the application of the techniques described, in a greater diversity of settings, will confirm their worth by increasing understanding and providing knowledge and practical benefits. For example, comprehensive analysis of hospital water system components (such as TMVs) using techniques which extend beyond the oft-employed culture-based analysis would provide a useful platform on which to apply these techniques on a larger scale. This research has provided a useful framework upon which further projects may be built, and it is hoped that it may serve to generate ideas as to the direction future projects could take. Table 7.2 presents a brief summary of potential future projects, encompassing a broad range of microbiological disciplines, from molecular microbial ecology to studies aimed at providing practical solutions to the control and management of biofilms and the primary pathogens closely associated with them.

A great deal remains to be learned about the driving mechanisms of biofilm formation within both the test rig, and within WDSs in general. The future

practical benefits to society of improved management of WDS biofilms, such as the reduced incidence of waterborne nosocomial infections, will only be achieved through the continual improvement of scientific understanding of both the governing mechanisms of their formation, proliferation and survival, and the application of this knowledge towards the development of novel control technologies. It is clear that many exciting challenges remain ahead for biofilm researchers and their industrial partners/ counterparts alike, if this goal is to be achieved.

Table 7.2 Examples of potential future research projects

Investigation type	Summary of potential future research projects
Continuation of test rig studies on stagnation/ pH	• Further investigation into the role of stagnation on long-term biofilm development and the possible use of pH as a possible control measure
Continuation of test rig studies on thermal purging (TP)/ and other control measures	• Optimization of thermal purging (TP) on biofilm populations within the test rig. • To investigate how biofilm-bound *Legionella* respond to frequent short-term exposures to rapid increases in temperature • To investigate necrotrophic behaviour within a thermally purged biofilm • To investigate the effectiveness of alternative control methods within the test rig
Continuation of test rig studies on plumbing materials and fittings e.g. thermal mixing valves (TMV's), tap outlet fittings	• How are TMV components/ tap outlet fittings components colonized by microbes (incl. bacteria, fungi yeasts and protozoa), with particular emphasis on *Legionella* and *Pseudomonas aeruginosa*. To what extent are they responsible for downstream contamination events? • Continued investigation of novel plumbing materials and fittings
Microbial ecology investigations of biofilm microorganisms including potential pathogens	• To screen tap water biofilm/ tap water for *Legionellal Pseudomonas* and associated phages using novel molecular microbiology methods. • To investigate interactions between potential pathogens and isolates taken from the test rig, by way of well plate biofilm formation/ viability/ co-aggregation studies.

APPENDIX I

Buffer solutions and acrylamide mixtures used during PCR-DGGE analyses as described in Chapter 6

All chemicals were purchased from Sigma-Aldrich (Poole, U.K.).

TE Buffer

10 mM (1.57 g/l) Tris/Cl, 1 mM EDTA (0.37 g/l)

Adjusted to pH 8.0 with concentrated HCl

Lysis Buffer

50 mM Tris/Cl, 25 mM EDTA pH 8.0, 3% SDS, 1.2% PVP

Extraction buffer

10 mM Tris/Cl, 1 mM EDTA pH 8.0, 0.3 M sodium acetate, 1.2% PVP

50 x TAE buffer

2 M Trizma base, 30 mM EDTA, 250 mM sodium acetate

pH 7.8 with concentrated acetic acid

6 x gel loading buffer

40% glycerol, 0.25% Bromophenol Blue

Made up in 1x TAE buffer

Stock 0% denaturant acrylamide solution (for an 8% polyacrylamide gel)

26.7 mls 30% acrylamide solution

2 mls 50 x TAE

71.3 mls ultra pure water

Store refridgerated in the dark

Stock 80% denaturant acrylamide solution (for an 8% polyacrylamide gel)

26.7 mls 30% acrylamide solution

2 mls 50 x TAE

32 mls molecular grade formamide

5.6 M (34 g) molecular grade urea

to 100 mls with ultra pure water

Store refridgerated in the dark

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