

**Investigating contamination of dental-unit waterline
systems and microbial biofilm ecology**

By

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Abstract

Introduction: Biofilms within dental-unit waterlines (DUWLs) are acknowledged sources of contamination in the dental clinical environment and affect the quality of clinical treatment water. As a standard for reducing exposure to potentially harmful microorganisms, the Department of Health (DoH), UK suggests that water discharged from DUWL should contain 100 to 200 CFU/mL. However, local audits suggest that the quality of clinical treatment water often fails to meet the standards required.

The aim: The aim was to be able to readily identify waterlines with higher levels of contamination via validation of a rapid existing “in-office” test and subsequently understand biofilm ecology.

Materials and Methods: Water samples from 31 DUWLs in general dental practices were taken during the working day and cultured using the Petrifilm™ AC plate test as per manufacturer’s instructions and for extended incubation periods under laboratory conditions. The samples were also cultured using the laboratory based benchmark R2A agar. Further culture methods were employed for investigating spread of human pathogens with aerosolization and splatter of DUWL water; retraction valve failure; waterborne biofilm ecology and environment within a simulated laboratory DUWL (sDUWL) and whether amoebae were harboring nosocomial bacteria.

Results: The bacterial concentration of the water samples cultured on R2A agar varied significantly (1×10^1 to 4.3×10^6); in surgeries (48%) which met DoH standards and those that failed (52%). A retest of water from surgeries which delivered safe and contaminated water revealed that approximately 55% of practices met the recommended threshold values whilst around 45% failed. The Petrifilm™ AC Plate method gave variable sensitivity values on different occasions with 100% specificity. Only the nosocomial clinical isolate of *Serratia marcescens* was recovered from one clinical water sample. The opportunistic yeast, *Candida parapsilosis* from 1 sample indicated possible retraction valve failure. The *in-vitro* sDUWL output water demonstrated a fully established biofilm community by day 2 consisting of bacteria, a fungus (*Cladosporium cladosporioides*), and one amoeba (*Vermamoeba vermiformis*) as the main organisms. When tested under laboratory culture conditions, *V. vermiformis*, appeared to feed on *S. marcescens* isolated from clinical water. Electron microscopy confirmed bacterial adherence characteristics for biofilm formation, and altered pattern of cell division in one Gram positive isolate from the *in-vitro* sDUWL. Despite the detection of a *Legionella* species, no metabolically active opportunistic human pathogens were observed within *V. vermiformis* in the sDUWL biofilm.

Conclusions: This study demonstrates the importance of regular monitoring of DUWL water because even clean DUWLs can quickly become contaminated. One aim of this study was to find an in-office testing method for dental needs but it appears that improving the sensitivity of in-office tests is a challenge that needs addressing in the first instance. A more positive outcome was that, on the whole, clinical output water was not harbouring opportunistic human pathogens at the time of testing and that clinical surfaces were clean. Also when dental units are used there was no evidence that contaminants were being drawn back into the DUWLs. Overall, achieving a low level of microbial contamination consistently in water to 100 - 200 CFU/mL appeared to be difficult. In the short-term, if water could be tested more often this would help to understand the related challenges associated with conforming to national standards of delivering clean treatment water. The laboratory sDUWL model showed defective cell division and altered phenotype of specific bacterial species, and that *V. vermiformis* appeared unlikely to be harboring the late coloniser *L. pneumophila*, as it was out-with the size-range of bacteria, amoebae choose to feed upon. As the laboratory sDUWL model closely mimicked the heterogeneous biofilm development including the type of main microorganisms as those of the clinical DUWL it can be used to accurately assess commercial biocides in the control of the biofilm independently as literature continues to question the efficacy of commercial disinfections in waterline cleansing protocols that fail to meet the required standards.

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List of Abbreviations and Symbols

ADA	American Dental Association
AIDS	Acquired immune deficiency syndrome
APS	Ammonium persulfate
ATP	Adenosine triphosphate
AIs	Auto-inducers
bp	Base pairs
BAP	Biofilm associated proteins
BSA	Bovine serum albumin
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CQC	Care quality commission
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DoH	Department of Health
DUs	Dental units
DUWLs	Dental-unit waterlines
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
EPS	Extracellular polymeric substance
FISH	Fluorescence <i>in-situ</i> hybridization
FDA	Food and drug administration
FLA	Free living amoebae
g	Grams
g/L	Grams per litre

GVPC	Glycine-Vancomycin-Polymyxin-Cycloheximide
h	Hour
HPC	Heterotrophic Plate Count
HIV	Human Immunodeficiency Virus
Ig	Immunoglobulins
Kb	Kilobase
kDa	Kilodaltons
L	Litre
LPS	Lipopolysaccharide
M	Molarity
mA	Milliamps
mg	Milligram
mL	Millilitre
mm	Millimetre
mM	Millimolar
mm ²	Millimetre squared
ng	Nanogram
NHS	National Health Service
No	Number
<i>p</i>	Probability
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
ppm	Parts per million
PVDF	Polyvinylidene difluoride
QS	Quorum sensing
RLU	Relative light units
rpm	Revolution per minute

rRNA	Ribosomal ribonucleic acid
S.D	Standard deviation
sDUWL	Simulated dental-unit waterline
SDA	Sabouraud Dextrose agar
SDS	Sodium dodecyl sulphate
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SOP	Standard Operating Procedure
TAE	Buffer solution containing a mixture of Tris base, acetic acid and EDTA
TEM	Transmission electron microscopy
TEMED	Tetramethylenediamine
TNTC	Too numerous to count
TVC	Total Viable Count
UCLan	University of Central Lancashire
µg	Micrograms
UK	United Kingdom
µL	Microliter
µm	Micrometre
µM	Micromolar
UV	Ultraviolet
V	Volts
VBNC	Viable but non-culturable
W	Watts
WCS	Waterline Cleaning System
WMS	Waterline Management System
w/v	weight per volume

List of prizes, abstracts and publications

Prize and nomination

- Don Claugher bursary prize 2013 by Society of Electron Microscope Technology.
- Poster nomination in top three at University of Central Lancashire postgraduate research conference, Preston, December 2014.

Abstracts

- Lal, S., Singhrao, S.K., Achilles-Day, U., Morton, L.H.G., Pearce, M., Crean, S., (2014). Does *Vermamoeba vermiformis* support the proliferation of nosocomial bacterial pathogens in the ecological niche of dental-unit waterlines? University of Central Lancashire postgraduate research conference, Preston, December 2014 – poster presentation (Poster nominated in top three).
- Lal, S., (2014). Microbial analysis of dental-unit waterlines biofilm ecology. SEMT one day meeting, UCL School of Pharmacy.

Publications

- Lal S, Singhrao SK, Bricknell M, Pearce M, Morton LHG, Ahmed W, Crean S (2014). Monitoring dental-unit water line output water by current in-office test kits. *Current Microbiology*, 69(2), 135-142.
- Lal, S., Singhrao, S.K., Achilles-Day, U., Morton, L.H.G., Pearce, M., Crean, S., (2015). Risk assessment for the spread of *Serratia marcescens* within dental-unit waterline systems using *Vermamoeba vermiformis*. *Current Microbiology*, 71, 434-44.
- Lal, S., Singhrao, S.K., Achilles-Day, U., Morton, L.H.G., Pearce, M., Crean, S., (submitted). The biofilm ecology of a dental-unit waterline environment.

Chapter 1. General Introduction

1.1. Introduction

1.1.1. Origin of dentistry

Dentistry evolved from medicine early on in the history of human health welfare. Early practices of dentistry around 500 B.C, centred on treating the excruciating pain associated with tooth decay. Tooth decay, now known as dental caries, was thought to be caused by “tooth-worms” (Fig. 1.1) (Thompson, 1926; Leix, 1940).

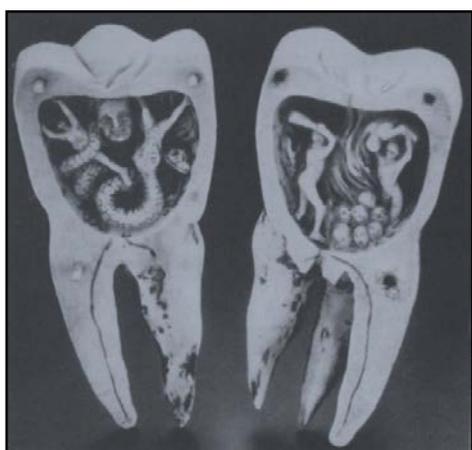


Figure 1.1: Illustration of the mythical aetiological agents of dental caries as being tooth-worms. (Ring, 1992).

Evidence from archaeological and anthropological finds suggests that the two most common oral diseases, now known as caries and periodontal disease(s) have affected humans through all ages (Suddick and Harris, 1990). However, post mid 1800s cane sugar and refined flour became readily available to everyone, rather than just the rich, and as a consequence dental caries became more prevalent leading to toothache and infection of the associated supporting tissues. Thus by the end of the 19th century, there was an increase in demand for dental professionals. As at this time in human civilisation, there was no formal regulated teaching in dentistry and the early providers of dental treatment acquired the necessary skills as apprentices and/or barbers by virtue of their other “pseudo-surgical” (shaving skin, bloodletting) skills. Clinicians of the day became known as barber-surgeons (Campbell, 1958). The training for and practice of

dentistry encompassed great variation in standards, but gradually in the 19th century through Europe and in North America a more formal university based dental education evolved.

1.1.2. Role of scholarly education

Providing health care to patients requires fully trained clinicians. The teaching of evidence- based medicine/dentistry to undergraduates, increases understanding of the most appropriate treatments and practices in their chosen profession. In addition, the teaching emphasises the fundamental needs of an undergraduate curriculum in promoting the learning of skills for that subject (in this case dentistry) as well as providing qualifications of the profession. Thus the scholarly recognition of the profession that now encompasses a programme of study via university education began with the worlds' first dental college “the Baltimore College of Dental Surgery” which opened in 1840 (Garfield, 1969). The degree awarded was Doctor of Dental Surgery (DDS). The college merged, in 1923, with the University of Maryland. This in time led to a requirement that a dentist hold a recognisable degree or pass a formal test to gain entry on to a register of those permitted to practice dentistry legally. Dentists who had been taught under the previous apprenticeship arrangements were allowed dispensation to join the register (Suddick and Harris, 1990).

Scholastic based education aims to dispel myths in an environment of evidence based learning. Hence Willoughby D. Millar, one of the early universities educated dental scholars, eventually put an end to the myth of the “tooth-worm” theory of dental caries by suggesting correctly that the disease was caused by bacteria (Miller, 1973). Further developments in dentistry began as new university educated dental surgeons (G. V. Black and others to present day) went on to explore and develop ideas in restoring teeth and developing apparatus for examination and dental treatment. As the practice of dentistry evolved, “the dental chair”, a prominent feature of dental

examination/treatment, also matured symbolizing the emergence of dentistry as a separate profession. (Field, 1995)

1.1.3. Evolution and history of the dental chair

The earliest roots of clinical medicine and dentistry trace back to the seventeenth century when, as mentioned earlier, barbers routinely performed operations such as dental extraction and bloodletting whilst the individual sat in the chair. However, the barber's chair did not fully serve the needs of dental protocols for examination and treatment and as a consequence, the dental chair began to evolve and emerge in its own right. The first dental chair was known as the "Windsor chair" and was made of solid wood (Fig. 1.2) (<http://www.parsdental.com/blog/invention-dental-chair>). It encouraged the patient to lean back so that the then barber-surgeon could access the oral cavity more easily, for extracting teeth. However, there was a need to support the head while the dental health provider operated with basic tools.



Figure 1.2: The first dental "Windsor chair", specifically made for dental treatment used by Josiah Flagg in 1790. Note the headrest for the patient to lean back and the arm extension to hold instruments. This chair is now in the Edward and Trudy Weaver Historical Dental Museum at the Kornberg School of Dentistry of Temple University, Philadelphia, USA (<http://www.parsdental.com/blog/invention-dental-chair>).

1.1.3.1. The first reclining dental chair

Around 1797-1832 James Snell designed the first reclining chair which aimed at providing comfort to the patient (Fig. 1.3). The seat, back, arm and head rests were upholstered in plush velvet. Together with the upholstered seating, and a separate rubber footrest, the fully adjustable dental chair was firmly held in place by the cast iron base.



Figure 1.3: The first reclining dental chair invented by James Snell in 1832.

1.1.3.2. The earliest hydraulic dental chair

In 1877, the prototype hydraulic dental chair was invented and became known as the Wilkerson chair (Fig. 1.4). The chair incorporated the first pump-type hydraulics to adjust its height. Hence the chair could be raised up or lowered down using a hand-cranked mechanism. This was made by the S. S. White Dental Manufacturing Company of Philadelphia, USA.



Figure 1.4: The Wilkerson chair (1877) - incorporated the first pump-type hydraulic dental chair which allowed easier adjustments to be made than older wooden dental chairs.

1.1.3.3. 1958 -A fully reclining dental chair is introduced with a “sink”.

During and following treatment, mouth rinse and spitting necessitated a “sink” which is commonly known as the spittoon or a “cuspidor” to be included as an essential part of in-office dental care and treatment (Fig. 1.5).



Figure 1.5: A fully reclining dental chair (1958) with a “sink” and a separate light in the dental surgery together with assistance from a nurse. Note the tools are still very basic and do not appear to require water for cooling them during treatment.

1.1.3.4. Other ancillary tools

The functional ‘workstation’ incorporates space for holding ancillary tools to aid the examination and treatment processes. These include fully integrated dental examination

lighting to enable accurate diagnosis and facilitate subsequent surgical procedures. The inclusion of a dental light became possible between the late 19th century and early 20th century when electricity became widely available. This coincided around the time of the fully reclining dental chair being built in 1958. The modern dental chair can incorporate other devices (monitor, digital camera) to take images of the mouth and teeth during examination and to display them, so that the pathological lesions can be recorded and shown to the patient to better explain the treatment plan (Field, 1995). Dental lighting, the spittoon, monitor and digital camera remain outside of the scope for this project and will not be described further.

1.1.3.5. High speed air rotor drill and the need for waterlines

The high speed air rotor drill (also known as a hand-piece) is an excellent example of a dental tool which has evolved overtime for efficient dental treatment. It was found that when it was used without water to cool the drill bit sufficient heat was generated to cause injury of soft dental pulp tissues (Stanley, 1971; Langeland, 1972). To reduce overheating, water was introduced via a series of waterlines (see Fig. 1.6), to maintain a cool temperature whilst cutting teeth with the high speed drills and scaling teeth with ultrasonic scalers using water and/or air and water at the same time. These are essential tools in dental treatment and are of major relevance to this project.



Figure 1.6: A modern dental chair with high speed dental drills and scalers as well as the waterlines that supply water to the hand-pieces.

This chapter will further introduce service evaluation and research aspects of this study in two parts.

1.2. Part I: Service evaluation

1.2.1. Dental-unit waterlines

All surgeries now depend on dental-unit waterlines (DUWLs) to supply water to hand-pieces for use with dental drills during clinical treatment. A DUWL is essentially a complex water circulation device that supplies clean water from a reservoir at one end to a dental drill for cooling purposes during clinical use (Fig. 1.7).

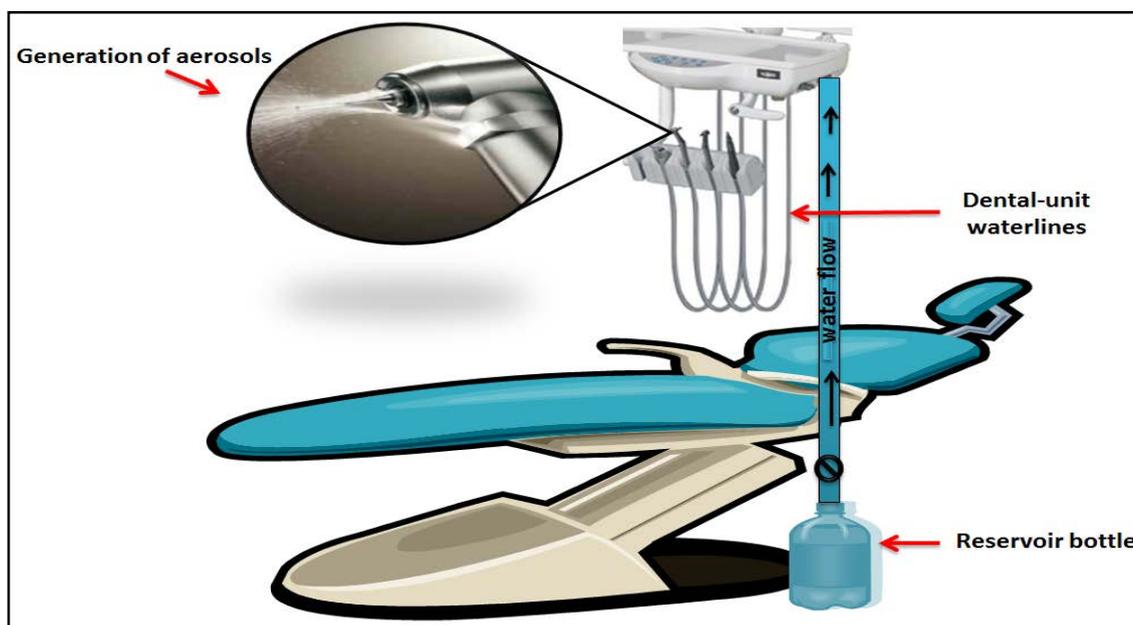


Figure 1.7: A typical dental chair with waterlines.

Earlier DUWL models were directly plumbed into municipal water (Singh and Coogan, 2005), which is not sterile. The intermittent operation of the dental-unit in the clinical setting, results in the water within the long lengths of small bore, of polyurethane tubing remaining stagnant typically for periods of around 16 h (overnight equivalent) and 64 h (over weekends). Polyurethane supplies carbon as a source of food for bacteria (Nakajima-Kambe *et al.*, 1995) thereby creating conditions conducive to microbial colonisation. Considering that the introduction of DUWLs in the sixties was fairly

novel, it is interesting to note that, Blake in 1963, and McEntegart and Clark, (1973) both initially reported the development of a biofilm within the tubing.

In the 1960s the older belt-driven hand-pieces were replaced by high speed and low speed air-driven rotor hand-pieces (Christensen, 2002) for provision of more efficient clinical treatment. However, the drills continue to use biofilm prone water from the dental-unit waterlines (Szymanska, 2007; Artini *et al.*, 2008; Chate, 2010; Pearce *et al.*, 2013; Arvand and Hack, 2013; Ditommaso *et al.*, 2016) and sprays, splatter and aerosols containing contaminants from the waterlines are generated in the immediate clinical environment (see Fig. 1.7) (Barbot *et al.*, 2012). In addition, there is retraction valve failure which suggests oral fluids are mixing with the dental treatment water and contributing to the biofilm (Bagga *et al.*, 1984; Lewis *et al.*, 1992; Panagakos *et al.*, 2001; Al Shorman *et al.*, 2002^a; Berlutti *et al.*, 2003; Montebugnoli *et al.*, 2005; Petti *et al.*, 2013; Ji *et al.*, 2016). All of these factors are seen as a biohazard to both the dental professionals and their patients (Clark, 1974; Lohr *et al.*, 1978; Wallace *et al.*, 1983; Martin, 1987; Reinthaler *et al.*, 1988; Atlas *et al.*, 1995; Pankhurst *et al.*, 1998; Putnins *et al.*, 2001; Pankhurst *et al.*, 2005; Pankhurst and Coulter, 2007; Barbeau, 2007; Iatta *et al.*, 2009; D'Ovidio *et al.*, 2011; Ricci *et al.*, 2012). This potential problem forms the subject of this investigation from the service evaluation aspects of the dental profession in part I and related research aspects in part II of this chapter.

1.2.2. Potential sources of microbial contamination of dental-unit waterlines

There are several potential sources of microorganisms that may contaminate the DUWL as listed below.

- An identical water supply is shared by both domestic users and the healthcare providers.
- The patient's oral fluid is sucked back into the waterlines due to retraction valve failure.

- Personal skin flora: microbes from the hands of dental staff.
- Protozoan vectors: free living amoebae as transporters of bacteria.

1.2.2.1. Water supply to the dental-unit waterlines

There is no doubt that specific microorganisms originate from the municipal/domestic water supply (Szymanska *et al.*, 2008). These include those bacteria that have characteristics for surface attachment, adherence and are able to grow and spread rapidly. In addition, there are opportunistic pathogens incoming from the same water source such as *Legionella* spp. and *Pseudomonas* spp. (Mayo *et al.*, 1990; Barben *et al.*, 2009; D'Ovidio *et al.*, 2011; Arvand and Hack, 2013; Szymanska and Sitkowska, 2013; Leoni *et al.*, 2015; Ditommaso *et al.*, 2016). As a standard for reducing exposure to potentially harmful water-borne microorganisms, the Environmental Protection Agency (EPA) (<https://www.gov.uk/government/organisations/environment-agency>) suggests the colony forming units/millilitre (CFU/mL) of water supplied for household use should be ≤ 200 CFU/mL and that any water intended for drinking should contain faecal and total coliform counts of 0 in any 100 mL sample.

1.2.2.2. Oral fluids cross-contaminating dental-unit waterlines

The oral cavity is a semi-aqueous, natural reservoir housing matrices derived from organic and inorganic components. As per any aqueous reservoir it harbours a diverse, indigenous taxa of microorganisms (Paster *et al.*, 2001; Aas *et al.*, 2005; Dewhirst *et al.*, 2010; Bik *et al.*, 2010; Ahn *et al.*, 2011; Human Oral Microbiome Database (<http://www.HOMD.org>). Dental plaque on teeth (Fig. 1.8) is a biofilm made up of a complex community of microorganisms embedded within an extracellular matrix of polysaccharides, proteins and inorganic compounds. This allows bacterial cell growth while affording protection from host defence mechanisms (Potempa *et al.*, 2000; Chandki *et al.*, 2011).



Figure 1.8: Dental plaque (<https://www.google.co.uk/search?q=image+of+dental+plaque>)

However, in view of a high prevalence of related dental diseases (caries, gingivitis, aggressive and chronic periodontitis, periodontal-endodontic lesions; peri-implantitis and mucositis), in all age groups, the pathogens in the oral cavity keep changing (Axelsson *et al.*, 1991; Flemmig, 1999; Armitage, 1999; Holt and Ebersole, 2005; Colombo *et al.*, 2009; Preza *et al.*, 2009; Torlakovic *et al.*, 2012). Additionally, unrelated medical conditions in medically compromised individuals such as immunocompromised victims, HIV/AIDS and old age may also increase an individual's susceptibility to infection and influence the type of microbiota taking up residency on the oral surfaces. During dental treatment, cross contamination due to aspiration of oral fluids back into the waterlines (Bagga *et al.*, 1984; Witt and Hart, 1990; Lewis *et al.*, 1992; Watson and Whitehouse 1993; Walker *et al.*, 2000; Montebugnoli *et al.*, 2002; 2005; Petti *et al.*, 2013; Ji *et al.*, 2016) is thought to contribute to the DUWL biofilm. The oral fluids may contain potential human pathogens. The implication here is that human pathogens will be transferred from patient to patient via the DUWL output water during dental visits.

1.2.2.2.1. Retraction valves in hand-pieces

When a high speed dental hand-piece is in use, air is being forced over a rotor to turn the bur. Water is simultaneously, sprayed over both the hand-piece to cool it as well as the tooth being treated. When the rotary action of the drill is stopped, valves close in the dental-unit and the supply of water and air to the hand-piece is stopped abruptly. This creates a short-lived, partial vacuum in the drill and has the potential to suck back oral fluids and other contaminants into the hand-piece (Bagga *et al.*, 1984; Witt and Hart, 1990; Lewis *et al.*, 1992; Watson and Whitehouse 1993; Walker *et al.*, 2000; Montebugnoli *et al.*, 2002; 2005; Petti *et al.*, 2013; Ji *et al.*, 2016). According to the American Dental Association (ADA)/American National standards specification # (ISO 7494) 40 µL of retracted volumes is acceptable (Anon, 1996). Retraction volume above 40 µL denotes retraction valve failure. This is the point of potential for oral fluid from the hand-piece to enter the waterlines and contribute to biofilm formation.

1.2.2.3. Personal skin flora

Skin microbes can also be transferred from the hands of dental staff while treating patients (Walker *et al.*, 2000). This is because the skin and fingernails harbour number of bacteria. These can be mobilised during inadequate hand washing procedures and when changing gloves. In addition skin is prone to being shed at all times (Meers and Yeo, 1978). Thus dislodged contaminated dead skin cells and bacteria from fingernails can contaminate the clinical environment. Such contaminants can enter the DUWLs during filling of the reservoir water and during general treatment procedures.

1.2.2.4. Protozoan vectors

Freshwater eukaryotic protozoa are ubiquitous in freshwater domestic/healthcare supply but can also proliferate in artificial water systems (Valster *et al.*, 2009). Drinking water treatment does not completely eliminate protozoa as they can be isolated quite readily

from tap water systems (Lau and Ashbolt, 2009; Loret and Greub, 2010; Thomas *et al.*, 2010). It is also recognised that the concentration of amoebae increases up to 300 times higher in DUWL output water than in tap water from the same source (Barbeau and Buhler, 2001). Free-living protozoa such as amoebae and some ciliates feed on mixed communities of bacteria, e.g. amoebae have been shown to ingest *S. aureus* and subsequently release it elsewhere, undigested and intact (Pickup *et al.*, 2007^a). *Paramecium* spp. can transmit *Mycobacterium* spp. and thereby act as a means of transport carrying live bacteria from different ecological niches (Peterson *et al.*, 2013). Amoebae are natural predators of bacteria and under appropriate conditions, will act as vectors to support the life cycle of potentially pathogenic bacteria, especially *Legionella*; fungi and viruses (Rowbotham, 1980; Barker and Brown, 1994; Brown and Barker, 1999; Molmeret *et al.*, 2005; Thomas *et al.*, 2010; Scheid, 2014).

1.2.3. Role of warm water in fouling of dental-unit waterlines

Some older dental-chair units were fitted with heaters that delivered warm water to provide more comfort to patients following treatment (Coleman *et al.*, 2007). However, it was soon realised that, heating DUWL water may actually encourage the proliferation of pathogenic *Legionella* spp. as their amoebal vector (with potential to support their life cycle) also resides in the same ecological niche (Wadowsky *et al.*, 1985). Naturally occurring *Legionella pneumophila* multiply readily at temperatures between 25 °C and 37 °C (Wadowsky *et al.*, 1985). It is also possible that heating DUWL water may promote the multiplication of human derived microbes that grow better at higher temperatures, compared with the environmental microbes that grow at lower temperatures (Coleman *et al.*, 2009). The manufacturers are aware of this biohazard facing clinical practitioners and have stopped making dental-units that use warm water.

1.2.4. The official guidelines for maintaining quality of DUWL output water

The US federal government agency, Centres for Disease Control and Prevention (CDC) and the ADA, accept that bacteria will always persist in the dental treatment water. The measurement unit for microbial contamination is CFU/mL of water. The USA emphasis now focuses on reducing the microbial loading of the discharged water to meet the recommended level of ≤ 500 CFU of aerobic mesophilic heterotrophic bacteria/millilitre, for infection control in dental health care system (Kohn *et al.*, 2003; ADA, 2004). Although, the ADA has set its own heterotrophic bacterial load of ≤ 500 CFU/mL for water delivered from DUWLs (ADA, 2004), the ADA also endorses the CDCs recommendation that patient treatment water should be the same quality as the EPAs standard of ≤ 200 CFU/mL (Kohn *et al.*, 2003; ADA, 2004). The UK has its own guidelines set by the Department of Health (DoH) which states that bacterial load in DUWL must lie in the range of 100 to 200 CFU/mL (HTM 01-05: <http://www.dh.gov.uk>; Anon., 1993; Al Shorman *et al.*, 2002^a; Kohn *et al.*, 2003). The Western government organizations such as CDC and DoH (UK) have recommended the routine monitoring of DUWL water in order to maintain bacterial counts within the recommended levels.

The UK guidelines set by the DoH for England on occupational health, infection control and the law can be found in the Health and Social Care Act 2008 in England on the following web sites (HTM 01-05; HTM 07-01 <http://www.dh.gov.uk>). In brief, these guidelines discuss immunisation, maintenance of DUWL's in relation to *Legionella* spp. by routine monitoring of DUWL output water, safe disposal of sharps, disposal of clinical waste, single use of surgical gloves and face masks etc. The care quality commission (CQC) is an independent body that inspects all health care related establishments in England (<http://www.cqc.org.uk/>). One of their duties is to check standards in dentistry including cross infection procedures.

1.2.5. Infection controls in dentistry

The infection control regimes employed in general dental practice have undergone many changes in the past 100 years. When local anesthetics were first introduced, the hypodermic needles were used on several patients, being disposed of only when they became blunt. The use of gloves by the dental team did not become routine until the late 1980s, which arose in part by the perceived risks associated with treating human immunodeficiency virus infection/acquired immune deficiency syndrome (HIV/AIDS) patients (Burke *et al.*, 1990). Again the sterilisation of hand-pieces between patients was not common in place until a television program exposed this serious flaw in cross infection control and shamed the profession into changing its ways. However, research has demonstrated how difficult it is to clean certain dental instruments (Lowe *et al.*, 2002). It is vital that all debris be removed if effective sterilisation is to be achieved. This has, in recent years, led to the use of more disposable items (e.g. endodontic files) within dental practice (Cockercroft, 2007).

Research has also demonstrated the importance in maintaining clean DUWLs in relation to infection control. The seminal publication by Martin (1987) reported the hospital admission of two patients having been infected with *Pseudomonas aeruginosa* following dental treatment. Martin (1987) demonstrated that a DUWL in the dental practice where the patients were treated was the source of the infection. Therefore, the CDC recommended that DUWL be cleaned and the daily use of biocides becomes essential (Williams *et al.*, 1994).

1.2.6. Infection control based on specific recommendations made by CDC and ADA board of trustees

- Independent removable water reservoirs for inclusion of biocides.
- DUWL to be cleaned using biocides daily.
- Regular monitoring of DUWL output water.

- Manufacturers' of the dental chairs to include anti-retraction valves in the high speed hand-pieces.

1.2.6.1. Use of independent reservoirs

Independent or removable water reservoir systems are now available as an integral part or as an accessory for most dental-units. By isolating the dental-unit from the municipal water system the quality of water introduced in the system can be controlled (and in addition the municipal water system is protected from contamination). This separate reservoir allows chemical agents to eliminate or inactivate biofilm organisms to be readily introduced into the DUWL.

1.2.6.2. Biocide cleansing of dental-unit waterlines

The manufacturers of each dental chair appear to have adopted their own specific biocide and cleansing regime compatible with their system. Hence the dental surgeries commonly use biocides recommended for use specific to manufacturers of the equipment installed in the practice. Some examples of the various biocides in use in dental practices are ICX (A-dec, Dental UK Ltd), Sterilox (Puricore UK), Alpron (Alpro medical GmbH) and Oxygenal 6 (KaVo Dental GmbH), Dentosept P (METASYS Medizintechnik GmbH). Active ingredients of these biocides and others are listed in Table 1.1.

Table 1.1: Biocides and their active agents to control microbial contamination in DUWLs.

Biocide	Active agent	Reference
Alpron	Sodium hypochlorite 1–2%, sodium-p-tolulsulfonechloramide < 0.2% and EDTA 1–5%	Smith <i>et al.</i> , 2002; Walker <i>et al.</i> , 2003; Schel <i>et al.</i> , 2006
ICX	Sodium percarbonate, silver nitrate and cationic surfactants	McDowell <i>et al.</i> , 2004
Oxygenal 6	Hydrogen peroxide 0.4%	O'Donnell <i>et al.</i> , 2005; Costa <i>et al.</i> , 2016
Sterilox	Superoxidized water 2.5% and 5%	Selkon, 2001; Martin and Gallagher, 2005
Sterilex Ultra	Alkaline peroxide 5%	Smith <i>et al.</i> , 2002; Meiller <i>et al.</i> , 2001; Tuttlebee <i>et al.</i> , 2002
Dioxiclear	Chlorine dioxide	Wirthlin and Marshall, 2001; Smith <i>et al.</i> , 2002
Bioblue, Bio2000	Chlorhexidine 0.12%, glycerol 0.12% and alcohol (undiluted)	Walker and Marsh, 2007
Dentasept	Hydrogen peroxide 1%	Tuttlebee <i>et al.</i> , 2002; Linger <i>et al.</i> , 2001; Shepherd <i>et al.</i> , 2001
Planosil	Hydrogen peroxide and silver	Montebugnoli <i>et al.</i> , 2004; Schel <i>et al.</i> , 2006
Sanosil super 25	Hydrogen peroxide and silver ions	O'Donnell <i>et al.</i> , 2007
Bilpron	Hydroxy benzoin acid ester, polyhexamethylenebiguanide ethylene, diamine tetra-acetate phenylalanine (undiluted)	Walker and Marsh, 2007
Dentapure	Iodine cartridge	Mills <i>et al.</i> , 1986
HealOzone unit	Ozone 2100 ppm	Pankhurst <i>et al.</i> , 1990; Al Shorman <i>et al.</i> , 2002 ^b
Ster4spray	Peracetic acid	Montebugnoli and Dolci, 2002; Larsen and Fiehn, 2003; O'Donnell <i>et al.</i> , 2007
Bleach	Sodium hypochlorite	Pankhurst <i>et al.</i> , 1990; Karpay <i>et al.</i> , 1999; Montebugnoli and Dolci, 2002
Alpron BRS solution & Alpron Mint	Sodium hypochlorite, Sodium-p-toluene-sulfonechloramide and EDTA 1–2%	Smith <i>et al.</i> , 2002
Tetrasodium EDTA	Tetrasodiun EDTA	Walker and Marsh, 2007
Cavicide	Isopropyl alcohol, Sodium hydroxide, 2-butoxyethanol, Methyl Salicylate	Meiller <i>et al.</i> , 2001
Listerine Antiseptic	Eucalyptol, Menthol, Methyl salicylate, Thymol, Water, alcohol, benzoic acid, poloxamer 407, sodium benzoate and caramel	Meiller <i>et al.</i> , 2001
Peridex	Chlorhexidine Gluconate 0.12%	Meiller <i>et al.</i> , 2001
N/A	Povidone-iodine	Mills <i>et al.</i> , 1986
N/A	electrochemically activated water	Marais and Brozel, 1999
Sterispray	Unknown	Costa <i>et al.</i> , 2016
Calbenium	EDTA, Chloramine, Benzalkonium, Allantoin, Aspartame	Costa <i>et al.</i> , 2016

1.2.6.3. Regular monitoring of dental-unit waterline output water

The US Government organization such as CDC and ADA, and the UK DoH have recommended the routine monitoring of DUWL water in order to maintain bacterial counts within a safe range and to assess the effectiveness of DUWL disinfection protocols. For measuring bacterial contamination in water samples, the conventional laboratory microbiological culture on R2A agar plates is favoured by EPA (Anon, 2012). This requires water samples being analysed under laboratory conditions.

1.2.6.4. Anti-retraction valve inclusion and failure

To prevent the suck back taking place, anti-retraction valves were introduced within the hand-pieces (Fig. 1.9). However, research has since demonstrated that a high proportion of these valves will fail and thus cannot be relied upon to prevent microbial contamination of dental-unit waterlines from oral fluids (Bagga *et al.*, 1984; Lewis *et al.*, 1992; Panagakos *et al.*, 2001; Al Shorman *et al.*, 2002^a; Berlutti *et al.*, 2003; Montebugnoli *et al.*, 2005; Petti *et al.*, 2013; Ji *et al.*, 2016). An investigation conducted in the Tianjin province of China, by Ji *et al.*, (2016) designed a detector to assess true functioning of the retraction valve in their local dental-unit waterlines. The study described 40 µl of trapped fluid as cut off for being acceptable, but denoted retraction valve failure if above this volume. By using the device, Ji *et al.*, (2016) concluded that retraction valve failure was more common than realised in the stated Chinese province.

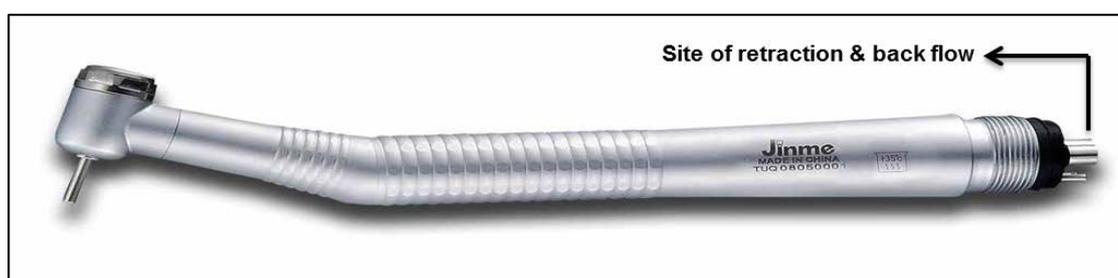


Figure 1.9: Dental hand-piece. Note the site of retraction and back flow.

(<http://dentalcareinf.blogspot.co.uk/2015/01/the-dentistry.html>)

1.2.7. Occupational health risks

1.2.7.1. Bioaerosols

Bioaerosols are defined as suspension of airborne biological particles. The biological particles include bacteria, viruses, fungi, protozoa, and their metabolites (exotoxins) (Dutil *et al.*, 2008). Bioaerosols can remain in the air for long periods during which time they may become transported to different locations via the flow of air. Aerosols of size $\leq 5 \mu\text{m}$ can after inhalation penetrate deep into the respiratory system, reaching as far down as pulmonary bronchi and alveoli (Harrel and Molinari, 2004; Decraene *et al.*, 2008). Depending on their nature and concentration, these bioaerosols have been shown to cause a variety of infections or sensitization leading to conditions such as ocular irritation, rhinitis, nasal congestion, asthma, allergic alveolitis, tuberculosis and legionellosis (Dutil *et al.*, 2008).

Bioaerosols are ubiquitous and their presence is highly influenced by human activity. In the dental surgery environment, aerosols are generated by the use of dental instruments including high speed dental hand-pieces, 3 in 1 syringes (air/water syringes), and ultrasonic scalers (Fig. 1.10) (Leggat and Kedjarune, 2001; Harrel, 2004; Szymanska, 2004; 2007) that affect the microbiological quality of the indoor air (Kadaifciler and Cotuk, 2014).



Figure 1.10: Various dental instruments, **A)** 3 in 1 syringe, **B)** Scaler, **C-E)** Handpieces.

Due to bioaerosols the environment in dental clinics is likely to be contaminated with multiple microorganisms to which dental staff are potentially exposed (Bennett *et al.*, 2000; Al Maghlouth *et al.*, 2004). The dental bioaerosols may contain both oral bacteria from a patients' mouth and bacteria colonizing the luminal surfaces of DUWLs.

Dental aerosols affect the microbiological quality of air in any clinical environment, and the factors forming dental aerosols exert an important influence on the composition of the microbiota of the clinic (Kedjarune *et al.*, 2000). Quantitative and qualitative studies conducted (Grenier, 1995; Al Maghlouth *et al.*, 2004) on air in the dental clinic demonstrate an increase in the levels of bacteria in the air during working hours and immediately after finishing treatment that follows a pattern of decrease in bacterial contamination by 50-70% overnight (Al Maghlouth *et al.*, 2004). Not surprisingly, *Staphylococcus* spp. and *Streptococcus* spp., which are prevalent in the oral cavity, appear in the air of a typical dental surgery (Osorio *et al.*, 1995; Bennett *et al.*, 2000). Grenier (1995) demonstrated the presence of additional bacteria including *Staphylococcus aureus* (0.6%), *Staphylococcus epidermidis* (37.1%), non-diphtherial corynebacteria (28.2%), *Micrococcus* spp. (32.6%), *Pseudomonas* spp. (0.6%), and fungi (0.9%). These authors therefore, recommended that the quality of air in dental surgeries should also be monitored regularly.

Aerosols generated during dental treatment are a major risk factor to the health of dental staff as particles ($\leq 5 \mu\text{m}$) can be inhaled, whilst larger particles settle easily onto working surfaces (Harrel and Molinari, 2004; Decraene *et al.*, 2008). In the dental practice, surfaces such as dental-unit switches, drawer knobs, and light handles, which are most frequently touched, can act as reservoirs of microorganisms.

A high titre of antibodies against *Legionella* in the serum of dentists compared to non-dental practitioners has been reported (Reinthalder *et al.*, 1988). In the Fotos *et al.*, (1985) study, a group of dental staff with more than 2 years clinical experience

revealed significant neutralising antibodies IgM (20%) and IgG (16%) for *L. pneumophila*, compared with a lower 8% (IgM) to 10% (IgG) titres in individuals who had no clinical experience. In another study significant difference in nasal flora of 50% of dentists was observed when compared with the nasal flora of the dental surgery assistants (Clark, 1974). This indicated a positive correlation between bacteria present in the nasal sinuses of dentists to that of the control group. The altered nasal flora in 14 out of 30 dentists was largely *Pseudomonas* and/or *Proteus* spp. as well as water-borne bacteria, whereas 3 out of 29 dental surgery assistants (control group) had altered flora consisting of *Proteus*, *Aeromonas* and *Klebsiella* spp. (Clark, 1974).

1.2.8. Risk factors for disease transmission from DUWLs

Despite the high levels of microbes often reported, there are very few clinical case reports published in association with contamination of DUWLs (Table 1.2). This lack of evidence may reflect very low rate of disease transmission. However, it may also reflect the difficulty in establishing epidemiological links between infections with long incubation times and preceding dental procedures (Shearer, 1996). This may result in low documented incidence of infections from exposure to DUWL water. Exposure of host to pathogen does not always cause disease; it depends upon the virulence, the dose of microbes and the host's resistance (Willey *et al.*, 2014).

Table 1.2: Summary health risks associated with dental-unit waterlines.

Organism	Associated with	Reference
<i>Pseudomonas</i> and <i>Proteus</i> spp.	Rhinitis	Clark, 1974
<i>P. aeruginosa</i>	Oral abscesses	Martin, 1987
<i>L. pneumophila</i>	Humoral responses initiated	Reinthalter <i>et al.</i> , 1988
<i>Legionella dumoffi</i>	Legionnaires' disease	Atlas <i>et al.</i> , 1995
<i>Mycobacterium gordonae</i>	Endocarditis	Pankhurst <i>et al.</i> , 1998
Non tuberculosis <i>Mycobacterium</i>	Cervical lymphadenitis	Lohr <i>et al.</i> , 1978; Wallace <i>et al.</i> , 1983
Bacterial endotoxins	Asthma, inflammation due to acute phase cytokine release, hypersensitivity pneumonitis	Putnins <i>et al.</i> , 2001; Pankhurst <i>et al.</i> , 2005; Pankhurst and Coulter, 2007
<i>Acanthamoeba</i>	Ocular keratitis	Barbeau, 2007
<i>Aspergillus</i> spp.	Oral aspergillosis	Iatta <i>et al.</i> , 2009
<i>P. aeruginosa</i>	Acute purulent maxillary sinusitis	D'Ovidio <i>et al.</i> , 2011
<i>L. pneumophila</i>	Legionnaires' disease	Ricci <i>et al.</i> , 2012

1.2.9. Risk groups

Although the majority of waterborne bacteria pose no risk of infection, the opportunistic nosocomial pathogens including *Legionella*, non-tuberculosis *Mycobacterium* spp., *Klebsiella pneumoniae*, *P. aeruginosa* and *Serratia marcescens* may cause infections in immunocompromised individuals (elderly and HIV), smokers, alcoholics, diabetics, sufferers of chronic lung diseases (bronchitis), heart disease, renal diseases and many other conditions (Willey *et al.*, 2014)

1.2.10. Occupational health protection

The basis of dental infection control is to create, and maintain, a safe clinical environment and to remove, or reduce, as much as possible, the risk of disease transmission between patients and dental health care workers. This has brought about immunisation of the dental professionals for hepatitis B, measles mumps and rubella.

Further recommendations are to decrease the exposure of patients and dental staff to microbes present in dental treatment water. To achieve this goal, continuous monitoring of the DUWL output water is required (HTM 01-05: <http://www.dh.gov.uk>).

1.2.11. Monitoring of DUWL output water contamination

For measuring heterotrophic bacteria in water samples, the benchmark is the use of R2A agar plates (Reasoner and Geldreich, 1979; Reasoner and Geldreich, 1985). The limitations of this method are that a standardised set of conditions and apparatus are required that are not suitable, or convenient for frequent use in an in-office setting. As the water samples have to be analysed under laboratory conditions, the DUWLs treated with biocides may go unmonitored, during which time the dental practices may be unaware of the gross contamination taking place in their DUWL output water. Therefore, it would be more convenient and practical to monitor the quality of DUWL water using a rapid test method in the dental practice premises so that if contamination is apparent, remedial action can be taken immediately.

1.2.12. Commercial in-office rapid methods for testing DUWL output water

Since there is no rapid, in-office test developed specifically for dentistry, dentists have currently adopted the use of various existing in-office rapid testing systems for monitoring contaminated DUWL output water (Table 1.3). The test kits, that have been used for the in-office testing of DUWL output water include the Heterotrophic Plate Count (HPC) Sampler (Millipore); the ATP test (3M Food Safety); Aquasafe™ water test (Pall corporation); the 3M™ Petrifilm™ Aerobic Count Plates (3M Food Safety); and the Dip Slide™ test (Accepta Ltd., UK). The Millipore HPC Sampler, Aquasafe™ water test, 3M™ Petrifilm™ and Dip slides™ all rely on the release of dehydrogenase enzymes by bacteria that reduce a colourless tetrazolium salt impregnated in the test to a

red compound called formazan. Only viable bacterial cells convert tetrazolium to formazan which makes the colonies readily visible to the naked eye.

1.2.12.1. Heterotrophic Plate Count (HPC) Sampler

The HPC Sampler also called Millipore HPC Sampler is a rapid method for the microbiological analysis of water in the environment. This includes the water used in cooling towers and waste water from a range of industries, including the electronics industry and processed water from the beverage and food industry and waste, laboratory grade water and dialysis water. The HPC Sampler is also used in dental surgeries as it has a threshold of < 200 CFU/mL. It consists of a plastic paddle, a Millipore membrane filter with a pore size of 0.45 µm which is in close contact with a nutrient pad, an air-vent on the back of paddle and a plastic case for sampling and incubating. Total volume (1 mL) can be inoculated on this test. The Millipore HPC Sampler test has been evaluated for DUWL output water but with differing sensitivity and specificity values from each investigators laboratory (Karpay *et al.*, 1998; Smith *et al.*, 2004; Bartoloni *et al.*, 2006; Morris *et al.*, 2010; Momeni *et al.*, 2012). See Table 1.3.

1.2.12.2. Adenosine Triphosphate test

ATP is an energy carrying molecule present in all living cells including bacteria and can be used indirectly to measure bacterial counts. In this method ATP produced by bacteria is measured in a bioassay using luciferase enzyme (from the firefly *Photinus pyralis*), catalysing D-luciferin to utilise ATP as the energy source. During the process, free energy in the form of light is emitted and is measured as relative light units (RLU) and converted to ATP values by plotting a calibration curve (Rolf and Lee, 2001). Fulford *et al.*, (2004) failed to find any strong correlation of ATP concentration with the TVC data and thus, suggested that this method could not be recommended. Limitations of the ATP test include loss of sensitivity, if the organism being tested is present in low copy numbers, and the variability that exists among microorganisms in their ATP content.

This makes it difficult to correlate CFU/mL with the RLU values. Bacterial counts can only be related to RLU values when dealing with pure cultures. Thus its application as an in-office test for dental needs is limited.

1.2.12.3. Aquasafe™ water test

The Aquasafe™ water test kit is a ready to use disposable, filtration monitoring system for the microbial analysis of heterotrophic bacteria in water from DUWLs. Aquasafe™ water test consists of a 0.45 µm membrane overlying a media-pad impregnated with a dehydrated growth medium. Water sample (1 mL) from DUWLs is allowed to pass through the grid-marked membrane. Medium becomes hydrated and supports the growth of microorganisms on the surface of membrane. Microbes are counted at room temperature after 72 h of incubation period (Momeni *et al.*, 2012).

1.2.12.4. The Dip Slide™ test

The Dip Slide™ test was developed primarily for testing the quality of water in cooling towers but is marketed in the UK as an aid to monitoring DUWL output water. Manufacturers of the Dip Slide™ test (Table 1.3) maintain that the range of sensitivity lies between 1,000-100,000 CFU/mL which, from the outset suggests that it is unsuitable for estimating the required threshold for dentistry. However, it does suffice a much higher threshold set for cooling towers as it was developed specifically for that use. Pearce *et al.*, (2013) evaluated the applicability of the Dip Slides™ to use in the dental premises and found longer incubation time increased the sensitivity without compromising the specificity. Pearce *et al.*, (2013) concluded that the test is applicable as a practical means of monitoring general levels of planktonic bacteria in water systems and can be used to screen for gross contamination of dental waterlines if used over five days; though it is not sufficiently sensitive to meet the threshold set by the DoH in the UK, as it can give false negative results.

1.2.12.5. Petrifilm™ AC Plate

The 3M™ Petrifilm™ is a rapid test for the quantitative microbial analysis of water from DUWLs. Manufacturers of the 3M™ Petrifilm™ test (3M Food Safety) maintain that the optimal counting range lies between 30-300 CFU/mL and therefore it appeared suitable for adopting for dental use. The 3M™ Petrifilm™ is a ready to use thin paper and plastic film which has dehydrated culture medium bound to it. Constituents of culture medium vary from plate to plate depending upon the microorganisms to be cultured. Generally 3M™ Petrifilm™ contains nutrients, a cold-water soluble gelling agent, and indicators to show the activity of microorganisms. The 3M™ Petrifilm™ is inoculated with 1 mL neat water sample and incubated to allow the growth of microorganisms present in it (Momeni *et al.*, 2012). Both Morris *et al.*, (2010) and Momeni *et al.*, (2012) evaluated the 3M™ Petrifilm™ Aerobic Count Plate method and concluded that it was unsuitable at the required threshold for dental needs due to its poor sensitivity/specificity values. Discussions with the manufacturers (3M Food Safety) did not elicit a clear explanation for the variability in the results published by Morris *et al.*, (2010) and Momeni *et al.*, (2012). One reason for the disagreement between the published reports and the manufacturers was that the false negative results may have arisen from overwhelming contaminated water with a high content of biocides in which majority of bacteria may have been stressed. It was therefore, suggested to test serially diluted water samples and plating them on multiple plates. This would clarify if the test could meet the standards required for use in the dental setting.

All of the above mentioned tests have been evaluated for use with DUWL contaminated water (Karpay *et al.*, 1998; Fulford *et al.*, 2004; Morris *et al.*, 2010; Momeni *et al.*, 2012; Pearce *et al.*, 2013), and universally suggest that discrepancies in bacterial recovery between R2A agar plates and medium based rapid in-office test kit remain (Karpay *et al.*, 1998; Morris *et al.*, 2010; Momeni *et al.*, 2012; Pearce *et al.*,

2013). These discrepancies may be due to differences in the nutrient media, counting area and preference of certain types of bacterial colonies to grow on some media over others. As DUWL output water contains a biocide, samples transferred directly to R2A agar plates appear to allow the growth of the culturable, low nutrient requiring bacteria. This is because the larger volume of agar present in the Petri dish allows for the permeation of any residual biocides in the diluted sample (several fold dilution of original sample) away from the smaller inoculum size (100 µL), of the already stressed bacteria and reduces the on-going toxic effect on the growing organisms. With the in-office test kits such as the Millipore HPC Sampler, the 3M™ Petrifilm™ Aerobic Count Plate, and the Dip slide™ test, this effect is reduced due to the smaller volume of agar and the larger inoculum size (1 mL) from neat sample, ultimately increasing the concentration of biocides in contact with bacteria compared to R2A plates. Unless an alternative comparator exists, the acceptability of an in-office test must be confirmed against the current conventional accepted methodology (R2A) when determining the contamination levels within and/or above the threshold for sensitivity and specificity.

Table 1.3: Summary of tests available for monitoring quality of water.

Test	Optimal counting range/sensitivity	Sensitivity/Specificity/Correlation	Supplier
R2A conventional microbiology lab test	30-300 for enumerating 0–10 ⁹ CFU/mL in general	N/A	Lab M Ltd
Heterotrophic Plate Count Sampler: Cooling towers, renal dialysis units and food industry. It is also recommended for use to monitor DUWL output water	0-200 CFU/mL	Sensitivity/Specificity: 98.3/77.3% (Karpay <i>et al.</i> , 1998); 50/100% (Momeni <i>et al.</i> , 2012); 54/95% (Morris <i>et al.</i> , 2010)	Millipore
Adenosine triphosphate (ATP) test: Food beverages, Brewery industry.	RLU does not equate to CFU/mL	Correlation: No correlation between RLU with the TVC data (Fulford <i>et al.</i> , 2004)	3M Food Safety
Estimation of endotoxin level	Endotoxin unit (EU) does not equate to CFU/mL	Correlation: No correlation between endotoxin with the TVC data (Fulford <i>et al.</i> , 2004; Szymanska, (2005 ^a); Spearman correlation coefficient of $p = 0.94$ between endotoxin and bacterial load (Huntington <i>et al.</i> , 2007)	N/A
Aquasafe™ water test, For dental use	unknown	Sensitivity/Specificity: 21/100% (Momeni <i>et al.</i> , 2012)	Pall corporation
3M™ Petrifilm™ Aerobic Count Plates: Developed for the food industry	30-300 CFU/mL	Sensitivity/Specificity: 57/100% (Momeni <i>et al.</i> , 2012); 79/98% (Morris <i>et al.</i> , 2010); 7 days incubation	3M Food Safety
Dip Slide™ test: Developed for testing water contamination in Cooling towers. Commonly used by dentists in the UK	1000-100,000 CFU/mL	Sensitivity/Specificity: 66/83% at 2 days and 95/85% 5 days incubation Pearce <i>et al.</i> , 2013	Dimanco Ltd Distributed by Accepta Ltd., UK and 3M Food Safety

1.2.13. Neutralization of water samples prior to testing

Due to use of biocides in DUWLs organisms become stressed. This can make them more difficult to culture in the laboratory; a prior neutralisation of biocides in sodium thiosulphate is required. Failure to neutralize the chlorine from water samples results in bactericidal action prior to sample processing resulting in lower bacterial count.

The chemical reaction of sodium thiosulphate and chlorine is given below.



According to the Environmental Protection Agency (Anon, 2012) 18 mg/L of sodium thiosulphate is required to neutralize chlorine residues in municipal water. This equates to 100 µL of 1.8% m/v solution of sodium thiosulphate/100 mL water sample.

1.2.14. Sensitivity and specificity measurements

Sensitivity of a clinical test is defined as the ability of the test to correctly identify the positive cases, whereas specificity refers to the ability of the test to correctly identify negative cases (Lalkhen and McCluskey, 2008). Sensitivity and specificity of in-office tests is assessed using their lowest threshold cut-off value (and according to threshold set by local Governments for DUWL output water) and comparing the results with accepted independent method (e.g. R2A agar plates). Therefore sensitivity will be proportional to samples with bacterial counts exceeding the threshold that were correctly identified by the test and specificity will be proportional to samples with bacterial counts below the threshold that were correctly identified by the test (Bartoloni *et al.*, 2006). A highly sensitive test would be more useful clinically to confirm true positive results; a more specific test would be useful to confirm true negative results.

To fully understand and manage biofilm associated problems with DUWLs, it is important to examine the factors that control biofilm communities and are described in part II of this chapter.

1.3. Part II: Research aspects of the study

1.3.1. Microbial biofilm

Microbial biofilms exist in almost every conceivable environment (Parsek and Singh, 2003), and can be found in both biotic and abiotic surfaces (Cortes *et al.*, 2011), on liquid surfaces as a floating mat and in submerged state (Vasudevan, 2014). For example, biofilms exist in the human mouth, skin, and water reservoirs to microprocessors and even in kerosene fuel lines (Donlan and Costerton, 2002). Artificial medical/dental interventional devices are commonly used to restore function to the patient who may have initially lost it due to disease. Such devices can be those that are implanted in the body (e.g. stents, pacemakers and other prosthetic implants) and as a consequence are in constant contact with the biological system of the individual. Other interventional devices are those that are attached to an intermediate machinery for use in medicine and dentistry (e.g. kidney dialysis tubing, dental-unit waterlines). All of these artificial systems are prone to biofilm formation and can cause clinical infections (Blake 1963; McEntegart and Clark, 1973; Kokare *et al.*, 2009; Otto, 2009; Zhang *et al.*, 2015; Murugan *et al.*, 2016).

1.3.1.1. What is biofilm?

A biofilm is defined as a sessile, and organized consortium of either homogenous or heterogeneous groups of microorganisms living together within a self-secreted matrix of extracellular polymeric substance (EPS) whilst attached to their specific substrate (Watnick and Kolter, 2000; Donlan and Costerton, 2002; Hurlow *et al.*, 2015; Gupta *et al.*, 2016).

1.3.1.2. Factors leading towards biofilm formation

Genetic factors that contribute to the formation of biofilms and their environment are still under investigation (Maric and Vranes, 2007). However, it appears that bacteria prefer residing in biofilms for a number of reasons as stated briefly below.

1.3.1.2.1. Acquiring new genetic traits

Biofilms provide genetic diversity to bacteria via sharing the same niche in which exchange of extra chromosomal DNA (plasmid) within or between populations can take place by the mechanism of conjugation (Wozniak and Waldor, 2010). By acquiring new genetic material, microbes can transcribe necessary genes to become part of a biofilm forming community (Kokare *et al.*, 2009). Biofilm associated cells express different phenotypic characters from planktonic counterparts and increase the rate of transcription of certain genes that help to strengthen the biofilm infrastructure. For example, transcription of *algC* gene which has a role in production of alginate is increased approximately fourfold in biofilm associated cells compared to planktonic cells (Coserton *et al.*, 1995). The main reason for enhanced conjugation within biofilm is that the biofilm environment provides least shear and closer cell-cell contact (Ghigo, 2001; Jefferson, 2004).

1.3.1.2.2. Nutrient trapping

In a heterogenous biofilm, multispecies of microorganisms live in metabolically co-operative environment exchanging metabolic products that aid their removal and utilisation by others (Davey and O'Toole, 2000). For example, degradation of complex organic material into carbon dioxide and methane during anaerobic digestion requires interaction of at least three bacterial species. Fermentive bacteria initiate the catabolism producing acids and alcohols, which are then used as substrate by acetogenic bacteria.

Methanogen bacteria on the other hand convert acetate, carbon dioxide and hydrogen to methane (Davey and O'Toole, 2000).

1.3.1.2.3. Defence mechanism

EPS of a biofilm provides defence and protection from the external environment. For example, it provides protection from environmental stressors such as desiccation, osmotic shock, UV radiation and pH shift (Kokare *et al.*, 2009). EPS prevents entry of certain antimicrobial agents into the biofilm by acting as an anion exchanger (Kokare *et al.*, 2009) and has a role in removing metal ions, cations and toxins (Nichols *et al.*, 1989).

1.3.1.3. Structure and the main component of biofilms

Confocal scanning laser microscopy (CSLM) is commonly used to monitor the development of a biofilm as this technique allows three-dimensional visualization of the arrangement of microorganisms *in-situ*. This technique has demonstrated that a biofilm is a three-dimensional microscopic structure (Hall-Stoodley and Stoodley, 2002) held together by EPS. Depending on the substrate on which the biofilms form, the initiating organisms can be Gram-positive bacteria as in the case of an oral biofilm (e.g. *Streptococcus* species of bacteria) (Chandki *et al.*, 2011) or by Gram negative bacteria as seen in waterborne biofilms attaching to plastic tubing (Barben *et al.*, 2009; D'Ovidio *et al.*, 2011).

The main constituent of biofilms is EPS, which is largely polysaccharide matrix mixed with proteins, lipids and nucleic acids (Cortes *et al.*, 2011). The role of lipids, lipopolysaccharides and glycopeptides is to form a framework that holds the biofilm in place (Flemming and Wingender, 2010). The positively charged biomolecules such as uronic acids or ketal linked pyruvate within the main framework bind divalent cations such as calcium and magnesium to cross-link the polymer strands and provide greater

binding force in the mature biofilm (Davey and O'Toole, 2000). EPS present in biofilm exhibits viscoelastic properties (Stoodley *et al.*, 2002^a; Hall-Stoodley *et al.*, 2004) that allow the biofilms to withstand mechanical stress within their environment. The amount of EPS produced by different microorganisms may vary but it does increase with the aging of the biofilm (Kokare *et al.*, 2009).

1.3.1.4. Biofilm initiation

A biofilm may be initiated when bacteria sense favourable environmental conditions that include availability of usable nutrients, temperature, moisture, pH, iron and oxygen (O'Toole *et al.*, 2000). Biofilm formation occurs through successive stages including an initial stage involving reversible and/or irreversible attachment, maturation, and dispersion (Sauer *et al.*, 2002; Stoodley *et al.*, 2002^b; Garrett *et al.*, 2008). These stages are common to all types of microbial biofilms and are discussed more fully below.

1.3.1.4.1. Reversible attachment

Physical forces that help bacterial adhesion to surfaces include the van der Waals forces, electrostatic interactions and steric hindrance interactions (Garrett *et al.*, 2008). However, if repulsive forces are greater than the attractive forces, the bacteria will detach from the surface. This is more likely to occur before the formation of the conditioning layer and is called a reversible adhesion phase (Garrett *et al.*, 2008).

1.3.1.4.2. Irreversible attachment

Succession of microbes can either begin with the formation of a conditioning layer (organic and inorganic base), which facilitates the attachment of planktonic microbial cells; or without an initial conditioning layer. In the latter case, bacteria irreversibly attach to their substrate either by physical forces to the substrate or by bacterial appendages such as pili, fimbriae or flagella to counteract the physical repulsive forces working against their adhesion (Maric and Vranes, 2007; Garrett *et al.*, 2008). Some

microbial cells contain biofilm associated proteins (BAP) on their surface membranes. BAPs aid primary attachment of microbial cells to their substrate and to any other cells and strains with inadequate BAP in the same niche (Lassa and penades, 2006). In addition, the hydrophobic microbial cell surface plays an important role during adhesion to its substrate as hydrophobic interaction between these two surfaces reduces the repulsive forces between them (Tribedi and Sil, 2015). Attachment of a microbial cell to a surface is termed as adhesion, whereas the attachment among microbial cells is known as cohesion. The biofilm develops with two kinds of co-aggregation interactions of microbial cells, **a)** genetically identical, single cells in suspension that attach to mature biofilm cells, **b)** secondary colonizers co-aggregating in suspension attach to the growing biofilm (Rickerd *et al.*, 2003).

1.3.1.4.3. Maturation stage

Following their initial adherence, with/without the conditioning layer, bacteria enter the surface associated lag phase. In this phase bacteria prepare themselves for different types of adaptations. Any changes in gene expression are accomplished in this phase (Sauer and Camper, 2001; Cvitkovitch *et al.*, 2003). Once cell division and any accompanying phenotypic changes have taken place, bacteria enter the log phase of growth. To determine the cell population density, microbial cells communicate with each other and within the consortium by using chemicals known as auto-inducers (AIs) or signal molecules; this process is called quorum sensing (Bassler, 1999; Schauder and Bassler, 2001; Del Pulcini, 2001; Ng and Bassler, 2009; Heilmann and Gotz, 2010; Bordi and de Bentzmann, 2011; Vasudevan, 2014; Scutera *et al.*, 2014). Quorum sensing was first identified in the regulation of bioluminescence in *Vibrio fischeri* and *Vibrio harveyi* (Nealson *et al.*, 1970; Nealson and Hastings, 1979), but was later recognised as a widespread mechanism of gene regulation in bacteria that senses their cell numbers and express phenotypes that are beneficial for the community. Bacteria

synthesize and release AIs either by actively transporting them across their cell membrane or via passive diffusion. When a certain AI threshold, that is cell population, is reached, the gene expression system of bacterial cells becomes altered and the transcription of certain genes is switched on or off. Thus, bacteria can regulate genes that are advantageous for their survival (Reuter *et al.*, 2016). Such cell-to-cell communication has a role, for example, in forming biofilms, expressing virulence factors, producing antibiotics, transferring genetic material, and exhibiting bioluminescence (Ng and Bassler, 2009; Bordi and de Bentzmann, 2011; Scutera *et al.*, 2014).

There is a different mechanism of quorum sensing in Gram negative and Gram positive bacteria. Gram negative bacteria primarily use N-acyl L-homoserine lactones (AHLs), which are homoserine lactone (HSL) rings with an additional fatty acid side chain (Ng and Bassler, 2009; Bordi and de Bentzmann, 2011), whereas Gram positive bacteria generally use oligopeptides as autoinducers (Miller and Bassler, 2001; Bordi and de Bentzmann, 2011).

During the maturation stage of the biofilm, EPS binds cells and protects them from shearing forces of the fluid. The water present in EPS is efficiently trapped by hydrogen bonding with the hydrophilic polysaccharides (Kostakioti *et al.*, 2013). In this perspective, it was reported that *P. aeruginosa* secretes three polysaccharides, namely alginate, Psl (pentasaccharide) and Pel (glucose rich polysaccharide) which together provide stability to the biofilm. Alginate supplies hydrated nutrients to the biofilm (Rasamiravaka *et al.*, 2015), and Psl and Pel act as structural support (Colvin *et al.*, 2011; Franklin *et al.*, 2011). In addition to EPS, DNA from extraneous sources appears to play a role in stabilization of *P. aeruginosa* biofilms (Gloag *et al.*, 2013). Thus co-aggregation of different types of microbes with each other leads to increase in the matrix and the depth of the biofilm (Rickard *et al.*, 2002). Biofilms can be in the form

of a monolayer or multilayer depending on the interaction between the constituent cells and surface of the substrate (Karatan and Watnick, 2009). Over time the biofilm becomes established with its structure, physiology and metabolism being dependent on the substrate. Presence of macro-colonies with water channels embedded in EPS, is an indication of a mature biofilm (Dufour *et al.*, 2012).

1.3.1.4.4. Dispersion

Over time, biofilm shedding takes place. Members of specific bacterial communities begin to produce enzymes that breakdown polysaccharides holding the biofilm together and thereby release bacteria residing on the top of a biofilm for colonization to a new surface. In this way sessile microbial cells return to the motile form (Hall-Stoodley *et al.*, 2004). For example, *Streptococcus equi* secretes hyaluronidase; *P. aeruginosa* and *Pseudomonas fluorescens* secrete alginate lyase and *Escherichia coli* secrete N-acetyl-heparosan lyase to breakdown the biofilm matrix (Sutherland, 1999). The detached microorganisms and sections of planktonic biofilm organisms may form biofilm elsewhere in the flow system (Lazar, 2011). Clinical significance of the dispersion phase of a biofilm is that any opportunistic human pathogens that may be present as planktonic bacterial clusters are a potential source of infection in vulnerable hosts (Otto, 2013).

With dental waterlines in mind, part I of this chapter highlights the importance of research in understanding the waterborne biofilm control and its management. Patients coming into contact with these devices risk health complications from opportunistic pathogens, if not monitored regularly and/or replaced.

1.3.2. Dental-unit waterline specific conditions conducive to biofilm formation

Typical dental-units are equipped with different types of plastic tubing that can extend for up to 10 metres. The internal diameter of this tubing is usually approximately 2

millimetres, and inside the small lumen of such narrow tubing, water flows freely at the centre leaving a thin layer of undisturbed water around the walls. This allows the formation of a conditioning pellicle of chemicals on the inner walls of plastic tubing, and over a very short time (days) promotes the attachment of microorganisms (Al Shorman *et al.*, 2002^a; Barbot *et al.*, 2012; Dallolio *et al.*, 2014). In addition, high surface area to volume, suitable temperature and long-term stagnation of water in tubing, provides an active planktonic population of bacteria, which together with the new incoming bacteria (e.g. from water reservoir or oral if retraction valves have failed), results in an active microbial population which can flourish, leading to the rapid development of biofilms (Al Shorman *et al.*, 2002^a, Barbot *et al.*, 2012; Dallolio *et al.*, 2014). See Fig. 1.11.

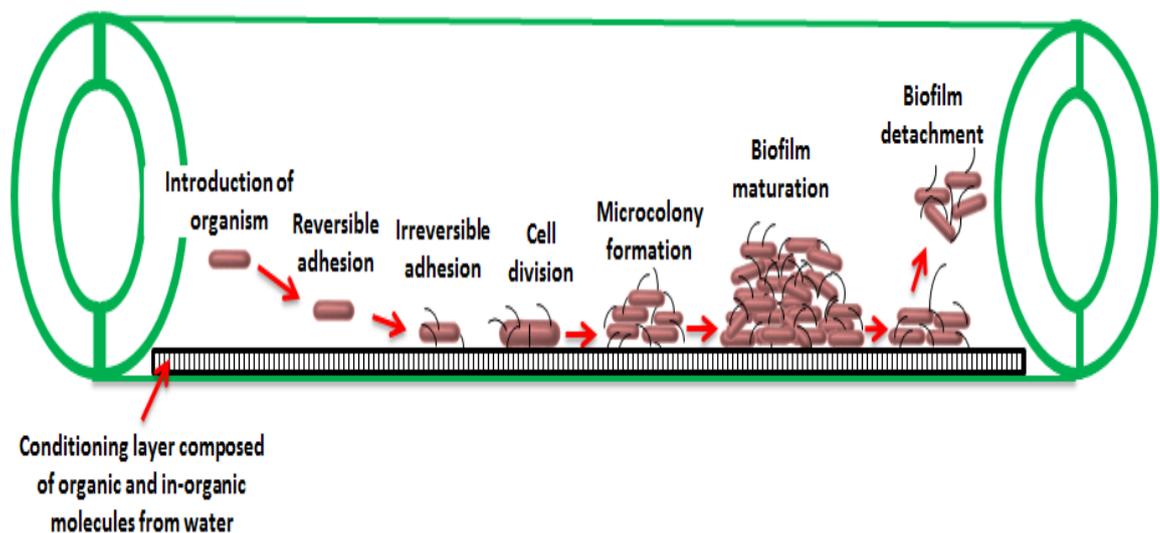


Figure 1.11: Schematic diagram of a homogenous biofilm formation within tubing.

1.3.3. Typical microbes found in the dental-unit waterline biofilm

As the DUWL biofilm is a heterogeneous community a diverse range of microorganisms, have been isolated from DUWL output water by various scientists

globally (Al Shorman *et al.*, 2002^a; Singh and Coogan, 2005; Szymanska, 2005^a; Pankhurst and Coulter, 2007; Goksay *et al.*, 2008; Petti *et al.*, 2013; Dillon *et al.*, 2014^a; Kadaifciler and Cotuk, 2014; Hikal *et al.*, 2015; Ditommaso *et al.*, 2016). They basically fall into the following categories.

- Aerobic heterotrophic bacteria
- Protozoa
- Fungi

1.3.3.1. Aerobic heterotrophic bacteria

Most frequently found are Gram-negative aerobic heterotrophic bacteria (see Table 1.4) of very low pathogenicity (Singh *et al.*, 2003; O'Donnell *et al.*, 2007; Pankhurst and Coulter, 2007). However, it is the human pathogenic bacteria identified from DUWLs that are of importance from the public health aspects. These include *Legionella* spp., *Pseudomonas* spp., non-tuberculosis *Mycobacterium* spp., *K. pneumoniae* and *S. marcescens* and are discussed separately.

1.3.3.1.1. *Legionella* species

There are 52 *Legionella* species (Gobin *et al.*, 2009) and 70 serogroups (Fields *et al.*, 2002). Of these species, 25 species are known to cause human disease. Most human infections are caused by *L. pneumophila* (Muder and Yu, 2002), and the predominant serogroup is serogroup 1 (Luck, 2010). *Legionella* spp. causes Legionellosis (Pontiac fever or Legionnaires' disease), a respiratory infection. Pontiac fever is a self-limiting influenza-like syndrome; Legionnaires' disease is more severe with pneumonia as the predominant clinical finding, and is a potentially fatal illness (Lam *et al.*, 2011). As *Legionellae* also colonize DUWLs (Pankhurst and Philpott-Howard, 1993; Williams *et al.*, 1993; Atlas *et al.*, 1995; Williams *et al.*, 1996; Pankhurst *et al.*, 1998; Singh and Coogan, 2005; Castiglia *et al.*, 2008; Ajami *et al.*, 2012; Pasquarella *et al.*, 2012;

Arvand and Hack. 2013; Szymanska and Sitkowska, 2013; Leoni *et al.*, 2015; Ditommaso *et al.*, 2016), the output water from dental-units represents a potential source of infection for both dental health care personnel and their patients via aerosolisation of water (Gross *et al.*, 1992; Bentley *et al.*, 1994; Szymanska, 2004; 2007). High speed drills being cooled under a stream of water is an inevitable part of many dental treatments. The implication of a droplet size of $\leq 5 \mu\text{m}$, generated by the aerosols, carrying around 1,000 CFU of *L. pneumophila* is profound. If inhaled, these have the capacity to penetrate deeply into the lungs and thereby represent an occupational health hazard (Fitzgeorge *et al.*, 1983; Rowbotham, 1986).

Domestic hot water systems harbour 6-30% *Legionella* (Strauss *et al.*, 1996) suggesting mains water supply may be a typical source of *Legionella* within DUWLs (Singh and Coogan, 2005). *Legionellae* require a temperature range of 20-45 °C to multiply in the DUWL environment, and the CDC advises ambient temperature to be maintained in dentistry whereas previously it was quite common for the water supply to 3 in 1 syringes to be heated to make its use more comfortable for the patients. Their incidence is increased by the presence of a host, usually amoebae, which browse on microbial biofilms containing *Legionellae* as a source of nutrients (Wadowsky *et al.*, 1985). Although free living amoebae are also found in fresh water environments, they appear to have an increased predilection for the DUWL niche, than for example, tap water from the same source (Barbeau and Buhler, 2001). Once established, presence of *Legionellae* may persist for years (Rangel-Frausto *et al.*, 1999). In relation to DUWL contamination, Atlas *et al.*, (1995) reported the case of a dentist in San Francisco, USA, who became seriously ill from Legionnaires' disease. More recently, this organism has been shown to be responsible for the death of an elderly patient following dental treatment from contaminated waterlines (Ricci *et al.*, 2012).

1.3.3.1.2. *Pseudomonas* species

Among the Pseudomonads, *P. aeruginosa* is the most frequently found bacterium in DUWL water and it was responsible for the initial public outbreak of disease following dental treatment (Martin, 1987). The DUWL environment is conducive to its existence (Martin, 1987; Pankhurst *et al.*, 1998; Barbeau *et al.*, 1996; 1998; Monarca *et al.*, 2002; Tambekar *et al.*, 2007; Barben *et al.*, 2009; D'Ovidio *et al.*, 2011; Ouellet *et al.*, 2014), because it can survive and grow in a low nutrient environment such as water. In addition, this organism is resistant to a wide range of biocides and antibiotics (Atlas *et al.*, 1995, Barbeau *et al.*, 1996; Vogwil *et al.*, 2016). It can cause pneumonia-like disease in elderly or immunocompromised individuals. The infective dose of this bacterium for colonization in a healthy individual is $> 1.5 \times 10^6$ CFU/mL (Pankhurst and Coulter, 2007).

1.3.3.1.3. Non-tuberculosis species of *Mycobacteria*

These are opportunistic pathogens which may cause respiratory, cutaneous and systematic infections. These organisms are frequently transmitted through environmental sources such as the ingestion or inhalation of water, particulate matter via aerosols, or through physical trauma (Falkinham, 2003). Presence of these organisms in DUWL water (Schulze-Robbecke *et al.*, 1995; Porteous *et al.*, 2004) reflects the original source of mains water supply (Walker *et al.*, 2000; Pankhurst *et al.*, 2003). Many studies have presented the prevalence and health risk from non-tuberculosis *Mycobacteria* spp. present in DUWL water. It has been reported that the number of non-tuberculosis *Mycobacteria* in DUWL water exceeds that of drinking water by a factor of 400 (Schulze-Robbecke *et al.*, 1995). The matter of concern is that a large number of non-tuberculosis *Mycobacteria* present in DUWL water may be inhaled. They may also contaminate oral wounds of immunosuppressed patients especially if at the time of dental treatment; they are undergoing additional therapy

and/or are HIV-positive (Falkinham, 2003). Two cases of cervical lymphadenitis following dental extraction and prosthetic heart valve infection with *M. gordonae* have been reported (Lohr *et al.*, 1978; Wallace *et al.*, 1983).

1.3.3.1.4. *S. marcescens*

S. marcescens is a Gram negative bacillus classified as a member of the Enterobacteriaceae family. It is known to be a nosocomial pathogen which can acquire antimicrobial resistance (Maseda *et al.*, 2009). This bacterium can cause a variety of infections in humans including septicaemia, meningitis, endocarditis and blindness in the susceptible host (Hejazi and Falkiner, 1997; Equi and Green, 2001; Tan *et al.*, 2014). Environmental strains of *S. marcescens* characteristically produce a red pigment described as prodigiosin. The function of prodigiosin is unclear because clinical isolates are rarely pigmented (Hejazi and Falkiner, 1997). The presence of *S. marcescens* in DUWL water has been documented by many researchers (Michel and Just, 1984; Williams *et al.*, 1993; Rowland and Voorheesville, 2003).

1.3.3.2. Protozoa

In biofilms, amoebae may be considered the dominant protozoan predator due to their surface associated lifestyle (Pickup *et al.*, 2007^b); Amoebae graze on mixed communities of bacteria within biofilms including pathogenic bacteria such as species of *Legionella*, *Mycobacterium*, *P. aeruginosa*, *Vibrio cholerae*, *Helicobacter pylori* and even *Staphylococcus aureus* (Barbaree *et al.*, 1986; Henke *et al.*, 1986; Fields *et al.*, 1989; Wadowsky *et al.*, 1988; Winiecka-Krusnell *et al.*, 2002; Pickup *et al.*, 2007^a; Cateau *et al.*, 2008; Salah *et al.*, 2009; Sandstrom *et al.*, 2010; Thomas *et al.*, 2010). Amoebae feed by creating digestive vacuoles inside their cell. The interaction between protozoa and bacterial prey is complex, as some bacteria are preferred over others (Pickup *et al.*, 2007^b). Once inside the amoebal cell, some bacteria will survive the

adverse conditions presented by digestive vacuoles, find sanctuary from unfavourable environmental conditions and exploit their protozoan hosts as vectors for multiplication (Marciano-Cabral *et al.*, 2010). Furthermore, bacteria such as *S. aureus* may be ingested but released undigested (Pickup *et al.*, 2007^a) implying that amoebae can also transport certain species of nosocomial bacteria (Wenzel, 1994; Scheid, 2014) within their ecological niches.

In hostile conditions amoeba cells can transform from an active trophozoite stage to a cyst dormant stage (Loret and Greub, 2010). Amoebal cysts can protect engulfed bacteria from adverse environmental conditions, earning them the title of “Trojan horses of the microbial world” (Barker and Brown, 1994). These cysts have a thick double layered wall that is resistant to many chemicals and drugs, including chlorine-based disinfectants and biocides (Rowbotham, 1986; King *et al.*, 1988; Marciano-Cabral and Cabral, 2003; Valster *et al.*, 2009). Encystment also guards amoeba cells and any bacteria inside them from high temperatures (Harb *et al.*, 2000).

Singh and Coogan (2005) have reported the presence of *Legionella* loaded amoebae in a DUWL in a hospital in South Africa. Amoebae harbouring potentially pathogenic bacteria may be present within the planktonic microorganisms from the DUWL output water, and direct inhalation via splatter and aerosols (Bently *et al.*, 1994; Szymanska, 2007) has the potential to infect both the patient and the dental staff. This bears impact on healthcare providers and responsible regulatory bodies to take appropriate preventative measures. Protozoa isolated from DUWLs to date are listed in Table 1.4.

1.3.3.3. Fungi

Studies on DUWLs have mainly focused on bacterial contamination, resulting in mycological contamination studies falling behind. Detailed studies on the concentration and composition of fungal flora in DUWLs (Szymanska, 2005^b; Goksay *et al.*, 2008;

Nikaeen *et al.*, 2009) indicate that compared to bacterial contamination, mycological contamination is less widespread. However, the existence of opportunistic fungal pathogens in DUWL water is cause of concern. Two cases of oral aspergillosis have been reported in which a periodontal infection associated with *Aspergillus* spp. has been recognized in a neutropenic patient and a mandibular bone infection by *Aspergillus* spp. has been documented in a diabetic patient after tooth extraction (Iatta *et al.*, 2009). Other studies (Singh, 2005; Tasic and Tasic Miladinovic, 2007) have reported the hazardous effects of microfungi including asthma and allergic reactions. The species/genera of fungi identified so far in DUWL water are shown in Table 1.4.

Table 1.4: Bacteria, protozoa and fungi isolated from dental-unit waterlines.

Bacteria	References
<i>Achromobacter xyloxidans</i>	Pankhurst <i>et al.</i> , 1998
<i>Acinetobacter calcoaceticus</i>	Barbeau <i>et al.</i> , 1996
<i>Acinetobacter</i> spp.	Williams <i>et al.</i> , 1996; Pankhurst <i>et al.</i> , 1998; Shepherd <i>et al.</i> , 2001
<i>Actinomyces</i> spp.	Barbeau <i>et al.</i> , 1996; Pankhurst <i>et al.</i> , 1998
<i>Alcaligenes denitrificans</i>	Williams <i>et al.</i> , 1996; Pankhurst <i>et al.</i> , 1998
<i>Alcaligenes faecalis</i>	Meiller <i>et al.</i> , 1999
<i>Alcaligenes</i> spp.	Shepherd <i>et al.</i> , 2001
<i>Bacillus</i> spp.	Barbeau <i>et al.</i> , 1996; Williams <i>et al.</i> , 1996; Pankhurst <i>et al.</i> , 1998; Meiller <i>et al.</i> , 1999; Shepherd <i>et al.</i> , 2001; Al-Saif <i>et al.</i> , 2007
<i>Bacterioides</i> spp.	Pankhurst <i>et al.</i> , 1998
<i>Brevundimonas vesicularis</i>	Barbeau <i>et al.</i> , 1996; Williams <i>et al.</i> , 1996; Meiller <i>et al.</i> , 1999; Szymanska <i>et al.</i> , 2008
<i>Brevibacterium epidermidis</i>	Szymanska <i>et al.</i> , 2008
<i>Burkholderia cepacia</i>	Pankhurst <i>et al.</i> , 1998; Meiller <i>et al.</i> , 1999; Uzel <i>et al.</i> , 2008
<i>Caulobacter</i> spp.	Pankhurst <i>et al.</i> , 1998
<i>Chryseomonas luteola</i>	Uzel <i>et al.</i> , 2008
<i>Flavobacterium indologenes</i>	Williams <i>et al.</i> , 1996
<i>Flavobacterium</i> spp.	Williams <i>et al.</i> , 1993; Pankhurst <i>et al.</i> , 1998; Shepherd <i>et al.</i> , 2001
<i>Fusobacterium</i> spp.	Pankhurst <i>et al.</i> , 1998
<i>K. pneumoniae</i>	Williams <i>et al.</i> , 1993; Pankhurst <i>et al.</i> , 1998
<i>Klebsiella</i> spp.	Williams <i>et al.</i> , 1993
<i>Lactobacillus</i> spp.	Pankhurst <i>et al.</i> , 1998
<i>Legionella anisa</i>	Singh and Coogan, 2005; Barben <i>et al.</i> , 2009
<i>Legionella bozemanii</i>	Singh and Coogan, 2005
<i>Legionella gormanii</i>	Singh and Coogan, 2005
<i>Legionella micdadei</i>	Singh and Coogan, 2005
<i>L. pneumophila</i>	Pankhurst <i>et al.</i> , 1998; Singh and Coogan, 2005; Rice <i>et al.</i> , 2006; Barben <i>et al.</i> , 2009; Dahlen <i>et al.</i> , 2009; Ajami <i>et al.</i> , 2012
<i>Legionella</i> spp.	Fotos <i>et al.</i> , 1985; Pankhurst and Philpott-Howard, 1993; Atlas <i>et al.</i> , 1995; Williams <i>et al.</i> , 1993; 1996; Pankhurst <i>et al.</i> , 1998; Singh and Coogan, 2005; Rice <i>et al.</i> , 2006; Tanzi <i>et al.</i> , 2006; Dutil <i>et al.</i> , 2007; Pankhurst and Coulter 2007; Veronesi <i>et al.</i> , 2007; Castiglia <i>et al.</i> , 2008; Pasquarella <i>et al.</i> , 2012; Arvand and Hack, 2013; Szymanska and Sitkowska, 2013; Leoni <i>et al.</i> , 2015; Ditommaso <i>et al.</i> , 2016
<i>Methylobacterium mesophilicum</i>	Barbeau <i>et al.</i> , 1996
<i>Micrococcus luteus</i>	Williams <i>et al.</i> , 1996; O'Donnel <i>et al.</i> , 2006; Szymanska <i>et al.</i> , 2008
<i>Micrococcus</i> spp.	Pankhurst <i>et al.</i> , 1998
<i>Micrococcus lylae</i>	Szymanska <i>et al.</i> , 2008
<i>Moraxella lacunata</i>	Szymanska <i>et al.</i> , 2008
<i>Moraxella osloensis</i>	Meiller <i>et al.</i> , 1999
<i>Moraxella</i> spp.	Pankhurst <i>et al.</i> , 1998; Goksay <i>et al.</i> , 2008; Szymanska <i>et al.</i> , 2008
<i>Mycobacterium avium</i>	Pankhurst <i>et al.</i> , 1998

<i>Mycobacterium gordonae</i>	Pankhurst <i>et al.</i> , 1998
<i>Mycobacterium</i> spp.	Schulze-Robbecke <i>et al.</i> , 1995; Pankhurst <i>et al.</i> , 1998; Porteous <i>et al.</i> , 2004
<i>Myroides odoratum</i>	Meiller <i>et al.</i> , 1999
<i>Nocardia</i> spp.	Williams <i>et al.</i> , 1996; Pankhurst <i>et al.</i> , 1998
<i>Ochrobactrum anthropi</i>	Goksay <i>et al.</i> 2008; Williams <i>et al.</i> , 1996; Kadaifciler and Cotuk, 2014
<i>Pasteurella haemolytica</i>	Williams <i>et al.</i> , 1996; Goksay <i>et al.</i> , 2008
<i>Pasteurella</i> spp.	Williams <i>et al.</i> , 1996; Pankhurst <i>et al.</i> , 1998; Kadaifciler and Cotuk, 2014
<i>Photobacterium damsela</i>	Goksay <i>et al.</i> , 2008
<i>Proteus vulgaris</i>	Pankhurst <i>et al.</i> , 1998
<i>Pseudomonas acidovorans</i>	Williams <i>et al.</i> , 1996
<i>Pseudomonas aeruginosa</i>	Martin, 1987; Pankhurst <i>et al.</i> , 1998; Barbeau <i>et al.</i> , 1996;1998; Monarca <i>et al.</i> , 2002; Tambekar <i>et al.</i> , 2007; Barben <i>et al.</i> , 2009; D'Ovidio <i>et al.</i> , 2011; Ouellet <i>et al.</i> , 2014
<i>Pseudomonas cepacia</i>	Williams <i>et al.</i> , 1996
<i>Pseudomonas fluorescens</i>	Williams <i>et al.</i> , 1996; Barbeau <i>et al.</i> , 1996; Uzel <i>et al.</i> , 2008; Kadaifciler and Cotuk, 2014
<i>Pseudomonas paucimobilis</i>	Williams <i>et al.</i> , 1996
<i>Pseudomonas putida</i>	Barbeau <i>et al.</i> , 1996; Kadaifciler and Cotuk, 2014
<i>Pseudomonas stutzeri</i>	Williams <i>et al.</i> , 1996; Meiller <i>et al.</i> , 1999
<i>Pseudomonas testosteroni</i>	Williams <i>et al.</i> , 1996
<i>Pseudomonas</i> spp.	Williams <i>et al.</i> , 1996; Shepherd <i>et al.</i> , 2001; Al-Saif <i>et al.</i> , 2007; Castiglia <i>et al.</i> , 2008; Mungara <i>et al.</i> , 2013; Uzel <i>et al.</i> , 2008
<i>Psychrobacter phenylpyruvica</i>	Meiller <i>et al.</i> , 1999
<i>Ralstonia pickettii</i>	Williams <i>et al.</i> , 1996; Meiller <i>et al.</i> , 1999; Uzel <i>et al.</i> , 2008; Szymanska <i>et al.</i> , 2008
<i>S. marcescens</i>	Michel and Just, 1984; Williams <i>et al.</i> , 1993; Williams <i>et al.</i> ,1996; Rowland and Voorheesville, 2003
<i>Sphingomonas paucimobilis</i>	Barbeau <i>et al.</i> , 1996; Meiller <i>et al.</i> , 1999; Uzel <i>et al.</i> , 2008; Szymanska <i>et al.</i> , 2008; Kadaifciler and Cotuk, 2014
<i>Sphingomonas</i> spp.	Shepherd <i>et al.</i> , 2001; O'Donnel <i>et al.</i> , 2006
<i>Staphylococcus aureus</i>	Pankhurst <i>et al.</i> , 1998; Meiller <i>et al.</i> , 1999; Lancellotti <i>et al.</i> , 2007
<i>Staphylococcus capitis</i>	Williams <i>et al.</i> , 1996
<i>Staphylococcus cohnii</i>	Szymanska <i>et al.</i> , 2008
<i>Staphylococcus epidermidis</i>	Lancellotti <i>et al.</i> , 2007
<i>Staphylococcus hominis</i> ss <i>novobiosepticus</i>	Szymanska <i>et al.</i> , 2008
<i>Staphylococcus lentus</i>	Szymanska <i>et al.</i> , 2008
<i>Staphylococcus pulvereri/vitulus</i>	Szymanska <i>et al.</i> , 2008
<i>Staphylococcus saprophyticus</i>	Williams <i>et al.</i> , 1996
<i>Staphylococcus warneri</i>	Williams <i>et al.</i> , 1996
<i>Staphylococcus</i> spp.	Williams <i>et al.</i> , 1996; Meiller <i>et al.</i> , 1999; Szymanska <i>et al.</i> , 2008
<i>Stenotrophomonas maltophilia</i>	Barbeau <i>et al.</i> , 1996; Williams <i>et al.</i> , 1996; Meiller <i>et al.</i> , 1999; Szymanska <i>et al.</i> , 2008
<i>Streptococcus</i> spp.	Williams <i>et al.</i> , 1996; Pankhurst <i>et al.</i> , 1998; Shepherd <i>et al.</i> , 2001; Petti <i>et al.</i> , 2013

<i>Streptomyces albus</i>	Szymanska <i>et al.</i> , 2008
<i>Xanthomonas</i> spp.	Pankhurst <i>et al.</i> , 1998; Shepherd <i>et al.</i> , 2001
Protozoa	
<i>Acanthamoeba castellanii</i>	Hikal <i>et al.</i> , 2015
<i>A. griffin</i>	Hikal <i>et al.</i> , 2015
<i>A. hatchitti</i>	Hikal <i>et al.</i> , 2015
<i>A. lenticulata</i>	Hikal <i>et al.</i> , 2015
<i>Acanthamoeba</i> spp.	Michel and Just, 1984; Williams <i>et al.</i> , 1993; Barbeau <i>et al.</i> , 1996; Pankhurst <i>et al.</i> , 1998; Barbeau and Buhler, 2001; Barbeau, 2007; Leduc <i>et al.</i> , 2012
<i>Hartmannella</i> spp.	Barbeau <i>et al.</i> , 1996; Barbeau and Buhler, 2001
<i>Giardia</i> spp.	Pankhurst <i>et al.</i> , 1998
<i>Microsporidium</i> spp.	Pankhurst <i>et al.</i> , 1998
<i>Naegleria</i> spp.	Michel and Just, 1984; Barbeau <i>et al.</i> , 1996; Barbeau and Buhler, 2001; Michel and Borneff, 1989; Leduc <i>et al.</i> , 2012
<i>Vahlkampfia</i> spp.	Barbeau <i>et al.</i> , 1996; Barbeau and Buhler, 2001
<i>Vanella</i> spp.	Barbeau <i>et al.</i> , 1996; Barbeau and Buhler, 2001
<i>Vermamoeba vermiformis</i>	Dillon <i>et al.</i> , 2014 ^a
Fungi	
<i>Alternaria</i> spp.	Pankhurst <i>et al.</i> , 1998
<i>Aspergillus amstelodami</i>	Szymanska <i>et al.</i> , 2008
<i>Aspergillus flavus</i>	Goksay <i>et al.</i> , 2008
<i>Aspergillus fumigatus</i>	Szymanska <i>et al.</i> , 2008
<i>Aspergillus glaucus</i>	Szymanska <i>et al.</i> , 2008
<i>Aspergillus repens</i>	Szymanska <i>et al.</i> , 2008
<i>Aspergillus versicolor</i>	Kadaifciler and Cotuk, 2014
<i>Candida albicans</i>	Szymanska <i>et al.</i> , 2008
<i>Candida curvata</i>	Szymanska <i>et al.</i> , 2008
<i>Citromyces</i> spp.	Szymanska <i>et al.</i> , 2008
<i>Cladosporium cladosporioides</i>	Kadaifciler and Cotuk, 2014
<i>Cladosporium</i> spp.	Pankhurst <i>et al.</i> , 1998
<i>Exophiala mesophila</i>	Porteus <i>et al.</i> , 2003
<i>Geotrichum candidum</i>	Szymanska <i>et al.</i> , 2008
<i>Penicillium expansum</i>	Goksay <i>et al.</i> , 2008
<i>Penicillium frequentans</i>	Szymanska <i>et al.</i> , 2008
<i>Penicillium pusillum</i>	Szymanska <i>et al.</i> , 2008
<i>Penicillium turolense</i>	Szymanska <i>et al.</i> , 2008
<i>Penicillium</i> spp.	Pankhurst <i>et al.</i> , 1998; Kadaifciler and Cotuk, 2014
<i>Phoma</i> spp.	Pankhurst <i>et al.</i> , 1998
<i>Sclerotium sclerotiorum</i>	Szymanska <i>et al.</i> , 2008
<i>Scopulariopsis</i> spp.	Pankhurst <i>et al.</i> , 1998

1.3.4. Bacterial endotoxin levels in DUWL water

Bacterial endotoxin, lipopolysaccharide (LPS) released from the cell walls of live and dead Gram negative bacteria have also been found from DUWL water at levels ranging from 500 to 2560 endotoxin units/mL (Fulford *et al.*, 2004; Pankhurst and Coulter, 2007). The generally accepted range for irrigation devices in USA is 0.06 to 0.5 endotoxin units/mL and is regulated by the US federal government (USDHHS, 1987). Bacterial endotoxin is associated local inflammation, high grade fever and shock in sensitive individuals. Hypersensitivity pneumonitis has also been documented in patients following exposure of DUWL water contaminated with bacterial endotoxin (Pankhurst and Coulter, 2007). According to Michel *et al.*, (1996), the severity of asthma in patients is directly correlated with the concentration of endotoxin. Moreover, bacterial endotoxin found in DUWL water can encourage the release of pro-inflammatory cytokines in gingival tissue during dental surgery and adversely affect the healing process (Putnins *et al.*, 2001). At present, there seems to be no standards set for endotoxin in drinking or recreational sources of water.

1.3.5. Unculturable and Viable but non-culturable (VBNC) bacteria

1.3.5.1. Unculturable bacteria

Bacteria in DUWL biofilm niche are usually detected and quantified by microbiological culture based methodologies. This methodological approach is by no means fool proof in detecting every conceivable species of bacteria that may be present in any given specimen of water being analysed. Some of the possible reasons are that the culture medium itself is toxic, a required nutrient is not present in the culture medium, that other bacteria in the sample produce inhibitory substances to the target organism or lack of understanding of optimal laboratory growth conditions for the specific species under investigation (Stewart *et al.*, 2012). However, even if ideal growth conditions are

provided the reason why specific bacterial species that have not yet been cultured and identified *in-vitro* may be due to their initial low numbers and variable growth rates in a mixed culture sample. For example, faster growing bacteria may outcompete slower growing bacteria in favour of the available nutrients, pH conditions, temperatures and oxygen levels as Kopke (2005) highlighted. In addition, culture media that are rich in nutrients favour the growth of faster-growing bacteria, whilst, bacteria grown on nutrient poor environments (e.g. water, use of biocides), tend to be slow growing and if placed on rich media their growth may be hindered (Watve *et al.*, 2000). Extended incubation times at more ambient temperature on low nutrient solid media may be more suitable for the slow growing bacteria to enable a balance for the faster growing members to die off first. This also reduces the species competition barrier and allows previously unculturable bacteria to be cultivated (Davis *et al.*, 2005). Hence our knowledge of the diversity and complexity of the strains of bacteria from specific ecological niches remains incomplete.

1.3.5.2. VBNC bacteria

VBNC bacteria are characterized by a loss of culturability on routine media, on which they normally grow (Oliver, 2010; Li *et al.*, 2014). Many bacterial species have been reported to exist in VBNC state (Li *et al.*, 2014) and are also found in DUWLs. This may lead to an underestimation of the total viable count (TVC) in DUWL water samples. A typical example is *Legionella* species and others see Table 1.5.

VBNC bacteria possess physiological and molecular differences from their culturable state. These differences include adhesion properties, virulence potential, cellular morphology, cell wall and membrane composition, metabolism, physical and chemical resistances and gene expression (Li *et al.*, 2014).

It is likely that the VBNC state is a survival strategy in which bacteria enter into a temporary state of low metabolic activity, that may be mistaken for an absence of species due to no growth on solid microbiological medium (Nichols *et al.*, 2008).

For some species of bacteria, there are reasons why they cannot be detected beyond optimal growth requirements alone. These include interspecies competition due to the release of pigments or bacteriocins from genotypically similar bacteria (*P. aeruginosa*, *S. marcescens*). These pigments may inhibit the growth of competing bacteria (Li *et al.*, 2014).

Co-cultivation with helper strains is an option to culture VBNC bacteria due to mimicking the beneficial bacterial interactions within their culture environment in laboratory conditions (Nichols *et al.*, 2008). These beneficial interactions can be released in the form of factors, which often present as growth stimulants and can be utilised in cell culture media to grow VBNC bacteria (Tanaka *et al.*, 2004).

1.3.6. Health risks related to VBNC bacteria

L. pneumophila are known to enter the viable non-culturable state in response to low nutrient levels (Steinert *et al.*, 1997; Brown and Backer, 1999; Oliver, 2010). This leads to an underestimation of total viable cells in test samples, and in this way pose a risk to public health. *E. coli* and *V. cholerae* cells are also reported to be able to enter viable non-culturable state (Xu *et al.*, 1982). Oliver (2010) provided a list of pathogens known to foster a viable non-culturable state. Some of these pathogens have been detected in DUWLs including *P. aeruginosa*, *L. pneumophila*, *Mycobacterium* spp., *K. pneumoniae* and *S. marcescens*. Factors which induce viable non-culturable state in these bacteria are shown in Table 1.5. The presence of VBNC in DUWLs must be considered because the medical implications are potentially numerous. For example, viable non-culturable bacteria have a low metabolic rate, and as such antibacterial compounds that target activities or components of active cells would be less effective against them. In

addition, the viable non-culturable state may favour the development of drug resistance when strict biocidal treatment protocols are not followed (Li *et al.*, 2014). Specific to this project, bacteria in DUWLs may not only be present in the viable non-culturable phase, but may be carried intracellularly within free-living amoebae at the time of sampling (see section 1.2.2.4. and 1.3.6.2).

Table 1.5: The factors inducing VBNC state of pathogenic bacteria found in dental-unit waterlines.

Bacterial Species	VBNC state inducing factor	References
<i>K. pneumoniae</i>	Starvation	Byrd <i>et al.</i> , 1991
<i>L. pneumophila</i>	Starvation, chemicals (disinfectants NaOCl and NH ₂ Cl)	Garcia <i>et al.</i> , 2007; Alleron <i>et al.</i> , 2008; Buse <i>et al.</i> , 2013
<i>Mycobacterium tuberculosis</i>	Starvation, oxygen limitation	Downing <i>et al.</i> , 2005
<i>P. aeruginosa</i>	Starvation, low temperature, chemicals (copper)	Trevors, 1995; Dwidjosiswojo <i>et al.</i> , 2011
<i>S. marcescens</i>	Aerosolization	Heidelberg <i>et al.</i> , 1997

1.3.7. Alternative methodologies in identification of specific VBNC bacteria

Molecular biology techniques, such as the use of polymerase chain reaction (PCR) amplification of small fractions of genomic DNA, have been sequenced in order to aid the characterisation of bacterial populations from a wide range of habitats. However, molecular methods generally do not provide information on the viability of the organisms present in the samples, but they do indicate their presence (Snelling *et al.*, 2006; Thomas and Ashbolt, 2011).

Alternatively, oligonucleotide probes have been designed to target bacteria with no known cultivable references. Fluorescence *in-situ* hybridization (FISH) target specific probes have been used previously to visualise the cellular morphology of previously uncultivable bacteria; however this process requires fixation procedures and therefore is not conducive to subsequent culture methods.

1.3.8. Research efforts to reduce DUWL contamination levels

Researchers beginning with Blake (1963) have investigated treatment options proposed to maintain the quality of dental treatment water. The largest number of studies of waterline treatment published over the last 52 years has investigated various chemical agents to inactivate microorganisms, induce detachment of biofilms or both. Some studies have also examined non-chemical approaches to the disinfection of the DUWL water. Chemical-based disinfection has been effective to some extent in controlling microbial contamination. However, both approaches are not effective at eliminating biofilm.

1.3.8.1. Chemical methods

An ideal chemical agent for controlling biofilm in DUWL would be bactericidal but not toxic or irritating to humans and not interact with the materials used by dentists. It would remove biofilm and discourage subsequent reformation, while protecting the tubing of DUWLs from degradation. If delivered continuously in dental treatment water, it would have no effect on enamel or dentine bonding agents (Mills, 2000). Although such a chemical agent does not currently exist, there are some products possessing some of these desired characteristics. Chemical disinfectants may be introduced into water system continuously (Szymanska, 2006; Bansal *et al.*, 2011; Dallolio *et al.*, 2014) or intermittently (Smith *et al.*, 2001; Montebugnoli *et al.*, 2004; Schel *et al.*, 2006; Dallolio *et al.*, 2014).

1.3.8.1.1. Continuous chemical treatment

Continuous treatment uses either biostatic or lower concentration of highly effective biocidal agents. A study conducted by Costa *et al.*, (2016) demonstrate that continuous chemical treatment is more effective in eliminating and preventing biofilm inside DUWLs. However, it may damage the tubes and/or valves in the dental-unit. Since the

chemical agent is always present in water and may be aerosolized, the effects of chronic exposure on the health care staff must be considered (Roberts *et al.*, 2000). In addition, enamel and dentine bond strength of dental adhesive materials may also be affected (Roberts *et al.*, 2000).

1.3.8.1.2. Intermittent chemical treatment

The usual practice for intermittent treatment is to deliver the agent for a specified contact time and frequency using an independent water reservoir that isolates the unit from the municipal water supply. The advantage of this type of treatment is that the active agent is eliminated from the system before the treatment of the patient. Disadvantages include the adverse effect on tubes; exposure of dental staff, and the potential for surviving microorganisms to re-bond between treatments (Mills, 2000).

Numerous studies (Tuttlebee *et al.*, 2002; Larsen and Fiehn, 2003; Walker *et al.*, 2003; Walker and Marsh, 2004; Chate, 2006; O'Donnell *et al.*, 2006; O'Donnell *et al.*, 2007; Walker and Marsh, 2007; Coleman *et al.*, 2007) have shown that regular cleansing of DUWLs with a chemical disinfectant or biocide ensures that the DUWL output water is safe. Disinfection by chemical agents is recommended regularly because the microbes present in the supply water or coming from fluids retraced back by dental instruments readily colonize the DUWLs after disinfection (Pankhurst and Philpott-Howard, 1993; Mills, 2000; Tuttlebee *et al.*, 2002; Walker *et al.*, 2003; O'Donnell *et al.*, 2006; O'Donnell *et al.*, 2007; Coleman *et al.*, 2009). In some studies it has been demonstrated that treating the DUWLs continuously rather than treating only in the evening and weekend was efficient in both reducing the microbial contamination and controlling the microbial biofilms (Montebugnoli and Dolci, 2002).

A number of studies have suggested DUWL treatment with various disinfectant solutions (Table 1.1), including: acidic electrolyzed water; hydrogen peroxide; ozone; peracetic acid; chlorine dioxide; chlorhexidine; peroxides; sodium hypochlorite; citric

acid; chlorhexidine gluconate; povidone-iodine; electrochemically activated water and Listerine mouthwash. Super-oxidised water has also been investigated to be efficient in controlling microbial contamination (Zinkevich *et al.*, 2000; Martin and Gallagher, 2005). Schel *et al.*, (2006) tested a variety of disinfectants (Table 1.1) including Sterilex Ultra, BioBlue, Sanosil, Alpron, ster4Spray and Dentosept in DUWLs in the general dental practices in Europe including Germany, Ireland, Netherland, Denmark, Greece, United Kingdom and Spain. The general outcome was that, if the products were used continuously, efficacy was demonstrated, but not if they were used intermittently.

Although literature suggests that a wide range of chemical disinfectants are effective in removing biofilm and reducing bacterial level in DUWL output water to an acceptable level, most of these studies were carried out *in-vitro* and relatively few examined the effectiveness of chemical agents in routine general practice (Tuttlebee *et al.*, 2002; Walker *et al.*, 2003; O'Donnell *et al.*, 2006; O'Donnell *et al.*, 2007; O'Donnell *et al.*, 2009; McDowell *et al.*, 2004; Schel *et al.*, 2006). In addition, only a few studies have demonstrated long term efficacy of chemical agents when applied in general practice (O'Donnell *et al.*, 2006; O'Donnell *et al.*, 2007; O'Donnell *et al.*, 2009). Manufacturers of dental chair recommend specific treatment agents to be applied in their apparatus as compatibility of the chemistry with DUWL tubes is important for their longevity. This becomes a serious problem if the chemical agents suggested by manufacturers are not effective. Technical errors and non-compliance could also be the reason for failure.

1.3.8.1.3. Manufacturers DUWL cleaning devices

There are several suppliers of dental chairs which are manufactured by 3 main companies (Leoni *et al.*, 2015) and are listed in Table 1.6. Each different manufacturer of the dental chair unit has adopted their own specific biocide and cleansing regime compatible with their system. Hence the dental surgeries commonly use biocides

recommended for use specific to manufacturers of the equipment installed in the practice and their suggested waterline treatment protocols (Montebugnoli and Dolci, 2002; Spratt *et al.*, 2004; Leoni *et al.*, 2015), see Table 1.6.

Table 1.6. Manufacturer of the Main dental chair used in the UK and their DUWL treatment protocols.

Company	Waterline treatment protocol	Reference
A-dec company	<ul style="list-style-type: none"> • Continuous disinfection with ICX™ (concentration: 0.01%). • Weekly cycle of overnight treatment with an alkaline based peroxide agent (Sterilex Ultra at the concentration of 0.5%). 	Leoni <i>et al.</i> , 2015
Castellini company	<ul style="list-style-type: none"> • Continuous disinfection with hydrogen peroxide (concentration: 0.06%). • Daily 10 minute cycle of treatment with a disinfectant product generating peracetic acid, peracetyl ions and hydrogen peroxide equivalent to 0.26% of peracetic acid (Rely+On Peracilyse). <p style="text-align: center;"><u>Castellini Autosteril system</u></p> <ul style="list-style-type: none"> • This device integrated in dental unit automatically flushes a disinfecting solution (Tetraacetylenediamine (TAED) in association with sodium perborate/per salt) through the water system. 	Leoni <i>et al.</i> , 2015 (Montebugnoli and Dolci, 2002; Spratt <i>et al.</i> , 2004).
Eurodent company	<ul style="list-style-type: none"> • Continuous disinfection with Calbenium™ (concentration: 2%). 	Leoni <i>et al.</i> , 2015

1.3.8.1.4. Emerging DUWL semi-automated cleaning devices

Some manufacturers of dental chairs have been experimenting with integrated semi-automated DUWL cleaning systems, which are not currently in clinical use. For example, the Planmeca Waterline Cleaning System (WCS) and Planmeca Waterline Management System (WMS) use a semi or fully automated DUWL cleaning device in which the chair can be connected to either the mains water system or water from a removable reservoir. The advantage of using two modes to draw water into the unit is that the removable reservoir can be filled with the disinfectant at the end of the working

day and the isolator valve can be turned to the reservoir so that disinfection can take place overnight. Once the machine is switched on next day, the waterlines are flushed automatically with mains water (O'Donnell *et al.*, 2006). Such an investigation reported the long-term effectiveness of both the WCS and the WMS using the Planosil (a disinfectant containing hydrogen peroxide and silver ions) in maintaining the quality of DUWL output water below 200 CFU/ml of aerobic heterotrophic bacteria following weekly disinfection (O' Donnell *et al.*, 2006; O' Donnell *et al.*, 2007). The development and ongoing improvement of these automated DUWL cleaning systems has the potential to reduce the effect of constantly being non-compliant with the relevant authorities for keeping to the dental threshold of clean treatment water.

1.3.8.1.5. Disadvantages of DUWL treatment agents

Chemical treatment of DUWLs is not a universal remedy for microbial control as there is the problem associated with resistance to antimicrobials (Gilbert and Allison, 2000). Some studies (Roberts *et al.*, 2000; Taylor-Hardy *et al.*, 2001) have reported that few DUWL treatment agents such as bio 2000; a 0.12% chlorhexidine gluconate and 12% ethyl alcohol-containing product; a 1:10 dilution of Listerine mouth rinse; 3 ppm sodium hypochlorite; and 0.224% BioClear, a citric acid containing product, decrease the adhesion of resins to both dentine and enamel leading to dental fillings failing prematurely. Such adverse effects may become clinically relevant in the case of residual DUWL treatments. In other studies it has been shown that chlorine-containing biocides release more mercury from amalgam than some other products (Batchu *et al.*, 2006; Roberts *et al.*, 2005). In another report (Stone *et al.*, 2006), it was documented that iodine-releasing cartridges installed to dental chairs can increase dissolved mercury levels in dental-unit wastewater. Dental personnel should therefore be advised as to which biocides are prone to causing deposition of ions in the environment. Furthermore, some chemicals decrease the total viable count of biofilm significantly, but increased

number of planktonic microorganisms in water may be present in the treatment water being used (Walker *et al.*, 2000). Further issues of exposure of chemicals to the patient and health care workers also require consideration (Lee *et al.*, 2001).

1.3.8.2. Non chemical methods

1.3.8.2.1. Use of sterile water

Sterile water has been used in reservoir bottle in place of mains water supply to improve the quality of DUWL output water (O'Donnell *et al.*, 2011). This approach has been found to be ineffective.

1.3.8.2.2. Flushing

Another approach to reduce the number of microbes in DUWL output water involves mechanical flushing of DUWLs (Rice *et al.*, 2006; Coleman *et al.*, 2009); however, literature (Whitehouse *et al.*, 1991; Santiago *et al.*, 1993; Williams *et al.*, 1995) does not support this method of DUWL cleansing because it only reduces microbial load (Santiago *et al.*, 1993). Flushing in between patients may eliminate material that may have entered the waterline during the previous treatment.

1.3.8.2.3. Drying of DUWL

Flushing DUWLs with sterilised water after using and drying them with pressurized air has also been used to improve the quality of DUWL output water (Fiehn and Larsen, 2002). However, after allowing water through DUWLs it was noted that bacterial concentration did not reduce significantly (Fiehn and Larsen, 2002). A plausible reason could be that the biofilm contains enough moisture to withstand the desiccation process and thereby protect microbes for short periods of time.

1.3.8.2.4. Filtration

Microbial filters fitted to the dental chair unit water supply or to DUWLs near the dental instrument attachment sites, have also been used to provide good quality DUWL output

water (Pankhurst *et al.*, 1990; Murdoch-Kinch *et al.*, 1997; Copenhagen, 2006). In the dental surgeries, where dental-units are connected to municipal water supplies, the water may also contain impurities including minerals, organic compounds and bacterial endotoxins that are not always removed by filters. Therefore, for surgical procedures sterile water should not be replaced with bacteria free water produced by filtration in dental clinics. However, some studies suggest that filters can produce water that meets the CDC standards of ≤ 500 CFU/mL for nonsurgical procedures. Mayo and Brown (1999), found no bacteria in water samples collected immediately downstream from 0.2 μm filters; however, the level of bacteria in effluent water increased when they increased the distance at which filters were placed from the air water syringe.

The potential advantages of using filters include the reduction or even the necessity to use chemical agents to protect dental staff from exposure to chemical residues. While, filters are effective in removing suspended bacteria from dental treatment water, they will have no effect on the biofilm that continues to develop in pre-filtration parts of waterlines, unless simultaneous treatment is undertaken to remove biofilm (Mills, 2000). Also, the existence of biofilm in DUWLs can result in the release of endotoxins that may pass through the filters. Another problem of using filters is that they are readily clogged therefore require frequent changing and increase the cost of dental treatment (Mills, 2000).

1.3.8.2.5. Changing composition of DUWLs

Few studies have examined the effect of DUWL composition on biofilm formation and decreasing the microbial contamination in DUWL output water (Coleman *et al.*, 2007; Coleman *et al.*, 2009). One study from Japan (Yabune *et al.*, 2005) reported that polyvinylidene fluoride was more effective in resisting biofilm formation and reducing microbial contamination in DUWL output water than conventional DUWLs made up of

polyurethane. Nevertheless, concentration of microbes in DUWL output water remained high despite a significant reduction. Another study from Italy (Sacchetti *et al.*, 2007) reported that the aerobic heterotrophic bacterial plate count at 22 °C from polytetrafluorethylene was lower than output water from DUWLs made from polyethylene. These findings indicate that the development of novel DUWL materials with antimicrobial and /or anti-biofilm properties is needed to control DUWL biofilm. Use of copper pipes can be beneficial in improving the microbial quality of dental chair units supply water, as copper pipe has been shown to have significant antimicrobial properties over drinking water (Rogers *et al.*, 1994).

1.3.8.2.6. An autoclavable systems

Williams *et al.*, (1996) reported that in response to providing clean water and preventing DUWL contamination, a fully autoclavable assembly of water reservoirs (silicon multi lumen DUWL tubing and fittings and sterilisable between patients) has been approved by the Food and Drug Administration, in the USA. Such a system may be safer, as any contamination can be eradicated by prior autoclave sterilisation. However, the disadvantage of such a system is that a sufficient number of units must be purchased for in-between-patient sterilisation (Williams *et al.*, 1996).

1.3.8.2.7. Engineering/redesigning of dental chair unit

The role of manufacturing is critical in achieving improvements in water quality (Coleman *et al.*, 2007). One of the improvements in manufacturing that could be made is to build a fully automated dental-unit water disinfection system and some steps have been taken in this direction. In addition, reducing the length of the tubing and keeping the water flow continuous in DUWLs would bypass/slowdown biofilm formation.

1.3.9. Research related to biocide testing

Disinfectants can be tested for their efficacy in killing bacteria and/or controlling their numbers in the laboratory. The main everyday laboratory method is to use pure cultures of bacteria for direct contact with the specified biocide followed by recovery on appropriate solid growth media using the Miles and Misra (1938) method to enumerate viable cells. The viable cell numbers (CFU/mL) are compared with the viable counts obtained from control plates to evaluate the efficacy of antimicrobials. These studies often provide encouraging results however; the same efficacy does not appear to be reproduced when applied to cleansing clinical DUWLs. This is because a consortium of biofilm organisms is capable of surviving antibacterial agents. This necessitated *in-vitro* testing on biofilms giving rise to various simulated DUWL (sDUWL) models (Walker *et al.*, 2001; Spratt *et al.*, 2004; McDowell *et al.*, 2004; Zhang *et al.*, 2011). However, they also serve in understanding the developing ecology of heterogeneous water-borne biofilm communities too (Walker *et al.*, 2001; Dillon *et al.*, 2014^a). The earliest mixed species biofilm model was that of Walker *et al.*, (2001) that used a continuous-culture in a chemostat design and consisted of medical-grade silicone tubing and small-bore polyurethane DUWL tubing. The chemostat generated biofilms differ from those generated *in-situ* DUWL tubing. In addition, the Walker *et al.*, (2001) model did not mimic the clinical DUWL operational conditions and flushing was not possible. Further models have been developed that incorporated more realistic additions to the disinfection test protocols.

The Spratt *et al.*, (2004) model employed lengths of true DUWL tubes (polyurethane) to a water reservoir with a programmable peristaltic pump. The pump allowed simulated chair-side use with clinical downtime as stagnation periods. The Spratt *et al.*, (2004) model (Fig. 1.12) compared tap water flushing with TAED flushing

model in which human commensal bacteria were added to the system to determine the subsequent decontamination levels.

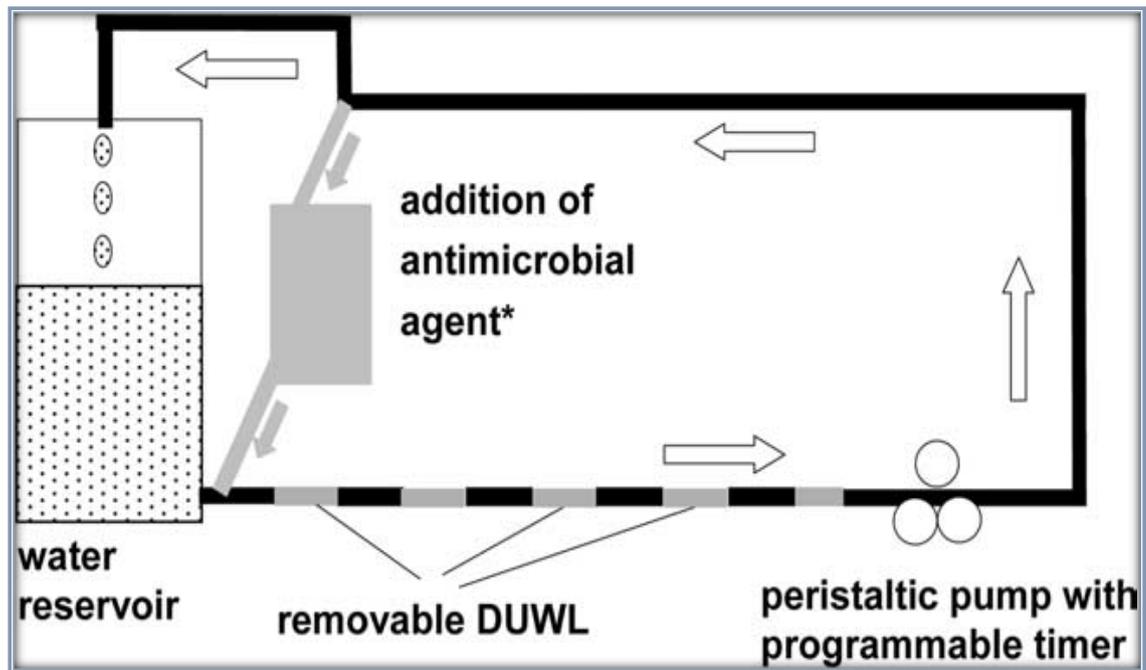


Figure 1.12: *In-vitro* model for DUWL biofilm formation and decontamination testing (Spratt *et al.*, 2004).

The Spratt *et al.*, (2004) DUWL model satisfies the CDCs recommendations for flushing and bacterial growth. This model is also very practical, relatively inexpensive and operates closely mimicking clinical waterline systems.

A more elaborate and expensive set up was constructed in the McDowell *et al.*, (2004) model in which multiple automated sDUWL systems operated to reproduce clinical DUWL conditions. Each system contained all of the components of a typical dental water delivery system that included a water bottle, polyurethane tubing, a control system, three high-speed hand-pieces water coolant lines and one air-water syringe line. An electronic controller was fitted which operated the system intermittently to mimic daily dental-unit usage, using approximately 60 mL per simulated patient. The test program comprised of 10 simulated patient treatment cycles per day and a flushing of all waterlines at the start of each day and after each patient, as per recommendation of the CDC. To create the environment of hard water, some dental systems were adjusted

with 200 mg/L of calcium carbonate (CaCO_3) in 1:100 dilution of phosphate-buffered saline (PBS). The main objective of this study was to test the effectiveness of ICX tablets in preventing microbial growth in DUWLs during an extended period of simulated use and to investigate how the ICX tablets effectiveness was affected by water hardness.

1.4. Rationale for the project

The significance of this research lies in providing safe water in clinical dental services and the prevention of potential occupational/public health outbreaks. Dental professionals have a duty of care to their patients to ensure adequate infection control policies and guidelines are adhered to, at all times.

Drinking water in the EU should not exceed 100 CFU/mL (Anon, 1998). The UK has its own guidelines set by the Department of Health (DoH) for water discharged from DUWL to be between 100 to 200 CFU/mL (HTM 01-05: <http://www.dh.gov.uk>) and similar guideline (≤ 200 CFU/mL) is set by the American Dental Association (Anon, 1996). However, literature suggests the CFU/mL will significantly exceed these figures in water discharges from untreated dental-units, often in excess of 200,000 CFU/mL (Smith *et al.*, 2002). One local audit performed in Lancashire, UK, demonstrated that these figures are also reached when protocols to clean waterlines are not working (Pearce *et al.*, 2013). A subsequent audit was performed in which reliability of repeated use of biocides in DUWL water was tested. The conventional methodological approach of that audit identified 85% of dental practices exceeded the DoH, UK recommended threshold of ≤ 200 CFU/mL whilst 15% conformed, of which 7% bettered the DoH threshold (Pearce *et al.*, 2013). Thus the rationale for this study was derived from the earlier audits that demonstrated the importance of continued monitoring of DUWL output water.

1.5. Aim of research

The main aim of the research is to address the question of how to provide dental treatment irrigated by clean water in patients' mouth which is consistently within the DoH recommended limit (≤ 200 CFU/mL) for dental treatment.

1.6. Objectives

1. Obtaining all ethical approvals and preparation/understanding of standard operating procedures.
2. Determine quality of clinical DUWL output water.
3. Investigate test kits for in-office monitoring of microbiological quality of water discharged from DUWLs.
4. Evaluate aerosols and splatter contamination in dentistry.
5. Investigate if *V. vermiformis* acts as a vector for pathogenic bacteria within dental-unit waterline systems.
6. Determine any evidence, from water samples, if retraction valves were failing in the participating dental practices.
7. Set up simulated laboratory model(s) of DUWL to understand a waterborne biofilm community in DUWL tubing.

1.7. Research Approach

This research uses the effective practice of microbiology to provide cultures for waterborne bacteria with which to validate in-office tests.

In office tests: The gold standard laboratory test, using microbiological cultures on R2A agar was chosen for this work. Commercial tests, Petrifilm™ AC Plate and the Dip test™ kits were tested for the reliability of measuring the quality of water discharged from clinical DUWLs.

Aerosols and splatter contamination: Identification of bacterial species was performed using 16S ribosomal (r) RNA gene primers and PCR, cloning and sequencing.

V. vermiformis study: This study involved the incidence of amoebal vectors. To determine if *V. vermiformis* acts as a vector for pathogenic bacteria, amoeba that are

frequently associated with DUWLs were fed, in the laboratory, on newly isolated nosocomial bacterial strains.

Retraction valve failure: This was investigated using a microbiological culture approach for the detection of opportunistic oral pathogen(s) (*Candida* spp.) and a biochemical assay (SDS-PAGE and immunoblotting) for the detection of albumin and IgG from oral sources.

A simulated laboratory model of a DUWL was constructed using the published methodology of Spratt *et al.*, (2004) to investigate the development of a heterogeneous biofilm over an extended period, explored the strategies developed by early colonizers that allow them to adhere, and spread onto the polyurethane tubing of DUWLs, tested occupational hazards associated with amoebic vectors, and identified potential pathogenic bacteria associated with DUWL ecology.

1.8. Ethical approvals

Approval from the relevant NHS authorities concerning research governance (R & D North West) was obtained (proposal No 310). Approval for this study was also obtained prior to commencing laboratory investigations in which all research procedures met the ethical guidelines of the Biological Safety Committee (BSO 1112-02) and my academic institute (STEM 100). For certification of approval see appendix A1-A3. A standard operating procedure was devised and agreed in a three-way collaboration involving consenting dental practices, the project advisor and the experimenter.

PART I: SERVICE EVALUATION

**Chapter 2. Investigating test kits for in-office monitoring of
microbiological quality of water discharged from dental-unit
waterlines**

2.1. Introduction

Service evaluation was undertaken in partnership with East Lancashire Primary Care Trust, UK (now no longer in existence), which conducted local audits to monitor compliance with delivering clean treatment water to the standard of the DoH (UK) in dental practices.

In these specific audits 73 surgeries belonging to 30 different practices were recruited to provide water samples between the period of June and September 2009 and again in July-August 2012. All samples were tested for bacterial contamination as part of an under graduate dissertation (1st audit) and summer intern project (2nd audit). The results indicated that, although the practices were actively making attempts to maintain clean supply of water (to 200 CFU/mL) from DUWL by the addition of biocides, a significant level of contamination was still detectable from various surgeries (Pearce *et al.*, 2013). This suggested that some dental practices fail to obtain clean water to the level within 200 CFU/mL from their DUWLs.

However, research has shown that DUWL biocides such as Alpron, Sterilox, Bio 2000, Dentosept, Oxygenal and sodium hypochlorite, if used with the specified protocols, are capable of maintaining clean DUWL discharged water to the standard recommended by the DoH (Smith *et al.*, 2002; Walker *et al.*, 2003; 2004; Martin and Gallagher, 2005; Chate, 2006; Schel *et al.*, 2006). Discussions with the principals or practice managers of appropriate practices did not elicit a clear explanation for the variability in the results. The East Lancashire audit study unequivocally highlighted that failure of all the various protocols occurred frequently and that the practices are unaware of the problem and were, as a consequence unable to take corrective measures to ensure that they met the standards for water quality set by the DoH (≤ 200 CFU/mL). To test the reliability of repeated use of biocides in DUWL water, a further audit (this study) was performed in the same locality.

In order to maintain high standards in clinical dentistry a rapid testing method for estimating the quality of water leaving the DUWL within each practice was considered as an advantage. However, there is no user-friendly, fully validated methodology for rapid testing of DUWLs for contamination. Sending samples to be cultured in a dedicated microbiology facility once a week, for example, is both expensive and involves a delay of several days while the test is carried out elsewhere. The concept of a rapid test for clinical use designed to measure DUWL bacterial contamination is appealing.

Therefore, in addition to estimating the quality of water leaving the DUWL, this study also set out to validate the use of two existing microbiology culture based commercial test kits (PetrifilmTM AC Plate and Dip slideTM) as possible rapid method of in-office testing DUWL contamination levels to an acceptable sensitivity for future use.

2.2. Materials and methods

Sources of material used in this chapter are listed in Table 2.1.

Table 2.1: Sources of material.

Material	Source	Material	Source
R2A	Lab M	Petrifilms™AC Plates	A gift from 3M Food Safety Ltd
Dip slides™ (Dimanco Ltd)	A gift from 3M Food Safety Ltd	Falcon tubes™ (50 mL)	Fisher Scientific
Eppendorf tubes™ (1.5 mL)	Fisher Scientific	Disposable spreaders	Fisher Scientific
Petri dishes	Fisher Scientific		

2.2.1. Sample collection

To honour the anonymity agreement with participating dental practices, collection of clinical DUWL output water and swab samples (described in chapter 3) from designated clinical surfaces a Standard Operating Procedure (SOP) (See section 2.2.2) was devised and agreed between the participating dental practices, the project advisor and the experimenter.

2.2.2. SOP

Part 1. To ensure compliance for clinical water collection

1. For the safe collection of DUWL water samples, participating dental practices located in East Lancashire were identified for the project.
2. Before commencing, NHS (R & D North West) service evaluation approval had been obtained.

3. Study approval from UCLan: Research Project Approval and approval from Biological Safety Committee was obtained (FM SHE 067 Procedural Guidance for Biological Safety, Section 4.5 work with hazardous biological agents).
4. The identified dental practices were asked to participate by sending out project information sheet through E-mail and thereafter, allowing time for their decision making without pressure.
5. Following a positive response, consent forms were sent out for their signature confirming a formal participation for the appropriate study and duration.
6. The participating dental practices were asked if they would volunteer information regarding: **a)** their waterline disinfection regime, **b)** if CFU counts were high, would they consider a re-test following revision of their waterline disinfection protocol? **c)** If a rapid in-office test was proposed, would they have a go at using it and comment on it? And, **d)** indicate if the participating practices required results at the time of providing written consent.
7. A check list was prepared for: if the Project Advisor had made arrangements with appropriate dental practice for collection of water samples on a given date and time; experimenter to ensure the project advisor has been provided with gloves, Falcon™ tubes (50 mL), swabs, sealable cool bag for transport; made arrangements with the project advisor to collect samples, anonymise them and deliver to UCLan within 2 h of collection; experimenter to anticipate delivery time and make himself available for handover of samples, and ensure experimenter is prepared to handle the specimens for culture straight away.

Part 2. SOP for actual clinical DUWL output water and swab collection

a) Collection of samples

1. Collect water around 10 am each time so that the DUWL is already in use.
2. Flush out stagnant water for 30 seconds.
3. Collect DUWL output water (50 mL) from the “3 in 1” air/water syringe in sterile 50 mL Falcon™ tubes whilst holding another 50 mL tube nearby containing sterile water as control from splashes and aerosols.
4. Place lids on tubes securely to prevent leaks and cross infection.
5. Collect Swab samples from pre-selected clinical surfaces.
6. Carefully label all tubes and swabs with study number to anonymise and keep records of practice identification code and the study number safe in a lockable filing cabinet in the advisors office for his use only.
7. Place the tubes and swabs in a sealable cool bag.
8. Zip lock the cool bag securely.
9. Contact the experimenter with expected delivery time to hand over the samples.
10. Transport samples and hand over to the experimenter within 2 hrs of collection.

b) Health and Safety

- The sample collector to travel by car (fully insured) to the destination by following UK driving laws (to avoid road accident).
- The experimenter to follow risk assessment and insurance according to UCLan guidelines.

c) Laboratory investigation

- Experimenter begins laboratory investigation directly after hand over using agreed conditions as stated in the biological safety officer’s approval application.

d) Data protection

The identification code, study code of samples and all data obtained from laboratory investigation to keep in locked cabinet in accordance with the Data Protection Act 1998.

2.2.3. Medium preparation

Low nutrient solid medium such as R2A is recommended for enumerating bacterial cells from stressed water sources. At the knowledge that specimen delivery was imminent R2A plates were poured by weighing out R2A powder (18 g/L) in distilled water prior to autoclave sterilisation at 121 °C at 15 lb/inch² for 15 min. After cooling to 45-50 °C, about 20 mL of the molten medium was poured into fresh Petri dishes and thereafter allowed to set. The composition of all media used, throughout this study is described in appendix A11.

2.2.4. Evaluation of the cleanliness of clinical water by R2A, Dip slidesTM and PetrifilmTM Aerobic Count (AC) Plate tests

All water samples (N=31) were processed within two hours of collection in a class II safety cabinet for the following tests.

1. Evaluating the quality of DUWL output water on R2A plates.
2. Inoculating (as per manufacturer's (Dimanco) instructions) same samples on the Dip slideTM whereby incubation time was varied for optimisation purposes.
3. Inoculating PetrifilmTM AC Plate tests closely following the manufacturer's (3M) instructions. Incubation temperature and time was varied for optimisation purposes.

2.2.4.1. R2A

Serial dilutions of DUWL water samples, in sterilised distilled water, were prepared, up to 10⁻⁷ and thoroughly mixed. Aliquots of 0.1 mL were inoculated onto R2A agar plates

in triplicate for each sample using the spread-plate method and incubated for 7 days at 22 °C.

2.2.4.2. Dip slides™

Dip slides™ were received as an assembly consisting of a paddle (Fig. 2.1, 1A) and an incubating chamber (Fig. 2.1, 1B). The paddle was two sided “slide” with agar based solid medium. The paddle was removed from the incubation chamber and each side was inoculated with one mL of undiluted sample (Fig. 2.1, 2) to keep in line with the procedures used in dental practices. The excess water was drained into the waste container containing 1% Virkon™ disinfectant (Fig. 2.1, 3). The paddle was re-inserted into the incubation chamber and allowed to incubate (Fig. 2.1, 4).

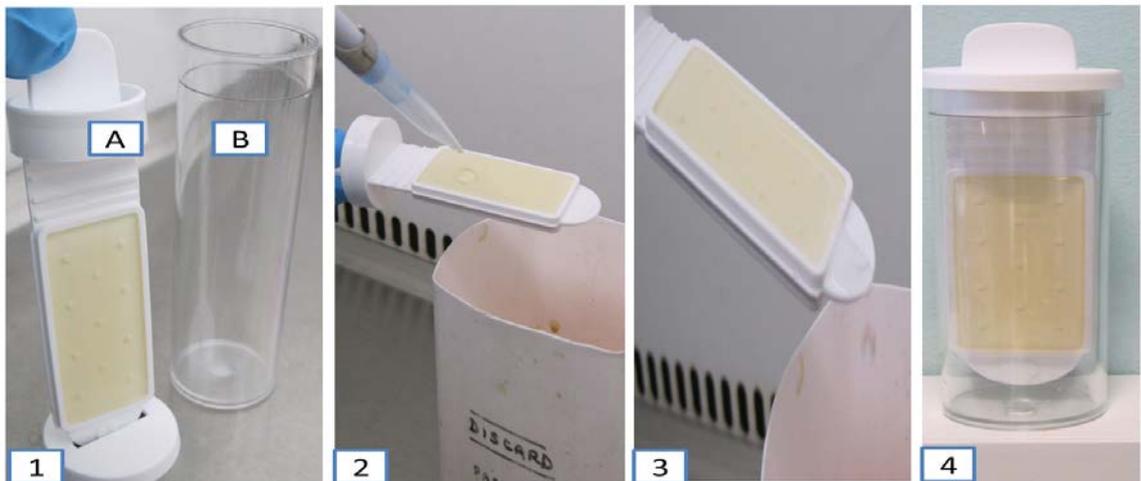


Figure 2.1: Steps 1-4 taken to inoculate the Dip slides™ **1)** two parts of dip slide test kit, A: Paddle with medium, B: incubation chamber, **2)** inoculation on both sides of the paddle was performed by delivering 1 mL/side of neat water sample, **3)** excess water was drained into a container containing Virkon™ disinfectant, **4)** the paddle was re-inserted into the incubation chamber and allowed to incubate.

Following inoculation, the Dip slide™ paddle was replaced in the incubation chamber and allowed to incubate, on a window ledge, as per manufacturer’s instructions for 2 days. Incubation of the Dip slide™, on a window ledge, was extended beyond the recommended time for 5 and 7 days (Fig. 2.1, 4) where temperature was measured during the day and recorded to lie in the range of 22-28 °C.

2.2.4.3. Petrifilm™ AC Plates

On receipt an aliquot of the water from each sample was withdrawn and treated with sodium thiosulphate (18 mg/L) (Anon, 2012) to neutralize the effects of any residual biocide on bacterial growth. The neutralised water and the original water samples were used to inoculate the Petrifilm™ AC Plate and the corresponding R2A plates.

To inoculate the Petrifilm™ AC Plate, it was placed on a flat bench surface and allowed to reach room temperature. The top film, of the multi-layered Petrifilm™ AC Plate was lifted and 1.0 mL of the test sample was delivered in the centre by holding a micropipette perpendicular to the Petrifilm™ AC Plate. Following inoculation, the top film was released and the sample was allowed to spread evenly with the aid of a “spreader tool” (included in each commercial kit). This involved placing the spreader on the bubble of water beneath the top film and applying gentle pressure, with the thumb or index finger (Fig. 2.2), at the centre of the tool to hold it down for further 30 seconds. Petrifilm™ AC Plate was left undisturbed for one min to allow the gel to set. Petrifilm™ AC Plates were incubated at 30 °C for 48 h in accordance with the manufacturer’s instructions. Further optimisation was performed by varying the temperature which were room temperature as measured on the days of the experiment to lie in the range of 22-28 °C, and constant temperature in the incubators set at 22 °C, 30 °C and 37 °C for 2, 5 and 7 days. The procedure for Petrifilm™ AC Plate inoculation is summarised in Fig. 2.2.

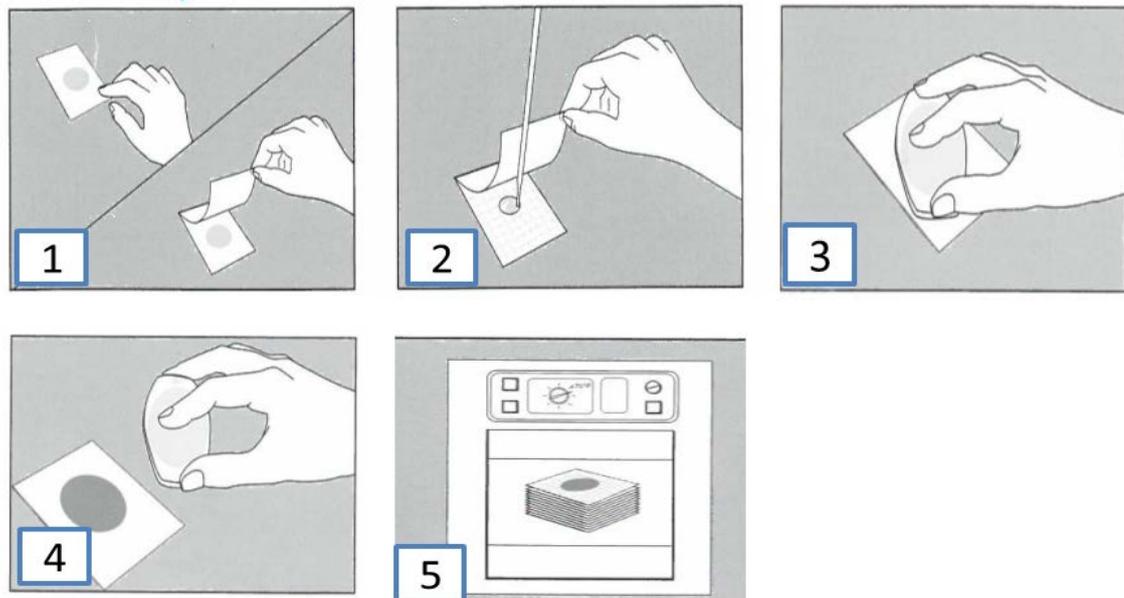


Figure 2.2: Diagrammatic summary of procedure for inoculating Petrifilm™ AC Plate. Step 1: After placing a Petrifilm™ AC Plate on a flat surface, top film was lifted up. Step 2: Sample (1 mL) was accurately delivered into the middle of the Petrifilm™ AC Plate, Step 3: and the top film was lowered onto the sample. The spreader tool was placed on top of the sample delivered site and held securely with the thumb and fingers whilst applying gentle pressure with index finger in the centre of the spreader for exactly 30 seconds. Step 4: The spreader tool was removed and the newly inoculated plate was left undisturbed for further 60 seconds. Step 5: All Petrifilm™ AC Plates were stacked on top of each other for incubation at desired temperature.

2.2.4.4. Colony enumeration

Following appropriate incubation times R2A and Petrifilm™ AC Plates and Dip slides™ were examined. Colonies on Dip slides™ were compared with the manufacturer's reference chart for an estimate of the CFU/mL. Whereas, CFU from R2A and Petrifilm™ AC Plates, were counted from plates displaying individual colonies in the range of 30-300. The CFU/mL was calculated using the mean of the triplicate R2A readings and adjusted for the dilution factor. If for example 30 colonies were counted from the plates (average of triplicate plates) from 1×10^{-4} inoculum dilution; then having applied 0.1 mL inoculum volume on the plate equals 300

colonies/mL divided by 10,000 (1×10^{-4}) equals 3×10^6 CFU/mL. This calculation (example only) was performed throughout the study to determine CFU/mL counts.

2.2.5. Testing the Petrifilm™ AC Plate for its in-office application

The Petrifilm™ AC Plate was tested for its reproducibility as an “in-office” application by offering it to practice staff for their DUWL water samples. Petrifilm™ AC Plate test was demonstrated to dental staff of each participating practice as per manufactures’ inoculation instructions and incubation at extended times at room temperature only.

A parallel Petrifilm™ AC Plate test was set up by using the remaining DUWL water under laboratory conditions as performed earlier by the same experimenter alongside of the R2A plating method (as described in section 2.2.4). This was necessary to calculate sensitivity, specificity and variation amongst different users.

2.2.6. Retesting of DUWL water

As part of the original service evaluation of this study, a re-test was offered to all surgeries that failed the original test (section 2.2.4); to see if a change in cleansing protocol had led to an improvement. Embedded in the re-test were some randomly selected surgeries that had met the DoH standards again from the original study (section 2.2.4). The water samples (N = 22) were inoculated on R2A plates as described in section 2.2.4. Petrifilm™ AC Plate tests were also set up and these are those performed in parallel to the in-office testing described above (section 2.2.5).

2.3. Results

2.3.1 Contamination level of dental-unit waterlines output water samples

The bacterial concentration of the water samples cultured on R2A agar varied significantly ranging from not detected to 4.3×10^6 CFU/mL. Out of 31 samples tested 16 (52%) exceeded the DoH, UK recommended threshold of ≤ 200 CFU/mL (Table 2.2). The remaining 15 samples (48%) met the DoH standards (Table 2.3). Frequency of the range of DUWLs in order of contamination is shown in Fig. 2.3. Control water (imported with the DUWL water collection sample) plated on R2A demonstrated no growth.

Table 2.2: List of samples exceeding DoH (UK) recommended threshold of ≤ 200 CFU/mL.

Sample ID	Biocide used by surgeries	CFU/mL	
		Mean (n = 3)	S.D
Control		Not detected	
DUWL 1	Alpron	1.3×10^4	$\pm 7.0 \times 10^2$
DUWL 3	Alpron	1.5×10^4	$\pm 2.2 \times 10^3$
DUWL 10	Alpron	2.0×10^4	$\pm 6.4 \times 10^2$
DUWL 11	Alpron	1.6×10^4	$\pm 3.2 \times 10^3$
DUWL 12	Alpron	1.5×10^4	n = 2
DUWL 25	Alpron	3.0×10^3	n = 2
DUWL 26	Alpron	3.1×10^3	$\pm 2.0 \times 10^2$
DUWL 27	Alpron	1.1×10^6	n = 2
DUWL 4	ICX	6.6×10^3	n = 2
DUWL 5	ICX	1.9×10^5	n = 2
DUWL 6	ICX	4.1×10^4	$\pm 7.3 \times 10^3$
DUWL 29	ICX	1.1×10^3	$\pm 5.3 \times 10^2$
DUWL 19	Sterilox	3.0×10^4	$\pm 6.1 \times 10^3$
DUWL 21	Sterilox	5.2×10^3	$\pm 6.0 \times 10^2$
DUWL 13	ICX & Sterilex Ultra purge	4.3×10^6	$\pm 5.1 \times 10^5$
DUWL 31	Unknown	6.4×10^4	$\pm 1.1 \times 10^4$

S.D = Standard deviation

Table 2.3: List of samples in compliance with DoH (UK) recommended threshold of ≤ 200 CFU/mL.

Sample ID	Biocide used by surgeries	CFU/mL	
		Mean (n = 3)	S.D
DUWL 2	Alpron	17	± 11
DUWL 7	Sterilox	7	± 6
DUWL 8	Sterilox	Not detected	
DUWL 9	Sterilox	Not detected	
DUWL 20	Sterilox	10	± 10
DUWL 14	ICX & Sterilex Ultra purge	Not detected	
DUWL 15	ICX & Sterilex Ultra purge	Not detected	
DUWL 16	ICX & Sterilex Ultra purge	Not detected	
DUWL 17	ICX & Sterilex Ultra purge	Not detected	
DUWL 18	ICX & Sterilex Ultra purge	Not detected	
DUWL 22	ICX & Sterilex Ultra purge	Not detected	
DUWL 23	ICX & Sterilex Ultra purge	Not detected	
DUWL 24	ICX & Sterilex Ultra purge	Not detected	
DUWL 28	Unknown	85	n = 2
DUWL 30	Unknown	103	± 6

S.D = Standard deviation

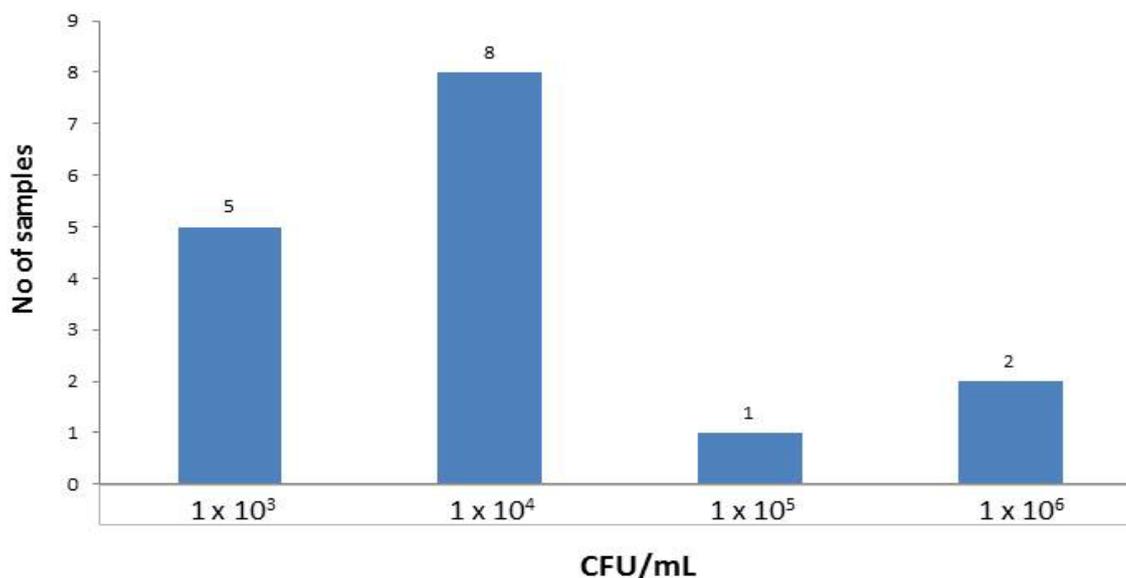


Figure 2.3: Summary chart of the frequency of the range of DUWLs in order of contamination.

2.3.2. Sensitivity and specificity of the Dip slide™ and Petrifilm™ AC Plate

2.3.2.1. Dip slide™

For validating the Dip slide™ the specificity remained at 100% throughout, whilst the sensitivity at two days, was 6%, after five days 25% and after seven days, 37% at the 1,000 CFU/mL thresholds (Table 2.4). Longer incubation periods (5-7 days) gave improved sensitivity than the shorter incubation (2 days) Fig. 2.4. False positives were not observed with the Dip slides™ test whereas false negative results equated to 15 out of 16 at 2 days, 12 out of 16 at 5 days and 10 out of 16 at 7 days.

Table 2.4: Sensitivity and specificity measurement of Dip slide™ at ≤ 1000 CFU/mL compared to R2A plating.

	Sensitivity (%)	Specificity (%)
Dip slide™		
Room temp for 2 days	6	100
Room temp for 5 days	25	100
Room temp for 7 days	37	100

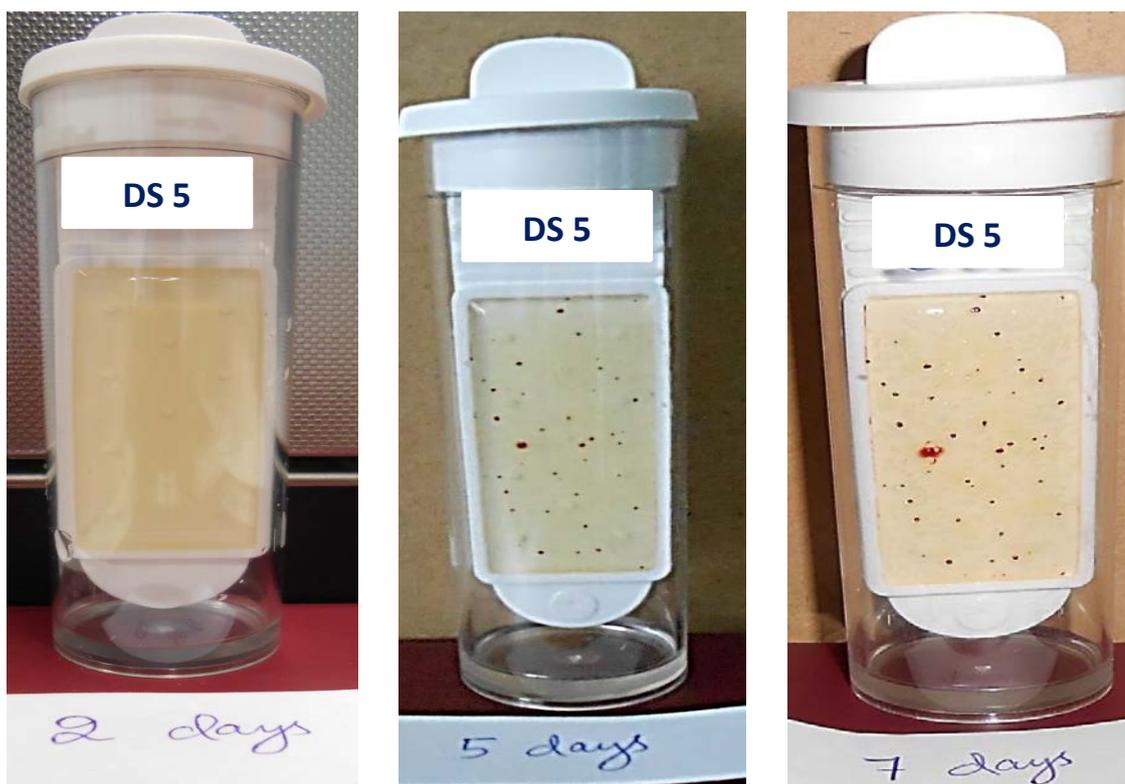


Figure 2.4: Dip slide™ test: longer incubation periods (5-7 days) gave improved sensitivity than the shorter incubation (2 days).

2.3.2.2. Petrifilm™ AC Plate

Validity of Petrifilm™ AC Plate compared to R2A agar was measured by calculating sensitivity and specificity values at the threshold of ≤ 200 CFU/mL (Table 2.5).

Where possible colonies were enumerated and it was noted that the Petrifilm™ AC Plate data failed to correlate with the CFU/mL values from R2A agar plates. When numerous, tiny colonies appeared on the plates, they were denoted as TNTC (too numerous to count). Sensitivity and specificity of Petrifilm™ AC Plates optimised under various conditions are given in Table 2.5. In general, there was no difference in the sensitivity and specificity when diluted samples were treated with/without sodium thiosulphate and incubated at room temperature (22-28 °C) after 5 and 7 days. Reduced sensitivity was recorded when Petrifilm™ AC Plates were incubated at 37 °C. Highest

sensitivity was observed following Petrifilm™ AC Plates incubation at 22 °C for 7 days.

Table 2.5: Validity measurement of Petrifilm™ AC Plate at ≤ 200 CFU/mL compared to R2A plating.

	Sensitivity (%)	Specificity (%)
Petrifilm™ AC Plate		
30 °C for 2 days (Manufacturer recommendations)	31	100
Room temp (22-28 °C) for 5 days (diluted sample <u>with</u> sodium thiosulphate)	37	100
Room temp (22-28 °C) for 7 days (diluted sample <u>with</u> sodium thiosulphate)	62	100
Room temp (22-28 °C) for 5 days (diluted sample <u>without</u> sodium thiosulphate)	37	100
Room temp (22-28 °C) for 7 days (diluted sample <u>without</u> sodium thiosulphate)	62	100
Room temp (22-28 °C) for 2 days (Neat sample)	19	100
Room temp (22-28 °C) for 5 days (Neat sample)	44	100
Room temp (22-28 °C) for 7 days (Neat sample)	50	100
37 °C for 2 days (Neat sample)	6	100
37 °C for 5 days (Neat sample)	19	100
37 °C for 7 days (Neat sample)	19	100
22 °C for 2 days (Neat sample)	31	100
22 °C for 5 days (Neat sample)	50	100
22 °C for 7 days (Neat sample)	69	100

No false positive results were recorded with Petrifilm™ AC Plates but false negative results were recorded and are given in Table 2.6.

Table 2.6: False negatives occurring with Petrifilm™ AC Plates.

Petrifilm™ AC Plate	False negatives (out of 16)
30 °C for 2 days (Manufacturer recommendations)	11
Room temp (22-28 °C) for 5 days (diluted sample <u>with</u> sodium thiosulphate)	10
Room temp for 7 days (diluted sample <u>with</u> sodium thiosulphate)	6
Room temp for 5 days (diluted sample <u>without</u> sodium thiosulphate)	10
Room temp for 7 days (diluted sample <u>without</u> sodium thiosulphate)	6
Room temp for 2 days (Neat sample)	13
Room temp for 5 days (Neat sample)	9
Room temp for 7 days (Neat sample)	8
37 °C for 2 days (Neat sample)	15
37 °C for 5 days (Neat sample)	13
37 °C for 7 days (Neat sample)	13
22 °C for 2 days (Neat sample)	11
22 °C for 5 days (Neat sample)	8
22 °C for 7 days (Neat sample)	5

2.3.2.2.1. Effect of incubation time

For Petrifilm™ AC Plate (Fig. 2.5) it was noted that longer incubation period of 7 days was better than 2 and 5 days.

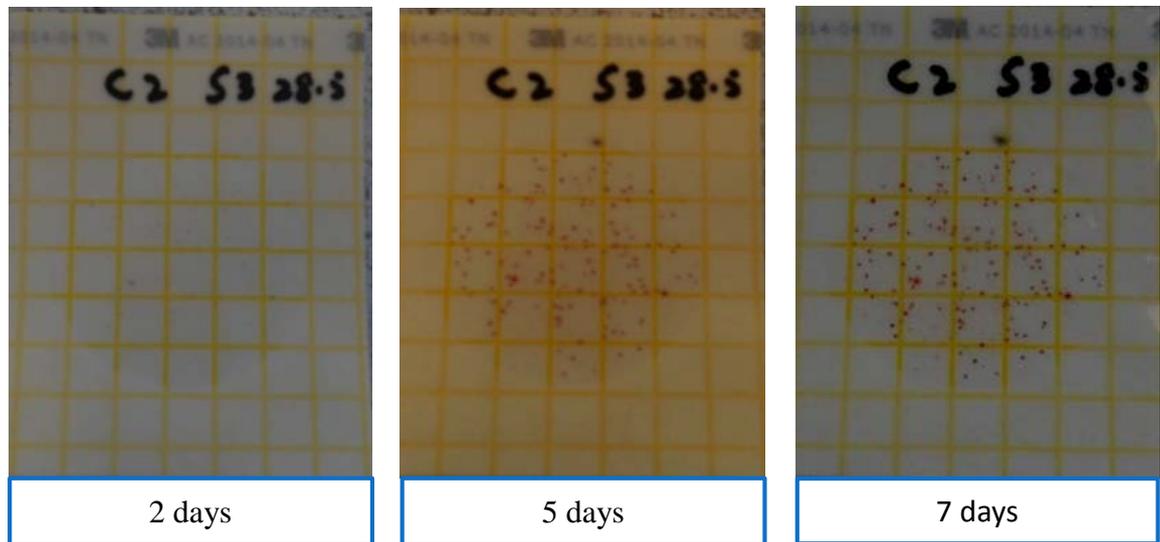


Figure 2.5: Effect of extended incubation time on the performance of Petrifilm™ AC Plate.

2.3.2.2.2. Effect of incubation temperature

Lower temperature (room temp and constant 22 °C) gave better results than at 37 °C for Petrifilm™ AC Plates (Fig. 2.6).

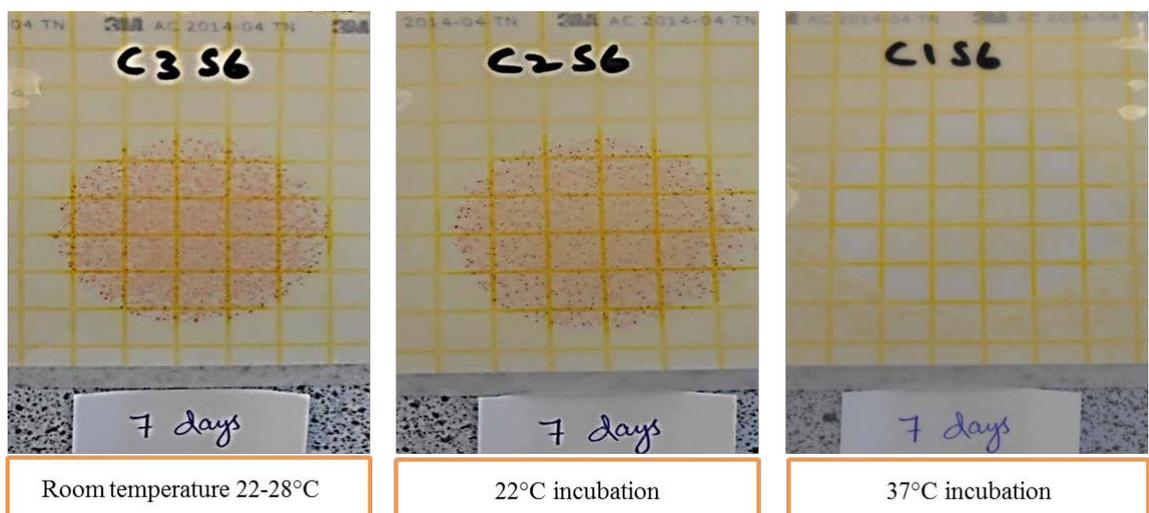


Figure 2.6: Effect of temperature on the performance of Petrifilm™ AC Plate.

2.3.2.2.3. Effect of biocide neutralisation

In this study, sodium thiosulphate neutralisation made no difference to the overall sensitivity of Petrifilm™ AC Plate (see Table 2.5).

2.3.3. Testing the Petrifilm™ AC Plate for its in-office application

The data for the Petrifilm™ AC Plate test performed at the dental premises is given in Table 2.7.

Table 2.7: CFU/mL obtained on Petrifilm™ AC Plate with test performed by dental staff, and a separate test performed in the laboratory by the experimenter alongside of the CFU/mL obtained on R2A plates.

Sample ID	Petrifilm™ AC Plate (CFU/mL)				R2A (CFU/mL)		
	Test performed by dental staff		Test performed in the laboratory by the experimenter		Mean (n = 3)	S.D	
	Mean (n = 3)	S.D	Mean (n = 3)	S.D			
DUWL 1	27	± 4	Not detected		1.3×10^3	±	1.1×10^2
DUWL 2	TNTC	N/A	TNTC	N/A	9.9×10^5	±	6.4×10^5
DUWL 3	22	± 4	1	± 1	7.0×10^2	±	7.6×10^1
DUWL 4	2	± 3	Not detected		Not detected		
DUWL 5	Not detected		Not detected		Not detected		
DUWL 6	Not detected		Not detected		Not detected		
DUWL 7	4	± 4	Not detected		2.8×10^2	±	9.7×10^1
DUWL 8	4	± 2	Not detected		7.3×10^1	±	5.7×10^0
DUWL 9	TNTC	N/A	TNTC	N/A	2.2×10^3	±	9.4×10^2
DUWL 10	4	± 0	12	± 2	8.4×10^2	±	7.1×10^0
DUWL 11	2	± 3	Not detected		1.3×10^2	±	3.6×10^1
DUWL 12	8	± 5	23	± 5	1.4×10^3	±	2.9×10^2
DUWL 13	1	± 1	Not detected		5.7×10^1	±	2.3×10^1
DUWL 14	Not detected		Not detected		6.7×10^1	±	2.5×10^1
DUWL 15	Not detected		Not detected		2.2×10^2	±	7.6×10^1
DUWL 16	3	± 2	Not detected		Not detected		
DUWL 17	5	± 1	Not detected		Not detected		
DUWL 18	2	± 2	Not detected		Not detected		
DUWL 19	3	± 3	Not detected		Not detected		
DUWL 20	6	± 5	Not detected		Not detected		
DUWL 21	4	± 1	Not detected		Not detected		
DUWL 22	14	± 6	TNTC	N/A	2.6×10^4	±	2.6×10^2
					S.D = Standard deviation		

When the results obtained with test performed by dental staff were compared with those obtained in the laboratory at room temperature, and the overall results compared with the R2A CFU/mL, for sensitivity and specificity (Fig. 2.7), only low sensitivity value was obtained compared to the original test performed by the experimenter. Specificity remained 100% as was achieved in the original test (Fig. 2.7).

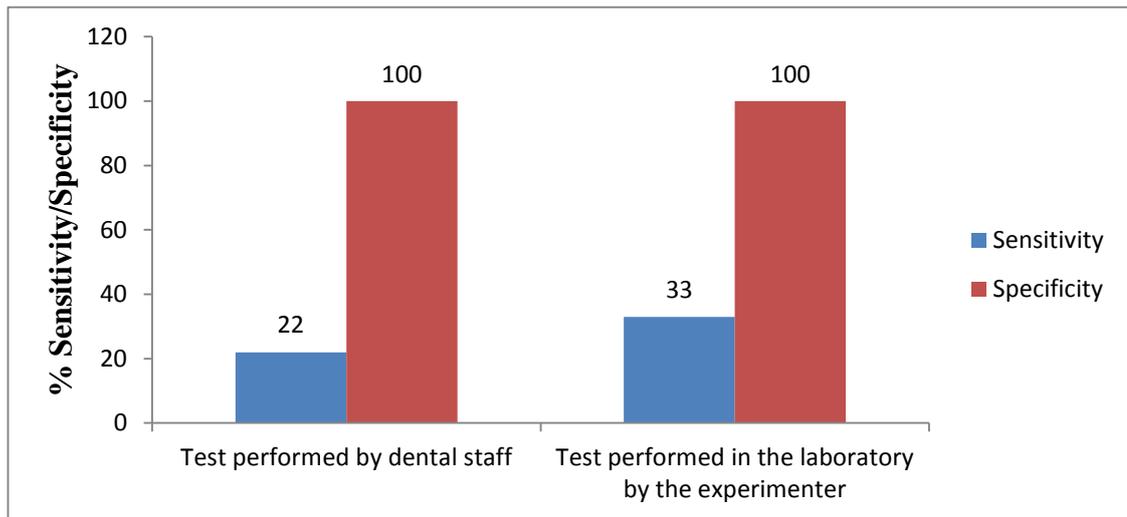


Figure 2.7: Sensitivity and specificity of Petrifilm™ AC Plate with test performed by dental staff, and a separate test performed in the laboratory by the experimenter.

2.3.4. Retesting the clinical DUWL water on R2A and Petrifilm™ AC Plate

2.3.4.1. Contamination level of DUWL water samples

The total number of samples for the re-test was 22. The bacterial concentration of the water samples cultured on R2A agar varied significantly ranging from not detected to 1.9×10^6 CFU/mL. Out of 22 samples tested 10 (45%) exceeded the DoH, UK recommended threshold of ≤ 200 CFU/mL (Table 2.8). Remaining 12 samples (55%) met the DoH standards (Table 2.8). After retesting it was noted that two samples (with ID DUWL 2 and DUWL 30 in the original study), which were in compliance with DoH, UK recommended threshold in original study now exceeded the DoH, UK recommended threshold (Table 2.8 red boxes).

Table 2.8: Contamination level of DUWL water samples after retesting.

Sample ID	R2A plating: with sodium thiosulphate (CFU/mL)		R2A plating: without sodium thiosulphate (CFU/mL)	
	Mean (n = 3)	S.D	Mean (n = 3)	S.D
DUWL 1	5.7×10^2	$\pm 1.1 \times 10^2$	1.3×10^3	$\pm 1.1 \times 10^2$
DUWL 2	2.0×10^6	$\pm 1.4 \times 10^6$	9.9×10^5	$\pm 6.4 \times 10^5$
DUWL 3	7.2×10^2	$\pm 4.0 \times 10^1$	7.0×10^2	$\pm 7.6 \times 10^1$
DUWL 4	Not detected		Not detected	
DUWL 5	Not detected		Not detected	
DUWL 6	Not detected		Not detected	
DUWL 7	3.8×10^2	$\pm 3.6 \times 10^1$	2.8×10^2	$\pm 9.7 \times 10^1$
DUWL 8	5.3×10^1	$\pm 2.5 \times 10^1$	7.3×10^1	$\pm 5.7 \times 10^0$
DUWL 9	3.4×10^3	$\pm 1.2 \times 10^3$	2.2×10^3	$\pm 9.4 \times 10^2$
DUWL 10	1.1×10^3	$\pm 1.2 \times 10^2$	8.4×10^2	$\pm 7.1 \times 10^0$
DUWL 11	1.4×10^3	$\pm 7.2 \times 10^1$	1.3×10^2	$\pm 3.6 \times 10^1$
DUWL 12	1.2×10^3	n = 2	1.4×10^3	$\pm 2.9 \times 10^2$
DUWL 13	9.3×10^1	$\pm 1.5 \times 10^1$	5.7×10^1	$\pm 2.3 \times 10^1$
DUWL 14	8.3×10^1	$\pm 2.3 \times 10^1$	6.7×10^1	$\pm 2.5 \times 10^1$
DUWL 15	2.2×10^2	$\pm 9.3 \times 10^1$	2.2×10^2	$\pm 7.6 \times 10^1$
DUWL 16	Not detected		Not detected	
DUWL 17	Not detected		Not detected	
DUWL 18	Not detected		Not detected	
DUWL 19	Not detected		Not detected	
DUWL 20	Not detected		Not detected	
DUWL 21	Not detected		Not detected	
DUWL 22	1.7×10^4	$\pm 4.4 \times 10^3$	$2.6. \times 10^4$	$\pm 2.6 \times 10^2$
	S.D = Standard deviation			

2.3.4.2. Sensitivity and specificity measurement of Petrifilm™ AC Plate

Sensitivity and specificity values obtained for Petrifilm™ AC Plate after retesting are given in table 2.9. Compared to original study sensitivity decreased in the retest study (Fig. 2.8).

Table 2.9: Sensitivity and specificity values after retesting of Petrifilm™ AC Plate.

Test performed in laboratory	With sodium thiosulphate		Without sodium thiosulphate	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Room temp (3-8 °C) for 7 days	30	100	33	100
22 °C for 7 days	40	100	44	100
30 °C for 7 days	40	100	33	100
37 °C for 7 days	20	100	22	100

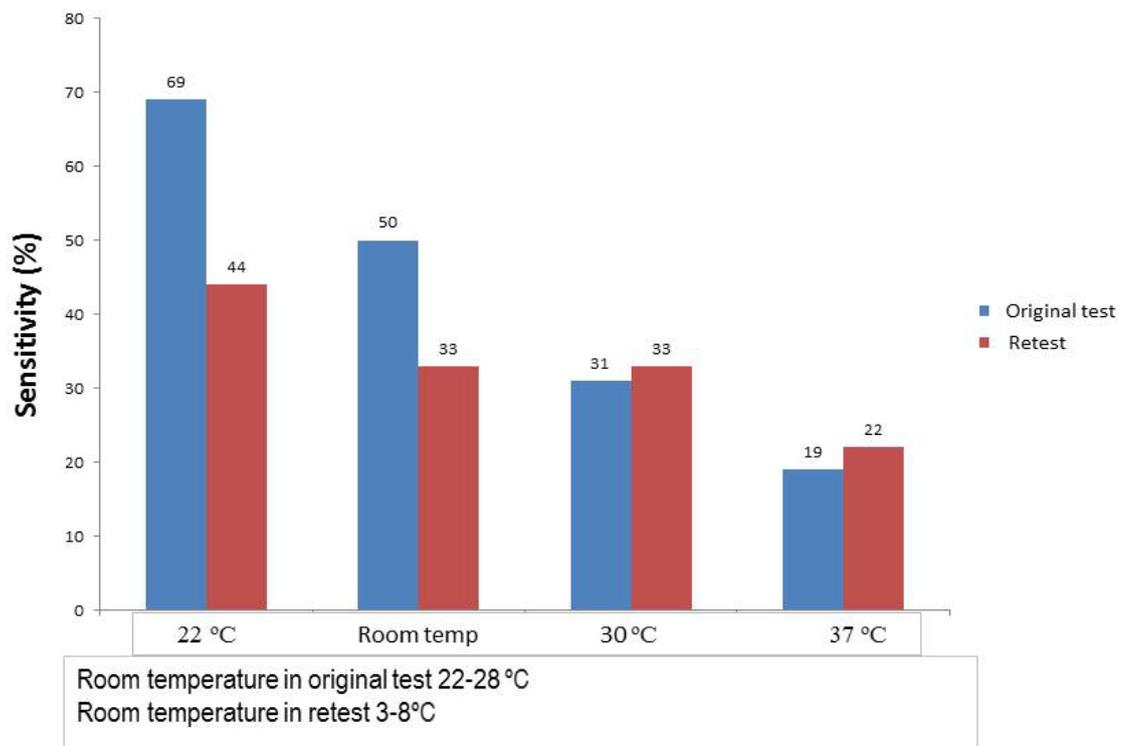


Figure 2.8: Sensitivity of Petrifilm™ AC Plate obtained in original testing and retesting.

2.4. Summary

This chapter demonstrates the importance of regular monitoring of DUWL water as even clean DUWLs can become contaminated in between periods of testing (four months, in this case). Use of an in-office test as a preliminary aid to monitoring planktonic bacterial levels in DUWL water as a cheap and practical option is appealing. However, the Dip slide™ has the cut-off value of 1000 CFU/mL suggesting its limited use in dentistry. The Petrifilm™ AC Plate test however, does have the threshold of ≤ 200 CFU/mL and demonstrated a potential for use as a rapid in-office test in dentistry. When Petrifilm™ AC Plate was tested for the first time (summer season), it demonstrated good sensitivity. When tested following its introduction to dental staff (winter season) its performance compared adequately with the results obtained in the laboratory by the experimenter. However, its overall sensitivity dropped when compared with R2A results. This could be due to many reasons but the seasonal variation in the type of bacteria in the water sample appears to be a factor affecting sensitivity. Factors other than seasonal change beyond the experimental approach may also be playing a role in the sensitivity of Petrifilm™ AC Plate including hardness/softness of water, level of contamination of water at source. Better understanding of the way commercial rapid in-office tests work, their design and factors beyond experimental control will eventually lead to manufacture of new tests exclusively for dental needs which can give consistent results close to those of the conventional microbiological culturing methods.

2.5. Discussion

It has repeatedly been demonstrated that DUWLs around the globe readily become contaminated (Walker *et al.*, 2000; Tuttlebee *et al.*, 2002; Pankhurst *et al.*, 2005; Singh and Coogan, 2005; Szymanska, 2007; Artini *et al.*, 2008; Chate, 2010; Pearce *et al.*, 2013; Arvand and Hack, 2013; Leoni *et al.*, 2015). The results from local audits had indicated that, although the practices were actively making attempts to maintain clean supply of water (to 200 CFU/mL) from DUWL by the addition of biocides recommended by the manufacturer of their dental-unit, a significant level of contamination was still detectable from various surgeries (Pearce *et al.*, 2013). This suggested that some dental practices fail to obtain clean water to the level within 200 CFU/mL from their DUWLs.

Alongside monitoring of water quality using the conventional laboratory microbiological culture based test on R2A agar plates, the aim of this chapter was to validate a rapid in-office testing method for estimating the quality of water leaving the DUWL for eventual use by dental staff. The desired features for such a test were, for it to be cheap, convenient and easy to use, and provide adequate sensitivity to satisfy dental threshold for water quality.

Service evaluation (original and retest studies)

The service evaluation in this chapter was performed using conventional R2A plating and the results demonstrated that despite all 31 DUWLs being treated with biocides, 16 (52%) exceeded the DoH, UK recommended threshold of ≤ 200 CFU/mL and the remaining 15 samples (48%) met the DoH standard for aerobic mesophilic bacterial contamination in the original study. High bacterial counts obtained from some DUWL water samples agree with published reports (Pankhurst *et al.*, 2005; Szymanska, 2007; Chate, 2010; Pearce *et al.*, 2013).

These results were reported to the clinical staff, and they revised the disinfection protocols and subsequent retesting of DUWL water (as part of the original service evaluation of this study) after four months was offered to all surgeries that failed the original test. As a control, randomly selected surgeries that conformed to the DoH standard mentioned above were also selected. The retest data revealed that from N = 22 retested samples 12 (55%) samples met the recommended threshold. Interestingly, during retesting 2 samples which were in compliance with DoH, UK recommended threshold in the original test failed to meet the standards when retested. This highlights that even clean DUWLs can become contaminated in between periods of testing (four months, in this case). High level of contamination in DUWLs despite receiving continuous chemical treatment supports the importance of regular monitoring of DUWL water. Thus, should one be asking, how often DUWL should be tested for contamination and would more frequent testing be indicated for those units that failed? In addition, there is a need to establish and understand why some disinfection protocols work and others fail. There is also a need to establish, if more rigorous cleansing methods need to be implemented and how often by working closely with the manufacturers of DUWLs. Previous local audits (unpublished data) have shown that about half of DUWL, treated with biocides but unmonitored, exceed the guidelines for contamination. Frequent testing of DUWL output water by conventional means (R2A agar plating), is considered costly, time consuming and largely impractical. This has led to the use of the current culture based; in-office commercial tests even though literature suggests that they are unsuitable for this purpose.

Choice of in-office test and its evaluation

This study validates an existing microbiology culture-based commercial test kit known as the Petrifilm™ AC Plate. This was performed as per manufacturers recommended temperature as well as at variable temperatures and extended incubation times. The

results of this test were compared with those of the R2A test performed alongside of testing DUWL contamination to an acceptable sensitivity.

In addition, the Petrifilm™ AC Plate test was compared to another test routinely used in dental practices in the UK namely the Dip slide™ as per manufacturers recommended temperature but at extended incubation times only. The results of these tests were compared with those of the R2A test as mentioned above for Petrifilm™ AC Plate test. The advantages for using the Petrifilm™ AC Plate test, if the results are comparable is, its current status as FDA approved test, immediate availability around the globe, low cost (cheaper than the Dip slide™ test), ease of use, optimal counting range between 30-300 CFU/mL and the option of being able to use a diluted sample (instead of the neat sample). In addition, use of sodium thiosulphate to neutralize the effect of residual disinfectants especially chlorine (Anon, 2012) was investigated where applicable.

Little difference was observed on the effect of sodium thiosulphate, when estimating bacterial numbers in 31 water samples using conventional means of testing with R2A. This could be because not all the surgeries used chlorine based disinfectants in their DUWL cleansing protocols. Therefore, information concerning the biocides used at the dental practices was requested. It became apparent that only 9 out of 31 water samples had come from the DUWLs being cleansed by a chlorine containing biocide (see Tables 2.2 and 2.3). On closer examination of the 9 out of 31 water samples of interest, 5 water samples were already below the recommended threshold for clean treatment water (< 100 and < 200). This means that any major differences would have been unnoticed. However, 3 out of the 4 remaining water samples containing chlorine based biocide, demonstrated higher CFU's compared to those without the use of thiosulphate in the same rapid test. The remaining 1 out of the 4 samples demonstrated

gross contamination making it difficult to establish any differences in the sensitivity whether thiosulphate was used or not.

Two different laboratories had previously tested the Petrifilm™ AC Plates in which the investigators concluded the test was not sensitive enough for dental needs (Morris *et al.*, 2010; Momeni *et al.*, 2012). However, Morris *et al.*, (2010) used incubation periods that were outside of those recommended by the manufacturers (7 days) and found that they were able to improve the sensitivity of the Petrifilm™ AC Plates. Momeni *et al.*, (2012) followed the manufacturer's protocol whereby shorter incubation time at higher temperature was used but with unsatisfactory outcome for sensitivity. In relation to these reports, the manufacturers of Petrifilm™ AC Plates (3M Food Safety) were approached and they maintained that the test was suitable for the UK threshold for dental needs, but agreed they had not tested it for its suitability to use with DUWL discharged water. The reason they suggested higher incubation time in their recommended guidelines is because the test was developed for the food industry, and food borne bacteria require higher temperature and shorter incubation time and rich media for optimal growth.

When Petrifilm™ AC plate was tested originally it was more reliable over the Dip slide™ test adopted by dental surgeries in the UK, because it demonstrated good sensitivity but it also gave false negative results. When the water samples were highly contaminated, the Petrifilm™ AC test became unresponsive (false negative), unlike the Dip slide™ test which continues to give a reading. Repeat testing of the Petrifilm™ AC test with serially diluted water demonstrated colony growth. We also found that comparable results could be obtained by incubation at room temperature and/or 22 °C constant temperature for 7 days. This was a huge variation from the manufacture's recommended conditions confirming previous findings that DUWL waterborne bacteria from low nutrient and chemical stress conditions grow much more slowly than those,

for example, from food sources as tested by Momeni *et al.*, (2010). Although in this study sensitivity values for Petrifilm™ AC Plates did not agree with those of Morris *et al.*, (2010) this may be because they measured the validity at the cut-off value of ≤ 500 CFU/mL (USA threshold for dentistry), while this study used cut-off value of ≤ 200 CFU/mL (DoH, UK threshold).

To observe the effect of sodium thiosulphate the water samples were inoculated on Petrifilm™ AC Plates, which unlike the Dip slide™ test, are amenable to the addition of thiosulphate. Petrifilm™ AC Plates, with and without addition of sodium thiosulphate were tested with no overall difference in sensitivity. Both methods gave the same sensitivity (62%) after incubation for 7 days and the plausible reasons are discussed above. Overall, the results of the original test indicated that compared to the Dip slide™, Petrifilm™ AC Plates offered higher sensitivity and specificity, while both in-office test kits accurately identified DUWLs with gross levels of contamination without/with dilution respectively. Both test kits gave false negatives whilst the Petrifilm™ AC Plates were less likely to underestimate the microbial levels compared to Dip slide™ with the diluted water samples. Both in-office test kits were unable to equal the results that R2A agar plating provided.

When Petrifilm™ AC Plate was tested following its introduction to dental staff its performance compared adequately with the results obtained in the laboratory by the experimenter. However, its overall sensitivity dropped when compared with R2A results. Plausible reasons could be different factors affecting the sensitivity of Petrifilm™ AC Plate including level of contamination of samples, type of organism from source of water, bacterial inoculation from low nutrient (water) to low nutrient R2A medium versus high nutrient medium on Petrifilm™ AC Plate, hardness/softness of water, the seasonal variability of the waterborne flora. This could be one reason why

each investigators laboratory has not been able to correlate results from these in-office test kits previously.

This study used R2A plating as the benchmark standard because R2A agar has been developed to study bacteria which normally inhabit potable water (Reasoner and Geldreich, 1979; Reasoner and Geldreich, 1985). Bacteria living in water tend to be slow-growing species and would be suppressed by faster-growing species on a richer culture medium. R2A agar is a low nutrient medium which, in combination with a lower incubation temperature and longer incubation time, stimulates the growth of stressed and chlorine-tolerant bacteria (Kelly *et al.*, 1983). In addition due to its less nutritious quality it has been shown to reduce the effects of substrate shock which can occur when microorganisms are taken from a low nutrient environment and cultured in a high nutrient laboratory medium (Mackerness *et al.*, 1991).

This chapter demonstrated the importance of regular monitoring of DUWL water as even clean DUWLs can become contaminated in between periods of testing. The search for a new test is warranted which can give consistent results to match those of the conventional microbiological culturing method and/or an equivalent biochemical test. The next chapter will evaluate the aerosols and splatter contamination in dental clinics.

Chapter 3. Evaluating aerosols and splatter contamination in dentistry

3.1. Introduction

Previous chapters have stressed the importance of clean DUWL output water for patient treatment and to protect the dental staff as they are at direct risk of being infected from contaminated aerosolised water. Aerosolised water during clinical procedures is likely to splatter and any contaminating organisms in it will disperse with air currents settling droplets anywhere within the clinical environment of the surgery. Contamination could also arise from for example, staff opening cupboards with splatter water covered hands and thereby transfer microbes to places away from the dental chair. DoH guidance on maintenance of acceptable levels of contamination in the clinical environment of the dental surgery is not clear. To my knowledge, there are no defined methods for evaluating how clean a dental environment should be.

Since *P. aeruginosa*, *K. pneumoniae* and *Legionella* spp. can be isolated from DUWL systems, they are the suspect species of planktonic bacteria posing a health hazard (Williams *et al.*, 1993; Pankhurst *et al.*, 1998; Porteous *et al.*, 2004; D'Ovidio *et al.*, 2011; Ricci *et al.*, 2012; Leoni *et al.*, 2015; Ditommaso *et al.*, 2016). This investigation evaluated the spread of planktonic opportunistic human pathogens in the clinical DUWL aerosolised water, by taking swabs from various in-surgery surfaces close to and away from the dental chair for culture and for their presence in the clinical DUWL output water. In addition, common biocides used in dentistry for cleansing DUWLs were tested for their effectiveness in controlling bacterial numbers on pure cultures.

3.2. Materials and methods

Sources of devices and reagents used in this chapter are listed in Table 3.1.

Table 3.1: Sources of devices and reagents.

Material/Device	Source	Material/Device	Source
<i>Pseudomonas</i> isolation agar	Sigma	Glycerol	Sigma
HiCrome™ <i>Klebsiella</i> selective agar	Sigma	<i>Klebsiella</i> selective supplement	Sigma
Falcon tubes™ (50 mL)	Fisher Scientific	Falcon™ tubes (15 mL)	Fisher Scientific
Eppendorf tubes™ (1.5 mL)	Fisher Scientific	Disposable wire loops	Fisher Scientific
Disposable spreaders	Fisher Scientific	Petri dishes	Fisher Scientific
Swabs (polypropylene tubes plastic sticks)	Fisher Scientific	Lysozyme	Sigma
Proteinase K	Qiagen	AL buffer	Qiagen
Phenol/Chloroform/Isoamyl Alcohol 25:24:1 saturated with 10 mM Tris, pH 8.0, 1 mM EDTA	Sigma	Glycogen	Sigma
Ethanol	Fisher Scientific	AE buffer	Qiagen
Nanodrop 1000 spectrophotometer	Thermo Scientific	PCR- buffer with MgCl ₂	Fisher Scientific
Mixed deoxynucleotide triphosphates	Fisher Scientific	D88- forward primer	Life technologies, UK
E94- reverse primer	Life technologies, UK	RNase free water	Fisher Scientific
Taq DNA polymerase	Fisher Scientific	Veriti thermocycler	Applied Biosystems
1 kb ladder	New England Biolabs	Gel loading dye	New England Biolabs
Ethidium bromide	Fisher Scientific	Gel red	NBS Biologicals
Gene snap software	Syngene, UK	BigDye® Terminator v3.1 cycle sequencing kit	Life Technologies

Glycine-Vancomycin-Polymyxin-Cycloheximide (GVPC) medium plates	Fisher Scientific	Bench top centrifuge (3-16PK)	Sigma
Ringer's solution tablets	Lab M	Scalpel blade	Fisher Scientific
Glutaraldehyde solution	Agar Scientific	Phosphate-buffered saline (PBS)	In-house
2% aqueous osmium tetroxide solution	Agar Scientific	Ethanol	Sigma
Propylene oxide	Sigma	Araldite Resin (CY212)	Agar Scientific
Polypropylene Beem® capsules	Agar Scientific	Ultracut E microtome	Leica, UK
Nickel grids	Agar Scientific	Uranyl acetate	Sigma
Lead citrate	Sigma	Freeze drier (Scanvac Coolsafe 110-4)	Fisher Scientific
Luria-Bertani (LB) broth	Fisher Scientific	LB agar	Fisher Scientific
Topo™ TA Cloning kit	Invitrogen	Kanamycin	Sigma
X-gal	Sigma	GeneJET™ Plasmid miniprep kit	Fisher Scientific
Freeze drying tubes	Fisher Scientific	Universal bottles	Fisher Scientific
Spreaders	Fisher Scientific	Pasteur pipettes	Fisher Scientific
Muslin cloth	Fisher Scientific	Nutrient broth	Fisher Scientific
Nutrient agar	Fisher Scientific	Glucose	Sigma

3.2.1. Source of bacterial cultures

P. aeruginosa (NCTC 10662) was maintained in a teaching microbiology laboratory C/O University of Central Lancashire (UCLan), UK; *K. pneumoniae*, a non-commercial strain isolated at UCLan (designated code: LP1 499) was a kind gift from Professor Glyn Morton; an avirulent *L. pneumophila* non-Sgp1 strain ST707 (from Dr Mandy Dillon) who originally obtained it from Dr Tim G. Harrison (Respiratory & Systemic Infection Laboratory, Health Protection Agency, Colindale, UK) for a related research

project at UCLan; *E. coli* strain DH5- α was maintained in a teaching molecular laboratory C/O University of Central Lancashire (UCLan).

3.2.2. Isolation and identification of opportunistic pathogens from swab samples and clinical DUWL water

3.2.2.1. Media preparation

All media were prepared according to the manufacturers' protocol (see appendix A11). R2A agar was prepared as described in chapter 2 section 2.2.3. Media specific to this chapter are described. *Pseudomonas* isolation agar powder (45.03 g/L) was suspended in distilled water containing 2% glycerol and making sure the powder completely mixed in the water, the mixture was autoclave sterilised at 121 °C at 15 lb/inch² for 15 min. After cooling to 45-50 °C, about 20 mL media was poured into pre-labelled Petri dishes and allowed to set. As per manufacturer's instructions HiCrome™ *Klebsiella* selective agar base powder (40.8 g/L) was suspended in distilled water and heated to boiling point in a microwave oven but not autoclaved. Following cooling to 45-50 °C, rehydrated contents of 2 vials of *Klebsiella* selective supplement/litre were added aseptically and thoroughly mixed prior to pouring in, pre-labelled, fresh plates.

Glycine-Vancomycin-Polymyxin-Cycloheximide (GVPC) medium plates were from a commercial source.

3.2.2.2. Specimen collection

3.2.2.2.1. Collection of swab samples

At the time of the original water sample collection, polyester fibre-tipped sterile swabs were sampled from two main areas from N=31 participating dental surgeries (see chapter 2). Hence their delivery was exactly the same as that described for water samples in chapter 2. The swabs specifically were taken from the dental chair parts

(denoted near), and from other clinical working surfaces (bench units, drawers, denoted far) that are likely to become coated with aerosolised water during clinical procedures.

3.2.2.2.2. Collection of DUWL water samples

The water samples (N=31) used for this investigation were the same as those described in chapter 2.

3.2.2.3. Inoculation of samples on selective bacterial media

3.2.2.3.1. Swabs

Swabs were either directly streaked on duplicate plates for each selective medium, or each swab head was suspended in 1 mL of sterilised distilled water to release the attached microbes. Following vigorous shaking it was inoculated (100 µL) in duplicate on *Pseudomonas* isolation agar for *Pseudomonas* spp., HiCrome™ *Klebsiella* selective medium for *Klebsiella* spp., and GVPC plates for *Legionella* spp.

3.2.2.3.2. DUWL water

Neat water (100 µL) of each DUWL sample was inoculated in duplicate on above mentioned selective media plates for *Pseudomonas* spp., *Klebsiella* spp., and *Legionella* spp.

3.2.2.4. Incubation of all plates

Pseudomonas isolation agar and HiCrome™ *Klebsiella* selective medium plates were incubated at 37 °C for 2 days and the GVPC plates were incubated at 30 °C in a humid environment for up to 7 days.

3.2.2.5. Maintenance of newly isolated bacterial colonies from water

Discrete bacterial glassy colonies growing on GVPC plates (suggestive of possible *Legionella* spp.), designated DUWL 9, 10 and 21 were picked and sub-cultured onto fresh GVPC medium and incubated at 30 °C in a humid environment for up to 7 days to

obtain pure cultures. Following incubation, colonies were tested for their Gram reaction and molecular identity. Subsequently, they were inoculated onto R2A medium and Nutrient agar at temperatures between 15 and 37 °C, for maintenance and to assess the incidence of pigmentation.

3.2.2.6. Identification of isolated microbes from water samples

3.2.2.6.1. Gram staining

Following the published method of Gerhardt *et al.*, (1994), Gram staining was performed on all the bacterial cultures smeared on clean glass slides. The procedure involved taking fresh bacterial culture (24 h) from the desired bacterial colony with a sterile disposable loop and mixing it in a drop of distilled water on a glass microscope slide. A thin smear was prepared by spreading the mixture evenly on the same slide and allowing it to air dry. Bacterial smear was heat fixed and stained with crystal violet solution for 1 min, and flooded with Gram's iodine solution for 1 min with washes in between. Ethanol (95%) was applied to the slide dropwise, until no more colour was released. Following further washings in distilled water, the slide was counter-stained with safranin for 30 seconds and washed prior to air drying and examination under oil immersion lens of a light microscope.

3.2.2.6.2. Molecular identification of bacterial colonies isolated from clinical DUWL water

i) DNA extraction

The colonies of interest were picked, immersed and incubated overnight at 37 °C in sterile lysis buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA, 1.2% Triton X-100) containing 20 mg/mL lysozyme. Following manufacturer's instructions (Qiagen DNA easy blood & tissue kit 69504) the bacterial colony mixes were further incubated overnight at 56 °C in proteinase K in AL buffer. Thereafter, genomic DNA was

separated manually in the phenol/chloroform/isoamyl alcohol gradient. Samples were centrifuged at room temperature for 5 min at 17,005×g. Upper aqueous phase was transferred to new centrifuge tubes and the rest discarded. DNA was precipitated by adding 1 µL of glycogen (20 µg/µL), 7.5 M ammonium acetate (pH 5.2) (half the volume of sample) and ice cold absolute ethanol (two and a half, the volume of sample) and incubation at -80 °C for 2 h. Samples were centrifuged at 4 °C for 30 min, and pellets washed (x 3) with 150 µL of 70% ice cold ethanol with centrifugation at 4 °C for 2 min at 17,005 ×g in between washes. Ethanol was removed and samples were dried for 10 min at room temperature. The resulting pellets were re-suspended in 50 µL of AE buffer by pipetting up and down. DNA was quantified using the Nanodrop 1000 spectrophotometer. All genomic DNA/colony were fully labelled prior to storage at -80 °C until needed.

ii) Chemically prepared competent cells

A discrete colony of *E. coli* strain DH5- α bacteria maintained previously on Luria Bertani (LB) agar plate was inoculated into a small volume (5 mL) of LB broth for an initial overnight pre-culture at 37 °C in a shaker set at 200 rpm. Next day, 1 mL of the overnight culture was inoculated in sterile LB (100 mL) and incubated at 37 °C in a shaker set at 200 rpm and monitored for growth until OD₆₀₀ approx. 0.5 was obtained. The cells were incubated on ice for 10-15 min and the culture was transferred in equal volumes into two sterile 50 mL conical centrifuge tubes (Falcon™ tubes) and centrifuged at 2504 ×g for 5 min at 4 °C. The supernatant was discarded and the remaining cells were re-suspended in 30 mL sterilised TBF1 (30 mM KOAc, 50 mM MnCl₂, 100 mM KCl, 10 mM CaCl₂ in 15% glycerol, pH 7.3) buffer/50 mL original culture. Following further incubation on ice for 15min, the cells were pelleted by centrifugation at the same conditions as before, but re-suspended in sterilised TBF2 buffer (10 mM MOPS, 75 mM CaCl₂, 10 mM KCl in 15% glycerol, pH 7.3). Aliquots (100 µL

volumes) of the chemically competent cells, on dry ice, were prepared in sterile Eppendorf™ tubes and stored at -80 °C until needed.

iii) Amplification

PCR

PCR was performed by targeting 16S rRNA gene and using universal bacterial gene primers designated D88 and E94 (Paster *et al.*, 2001). D88- forward (5'GAGAGTTTGATYMTGGCTCAG3') and E94-reverse (3' GAAGGAGGTGWTCCARCCGCA5'). Thermocycler was used to amplify the DNA for rRNA using the Taq PCR Master Mix including D88F and E94R primers (Paster *et al.*, 2001). The amplification conditions in the thermocycler were: an initial denaturation step at 95 °C for 8 min, followed by 35 cycles including denaturation (94 °C for 1 min), annealing (62 °C for 30 seconds), and elongation (72 °C for 1 min 45 seconds) followed by a final extension at 72 °C for 10 min as described elsewhere (Paster *et al.*, 2001). The positive control consisted of all reagents and DNA isolated from *P. aeruginosa* taken from a pure culture maintained in the laboratory. The negative control included all other reagents except for the test genomic DNA.

iv) Electrophoresis

The PCR product (5 µL of PCR product in 2 µL of loading dye) was examined for expected bands around 1500 base pairs (bp) using 1% agarose gel electrophoresis performed at 100 V in 1xTris acetate EDTA (1 x TAE) buffer (Final working dilution: 0.04 M Tris acetate, 0.001 M EDTA) made from laboratory stock of x50 TAE buffer (1L: 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA). The gel was stained with ethidium bromide and/or with gel red and the bands were visualised using a GENE GENIUS Bio imaging system and Gene snap software. At this stage two different sequencing methods were tested for the final identification of bacterial species.

The first method employed TopoTM TA cloning kit system and the second used direct sequencing from cleaned PCR product. Both methods are described in detail below.

v) *TopoTM TA Cloning*

The PCR product of interest was purified using 3M sodium acetate (pH 5.2)/100 mM EDTA/absolute ethanol). The cleaned PCR product (20 ng) was ligated into the PCR vector as per manufacturer's instructions. Transformation was performed using the chemically treated *E. coli* DH5- α strain to take up the cloned vector and the transformants were plated onto LB agar plates containing (50 mg/mL) kanamycin and (40 mg/mL) X-gal.

vi) *Colony screening*

A total of 10% of the discrete white colonies were picked randomly from the plates and screened by PCR using the M13 primers and only those colonies containing the expected kb size (~1600 bp) were chosen for plasmid isolation.

vii) *Plasmid isolation*

A small volume of the overnight culture (10 mL) in LB broth containing (50 mg/mL) kanamycin was set up for the appropriate bacterial colonies at 37 °C in a shaker set at 200 rpm. The plasmid was isolated using the GeneJETTM Plasmid miniprep kit according to manufacturer's instructions and the purified plasmid DNA was stored at -80 °C until further use.

viii) *Sequencing*

From plasmid DNA

Using the M13R primer, plasmid DNA was sequenced with the BigDyeTM Terminator v3.1 cycle sequencing kit according to the manufacturer's instructions. The reaction mixture (10 μ L) final volume, consisted of 3.6 μ L of reaction buffer, 0.8 μ L of reaction mix, 1.6 μ L of M13R primer (1:1000), variable amount of plasmid DNA template (50

ng/reaction), variable amount of RNase free (Molecular biology grade) water. The sequencing parameters were: an initial denaturation step at 96 °C for 1 min and 25 cycles involving (96 °C for 10 seconds), annealing (50 °C for 5 seconds), elongation (60 °C for 4 min) and final extension (60 °C for 4 min). The sequenced product was cleaned using 7.5 M sodium acetate (pH 5.2)/absolute ethanol.

From PCR product

a) Cleaning PCR product

DNA of interest was cleaned by adding 20 µL PCR product, 1 µL of glycogen (20 µg/µL), 1 µL of 3M sodium acetate (pH 5.2), 1 µL of 100 mM EDTA (pH 8.0) and 30 µL of absolute ice cold ethanol. The content was mixed by gently tapping the tube. The DNA was precipitated by incubating at -80 °C for 2 h. The tubes were centrifuged at 17,005 ×g for 30 mins at 4 °C, and pellet washed with ice cold 70% ethanol (x 3) with 2 min spins at 17,005 ×g. The pellet was dried at room temperature for 10 min and re-suspended in 20 µL of RNase free water. Concentration of the DNA was obtained using the Nanodrop 1000 spectrophotometer. The cleaned PCR product was stored at -80 °C until sufficient number of samples was generated for the sequencing reaction.

b) Sequencing

DNA sequencing was performed on cleaned PCR product by using the BigDye® Terminator v3.1 cycle sequencing kit as for plasmid DNA sequencing described above except E94-reverse primer (Paster *et al.*, 2001) was used.

All sequenced specimens were sent to the Institute for Translation, Innovation, Methodology and Engagement, Central Biotechnology services, Cardiff University, for nucleotide analysis. On receiving the nucleotide data, sequences were submitted to the European Nucleotide archive (ebi.ac.uk) and only those bacteria with 98-100% alignable match with > 200 bases were accepted.

3.2.2.7. Freeze drying of a selected bacterial culture

An isolated bacterial culture (suspected opportunistic human pathogen) was selected for longer term storage using the following methodology.

3.2.2.7.1. Media preparation

Nutrient agar was prepared as per standard manufacturers' protocol (see appendix A11). Nutrient broth (see appendix A11) was supplemented with glucose (3.75 g / 50 mL). Both media were autoclave sterilised (as described in section 3.2.2.1) and either poured into pre-labelled Petri dishes or 10 mL aliquots were prepared in sterilised universal bottles.

3.2.2.7.2. Preparation of bacterial lawn

Bacterial colonies were picked and individually mixed in 500 µL of sterilised distilled water. After vigorous shaking 100 µL of each suspension was spread onto separate Nutrient agar plates until dry. Plates were incubated at 37 °C for 24 h.

3.2.2.7.3. Preparation of fresh bacterial suspension

Under class II safety cabinet conditions, 5 mL of sterilised Nutrient broth containing glucose was dispensed over the bacterial culture in their individual plates to suspend the cells. The suspension was collected into sterilised universal bottles and mixed vigorously.

3.2.2.7.4. Inoculation of bacterial culture on filter paper identity labels

Filter paper identity labels were previously autoclave sterilised in a freeze drying tube. Drop (50 µL) of freshly prepared bacterial suspension from each plate was placed at one end of the tube containing the sterile filter paper carrying bacterial identity. Once the bacterial drop was completely soaked into the tip of the filter identity paper without

contaminating the labelled end, the tube was covered with sterilised “muslin cloth hats” prepared in the laboratory by the experimenter beforehand.

3.2.2.7.5. Partial freeze drying

A rack containing the specimen tubes was placed in the freeze drier and allowed to dry for 4 h. Following freeze drying, the tubes were transferred to the class II safety cabinet where the muslin cloth hats were replaced with sterilised cotton wool plugs. The plug was pushed close to but not in contact with the specimen using a sterilised Pasteur pipette nozzle.

3.2.2.7.6. Constriction of tubes

The specimen containing tubes were heated with a blow torch approximately midway of tube and the cotton wool plug, until the glass softened sufficiently to be pulled with tweezers to form a thin constriction in the tube.

3.2.2.7.7. Complete freeze drying

Following cooling of the tubes they were mounted on a specially designed arm suitable for freeze drying. Making sure the system was completely sealed, the arm was connected to the freeze drier and specimens were allowed to freeze dry overnight. Next day, the specimen tubes were collected by sealing them with the blow torch whilst still under vacuum. All specimen tubes are now stored under the bacterial culture archive (see results section).

3.2.2.8. Quality control test for selective media

3.2.2.8.1. Positive control cultures

Pure cultures of *P. aeruginosa* (NCTC 10662), *K. pneumoniae* (LP1 499) and an avirulent *L. pneumophila* non-Sgp1 strain ST707 were inoculated on their respective

selective media to observe the ability of these media to support their specific growth. The source of these pure cultures is given in section 3.2.1.

3.2.2.9. Effect of desiccation on bacteria (Laboratory strains)

Pure cultures of *P. aeruginosa*, *K. pneumoniae* and *L. pneumophila* non-Sgpl were suspended in sterilised distilled water separately and thoroughly mixed in the class II safety cabinet. Each suspension was inoculated on to fresh Petri dishes devoid of growth media and left to dry in the class II safety cabinet. Once the suspension had dried completely, the Petri dishes (fully covered) were left at room temperature for further 24 and 48 h. The “desiccated bacteria” were collected from each plate with moistened (sterile distilled water) swabs and streaked onto R2A plates to assess the re-growth of *P. aeruginosa* and *K. pneumoniae* and on GVPC medium for *L. pneumophila*.

3.2.3. The effect of DUWL biocides on bacteria isolated from DUWL water

Colonies isolated from DUWLs 9 and 10 together with *P. aeruginosa* were tested for sensitivity to standard DUWL cleansing biocides Sterilox™, ICX™, Alpron™ and Oxygenal 6™ at the recommended concentrations using the method described previously by Miles and Misra (1938), see below for details.

3.2.3.1. Bacterial liquid cultures

The antimicrobial testing was undertaken using 18 h culture in Nutrient broth at 30 °C in a shaker set at 200 rpm.

3.2.3.2. Dilution profiles/regimes

The log phase bacterial cultures were centrifuged using a bench top centrifuge at 2504 ×g for 20 min at 4 °C. The resulting pellets were washed and re-suspended three times in 10 mL of sterile Ringer’s solution made from ¼ strength Ringer’s solution tablets and the final suspension was held on ice until needed.

3.2.3.3. Assessment of resistance to DUWL biocides

DUWL biocides were used at the manufacturers' recommended concentrations. The dilution in this study was prepared within ¼ strength Ringer's solution containing 100 µL of each bacterial suspension (final concentration of bacteria at 1×10^8 CFU/mL) for laboratory use. The controls consisted of 100 µL of bacterial suspension added to Ringer's solution (900 µL). After approximately 12 h contact time with the biocide at room temperature, each suspension was serially diluted and inoculated on R2A agar plates using the Miles and Misra (1938) method. The plates were incubated at 30 °C for up to seven days and examined after 24 h, two days and seven days using a colony counter.

3.2.4. Morphological analysis of bacteria from clinical water identified by molecular techniques

3.2.4.1. Transmission electron microscopy (TEM) of isolated bacterial cultures

To observe the morphology of the isolated bacteria from clinical DUWL water samples pure colonies of each bacterium were processed for TEM.

3.2.4.1.1. Specimen collection and preparation

Selected areas of agar were cut out from the plate with large individual bacterial colonies using a sterile scalpel blade.

3.2.4.1.2. Fixation

For all bacterial cultures (colonies on agar slabs) were taken out of their respective growth media plates and directly immersed into 2.5% glutaraldehyde fixative diluted in phosphate-buffered saline (PBS) for up to 3 h at 4 °C. Only one prolonged wash was performed at 4 °C overnight, in excess PBS. Next day, the specimens were post fixed in 2% aqueous osmium tetroxide solution for 2 h at room temperature in a fume hood.

3.2.4.1.3. Dehydration

The specimens were dehydrated in graded alcohols from 70% ethanol to absolute alcohol (3x15 min each). The absolute ethanol wash times were extended (3x 30 min each). During the dehydration process, the bacterial colonies detached from the agar slabs making it easier to process and subsequently embed the individual bacterial colonies in resin.

3.2.4.1.4. Resin infiltration

The osmicated and fully dehydrated specimens were placed in propylene oxide (3x10 min each). A mixture of propylene oxide and Araldite CY212 in the ratio of 1:1 was used for 2 h to facilitate initial infiltration of resin into the specimen tissue at room temperature followed by 3 changes in fresh resin over 24 h whilst on a rotary device.

3.2.4.1.5. Embedding and polymerisation

All specimens were embedded in fresh Araldite using polypropylene Beem[®] capsules held in a metal rack, containing specimen identity labels. Once the tissue had sunk to the bottom of the capsule (within the Beem[®] of the capsule), the capsule was filled with more resin and lids were closed. The rack holding the capsules was placed into an oven to polymerise the resin at 65 °C for 48 h.

3.2.4.1.6. Sectioning

Thin sections of the specimens were cut using glass knives at 80-100 nm thickness on the Leica Ultracut E microtome. The sections were collected onto 300 mesh naked nickel grids.

3.2.4.1.7. Staining

In order to increase the overall contrast of the ultrathin sections the following solutions of electron opaque heavy metal salts were applied. The grids were fully immersed for

20 min in filtered, freshly prepared, saturated aqueous uranyl acetate solution on a sheet of dental wax and thoroughly washed in distilled water (3x2 min each). The grids were then treated for 5 min in Reynolds (1963) lead citrate solution followed by further washings in distilled water (3x2 min each) and finally air dried. The grids were stored in a grid box and their position was recorded for eventual TEM analysis.

3.2.4.1.8. Examination and image capture

The sections on grids were examined at The University College London C/O David McCarthy as part of the Don Claughar bursary prize (2013-2014) offer made to Sham Lal (the investigator). Images were captured using a Philips CM 120 BioTwin TEM with a Hamamatsu C8484-05G digital camera with AMT V601 software.

3.2.5. *Statistical analysis*

Where appropriate, data are presented as the mean \pm SD (N = 3), tested for normality and equal variances, and analysed by one-way ANOVA (Minitab 16 statistical software and the IBM SPSS statistics²⁰). Differences were considered significant at $p \leq 0.05$.

3.3. Results

3.3.1. Quality control test for selective media

All the three laboratory bacterial strains *P. aeruginosa* (NCTC 10662), *K. pneumoniae* (LP1 499) and *L. pneumophila* non-Sgp1 strain ST707 grew on their respective selective media (Fig. 3.1) demonstrating bacteria of interest, if present on the swabs or in water samples, would be able to grow on them.

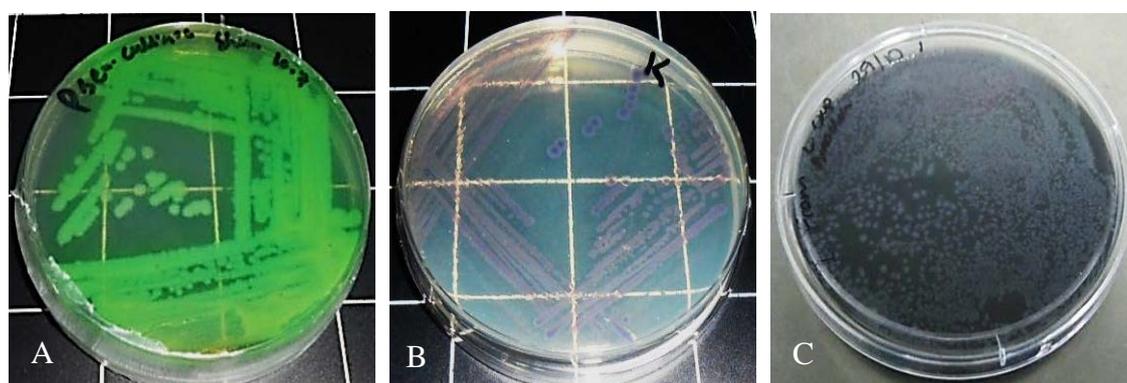


Figure 3.1: Pure cultures of bacteria **A)** *P. aeruginosa* (NCTC 10662), on *Pseudomonas* isolation agar, **B)** *K. pneumoniae* (LP1 499) on HiCrome™ *Klebsiella* selective medium, **C)** *L. pneumophila* non-Sgp1 strain ST707 on GVPC.

3.3.2. Isolation and identification of opportunistic bacterial pathogens from clinical surfaces

3.3.2.1. Opportunistic bacterial isolates from swabs taken from clinical surfaces

No colonies of bacteria grew from any of the plates streaked with swabs or inoculated with water following swab washes. In order to confirm if the splatter from contaminated aerosolised water had landed on working surfaces, leaving dry spots, then desiccation may have affected their laboratory growth.

3.3.2.2. Effect of desiccation on selected bacterial strains

Following desiccation, although not quantified both *P. aeruginosa* (NCTC 10662) and *K. pneumoniae* (LP1 499) were recovered after 24 and 48 h (Fig. 3.2) whereas *L. pneumophila* non-Sgp1 strain ST707 was not recovered on GVPC plates.

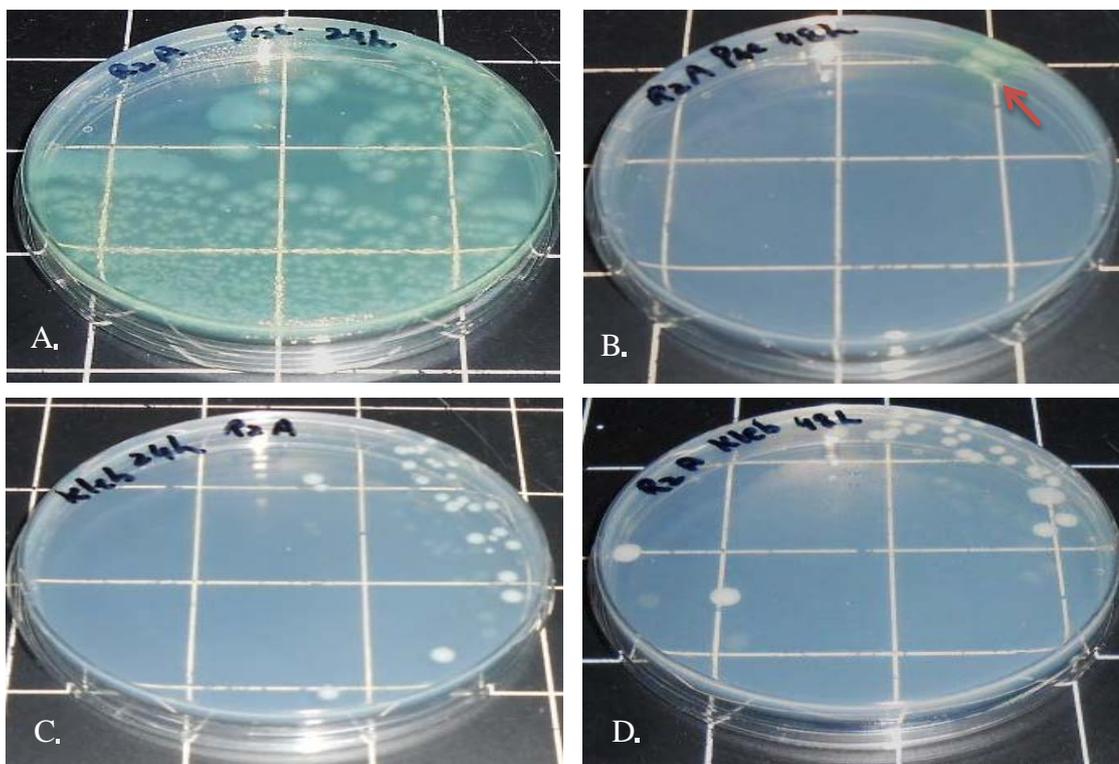


Figure 3.2: Qualitative measure of the effect due to desiccation on bacteria **A)** *P. aeruginosa* (NCTC 10662), recovered after 24 h, and in **B)** after 48 h the recovery was much more diminished see arrow, **C)** *K. pneumoniae* (LP1 499) recovered after 24 h, and in **D)** after 48 h.

3.3.3. Isolation and identification of opportunistic bacterial pathogens from DUWL water samples

3.3.3.1. Opportunistic bacterial isolates from clinical DUWL water samples

P. aeruginosa and *K. pneumoniae* were not recovered from DUWL water samples tested on their respective selective media. Glassy colonies were observed on GVPC plates from 3 out of 31 DUWL water samples.

3.3.3.2. Gram staining properties of the isolated bacterial colonies

The bacteria isolated from DUWL(s) 9 and 10 were Gram negative rods. The bacterium isolated from DUWL 21 was Gram variable.

3.3.3.3. Molecular identification of PCR DNA bands in agarose gel electrophoresis

The colony screen plasmid DNA demonstrated bands of variable sizes and occasionally no bands, as well as the band of interest at the 1600 bp (Fig. 3.3). The direct sequencing from the PCR product demonstrated bands of interest at the 1500 bp size (Fig. 3.4).

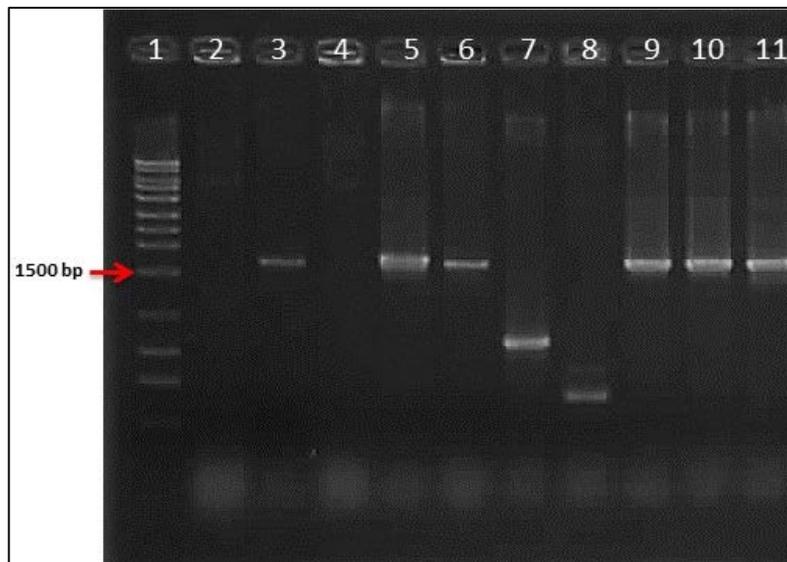


Figure 3.3: Agarose gel electrophoretogram for colony screen following cloning of PCR product (lane 1=1 kb ladder, lanes 2-11 contain single colonies isolated following cloning. Positive results were observed in lanes 3, 5, 6, 9, 10 and 11. Note variable size bands in lanes 7 and 8 and no bands in lanes 2 and 4).

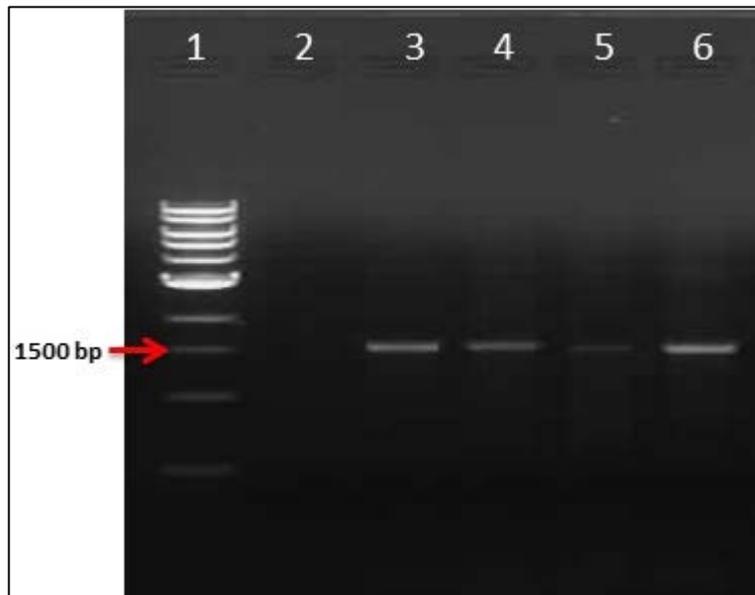


Figure 3.4: Agarose gel electrophoretogram for PCR product (lane 1=1 kb ladder, lane 2= Negative control, lane 3= Positive control, Lane 4= DUWL 9 (test sample), lane 5 = DUWL 10 (test sample), lane 6 = DUWL 21 (test sample). PCR product demonstrated bands of interest at the 1500 bp size.

3.3.3.4. Molecular identification of bacteria from GVPC plates

Molecular sequencing and subsequent Nucleotide BLAST search (ebi.ac.uk) identified the newly isolated bacteria with sequence similarities of 98-100% match with > 200 bp of submitted sequenced nucleotides (Dillon *et al.* 2014^b). These are listed in table (Table 3.2). No *Legionella* species were identified from any of the colonies using molecular identification.

Table 3.2: Final identity of bacterial species isolated from DUWLs on GVPC medium.

Sample ID	Bacterial species	% Match	Accession Number
DUWL 9	<i>S. marcescens</i>	98	ATOI01000027
DUWL 10	<i>Phyllobacterium myrsinacearum</i>	100	JX512224
DUWL 21	<i>Mycobacterium llatzerense</i>	99.3	AJ746070

3.3.3.5. Short term maintenance of bacterial cultures and assessment for the pigmentation production

Of the bacteria identified, *S. marcescens* from DUWL 9 was the only bacterium with possible pigmented colonies. Thus, pure cultures of *S. marcescens* were maintained on R2A and Nutrient agar at various temperatures (see section 3.2.2.5). This bacterium only produced white coloured colonies on both R2A and Nutrient agar medium at all temperatures tested when sub-cultured from GVPC medium (Fig. 3.5A).

P. myrsinacearum also grew on both R2A and Nutrient agar and on GVPC (Fig. 3.5B).

Following sub-culturing, *M. llatzerense* (Fig. 3.5C) failed to grow in the laboratory and further experimental work was not possible with this organism.

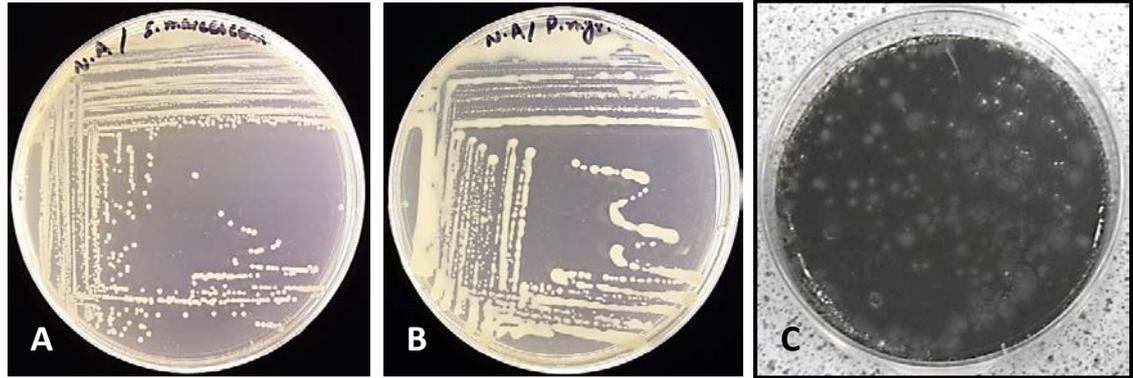


Figure 3.5: Pure cultures of bacteria isolated from clinical DUWL water, **A)** *S. marcescens* on Nutrient agar, **B)** *P. myrsinacearum* on Nutrient agar, **C)** *M. llatzerense* on GVPC.

3.3.3.6. Longer term storage of the newly isolated bacterium from DUWL 9

A unique identification code (UL 234 14) was assigned to *S. marcescens* and freeze-dried stocks were stored at 4 °C at the University of Central Lancashire, UK.

3.3.4. The effect of DUWL biocides on *S. marcescens*, *P. aeruginosa*, and *P. myrsinacearum*

Biocides tested on *S. marcescens*, *P. aeruginosa*, and *P. myrsinacearum* in 1×10^8 CFU/mL inoculum size demonstrated that ICX™ was less effective than the other biocides, although it still controlled the bacterial populations ($p = 0.0001$) (Fig. 3.6). Whereas, Sterilox™, Alpron™, and Oxygenal 6™ completely killed all of the bacteria.

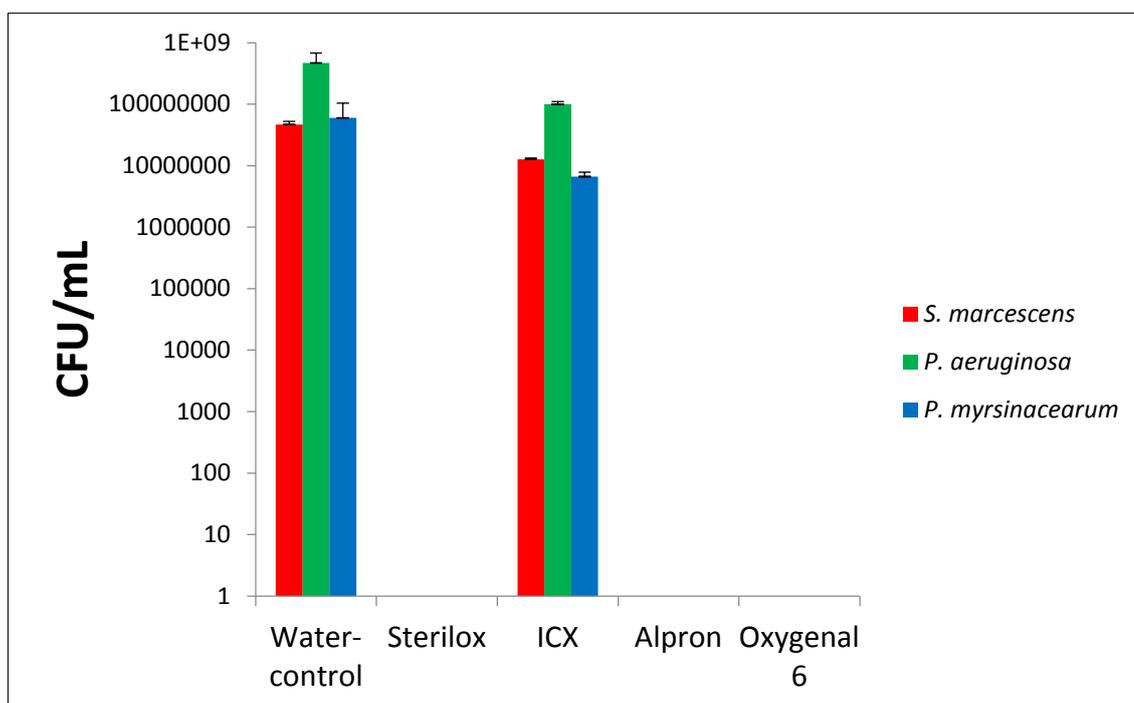


Figure 3.6: The effect of DUWL biocides on *S. marcescens*, *P. aeruginosa*, and *P. myrsinacearum* in 1×10^8 CFU/mL inoculum size.

3.3.5. TEM of isolated bacterial cultures

The ultrastructure of *S. marcescens* from DUWL 9 confirmed these bacteria to be rod shaped with an abundance of fimbriae (Fig. 3.7A). *P. myrsinacearum*, bacteria from DUWL 10 were also rod shaped (Fig. 3.7B) but were even smaller in size than *S. marcescens* and lacked fimbriae. *M. llatzerense* were also rod shaped, slender and larger (Fig. 3.7C) than *S. marcescens* and *P. myrsinacearum* and lacked fimbriae. The flagellum was not observed on any of the three bacteria examined.

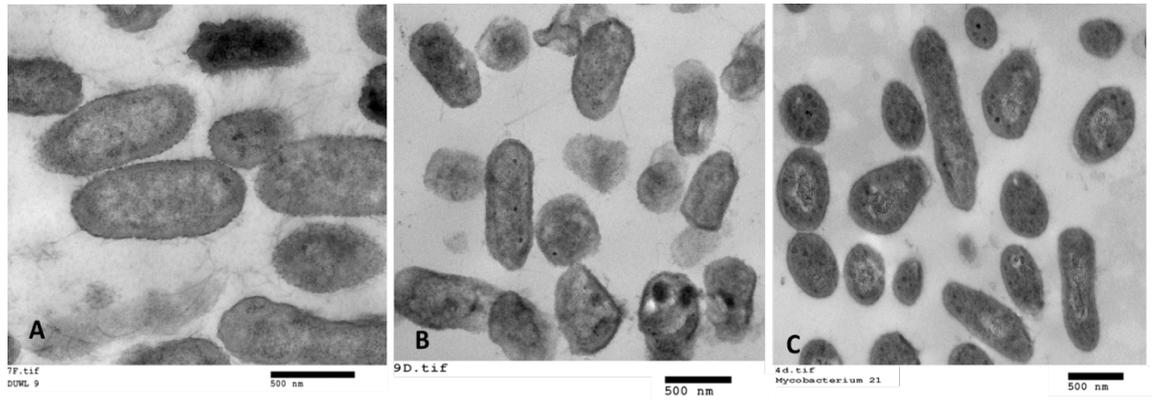


Figure 3.7: TEM images of the bacteria isolated from clinical DUWL water, **A)** *S. marcescens*, **B)** *P. myrsinacearum*, **C)** *M. llatzerense* (Images were taken at the University College London C/O David McCarthy as part of the Don Claughery bursary prize in the presence of the investigator).

3.4. Summary

This chapter set out to evaluate the presence of opportunistic pathogens in the clinical DUWL environment and output water due to aerosols and splatter contamination in dentistry. The results presented here suggest the DUWL output water was free of opportunistic pathogens and that the clinical environment was safe. The only opportunistic pathogenic bacterium identified by molecular biology techniques was *S. marcescens*, which is a typical nosocomial bacterium that can develop biocide resistance. This bacterium has abundance of fimbriae on its surface membrane and these allow the bacteria to attach to existing cells and surfaces in biofilms. The fact that *S. marcescens* was part of the planktonic clinical DUWL water suggests the DUWL 9 must have been heavily contaminated with these species of bacteria. It is therefore likely that the biocide was unable to control its populations.

3.5. Discussion

Although, very little is known about how clean a dental clinical environment may be, it is nevertheless, associated with risk of infections from a variety of pathogenic microorganisms that colonize or infect the oral cavity and the respiratory tract, or are carried in the water used during dental treatment for the “at risk” healthcare operators and patients. To our knowledge, there are no specifications for infection to which the clinical environment should conform to. HTM 01-05: <http://www.dh.gov.uk> website does not provide clear information on this subject and it does not specify which tests to perform when monitoring for the clinical environment for contaminants. This study has devised its own methodology for testing for *P. aeruginosa*, *K. pneumoniae* and *Legionella* spp., by taking swabs from various working surfaces suspected of having been splattered with the contaminated DUWL output water. The rationale being that if they were in the planktonic water, then swab data would provide correlation.

In this study, *P. aeruginosa*, *K. pneumoniae* and *Legionella* spp. were not recovered from dental surfaces. This may have been due to several reasons. One reason for their absence could be their initial desiccation as splatter and aerosolised water is likely to dry out after landing onto working surfaces during daytime. The effects of desiccation on *P. aeruginosa*, *K. pneumoniae* and *Legionella* spp. was tested on laboratory strains which demonstrated both *P. aeruginosa* and *K. pneumoniae* were resilient to desiccation and if these bacteria were present on surfaces of dental surgeries they should have been recovered from swabs. However, *Legionella* spp. did not recover following experimental desiccation conditions and such a result agreed with literature suggesting that *L. pneumophila* are difficult to recover after drying (Katz and Hammel, 1987). These bacterial species were also not recovered from DUWL water samples (N=31) on their selective growth medium. This may be because biocides used to cleanse DUWL systems may have suitably exerted their bactericidal activity. Attempts to

identify (by molecular biology) potential glassy colonies as being *Legionella* spp. resulted in identifying *S. marcescens*, *P. myrsinacearum* and *M. llatzerense* from DUWL water samples growing on GVPC plates. *S. marcescens* is a nosocomial pathogen whereas both *P. myrsinacearum* and *M. llatzerense* are environmental strains. Ultrastructure demonstrated abundance of fimbriae on surface membrane of *S. marcescens* and these allow this bacterium to attach to existing cells and surfaces in biofilms. *M. llatzerense* failed to grow in the laboratory following sub-culturing and further experimental work was not possible.

Recovery of *S. marcescens* was considered to be a clinical isolate and its likely survival in the clinical DUWL may have been related to the development of biocide resistance (Maseda *et al.*, 2009; 2011). Since the dental biocide used to treat the DUWL from which the water sample was taken was Alpron™, its efficacy was tested in the laboratory alongside with other dental biocides on the isolated strain of *S. marcescens*, *P. myrsinacearum* and laboratory strain of *P. aeruginosa*. The results demonstrated that all dental biocides were effective on pure cultures of *S. marcescens*, *P. myrsinacearum* and *P. aeruginosa* in the laboratory up to 1×10^8 CFU/mL. In a previous feasibility study (Pearce *et al.*, 2013), it was found that despite being treated with the same biocide, the planktonic bacterial counts of aerobic mesophilic bacteria were significantly higher in some DUWLs than those set for dentistry by government authorities. Other studies have demonstrated that a consortium of biofilm organisms is capable of surviving antibacterial agents at higher inoculum levels (Anwar and Costerton, 1990; Domingue *et al.*, 1994; Moskowitz *et al.*, 2004; Barbot *et al.*, 2012; Scheid, 2014). However, *S. marcescens* is prone to developing multidrug resistance in the presence of inappropriate concentrations of biocide and antibiotic usage (Maseda *et al.*, 2009; 2011). This study failed to confirm biocide resistance in *S. marcescens* under laboratory conditions suggesting the extracellular polymeric matrix environment of the

biofilm may have provided protection from Alpron™ within the DUWL. Since *S. marcescens* isolated from DUWL 9 is a typical nosocomial bacterium, next chapter will evaluate if free living amoeba found in DUWL systems alongside bacteria act as vector for *S. marcescens* within dental-unit waterline systems.

Chapter 4. *Is V. vermiformis* a vector for *S. marcescens* within dental-unit waterline systems

4.1. Introduction

Domestic tap is an acceptable source of water for DUWLs but this water is neither sterile nor should it contain any pathogenic bacteria (Health Technical Memorandum 01-05, 2013). However, opportunistic pathogens such as *P. aeruginosa*, *L. pneumophila* are linked to clinical DUWL systems (Martin, 1987; D'Ovidio *et al.*, 2011; Ricci *et al.*, 2012). Previous chapters have therefore, stressed the importance of clean DUWL output water for patient treatment and to protect the dental staff as they are at risk of being directly infected from aerosolised water if it is contaminated with opportunistic pathogens.

One reason for the presence of pathogenic bacteria in dental treatment water may be due to fresh water amoebae having internalised live bacteria at source (tap water). Amoebae can subsequently release the undigested pathogenic bacteria, post entry into the DUWL system. An alternative source of amoebae (*Vermamoeba*) is also found in the throat of human individuals (Wang and Feldman, 1967) meaning, even if dental practices were to rely on separate reservoirs of distilled water, DUWL biofilm will eventually succumb to harbouring protozoa in the biofilm consortium of microorganisms as retraction valve failure does occur (Bagga *et al.*, 1984; Montebugnoli *et al.*, 2005; Petti *et al.*, 2013; Ji *et al.*, 2016).

Disinfectants form a vital part of the cleansing regime of DUWL systems and this may act as a double-edged sword where amoebic vectors and human pathogens co-exist. For example, amoebae can survive chlorination, and bacteria growing inside their host are resistant to chemical disinfectants, and antibiotics (King *et al.*, 1988; Marciano-Cabral and Cabral, 2003; Scheid, 2014). Biocides are thought to aid in the selection of *Legionella* strains that prefer to grow and persist within amoebae and have the potential to become pathogenic (Lau and Ashbolt, 2009). Harb *et al.*, (2000) reported that *L. pneumophila* grown in amoebae is several fold more invasive for macrophages

compared to bacteria grown on agar. Other common water-borne bacteria such as *P. aeruginosa* and non-tuberculosis *Mycobacterium* spp. isolated from DUWL systems can also pose a health hazard (Porteous *et al.*, 2004; D'Ovidio *et al.*, 2011). As *V. vermiformis* is the dominant protozoan found in DUWL biofilm community (Barbeau and Buhler, 2001), this investigation was undertaken to evaluate if *V. vermiformis* was acting as a vector for specific pathogens found in clinical DUWL output water.

4.2. Materials and methods

Sources of devices and reagents used in this chapter are listed in Table 4.1.

Table 4.1: Sources of devices and reagents.

Material/Device	Source	Material/Device	Source
R2A	Lab M	Petri dishes	Fisher Scientific
Disposable wire loops	Fisher Scientific	Glutaraldehyde solution	Agar Scientific
Phosphate-buffered saline (PBS)	In-house	2% aqueous osmium tetroxide solution	Agar Scientific
Petri dishes	Fisher Scientific	Ethanol	Sigma
Propylene oxide	Sigma	Araldite Resin (CY212)	Agar Scientific
Beem [®] capsules	Agar Scientific	Ultracut E microtome	Leica, UK
Nickel grids	Agar Scientific	Uranyl acetate	Sigma
Lead citrate	Sigma		

4.2.1. Source of bacterial cultures

P. aeruginosa (NCTC 10662) were maintained in a teaching microbiology laboratory C/O University of Central Lancashire (UCLan), UK; *E. coli* (XL blue) culture was a gift from Culture Collection of Algae and Protozoa, Scottish Association for Marine Science, Scotland, UK and *V. vermiformis* (CCAP 1534/16) was obtained from Dr Mandy Dillon who originally isolated it from a simulated dental-unit waterline system as well as from a decommissioned clinical dental-unit waterline (Dillon *et al.*, 2014^a) at UCLan.

4.2.2. Media preparation

R2A agar was prepared as described in chapter 2 section 2.2.3.

4.2.3. Culture maintenance

E. coli, *P. aeruginosa*, *P. myrsinacearum* (isolated from DUWL 10) and *S. marcescens* (isolated from DUWL 9) were maintained by aseptically transferring cultures onto R2A plates and were incubated at 30 °C for two days. *V. vermiformis* was maintained on *E. coli* following the procedure described previously (Dillon *et al.*, 2014^a).

4.2.4. Phase-contrast and differential interference contrast microscopy

V. vermiformis culture was placed onto a glass slide containing sterile isotonic saline solution and examined directly under a Zeiss Axio Imager A2 microscope. Images were taken using a Zeiss AxioCam HRc digital camera. For the image acquisition, phase-contrast and differential interference contrast microscopy methods were employed.

4.2.5. Preparation of fresh, live bacterial feed

Strains of *E. coli*, *P. aeruginosa*, *P. myrsinacearum* and *S. marcescens* were cultured on R2A plates at 30 °C for two days.

4.2.6. *V. vermiformis* feeding on *E. coli*, *P. aeruginosa*, *P. myrsinacearum* and *S. marcescens*

After two days of incubation, each bacterium was taken and placed as food lines onto R2A plates to feed *V. vermiformis* at 22 °C for five days as described previously (Dillon *et al.* 2014^a). The density of cells growing on a 1 cm² plug of agar was calculated by detaching amoebae and suspending them in 2 mL of 1x PBS. A 10 µL aliquot was taken and used to count cells. This was carried out using a standard cell counter (haemocytometer) (Dillon *et al.* 2014^b). Plugs of agar with equivalent numbers of *V. vermiformis* on their respective bacterial feeds were taken weekly for up to eight weeks. This procedure was carried out in triplicates. The plates were incubated at 22 °C for 5 days. Following incubation, the plates were examined for growth and the area onto

which amoeba had migrated over the R2A agar plates was measured to calculate the total number of cells/unit area as described previously (Dillon *et al.*, 2014^b).

4.2.7. Establishing if *V. vermiformis* is a vector for potentially pathogenic clinical isolates

In order to establish whether *V. vermiformis* was acting as a vector for chosen bacteria, TEM was the method of choice. Plates (x3) with amoebae grown on their respective feed (*E. coli*, *P. aeruginosa*, *P. myrsinacearum* or *S. marcescens*) were incubated at 22 °C for five days. Amoebae were subsequently transferred from the plate by gentle re-suspension in a small volume of PBS. The cells were collected into a Falcon™ 15 mL conical centrifuge tube and pelleted by centrifugation at 352 ×g for 30 min. The pelleted cells were fixed, processed and embedded in Araldite resin using the procedure fully described in chapter 3.

4.2.8. Statistical analysis

Where appropriate, data are presented as the mean ± SD (N = 3), tested for normality and equal variances, and analysed by one-way ANOVA (Minitab 16 statistical software and the IBM SPSS statistics20). Differences were considered significant at $p \leq 0.05$.

4.3. Results

4.3.1. Phase-contrast and differential interference contrast microscopy

The *V. vermiformis* (CCAP 1534/16) under phase contrast and differential interference contrast microscopy demonstrated their limax (trophozoite) morphology and round cysts (Fig. 4.1).



Figure 4.1: Phase-contrast image of *V. vermiformis*. Small newly emerging trophozoite showing a rough surface and spherical cysts also with rough surface. Image captured C/O the curator at the Culture Collection of Algae and Protozoa, Scottish Association for Marine Science, Scotland.

4.3.2. Investigation to determine if *V. vermiformis* acts as a vector for clinical isolates

4.3.2.1. Growth statistics for *V. vermiformis*

As anticipated, *V. vermiformis* grew well on all freshly prepared, live bacterial feeds: *S. marcescens* ($p = 0.0001$), *P. myrsinacearum* ($p = 0.0001$), *P. aeruginosa* ($p = 0.0001$) and *E. coli* (control) ($p = 0.0001$) using one-way ANOVA (Fig. 4.2).

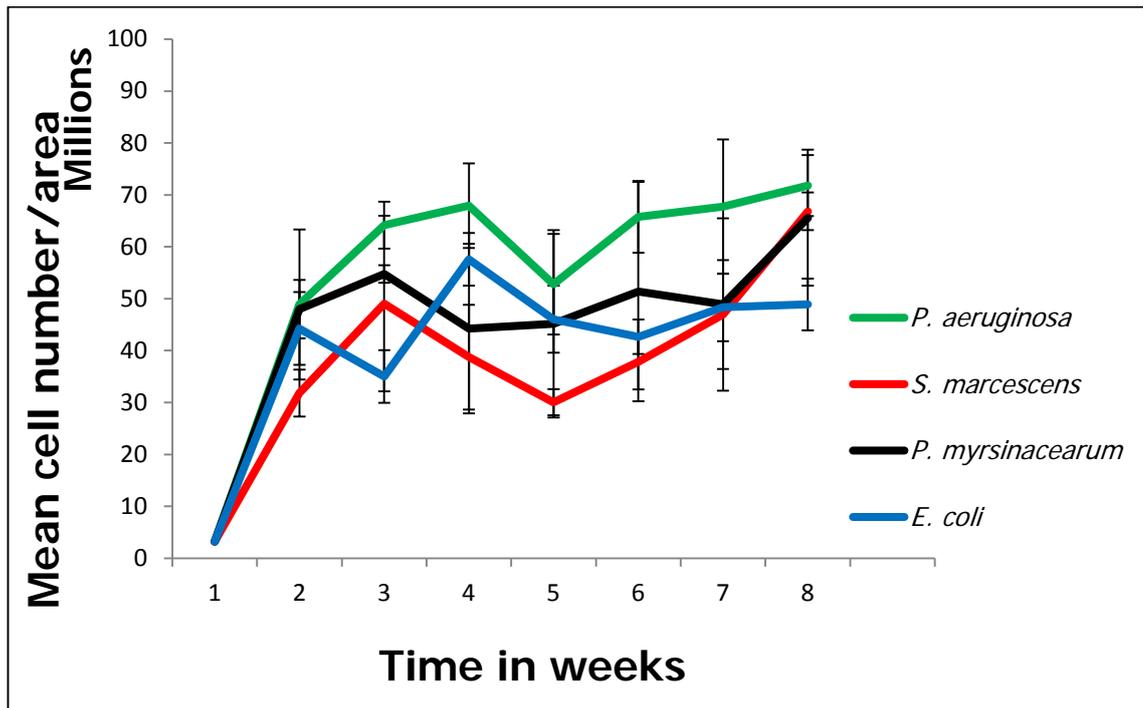


Figure 4.2: Growth curves of *V. vermiformis* fed on *P. aeruginosa*, *S. marcescens*, *P. myrsinacearum*, and *E. coli* (control) for 8 weeks.

4.3.2.2. TEM of *V. vermiformis* fed on pure freshly cultured live bacteria

To determine whether *V. vermiformis* supported growth of the Gram negative *S. marcescens* (from DUWL 9, Fig. 4.3 A), *P. myrsinacearum* (from DUWL 10, Fig. 4.3 B) and two laboratory strains *P. aeruginosa* (Fig. 4.3 C) and *E. coli* (XL blue, Fig. 4.3 D), samples of *V. vermiformis* cells were examined for internalised bacterial cells within the cytoplasm and/or within their encysted form, using high resolution electron microscopy. Neither the trophozoidal amoebae nor their encysted forms showed metabolically active bacterial cells within their cell bodies with the exception of the occasional *V. vermiformis* cell that fed on *P. aeruginosa* (Fig. 4.3 C, box).

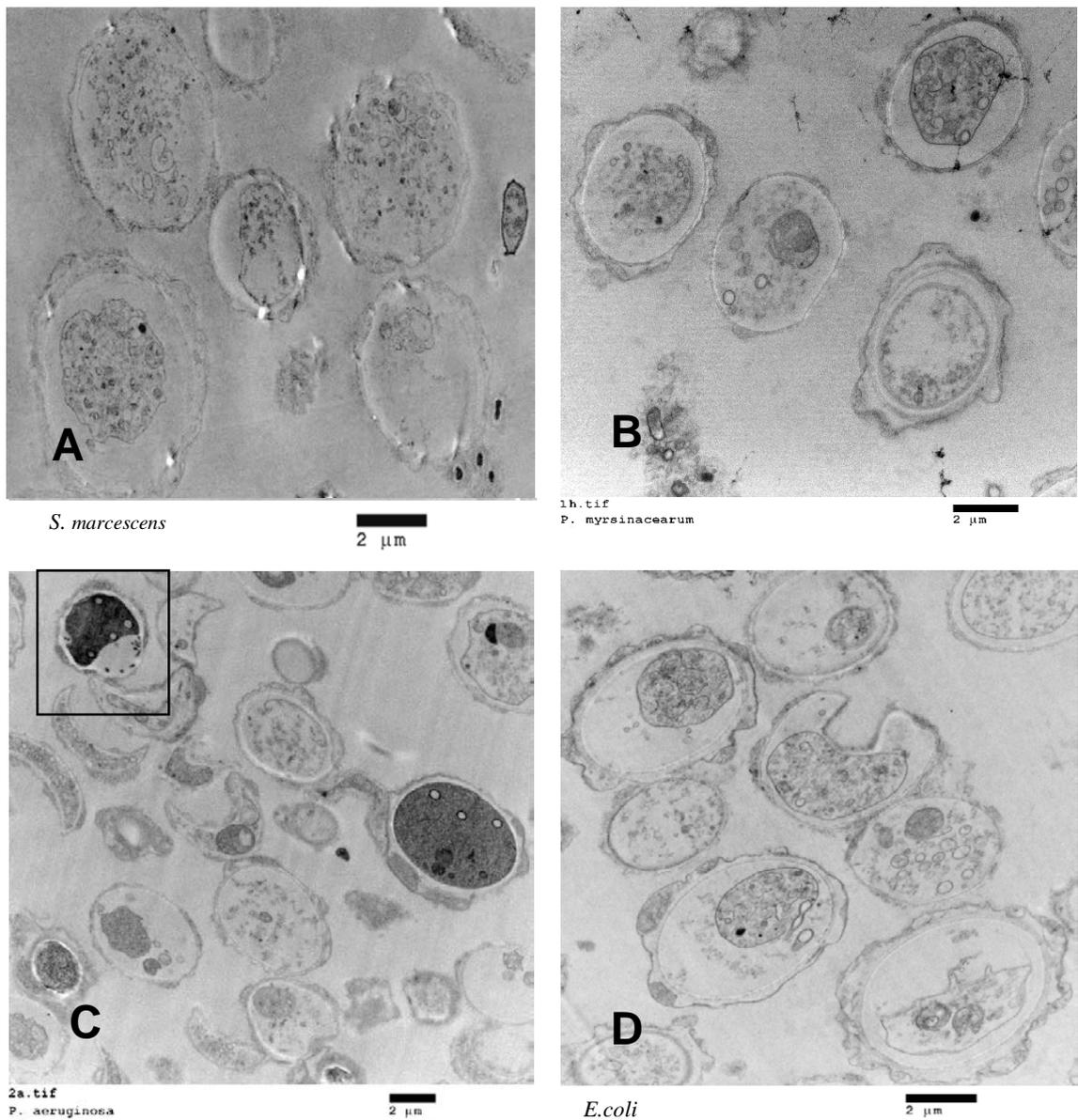


Figure 4.3: TEM micrographs of encysted *V. vermiformis* after feeding on *S. marcescens* (Fig. 4.3 A), *P. myrsinacearum* (Fig. 4.3 B), *P. aeruginosa* (Fig. 4.3 C) and *E. coli* (XL blue) (Fig. 4.3 D). No bacterial cells were observed within the amoebae or their encysted forms except, for one amoebal cell fed on *P. aeruginosa* (Fig. 4.3 C, box).

4.4. Summary

This study set out to characterise if free living amoebae grazed on *S. marcescens* found in DUWL systems alongside on these bacteria. Since the molecular identity of the bacterium from DUWL 9 was identified as *S. marcescens*, which is a typical nosocomial bacterium, this raised concerns for associated health risk with contaminated DUWL. No evidence for bacterial cells within the encysted amoebae was observed by ultrastructure suggesting that *V. vermiformis* prefers to feed on *S. marcescens* rather than acting as a vector to support its life cycle and/or transmit associated disease.

4.5. Discussion

Free living amoebae that graze on the pathogenic bacteria commonly found in DUWL systems pose a risk to both patients and dental staff because they appear to resist DUWL decontamination protocols. The aim of this investigation was to evaluate if *V. vermiformis* commonly found in the DUWL biofilm (Barbeau and Buhler, 2001) was capable of harbouring the clinical isolate *S. marcescens* within the same niche. The main reasons for the investigation was to determine if ingested pathogenic bacteria such as *S. marcescens* could manipulate the amoebal host for their own survival and multiplication, potentially leading to the death of their infected human host (Ricci *et al.*, 2012). Lawsuits can be brought against the dental practitioner if causal links between an infection and the dental treatment water are confirmed (Barbeau, 2007). Thus the importance of improving the quality of dental treatment water is essential, clinically, ethically and financially, to halt spread of disease from DUWL water to humans. Although infection rates in humans are low, continued preventative measures must be taken to decrease the possibility of contracting disease from contaminated DUWL output water.

Hartmannella vermiformis (now called *V. vermiformis*) dominates the DUWL environment (Barbeau and Buhler, 2001) and their initial introduction into this interventional device is likely to come from fresh water supplies used for the reservoir. The same source of water, which may supply domestic and clinical service providing premises, will also have nosocomial pathogens and amoebae prevalent within them (Henke *et al.*, 1986; Fields *et al.*, 1993; Wadowsky *et al.*, 1988; Marciano-Cabral *et al.*, 2010; Nazar *et al.*, 2012). Although this study reports of one clinical isolate of *S. marcescens*, it was nevertheless, a serendipitous find, given that the many glassy colonies analysed by sequencing were taken from *Legionella* selective growth medium plates.

As *V. vermiformis* is a much more cosmopolitan feeder than many other free living amoebae (Weekers *et al.*, 1993) this means that there is a greater likelihood of a pathogenic bacterium utilising this taxon of amoeba as an effective means of transport and dispersal in the DUWL environment (Pickup *et al.*, 2007^b). Previous work from our laboratory on grazing habits of *V. vermiformis* (Dillon *et al.*, 2014^b) indicated that small sized bacteria, from the simulated DUWL biofilm, were favoured as a food source. In addition, an earlier investigation suggested a permissive role of *V. vermiformis* for *P. aeruginosa* (Cateau *et al.*, 2008). Since *P. aeruginosa* was the organism influential in the introduction of control measures in dentistry after reports that it caused serious health problems to patients following dental treatment (Martin, 1987; Williams, 1994); this study also explored the likelihood of *V. vermiformis* supporting the life cycle of *P. aeruginosa* under laboratory conditions.

The results of this study demonstrate that *V. vermiformis*, which was fed on *E. coli* and *P. aeruginosa* ($p = 0.0001$), grew to the same extent as it did on the non-pigmented *S. marcescens* isolated from clinical DUWL water ($p = 0.0001$). These results agree with those of Singh (1942) in which free living amoebae were fed only on a non-pigmented *S. marcescens*. However, in this investigation, *V. vermiformis* also fed on *P. aeruginosa* ($p < 0.05$). These results strongly agree with the study conducted by Pickup *et al.* (2007^a), but disagrees with those of Groscop and Brent (1964) who suggested that *P. aeruginosa* was toxic to an unknown species of the genus *Hartmannella*.

S. marcescens is a known nosocomial pathogen and can cause a variety of infections in humans including blindness in the susceptible host (Hejazi and Falkiner, 1997; Equi and Green, 2001; Tan *et al.*, 2014). It is thus important to understand its proliferative mechanisms in relation to its existence in the DUWL environment to inform the future development of disinfection regimes. Since no evidence for bacterial

cells within the encysted amoebae was observed by ultrastructure, this suggests that *V. vermiformis* is not acting as a vector to support the proliferation of the nosocomial pathogen *S. marcescens* and disagrees with Cateau *et al.*, (2008) for *P. aeruginosa*, although strain differences may apply.

During the past few decades, infection control procedures in dentistry have changed significantly. The basis of dental infection control is to create and maintain a safe clinical environment and to remove, or reduce, the risk of disease transmission as much as possible between patients and dental health care workers. This study confirms that, despite the recommended and appropriate control measures being employed, bacteria such as *S. marcescens* can still be isolated in the laboratory from clinical DUWL water. Care must be taken to use biocides according to manufacturer's instructions to avoid multidrug resistance taking place. In addition, it is also important to adhere to the regular purging protocols recommended by the manufacturers' of the biocide.

This investigation confirms that *V. vermiformis* can actively feed on fresh *P. aeruginosa* and *S. marcescens*, both are small-sized bacteria of which the latter was isolated in this study. This is in agreement with the description of an ideal food source for amoebae suggested by Pickup *et al.*, (2007^a) i.e. ease of intake during phagocytosis/ingestion. Since amoebae appear to be genetically programmed to eat bacteria it is plausible to suggest that *V. vermiformis*, may be able to control bacterial populations by feeding on newly dividing *S. marcescens* providing a promising outcome for infection control in dental treatment.

Next chapter evaluates if anti-retraction valves fitted in hand-pieces are effective in preventing 'suck-back' of oral fluids into the DUWLs.

Chapter 5. Evaluating anti-retraction valve failure

5.1. Introduction

Since the introduction of anti-retraction valves in hand-pieces, differing opinion has been expressed for their efficacy in preventing 'suck-back' of oral fluids into the DUWLs (Bagga *et al.*, 1984; Witt and Hart, 1990; Lewis *et al.*, 1992; Watson and Whitehouse 1993; Walker *et al.*, 2000; Montebugnoli *et al.*, 2002; 2005; Petti *et al.*, 2013; Ji *et al.*, 2016). A failed valve is reported to have collected up to 1 mL of oral fluids rich with opportunistic oral flora/pathogens within them (Bagga *et al.*, 1984). It is implied that the contaminated water may either be transferred to the next patient and/or it may drain into DUWL tubing and thereby contribute to the biofilm community. Since release of oral human pathogens from aerosolised water would pose an occupational health risk for dental staff and patients. In this chapter, I tested the hypothesis that the detection of clinical isolates of known species of commensal or opportunistic microorganisms e.g. *Candida albicans* in DUWL output water from clinical dental-units using microbiological culture techniques and/or detection of albumin and/or immunoglobulin G by biochemical testing would indicate 'suck-back' problems.

5.2. Materials and methods

Sources of devices and reagents used in this chapter are listed in Table 5.1.

Table 5.1. Sources of devices and reagents.

Material/Device	Source	Material/Device	Source
Sabouraud Dextrose agar (SDA)	Lab M	Malt extract broth	Lab M
Petri dishes	Fisher Scientific	Falcon tubes (15 ml)	Fisher Scientific
Chloramphenicol	Sigma	API Candida	BioMerieux
Freeze drier (Scanvac Coolsafe 110-4)	Fisher Scientific	Scalpel blade	Fisher Scientific
Glutaraldehyde solution	Agar Scientific	Phosphate-buffered saline (PBS)	In-house
2% aqueous osmium tetroxide solution	Agar Scientific	Ethanol	Sigma
Desiccator	Fisher Scientific	Methanol	Fisher Scientific
Tween 20	Fisher Scientific	Bovine serum albumin (BSA)	Sigma
Protease inhibitors (cOmplete ULTRA Tablets)	Roche	Coomassie SafeBlue TM reagent	Sigma
Spectrophotometer (7315)	Jenway	Polyvinylidene difluoride (PVDF) membrane Immobilon-P	Millipore, UK
Tris base	Sigma	30% acrylamide, 0.8% N, N'-methylene bisacrylamide stock solution	GE Healthcare
N,N,N',N'-Tetramethylethylenediamine	Sigma	Laemmli reducing sample buffer (non-reducing Laemmli sample buffer BioRad 161-0737 with the addition of 5% β -mercaptoethanol)	Sigma
Gel loading tips	Elkay	Multicolor broad range protein ladder	Thermo Scientific
SuperSignal TM West Dura Extended Duration Substrate	Thermo Scientific	Human serum	Sigma

Primary antibody, Anti-human whole serum (raised in rabbits),	Sigma	Secondary antibody, Peroxidase conjugated affiniPure mouse anti-human IgG (H+L), Minimal cross-reaction to bovine, horse & mouse serum proteins	Jackson immunoResearch Laboratories
ChemiDoc™ XRS + with Image Lab™ Software	BioRad		

5.2.1. Dental-unit output water samples

Having completed the primary aim and objectives for water samples collected in chapter 2, the same water (N = 31) samples were used in this investigation.

5.2.2. Microbiological culture based screening of clinical water for Candida species

5.2.2.1. Preparation of SDA and malt extract broth

SDA and malt extract broth were prepared according to the manufacturer's protocol (see appendix A11). The powdered medium granules were weighed according to the supplier's instructions printed on each package/specified volume. Following addition of appropriate volume of distilled water to a Duran® glass bottle; the mixture was shaken to completely mix and then autoclave sterilised at 121 °C at 15 lb/inch² for 15 mins. After cooling to 45-50 °C, Chloramphenicol (0.1 g/L) was added to prevent any contaminating bacterial growth and then about 20 mL of molten SDA media was poured into fresh, pre-labelled, Petri dishes and allowed to set.

5.2.2.2. Inoculation of Dental-unit waterline output water samples in malt extract broth for possible yeast cultures

Using a class II safety cabinet, 1 mL aliquots of each of the neat water samples (N = 31) were inoculated into a sterile container with 9 mL of malt extract broth. All containers were incubated at 30 °C for 2 days in a shaking incubator set at 180 rpm speed. The

remaining DUWL water from each sample was freeze dried using a bench top freeze drier to concentrate any proteins originating from humans sources.

5.2.2.3. Sub-culturing and maintenance of yeast colonies

After incubation, malt extract broth containing vessels were examined visually for growth (turbidity). A small volume of the culture from the tube that showed up as being turbid compared to the control medium (Fig. 5.1) was streaked onto SDA plates. These were incubated at 30 °C for 3 days (Kadaifciler *et al.*, 2013). Yeast- like colonies which developed were subcultured on fresh SDA plates for purity. Plates were incubated at 30 °C for 3 days as before.

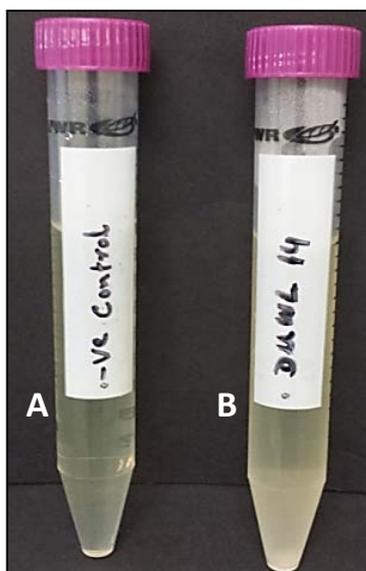


Figure 5.1: Malt extract broth tubes after incubation at 30 °C for 2 days in a shaking incubator set at 180 rpm, **A)** clear medium without inoculum, **B)** turbid medium after inoculation of DUWL 14 water sample.

5.2.2.4 Identification of yeast species

Scanning electron microscopy (SEM) was employed for high resolution morphological examination and API *Candida* test was employed for specific species identification.

5.2.2.4.1. SEM

The pure, isolated colonies were taken through the following steps involved with sample preparation.

i) Fixation

Colonies from SDA plate were immerse fixed in 2.5% glutaraldehyde fixative diluted in PBS for up to 3 h at 4 °C followed by 1 prolonged wash in excess PBS overnight at 4 °C. Next day, the specimen was post fixed in 2% aqueous osmium tetroxide solution for 2 h at room temperature in a fume hood.

ii) Dehydration

The specimen was washed briefly in distilled water and dehydrated in graded alcohols from 70% ethanol to absolute alcohol 3 times for 15 min each with absolute ethanol washes extended for 30 min each. The fully dehydrated specimens were placed in a bench top glass vacuum desiccator for up to 12 h. In order to impart contrast to the specimen under the electron beam, it was sputter coated with gold.

iii) Gold coating and examination

Specimen was adhered to pin stubs with carbon tabs and placed into the Emitech K550X sputter coater to deposit a thin layer of gold under an automated programme preset to high vacuum and voltage conditions. Examination and imaging of the specimens was performed using the FEI Quanta 200 SEM.

5.2.2.4.2. API Candida test

The API *Candida* test was performed according to the manufacturer's instructions. Briefly, the API *Candida* system consists of a single-use disposable plastic strip with 10 wells to perform 12 biochemical tests: five sugar assimilation tests (for glucose, galactose, sucrose, trehalose, and raffinose) and seven enzymatic tests (for β -maltosidase, α -amylase, β -xylosidase, β -glucuronidase, urea hydrolysis, N-acetyl β -glucosaminidase, and β -galactosidase). The inoculum was prepared by adding isolated colonies of interest in 0.85% saline provided by the manufacturer. Inoculation of the wells was performed by adding the test suspension to the dehydrated substrates. The results were read after incubation of the strip for 24 h at 37 °C. A four digit numerical profile was generated depending upon the reactions it produced. Identification of isolate was made by referring to the list of numerical profiles displayed on a computer program (apiweb™) provided by the manufacturer. Alongside the test organism, the laboratory strain of *C. albicans* NCYC 147 previously purchased from National Collection of Yeast Cultures was also tested by the API system for accuracy.

5.2.3. Biochemical methods for screening DUWL water samples for albumin and IgG

Sodium dodecyl sulphate polyacrylamide gel-electrophoresis (SDS-PAGE) was used to separate the proteins within each sample by their size in kilodaltons (kDa) under reducing and non-reducing conditions. The detection of specific proteins was performed either by directly staining the SDS-PAGE gels (for detecting bovine serum albumin) or by transferring the proteins from the gel matrix onto a PVDF membrane for immunoblotting using antibodies specific to the desired proteins.

5.2.3.1. Sample lysates

All freeze dried water samples were re-suspended in 200 μ L of lysis buffer containing 50 mM Tris pH 8.0, 1% NP-40, 150 mM NaCl, 5 mM EDTA with protease inhibitors.

Protease inhibitors prevent the degradation of the proteins by the protease enzymes released during cell lysis within the sample. Following incubation on ice for 30 min and frequent vortex mixing, the sample homogenate was collected in pre-labelled, sterile, 1.5 mL Eppendorf® tubes and 5 µL of each sample was taken and added with 45 µL of distilled water for protein measurement. The test sample lysates were stored at -20 °C until needed.

5.2.3.2. Controls

Along with the sample, a number of negative and positive controls were also generated as described below.

5.2.3.2.1. Negative controls

The lysis buffer only.

5.2.3.2.2. Positive controls

An aliquot (50 µL) of human serum, and BSA.

5.2.3.3. Protein assay

The total protein concentrations of all lysates (samples and controls) were determined using the Bradford colorimetric assay (Bradford, 1976). Protein concentration was obtained from a standard curve prepared using 100-400 µg/mL of BSA diluted in lysis buffer. Following addition of the Coomassie reagent to all standards and test samples, absorbance was measured at 595 nm wavelength using the Jenway 7315 spectrophotometer. The unknown concentration of each of the samples was calculated by comparing the absorbance values with the standard curve.

5.2.3.4. Optimization of Sodium dodecyl sulphate polyacrylamide gel-electrophoresis

5.2.3.4.1. SDS- PAGE conditions

i) Percentage gel

Selecting the correct percentage gel is important as this will determine the rate of migration and degree of separation between proteins. Lower percentage gels (7.5%) are used when trying to resolve proteins of a larger size, whereas higher percentage gels (12.5-15%) are required for resolution of smaller proteins. As this study was about investigating the presence of albumin and IgG, both of which are high molecular weight proteins, lower percentage gels (7.5%) were used.

5.2.3.5. Casting gels

Bio-Rad mini gel electrophoresis system was used to prepare gels for SDS-PAGE. Both short and spacer glass plates were cleaned using 70% ethanol and assembled in the apparatus according to manufacturer's instructions.

The required percentage gels were cast as per table 5.2 using the following reagents: Stacking gel buffer (upper buffer) 0.5 M Tris base and 0.4% SDS, pH 6.8; Resolving gel buffer (lower buffer) 1.5 M Tris base and 0.4% SDS, pH 8.8; freshly prepared 10% aqueous ammonium persulfate (APS); 40% acrylamide; 0.8% N, N'-methylene bisacrylamide stock solution and N, N, N', N'-tetramethylethylenediamine (TEMED). The lower (resolving) gel was added first, and then overlaid with a layer of 70% methanol until set; this was done to insure a smooth divide interface between gels with no air bubbles. Once the gel was set the methanol was removed using a series of washes in distilled water, then the upper (stacking) gel was poured on top of the resolving gel, and placing a comb to create 10 sample loading wells. Once the gel had set the combs were removed and the gels were arranged in the electrophoresis unit (Bio-Rad) as per manufacturer's instructions. Electrophoresis was performed in x1 running buffer made from 1/10 dilution of the laboratory stocks (x10 running buffer containing:

glycine 144 g, Tris base 30 g, SDS 10 g/L of distilled water pH 8.3), at 100 v for approximately 1 h in the Bio-Rad mini protean gel apparatus. Duplicate gels were prepared at equivalent conditions whereby one gel was destined for protein staining (see SDS-PAGE gel protein staining section below) and the other for immunoblotting.

Table 5.2: Quantity of reagents required for preparation of resolving and stacking gels for SDS-PAGE.

Reagents	Resolving gel (7.5%)	Stacking gel (4%)
40% bisacrylamide	2.72 mL	575 µL
1M Tris HCl, pH 8.8	3.63 mL	-
1M Tris HCl, pH 6.8	-	1.3 mL
10 % APS	75 µL	37.5 µL
dH ₂ O	8.16 mL	4.075 mL
TEMED	15 µL	7.5 µL

5.2.3.6. Sample preparation and electrophoresis

Initially all samples (positive controls and tests) were loaded with 30 µg of total protein. However, when using this amount of protein (30 µg) of the BSA and human serum, it became clear after protein staining in the gel (see SDS-PAGE gel protein staining section below) that these proteins were more than required. Therefore, the original stock of positive control proteins (BSA and human serum) was adjusted to ensure definitive discrete, tight bands were obtained at the appropriate molecular weight positions, whereas no bands were obtained while loading 30 µg of total protein for test samples therefore these were loaded with up to 150 µg of total protein. To maintain reducing conditions, 5% β-mercaptoethanol was added (in the fume hood) to the commercial non-reducing 1x sample buffer (Bio-Rad). Electrophoresis was performed at 100 v for approximately 1 h in x1 running buffer in the Bio-Rad mini protean gel apparatus until

the dye front (0.01% bromophenol blue present in the commercial sample buffer) was visible approximately 1 cm from the bottom of the gel.

5.2.3.7. SDS- PAGE gel protein staining

Following electrophoresis, one set of each of the two duplicate gels was stained with Coomassie SafeBlue™ reagent (as per manufacturer's instructions) for detection of bands representing BSA. The images were recorded using ChemiDoc™ imaging device (Bio-Rad) with Image Lab™ software programme.

5.2.3.8. Electrotransfer

Prior to protein transfer, PVDF membranes were made moistened by placing them in methanol for 30 seconds, followed by brief washings in distilled water and then left to equilibrate in 1x transfer buffer made from 1/10 dilution of the laboratory stocks (x10 containing: glycine 144 g, Tris base 30 g/L of distilled water pH 8.3) without methanol. Following electrophoresis, the other duplicate SDS-PAGE gel was used to transfer the proteins from the gel matrix to a "wet" PVDF membrane using the Bio-Rad trans-blot transfer cell as per manufacturer's instructions. Transfer buffer (x1) was diluted 1/10 from 10x stock in distilled water containing 10% methanol. This was used to fill the tank to the appropriate level. The required components were layered in a specific order inside plastic cassette – sponge, filter paper, wet PVDF membrane, gel from SDS-PAGE, filter paper and sponge (all were made moist using 1x transfer buffer without methanol). The cassette was placed in the transfer tank with black side of the cassette facing the black side of the holder. The electrodes were connected, then current of 180 mA/60 V was applied for 2 h to allow successful transfer of proteins from the gel (+) to the membrane (-).

5.2.3.9. Immunoblotting

Following the electro transfer of proteins to a PVDF membrane, the membranes were blocked in 5% w/v skimmed milk/PBS at room temperature for 30 min prior to overnight incubation at 4 °C in anti-human whole serum (developed in rabbit) antibody diluted 1/5 using 5% w/v skimmed milk/PBS. Following washes in PBS containing 0.2% tween 20 (3 ×15 min), the membranes were incubated in the secondary detection horse radish peroxidase (HRP) conjugated affiniPure™ mouse anti-human IgG (H+L), minimal cross-reaction to bovine, horse and mouse serum proteins, diluted 1/ 10,000 in 5% w/v skimmed milk/PBS for 2 h at room temperature. Membranes were washed in PBS/tween 20, three times followed by the detection of any positive bands using the SuperSignal™ West Dura Extended Duration Substrate in a chemi-doc imaging device (Bio-Rad) using the Image Lab™ software programme.

5.3. Results

5.3.1. Detection of *Candida* species in clinical dental-unit water samples

Yeast like colonies were recovered from one (DUWL 14) out of 31 separate water samples.

5.3.1.1. Identification of the isolated yeast

5.3.1.1.1. Colonial characteristics of isolated yeast

On SDA plates, colonies were white to cream coloured and smooth in appearance when viewed following 24 h incubation period (Fig. 5.2A). By 36 h incubation period, the colonies appeared to wrinkle (Fig. 5.2B, arrows).

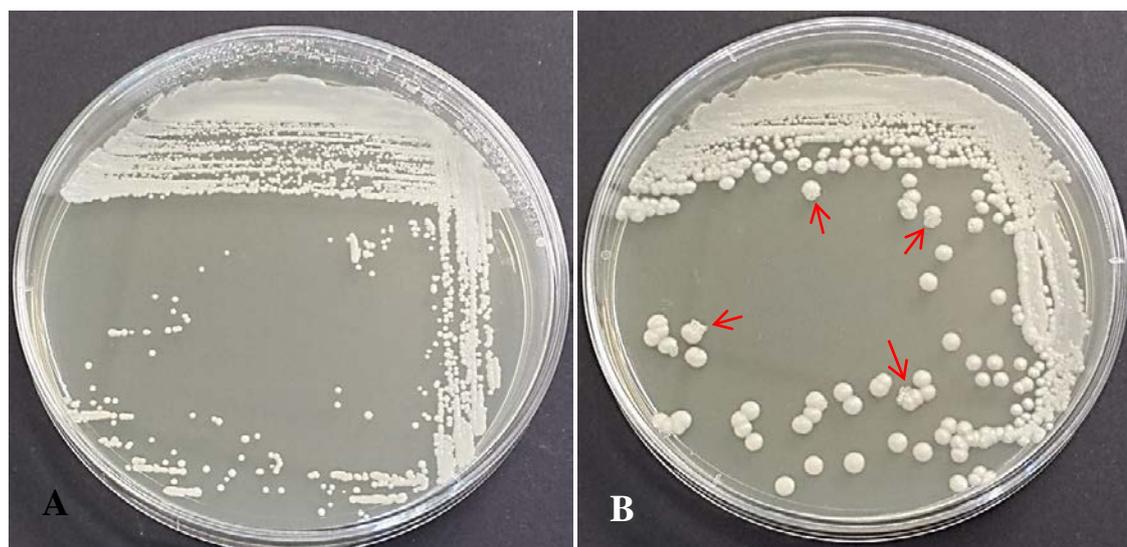


Figure 5.2: Isolated yeast growth on SDA plate. **A)** Smooth colonies after one day incubation, **B)** colonies started to wrinkle after 3 days of incubation (arrows).

5.3.1.1.2. SEM

SEM employed for high resolution morphological examination demonstrated globose to ovoid yeast-like cells with smooth surface (Fig. 5.3A). There were some cells actively budding and others with daughter cells (Figs. 5.3A and 5.3B). On higher magnification, distinct ring-like markings known as budding scars were observed (Fig. 5.3B box).

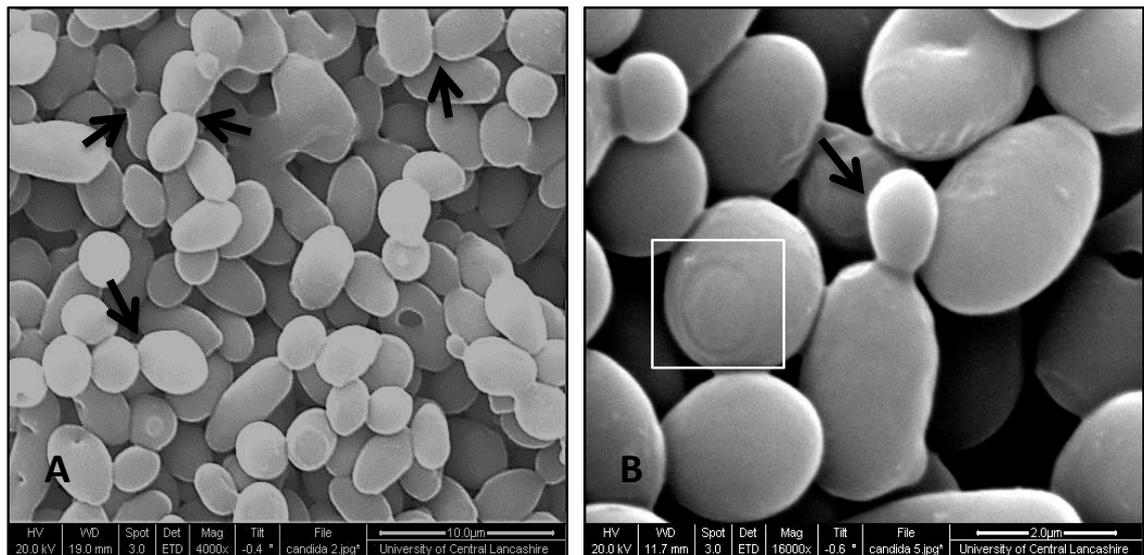


Figure 5.3: SEM images of isolated yeast, **A)** ovoid yeast cells, note the production of daughter cells from parent yeast cells (arrows), **B)** young developing bud from yeast cell (arrow) and distinct budding scars (box).

5.3.1.1.4. API Candida

Using the *API Candida* test, the yeast isolated from DUWL 14 water sample showed positive reaction for glucose, galactose and sucrose as indicated by production of yellow colour in the first three wells (Fig. 5.4). These results identified the unknown organism as being *Candida parapsilosis* with 92.7% certainty (Fig. 5.5). The quality control test confirmed the identification of laboratory strain of *C. albicans* with 99.9% certainty (Figs. 5.6 and 5.7).

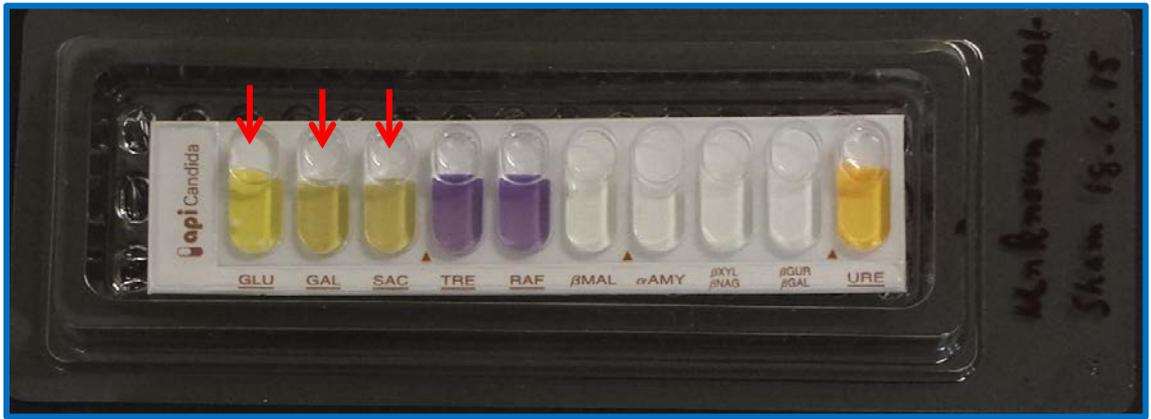


Figure 5.4: API Candida test for isolated yeast showing positive reaction for glucose, galactose and sucrose as indicated by production of yellow colour in first three wells.

API CANDIDA V2.2			
+	+	+	
1	2	4	
GLU	GAL	SAC	
7			
-	-	-	
1	2	4	
TRE	RAF	βMAL	
0			
-	-	-	
1	2	4	
αAMY	βXYL	βGUR	
0			
-	-	-	
1	2	4	
URE	βNAG	βGAL	
0			
REFERENCE	DATE		
	7/24/15		
COMMENT			
GOOD IDENTIFICATION			
Strip	API CANDIDA V2.2		
Profile	7 0 0 0		
Note			
Significant taxa	% ID	T	Tests against
Candida parapsilosis	92.7	1.0	
Next taxon	% ID	T	Tests against
Candida famata	5.1	0.73	TRE 93%

Figure 5.5: Final identity table generated by computer program (apiweb™) showing reasonable match for the identification of *C. parapsilosis*.

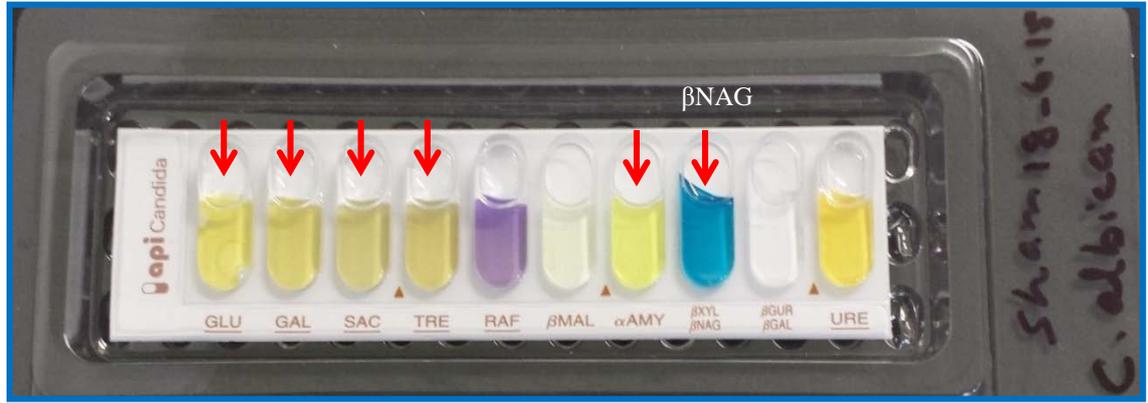


Figure 5.6: API Candida test for laboratory strain of *C. albicans* showing positive reaction for glucose, galactose, sucrose, trehalose, α -amylase and N-acetyl β -glucosaminidase (Arrows).

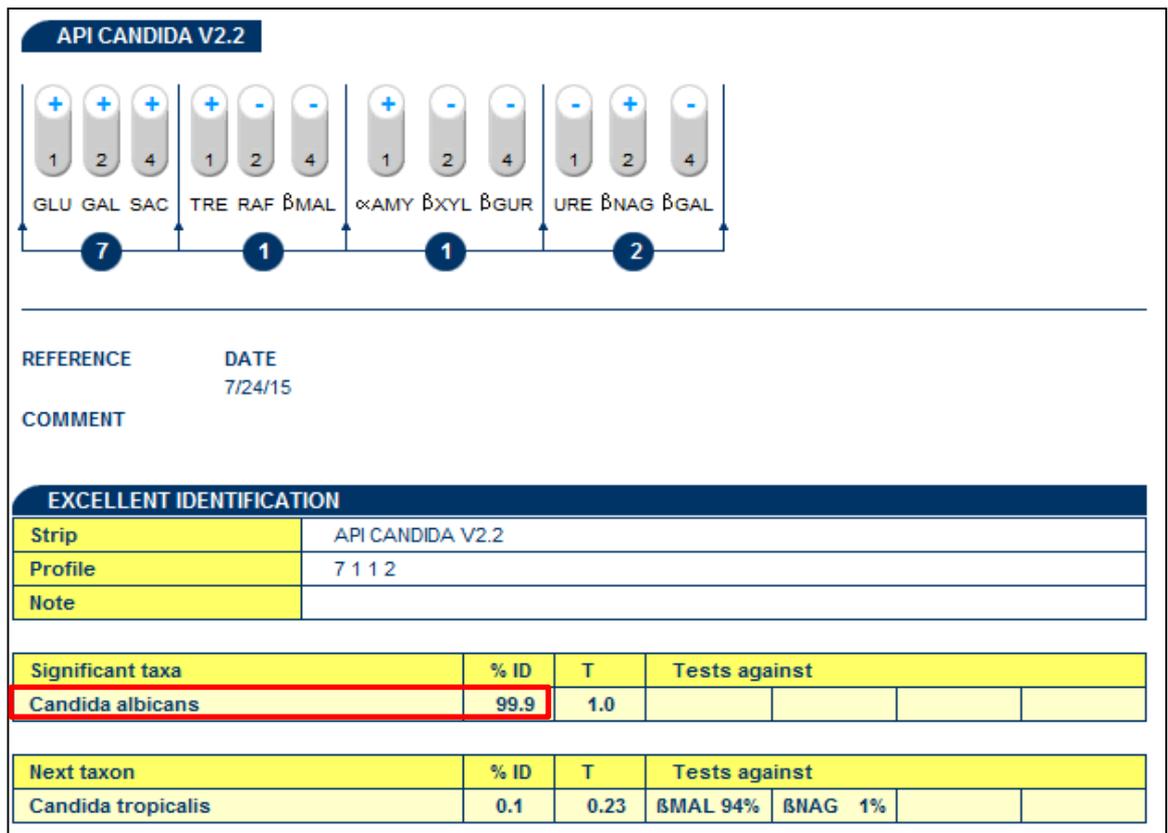


Figure 5.7: Final identity table generated by computer program (apiweb™) showing very good match for positive identification of *C. albicans*.

5.3.2. Detection of albumin and immunoglobulins in DUWL output water samples

5.3.2.1. Protein assay

Figure 5.8 shows a standard curve to find out the concentration of an unknown protein. The assay used various concentrations of BSA for a calibration curve to be plotted by using the Bradford colorimetric assay with mean values where $n=3$ readings with error bars. A straight line (Fig. 5.8) represents the linear regression that best describes the entire set of standard points. Linearity was observed over the entire range of standard solutions (0 to 400 $\mu\text{g/mL}$) with the regression coefficient (R^2) of 0.99. Equation ($y=0.0002x + 0.0028$) values generated from this graph were used to calculate protein concentration of test samples from their recorded absorbance. Table 5.3 shows the total protein in samples. Subsequently, the required volume of each lysate to load 30 μg of total protein for SDS-PAGE was calculated.

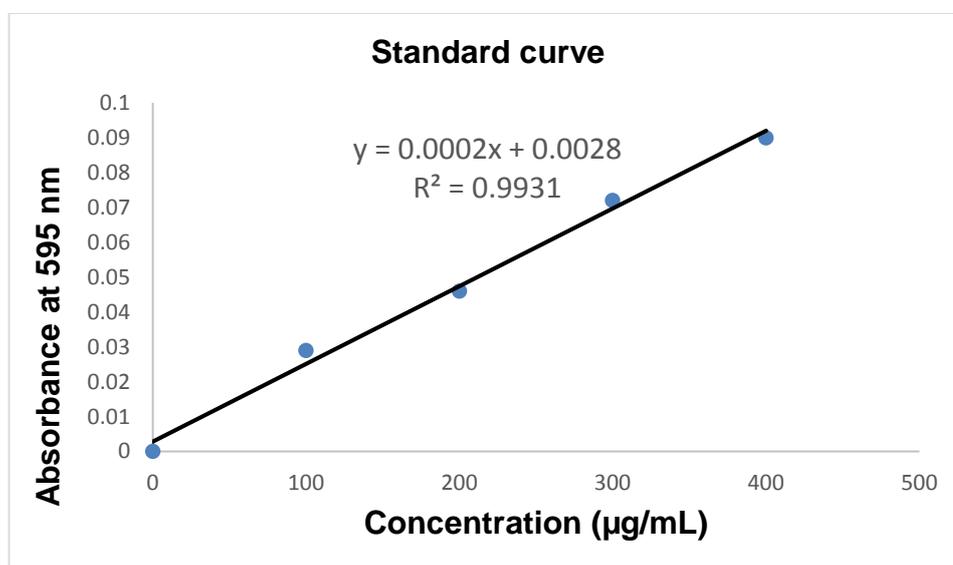


Figure 5.8: Standard curve for the protein assay was used to calculate the concentration of total proteins in all the test lysates.

Table 5.3: Total protein concentration in samples calculated using the data from the standard curve.

Sample	Total protein ($\mu\text{g}/\mu\text{L}$)	Sample	Total protein ($\mu\text{g}/\mu\text{L}$)
DUWL 1	30	DUWL 18	40
DUWL 2	37	DUWL 19	15
DUWL 3	16	DUWL 20	4
DUWL 4	1	DUWL 21	7
DUWL 5	2	DUWL 22	25
DUWL 6	1	DUWL 23	32
DUWL 7	6	DUWL 24	35
DUWL 8	39	DUWL 25	8
DUWL 9	39	DUWL 26	7
DUWL 10	39	DUWL 27	4
DUWL 11	4	DUWL 28	38
DUWL 12	6	DUWL 29	34
DUWL 13	2	DUWL 30	38
DUWL 14	24	DUWL 31	38
DUWL 15	7	Human serum	114
DUWL 16	40	BSA	100
DUWL 17	40		

5.3.2.2. SDS-PAGE

As expected, the lanes with the lysis buffer as a negative control were clear and the lane with BSA (positive control expected at 66 kDa) showed a band between the 72-52 kDa molecular weight. The lane with human serum showed a ladder of bands including a band corresponding to human albumin which was of the similar molecular weight as the BSA positive control. Some of the DUWL water samples (Fig. 5.9 lanes 8, 9 and 10) demonstrated degraded smears of possible lipoproteins/proteoglycans without any specific bands corresponding to the positive control (BSA) molecular weight in DUWL water samples. Fig. 5.9 represents typical results obtained from all coomassie stained gels following their capture in Chemi-Doc™ imaging device (Bio-Rad) with Image Lab™ software programme.

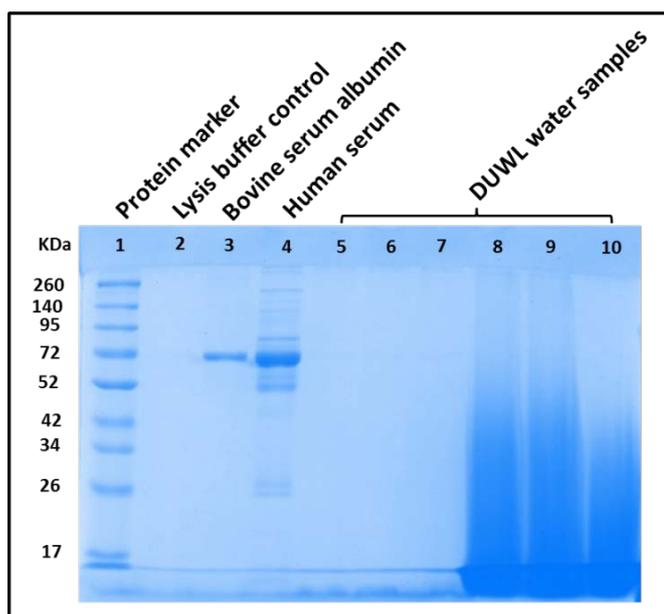


Figure 5.9: SDS-PAGE analysis following staining with Coomassie SafeBlue™ reagent. All lanes (1-10) are labelled to indicate the sample ID. Lysis buffer control remained negative. BSA showed a distinct band between 72-52 kDa sizes. A band for albumin was observed from the human serum in lane 4 corresponding to BSA. All DUWL water samples remained negative for the presence of albumin.

5.3.2.3. Immunoblot analysis

Immunoblot analysis confirmed the lysis buffer (negative control) containing lane remained clear. The lane with human serum (positive control) in both reduced and non-reduced blot showed band/bands for IgG. No bands were detected in the lanes with DUWL water samples indicating absence of IgG. See Fig. 5.10.

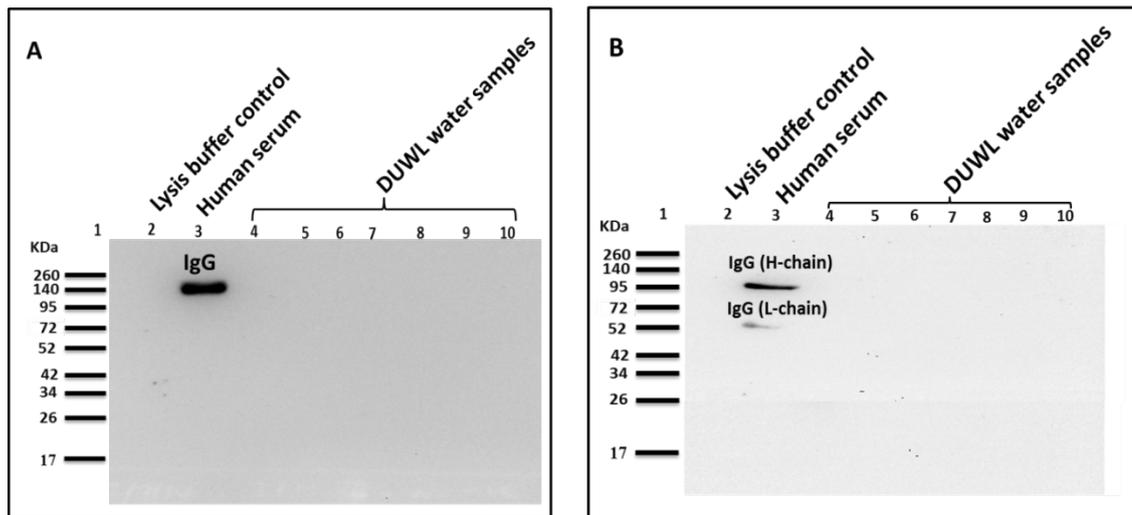


Figure 5.10: Immunoblot for anti-human IgG where **A)** is a blot performed under non-reducing conditions (one band at 150 kDa) typical for human IgG, and **B)** performed under reducing conditions (2 bands at 100 kDa and 50 kDa). In lane 2, lysis buffer control remained negative. A band(s) for IgG was not detected in any of the DUWL water samples tested.

5.4. Summary

Failure of anti-retraction valves in preventing bacterial contamination of DUWLs can be considered a serious hazard with the potential to cause iatrogenic cross infection. This study explored the hypothesis that failure of anti-retraction valves may lead to contaminated oral fluids leaking into the DUWL. Such an occurrence will add new species from human origins to the biofilm community. A microbiological cultural approach was tested for any opportunistic oral pathogen(s) (*Candida* spp.). SDS-PAGE assay was performed for detecting foreign albumin and immunoblotting for Ig. The rationale was that if retraction valves failed, the DUWL water could contain albumin and/or Ig from oral sources. Only one sample indicated the presence of possible human opportunistic yeast, *C. parapsilosis*. Although this is an interesting result, it is not statistically significant. However, in view of the fact that anti-retraction valve tubing does accumulate oral fluids suggests that the contaminants are not reaching the DUWL. If an anti-retraction valve fails within a hand-piece, oral material could be sucked back into the retraction tubing. However the hand-piece is removed and sterilised by autoclaving between each patient. (There is some controversy about how effective this sterilisation is within the actual tubing, but that is outside the scope of this study). The results from this study demonstrate that there appears to be no contribution of the oral contamination to the DUWL planktonic output water.

5.5 Discussion

Failure of anti-retraction valves in preventing bacterial contamination of DUWLs can be considered as a serious hazard for iatrogenic cross infection. However, it is not easy to measure the actual impact on human health because it is very difficult to establish a link between microbial contamination of DUWLs and occurrence of an infectious disease in host.

The efficacy of anti-retraction valves in preventing microbial contamination of the dental units has not been thoroughly investigated, however, previous investigations have recorded that a high proportion of them fail within days of being used (Bagga *et al.*, 1984; Lewis *et al.*, 1992; Montebugnoli *et al.*, 2002; 2005; Petti *et al.*, 2013; Ji *et al.*, 2016). This chapter explored the hypothesis that failure of anti-retraction valves may lead to contaminated oral fluids leaking into the DUWL thereby adding new species to the biofilm community.

Two qualitative tests were devised. A microbiological culture approach for the detection of opportunistic oral pathogen(s) (*Candida* spp.) and a biochemical assay (SDS-PAGE and immunoblotting) for the detection of albumin and/or Ig from oral sources were used. The rationale being that oral yeast infection represents a secondary opportunistic infection particularly involving *C. albicans*, but increasingly non-*albicans* species as well. Oral yeasts can be found in periodontal pockets, in root canals, on the mucosae and underneath dentures (Song *et al.*, 2005; Kumar *et al.*, 2015), areas, which are debrided during dental treatment. Therefore, the presence of *Candida* spp. and/or albumin and/or IgG in DUWL water could only be due to the retraction of a volume of inoculating contaminants into the DUWL supply water.

Yeast like colonies were recovered from one (DUWL 14) out of 31 separate water samples. Based on the morphological characteristics of the organism, SEM confirmed that it was a yeast. This observation was supported by the presence of

budding cells and budding scars on the cells. Final identity performed by API candida indicated unknown yeast as being *C. parapsilosis*.

Although this is an interesting result, it is not statistically significant. Hence it would be difficult to relate the one sample that tested positive for *C. parapsilosis* to retraction valve failure. Some researchers suggest that large volumes up to 1 mL of oral fluids, enriched with biofilm forming bacteria and yeast could be drawn up into DUWL systems (Bagga *et al.*, 1984; Lewis *et al.*, 1992; Panagakos *et al.*, 2001; Al Shorman *et al.*, 2002^a; Artini *et al.*, 2002; Montebugnoli *et al.*, 2002; Berlutti *et al.*, 2003; Montebugnoli *et al.*, 2005; Petti *et al.*, 2013; Ji *et al.*, 2016). If that was the case, they would not only be contributing to the biofilm community, but also become part of the planktonic DUWL output water.

Albumin and IgG were not detected in any of the 31 samples tested. This could be because fresh changes of DUWL water are more likely to dilute these out and their detection would be difficult. In the future it may be a good idea to detect oral bacteria as a culture based marker instead.

Overall, this study demonstrates that the contaminants are not reaching the DUWL possibly because the hand piece is removed and sterilised by autoclaving between each patient.

PART II: RESEARCH ASPECTS

Chapter 6. Microbial succession in an *in-vitro* laboratory model of a simulated dental-unit waterline system

6.1. Introduction

The in-service evaluation of the investigation has provided several good reasons as to why microbial quality of water in DUWLs is of considerable importance. Cleansing and maintaining the recommended level of $\leq 200\text{-}500$ CFU/mL of aerobic mesophilic heterotrophic bacterial counts low is difficult despite being compliant with the various authorities' recommendations (Anon, 1996; HTM 01-05; HTM 07-01 <http://www.dh.gov.uk>; <https://www.gov.uk/government/organisations/environment-agency>). The reason why it is more difficult to keep delivering clean water from some DUWLs rather than others is poorly understood. The findings of chapter 2 suggest that water from a supposedly clean DUWL can fail to meet recommended limits of quality. Thus, there is a need to understand the population dynamics of biofilm formation which includes the type of microbiota taking up residency within dental tubing. Only then can better strategies to control and/or eradicate the opportunistic pathogens in order to protect all those who come into contact with DUWL water, can be devised. To achieve this goal, biofilm formation in dental waterline tubing fitted to a simulated *in-vitro* DUWL was employed. This was based on the published designs of Spratt *et al.*, (2004) and Dillon *et al.*, (2014^a) with the aim of replicating the development of a heterogeneous biofilm and understanding the events occurring from the time of its initiation to the formation of a complex ecological community with possible opportunistic human pathogens produced in the laboratory, whereby no biocidal control mechanism was employed and no oral fluids could have contaminated the water.

6.2. Materials and methods

Sources of devices and reagents used in this chapter are listed in Table 6.1.

Table 6.1: Sources of devices and reagents.

Material/Device	Source	Material/Device	Source
R2A	Lab M	Malt extract agar	Lab M
Glycine-Vancomycin-Polymyxin-Cycloheximide (GVPC) medium plates	Fisher Scientific	Duran glass bottles	Fisher Scientific
Petri dishes	Fisher Scientific	Chloramphenicol	Sigma
Plastic container (5 L)	Fisher Scientific	DUWL tubing	A free gift from A-dec Dental UK Ltd
Peristaltic pump	HaiYang, China	Falcon tubes™ (50 mL)	Fisher Scientific
Falcon tubes™ (15 mL)	Fisher Scientific	Eppendorf tubes™ (1.5 mL)	Fisher Scientific
Swabs (Polypropylene swab tubes plastic sticks)	Fisher Scientific	Disposable wire loops	Fisher Scientific
Disposable spreaders	Fisher Scientific	Gram staining kit	Fisher Scientific
Lysozyme	Sigma	Proteinase K	Qiagen
AL buffer	Qiagen	Phenol/Chloroform/Isoamyl Alcohol 25:24:1 saturated with 10 mM Tris, pH 8.0, 1 mM EDTA	Sigma
Ethylenediaminetetraacetic acid (EDTA)	Sigma	Glycogen	Sigma
Nanodrop 1000 spectrophotometer	Thermo Scientific	Ammonium acetate	Sigma
Sodium acetate	Sigma	Ethanol	Fisher Scientific
AE buffer	Qiagen	Taq DNA polymerase	Fisher Scientific
Taq Buffer A	Fisher Scientific	Forward (D88) and Reverse (E94) primer	Life technologies
Deoxynucleotide triphosphates (dNTPs mix)	Fisher Scientific	Agarose	Fisher Scientific
RNase free water	Fisher Scientific	Veriti thermocycler	Applied Biosystems

1 kb ladder	New England Biolabs	Loading dye	New England Biolabs
gel red	NBS Biologicals	Gene snap software	Syngene, UK
BigDye® Terminator v3.1 cycle sequencing kit,	Applied Biosystems		

6.2.1. Preparation of media

R2A, *Pseudomonas* isolation agar and HiCrome™ *Klebsiella* selective agar media were prepared as described in chapter 3 section 3.2.2.1.

Malt extract agar powder was weighed according to the supplier's instructions printed on each package/specified volume. Following addition of appropriate volume of distilled water to a Duran® glass bottle and making sure the powder completely mixed in the water, it was autoclave sterilised at 121 °C at 15 lb/inch² for 15 min. After cooling to 45-50 °C, chloramphenicol (0.1 g/L) was added and then about 20 mL of molten media was poured into previously labelled fresh Petri dishes.

All media were prepared according to the manufacturers' protocol (see appendix A11).

6.2.2. Biofilm formation in dental-unit waterline tubing fitted to simulated in-vitro dental-unit waterlines

A simulated *in-vitro* dental-unit waterline system (sDUWL) was set up on the bench near a sink in the microbiology laboratory of my academic institute. The model consisted of a plastic container with total liquid capacity of 5 litres, to which a length of approximately 2 metres of dental-unit waterline tubing, of 4 millimetre (mm) bore was attached. The DUWL tubing was a free gift from A-dec, Dental UK Ltd. A peristaltic pump was attached to the tubing for a constant flow rate of the water at 6 L/h (Fig. 6.1 A). The water supply was from a header tank housed on top of Maudland building of my academic institute (University of Central Lancashire) at the Preston campus. To my knowledge the water was free of any additives. During the working days, fresh cold, tap

water circulated through the reservoir by a peristaltic pump filling the container up to the 3 litre volume mark to match the output flow rate. At the end of each ‘working day’ and at night and at the weekends water from the reservoir was circulated continuously through the unit via the connector tubing - as seen in (Fig. 6.1 B) to avoid desiccation. This procedure was maintained during the three year duration of this study.

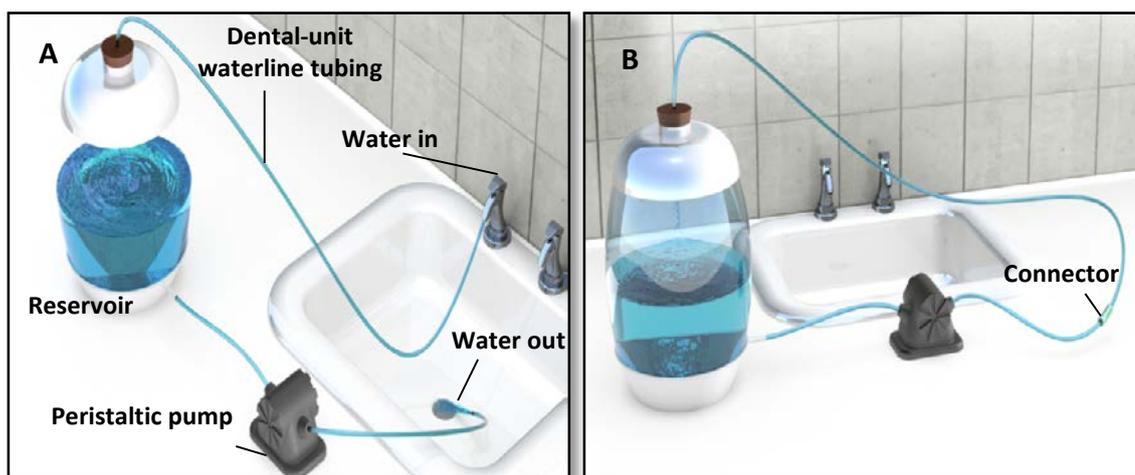


Figure 6.1: A simulated laboratory dental-unit waterline (sDUWL) system set up, **A)** Day model, **B)** Night and weekend model.

6.2.3. Establishment of biofilm microbes during first two weeks of the newly set up in-vitro simulated dental-unit waterline by testing quality of output water

The DUWL water samples (equivalent in volume to clinical output water) were tested on regular basis, daily up to 2 weeks and thereafter once a week for total of 180 days. Bacterial numbers were enumerated using R2A agar. To enumerate planktonic bacteria, samples of water from the reservoir (test) and the tap water at source (control), were collected first thing each morning in a sterile container. Using a class II safety cabinet, serial dilutions were prepared down to 10^{-7} in sterile container and mixed manually by vigorous shaking. A fixed volume (0.1 mL) of the diluted planktonic suspension and the tap water (control) was inoculated (in triplicate), onto freshly poured, pre-labelled R2A agar plates. All plates were incubated at 22 °C for 7 days.

6.2.3.1. Colony enumeration

Following incubation R2A plates were examined and only plates with colonies in the range of 30-300 were counted. The CFU/mL was calculated using the mean of the triplicate R2A plate readings and adjusted for the dilution factor as mentioned in section 2.2.5.4. of chapter 2.

6.2.4. Isolation of the heterogeneous biofilm community of microbes dwelling in simulated dental-unit waterline tubing

The method involved taking 1 cm length of the reservoir-DUWL tubing from the water outflow section. Using aseptic technique in a class II safety cabinet, the tubing was cut lengthwise to expose the lumen. The biofilm growing on the wall of the lumen of one half of the tube was swabbed using a sterile, commercial, swab (polypropylene swab tube plastic stick) and the attached microbes were dispersed in 2 mL of sterile water. Following vigorous shaking, serial dilutions were prepared down to 10^{-7} in sterile water. A fixed volume (0.1 mL) from each diluted biofilm suspension was inoculated (in triplicate) onto freshly poured, pre-labelled R2A agar plates. A fixed volume (0.1 mL) of the remaining of the neat biofilm suspension was used to inoculate malt extract agar plates for the isolation of fungi, and as an inoculum for preparing fresh food for subsequent isolation of protozoans, and for detecting any potential opportunistic, human pathogens.

For the isolation of protozoa, mixed colony, dense bacterial 3 day growth from the biofilm suspension (above) were used to serve as a “food line” (see chapter 4, section 4.2.7.). The other unused half of the cut side of the tubing was placed directly in contact with one end of the food line to encourage any amoebae to venture out of the tubing to graze on bacteria supplied as food on the plate.

6.2.5. Isolation of potential opportunistic, human pathogens, from the simulated dental-unit waterline

Neat biofilm suspension (0.1 mL) obtained from section 6.2.4, was inoculated on *Pseudomonas* isolation agar plates for *Pseudomonas* spp.; HiCrome™ *Klebsiella* selective medium for *Klebsiella* spp. and onto commercially prepared GVPC medium plates for *Legionella* spp. as described in chapter 3.

6.2.6. Incubation of all plates

R2A plates were incubated as described in chapter 2. Malt extract agar plates were incubated at 22 °C for 7 days. *Pseudomonas* isolation agar and HiCrome™ *Klebsiella* selective medium plates and the GVPC plates were incubated as described in chapter 3.

6.2.7. Sub-cultures and maintenance

6.2.7.1 Bacteria from R2A and GVPC plates

Of the many different colonies growing on R2A plates, selected isolates (based on colonial morphological characteristics) from each group of the colonies were sub cultured onto fresh R2A plates and incubated at 22 °C for 7 days. This method was repeated for all colonies that were initially observed on GVPC plates to determine whether they could also grow on R2A agar plates. Gram staining characteristics and molecular identification of the biofilm community of bacteria were subsequently determined.

6.2.7.2 Maintenance of fungi

Macroscopic examination of the malt extract agar plates containing chloramphenicol demonstrated blackish green coloured colonies. A pure culture of each colony type from each plate was obtained and maintained by sub culturing onto new malt extract agar plates without chloramphenicol to avoid any adverse effect on production of fungal spores which is a key factor for identification of fungi. Plates were incubated at 22 °C

for 7 days as before and were kept for macroscopic and microscopic identification of fungi.

6.2.7.3. Maintenance of protozoa

As described in chapter 4 (see section 4.2.3).

6.2.8. Identification of isolated microbes

6.2.8.1. Gram staining of bacterial colonies

Gram staining was performed as per chapter 3 section 3.2.2.6.1.

6.2.8.2. Molecular identification of bacterial colonies

All steps referring to molecular identification of bacteria (DNA extraction, primers, PCR electrophoresis, cleaning PCR products, sequencing and molecular identity) were performed as fully described in chapter 3, section 3.2.2.6.2.

6.2.8.3. Morphological Identification of isolated fungi

The isolated fungi were identified by macroscopic and microscopic methods, according to the morphological characteristics of hyphae and fruiting bodies previously described by Onions *et al.*, (1991). Using aseptic technique, small samples of mature peripheral fungal growth were removed from colonies growing on plates of malt extract agar (without chloramphenicol) and placed onto glass microscope slides for examination under phase-contrast and differential interference contrast microscopy (DIC). Images were obtained using a Zeiss Axio Imager A2 microscope and a Zeiss AxioCam HRc digital camera (100x magnification).

6.2.8.4. Identification of protozoa

6.2.8.4.1. Microscopy

Phase-contrast and differential interference contrast microscopy (DIC) was performed and images were taken using a Zeiss Axio Imager A2 microscope and a Zeiss AxioCam HRc digital camera (100x magnification).

6.2.8.4.2. Molecular identification of protozoa

The molecular identification described in this section was performed completely by the protozoan curator Dr Undine Achilles-Day, c/o Culture Collection of Algae and Protozoa, Scottish Marine Institute, Scotland. Several plates with protozoa growing on them were sent to the Culture Collection of Algae and Protozoa. The curator surveyed the plates under the phase contrast microscopy for single amoebal cells initially for sub culture on non-nutrient agar plates overlaid with a strain of *E. coli* XL1-blue (Stratagene) as a food source. Plates were then incubated at 20 °C for two weeks in the dark, by which time a lawn of a clonal culture of the amoeba had grown for molecular analyses.

i) DNA extraction, amplification, and sequencing

Stationary phase amoebae from a culture plate were removed using 5 ml of sterile distilled water and collected prior to centrifugation for 3 min at 2504 ×g. The supernatant was discarded and the cell pellet was retained for molecular profiling. Genomic DNA was extracted from the cell pellet using the DNeasy Plant Mini kit according to the manufacturer's instructions. DNA amplification and sequencing for ribosomal RNA gene was performed using the QIAGEN Taq PCR Master Mix using EAF3 and ITS055R as PCR primers (Marin *et al.*, 2003). For the amplification in the thermo-cycler the following protocol was used: an initial denaturation step at 95 °C for 2 minutes was followed by 30 cycles including denaturation (95 °C for 1 minute), annealing (55 °C for 2 minutes), and elongation (68 °C for 3 minutes). The amplified

product was visualised using electrophoresis (1.5% agarose gel) and purified using QIAquick PCR.

ii) ***Purification Kit following manufacturer's instructions.***

The sequencing was performed on an ABI-system and assembly of the sequence data was carried out using Gene Geneious Pro v6.1.5 (<http://www.geneious.com/>). To assess microbial sequence diversity a representative sequence was selected and submitted to the European Nucleotide archive (ebi.ac.uk) and the NCBI Basic Local Alignment Search Tool (GenBank) to allow comparison with bacterial 16S rRNA gene sequences present in this database. Only those sequence matches scoring 98-100% were considered for the identity of the organism (Stackebrandt and Goebel, 1994).

6.2.9. Statistical analysis

All analyses were performed using the Minitab 16 statistical software. Where appropriate, data are presented as the mean \pm SD (N = 3), tested for normality and equal variances, and analysed by a *t*-test for two independent samples and Pearson correlation coefficient. Differences were considered significant at $p \leq 0.05$.

6.3. Results

6.3.1. *The in-vitro simulated dental-unit waterline set up*

The in-house *in-vitro* sDUWL (Fig. 6.1) was an adaptation of former models (Spratt *et al.*, 2004; Dillon *et al.*, 2014^a). The waterline tubing used in this study was the same quality as that would be incorporated into the commercial dental chair units. For this study the tubing was a gift from A-dec (Dental UK Ltd, <http://gb.a-dec.com/en/>) a commercial supplier of dental equipment including dental chairs, dental drills, air/water syringes and DUWLs.

6.3.2. *Time span for the biofilm formation in the simulated dental-unit waterline tubing*

The planktonic bacterial counts from the *in-vitro* sDUWL output water in the early phase (1-10 days) demonstrated numbers of bacteria increased resulting in a fully established biofilm community by day 2 and onwards.

The Anderson-Darling normality test (Minitab 16) suggested the data were normally distributed, and the independent samples *t*-test demonstrated a statistically significant result from the sDUWL output water compared to the tap water used as control ($p = 0.0001$) (Fig. 6.2).

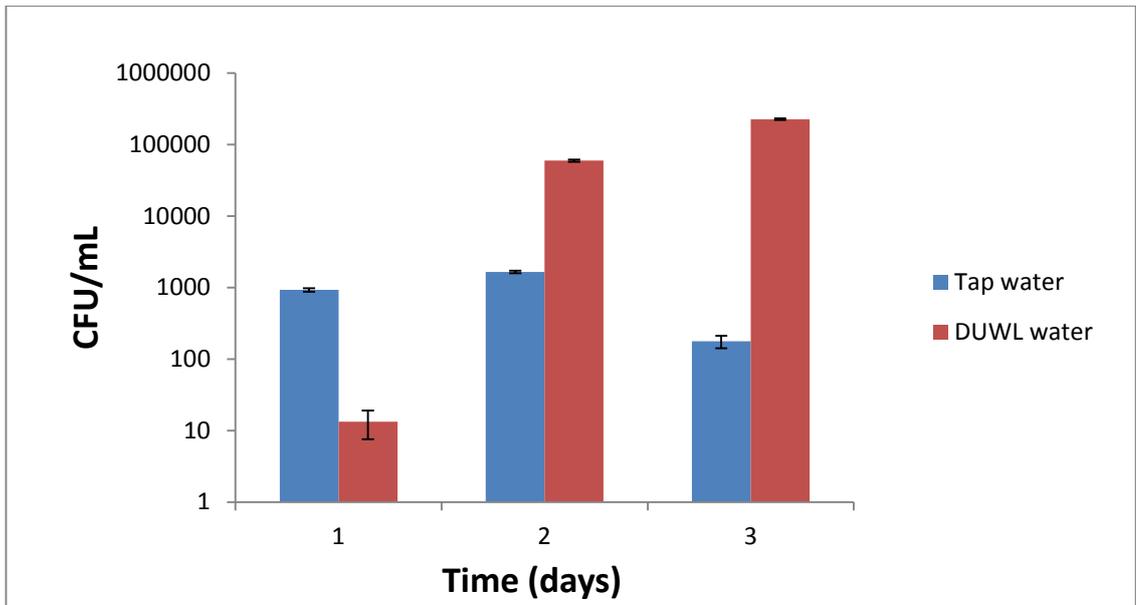


Figure 6.2: Planktonic bacterial counts from the sDUWL output water and tap water (source) during first three days. Difference in CFU/mL count was statistically significant ($p = 0.0001$).

6.3.3. Longer-term monitoring of simulated dental-unit waterline output water

In the early to middle (total 6 months) stages of the biofilm the counts dropped from 10 million CFU/mL to 10,000 CFU/mL and this fluctuation persisted for the entire 6 months (Fig. 6.3).

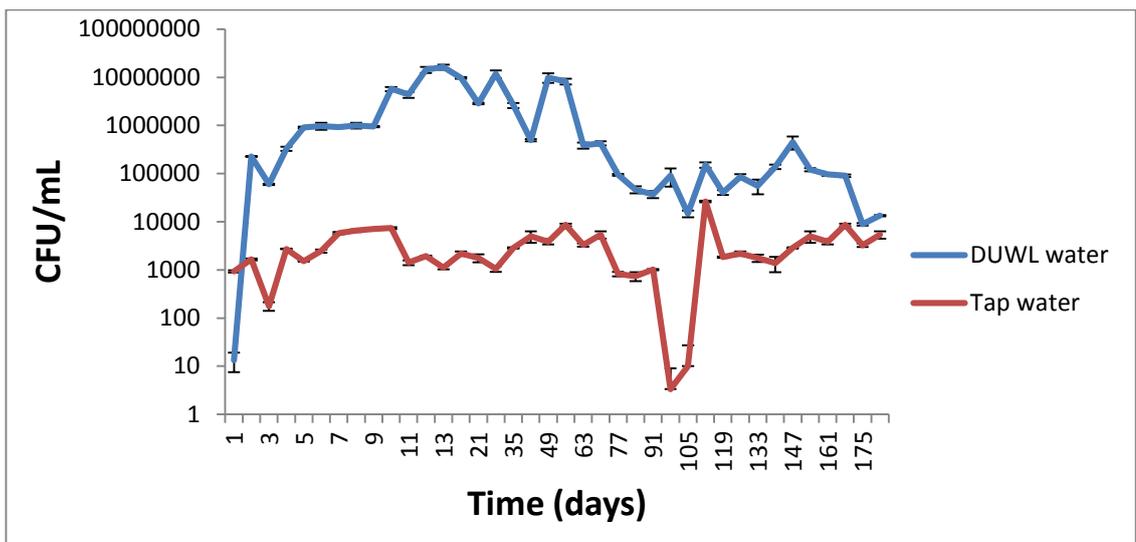


Figure 6.3: Planktonic bacterial counts from the sDUWL output water and tap water (source) for six months.

Next, the degree of linear dependence between CFU/mL count of test and control water samples was tested using the correlation coefficient test on normally (Anderson-Darling) distributed data. The results indicated a negative correlation between fluctuation in the numbers of bacteria from sDUWL output water and tap water samples ($r = -0.079$; $p = 0.402$).

6.3.4. Bacterial succession forming the microbial biofilm within the in-vitro simulated dental-unit waterline

Macroscopically, the plates demonstrated colonies of multiple colours (creamy, white and yellow on R2A medium plates) and glassy colonies (on GVPC) together with their variable size and numbers (not quantified). No growth was observed for *Pseudomonas* spp. on *Pseudomonas* isolation agar or for *Klebsiella* spp. on HiCrome™ *Klebsiella* selective agar media from the test water having initially tested these media on laboratory strains of *P. aeruginosa* and *K. pneumoniae* beforehand as described in chapter 3 section 3.3.1.

The earliest time of testing the biofilm formation was from day 1. The higher planktonic count started from day 2 onwards (Fig. 6.3). The microbes isolated by culture methods were easily maintained in the laboratory and grew well on standard media described.

6.3.5. Isolation and identification of early colonisers in the simulated dental-unit waterline tubing

A total of five early bacterial colonisers were isolated from the wall of the lumen of the tubing biofilm in the first 10 days. Of the 5 bacterial species recovered, three were Gram negative rods, one was Gram variable rod and the other one was Gram positive coccus (Fig. 6.4). The molecular identity of bacterial species isolated from sDUWL was *Acidovorax facilis*, *Leptothrix cholodnii* SP-6 strain, *Mycobacterium chelonae* strain B14, *Herminiimonas saxobsidens* and *M. luteus* (Fig. 6.4 A-E and Table 6.2).

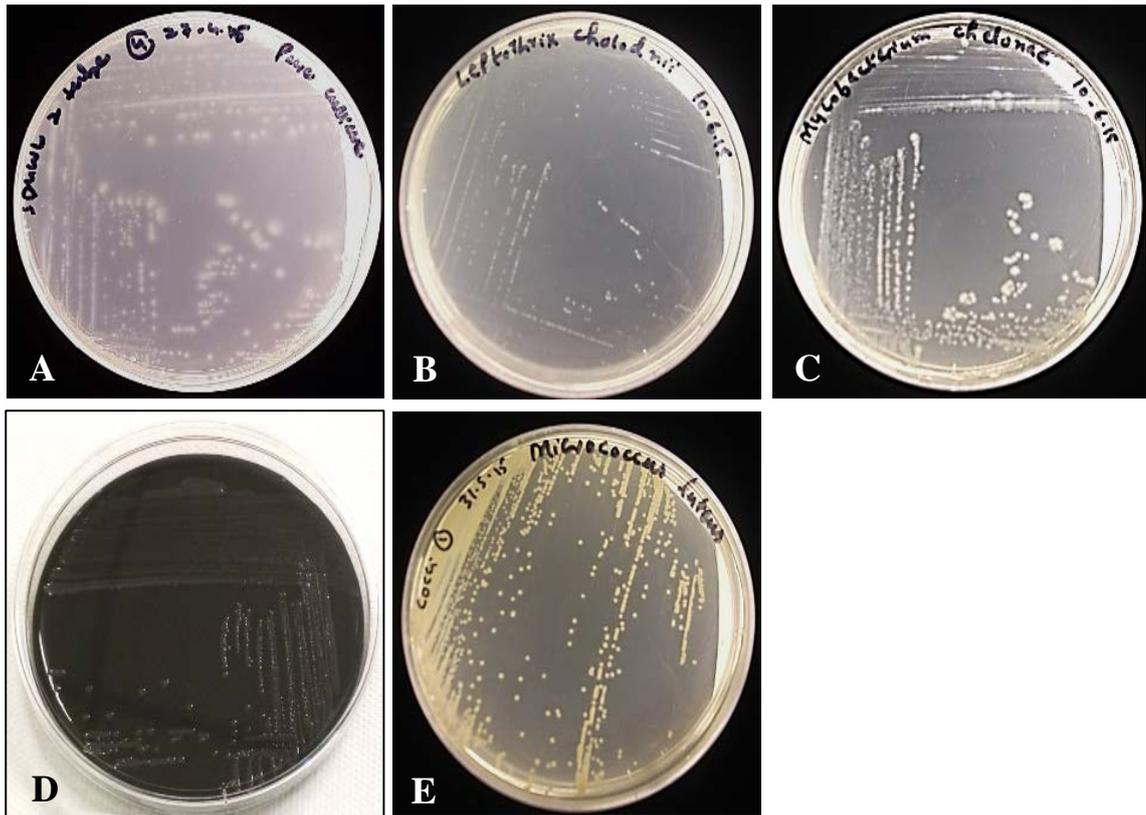


Figure 6.4: Pure cultures of bacterial species isolated from sDUWL. **A)** *A. facilis* on R2A agar, **B)** *L. cholodnii* SP-6 strain on R2A agar, **C)** *M. chelonae* strain B14 on R2A agar, **D)** *H. saxobsidens* on GVPC medium and **E)** *M. luteus* on R2A agar.

6.3.6. Isolation and identification of the opportunistic pathogens dwelling in simulated dental-unit waterline tubing

L. pneumophila (Fig. 6.5, Table 6.2) was isolated after the sDUWL had been running for at least 2+ years. Whereas *Pseudomonas* spp., and *Klebsiella* spp., were not recovered in the entire duration (2+ years) of this study.



Figure 6.5: Colonies from which *L. pneumophila* was identified.

At this stage the entire sDUWL system with its water content was initially decontaminated with 1% Virkon disinfectant overnight. The entire system was destroyed by autoclaving following strict Safety, Health and Environmental guidance rules outlined in the “SHE Intranet, FM SHE 067 Biological and GMO safety booklet (UCLan)”.

6.3.7. Identification of fungi

Macroscopic characteristics

Macroscopically, the colonial texture was velvety and became powdery overtime. The surface colonial colour was blackish green while on the reverse side it was black.

Microscopic characteristics

Microscopically, both hyphae and conidiophore were septate and dark in colour. Conidiophores produced branching acropetal chains of unicellular and smooth conidia (Figure 6.6). All the aforementioned features of the isolate agreed with the known features of *C. cladosporioides* (Onions *et al.*, 1991).

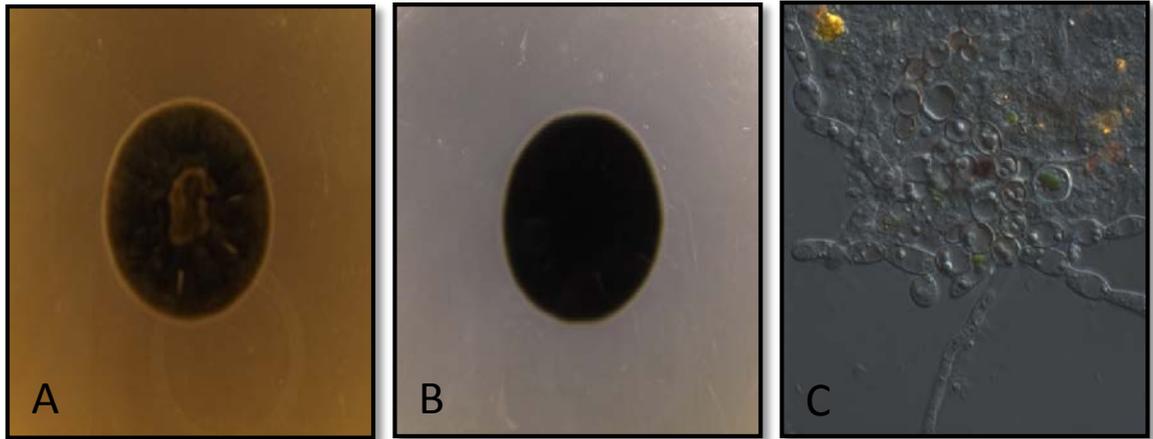


Figure 6.6: *C. cladosporioides* isolated from sDUWL. (A) Front view of colony on plate, (B) reverse view of colony on plate, (C) hyphae and conidial chains under phase-contrast light microscopy.

6.3.8. Identification of protozoa

The amoeba, *V. vermiformis*, (Fig. 6.7) was the same as that identified by Dillon *et al.*, (2014^a) and hence remained with the same accession number CCAP 1534/16 as that already deposited at the Culture Collection of Algae and Protozoa. They were worm-like and displayed motile trophozoites and non-motile encysted forms (Fig. 6.7).

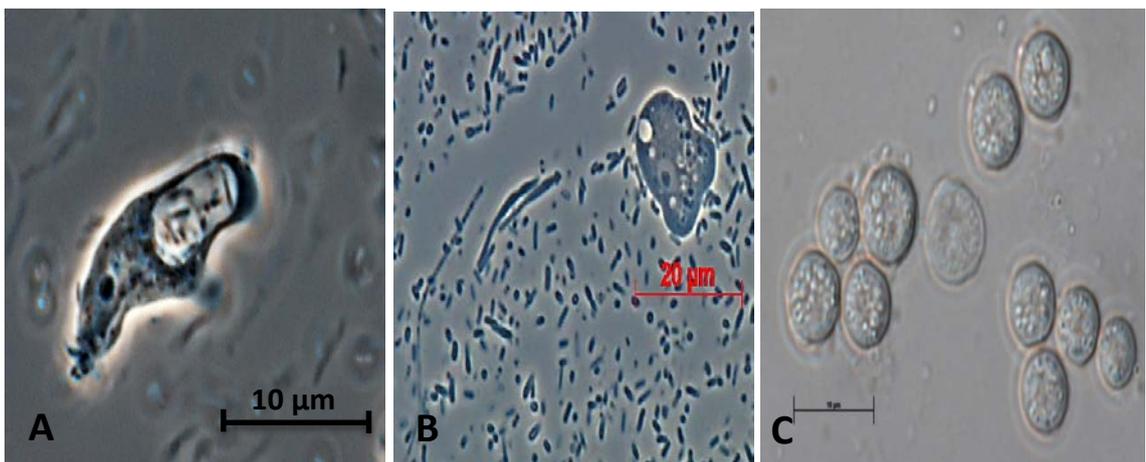


Figure 6.7: Phase-contrast and differential interference contrast microscopy images of possible *V. vermiformis*. (A) The motile trophozoite with bacteria internalised in its food vacuole, (B) a trophozoite showing tracks of its movement, (C) encysted non-motile form with spherical rough surface.

6.3.9. Order of isolation of microbial species from simulated dental-unit waterline

Many microbial species were isolated from sDUWL tubing. Using molecular methodology (for identification of bacteria), and light microscopy (fungi and amoebae) it was noted that the succession of microbial species in the sDUWL biofilm were in the order of **bacteria:** *A. facilis* (2 days), *L. cholodnii* SP-6 strain (3 days) *M. chelonae* strain B14 (5 days), *H. saxobsidens* (5 days), *M. luteus* (6 days); **fungi:** *C. cladosporioides* (13 days); **amoeba:** *V. vermiformis* (14 days); **bacterium:** *L. pneumophila* (after 2+ years) of initial biofilm formation) as summarized in Fig. 6.8.

Table 6.2: Final identity of microbial species isolated from sDUWLs.

	Organism	Gram reaction and Morphology	% Identity	Accession number
	Bacteria			
1	<i>A. facilis</i>	Gram negative rods	100	LC015536.1
2	<i>L. cholodnii</i> SP-6 strain	Gram negative rods	99	LN613119.1
3	<i>M. chelonae</i> strain B14	Gram variable rods	100	JX010972.1
4	<i>H. saxobsidens</i>	Gram negative rods	100	AB681896.1
5	<i>M. luteus</i>	Gram positive cocci	98	NC_012803.1
6	<i>L. pneumophila</i>	Gram negative rod with single polar flagellum	99	NC_002942.5
	Fungi			
1	<i>C. cladosporioides</i>			
	Amoeba			
1	<i>V. vermiformis</i>		99	CCAP 1534/16

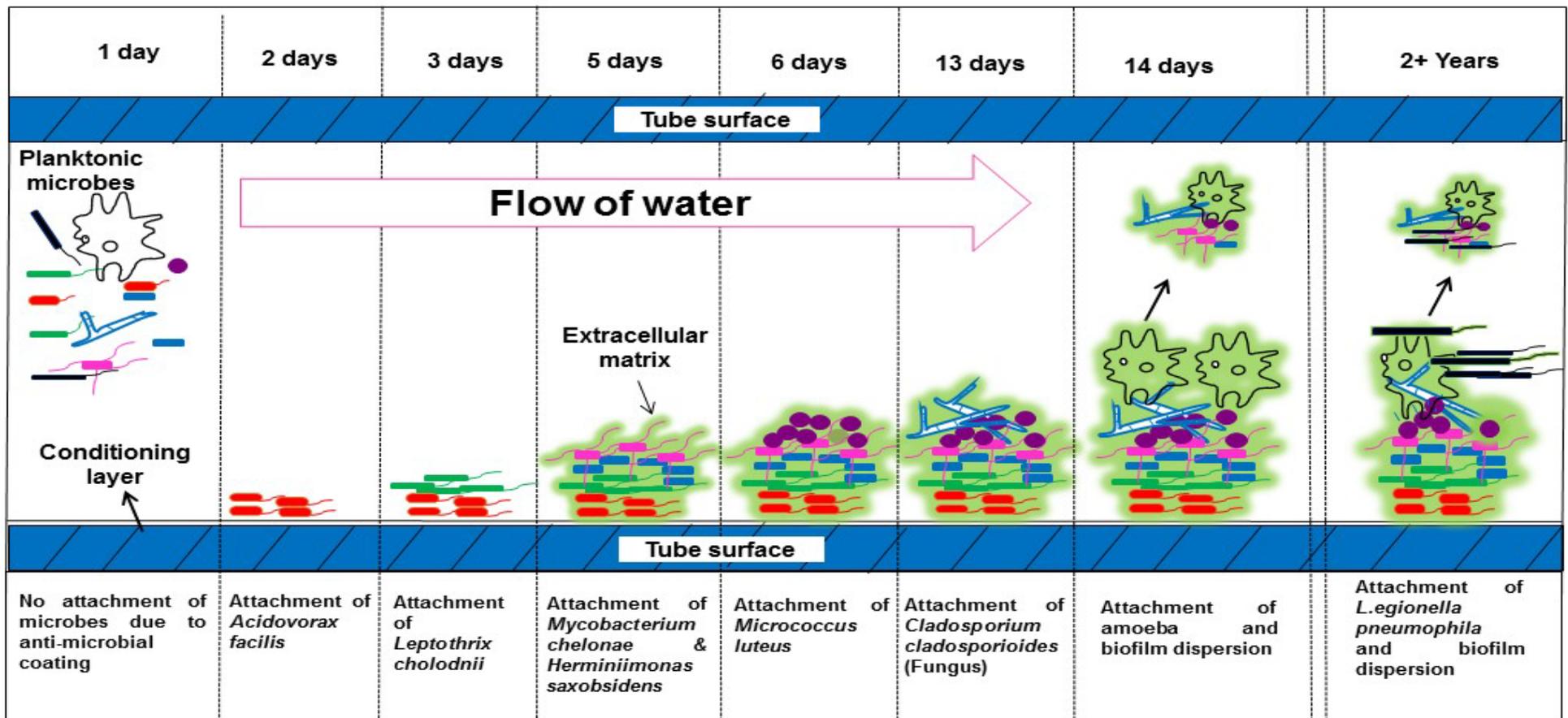


Figure 6.8: Stages of biofilm development in sDUWL. Fully established complex biofilm comprising of bacteria, extracellular matrix, fungi and amoeba.

6.4. Summary

A simulated laboratory dental-unit waterline (sDUWL) system was set up. Water was monitored and the biofilm growing on the wall of the lumen of sDUWL tubes was used to visualize, isolate and identify microorganisms employing microbiological culture and molecular biology, at various time points. The planktonic bacterial counts from the sDUWL output water showed that contamination had occurred by day two and a fully established heterogeneous biofilm community closely mimicking the ecology of clinical DUWLs consisting of bacteria, a fungus within an extracellular matrix with one free living amoeba species by day 14.

6.5. Discussion

The aim of this chapter was to understand waterborne biofilm ecology and environment within simulated DUWL.

The DUWL is an artificial aquatic system, in which the occurrence of a biofilm in relation to polyurethane tubing is to be expected because the tubing is a source of carbon (Nakajima-Kambe *et al.*, 1995) for bacteria that can utilize as a source of nutrients; non-sterile water usually passes through small bore diameter at ambient temperature, which stagnates for long periods when the drills are not being used (Szymanska, 2003; O'Donnell *et al.*, 2011; Barbot *et al.*, 2012; Dallolio *et al.*, 2014). The challenge for the manufacturers of the DUWL systems is to provide longer term solutions to overcome biofilm fouling. To this end, antimicrobial silver coatings (Johnson *et al.*, 1990; Saint *et al.*, 1998; Knetsch and Koole, 2011) have been applied to the polyurethane tubing. However, this investigation using commercial waterline tubing coated with an antimicrobial agent indicated that it was only effective for one day and thereafter failed to control the development of the biofilm. However, the coating may well have affected the cell division of *M. luteus* which appeared to have divided unevenly (as triads and six cells) rather than the expected doublets and tetrads (John *et al.*, 1993) (See chapter 7, Fig 7.5 E).

To measure the degree of linear dependence between sDUWL output water and tap water samples, correlation coefficient test (Minitab 16) was performed which demonstrated a negative correlation between fluctuations in a number of bacteria from test and tap water samples ($r = -0.079$; $p = 0.402$). However, the fluctuation of CFU count observed for the entire six months could be either because of self-cleansing of biofilm in DUWL tubing, or reduced detachment of bacteria from mature biofilm due to the sticky nature of exopolysaccharide which effectively acts as cement holding the biofilm in place (Gupta *et al.*, 2016).

The biofilm grown under laboratory conditions in polyurethane sDUWL tubing was heterogeneous, initiated by Gram negative bacteria made up of rods and cocci, in which one fungal and one amoebal species were the main microorganisms. A heterogeneous biofilm is composed of multispecies of microorganism (Barbot *et al.*, 2012) and is a known feature of the clinical DUWL biofilm community (Tall *et al.*, 1995). The laboratory sDUWL model set up thus, closely mimicked the heterogeneous biofilm development including the type of main microorganisms (bacteria, fungus, and amoeba) as those of the clinical DUWL (Tall *et al.*, 1995; Barbeau and Buhler, 2001; Dillon *et al.*, 2014^a; Kadaifciler and Cotuk, 2014). This suggests that the laboratory DUWL model tested could be used to accurately assess commercial biocides in the control of the biofilm independently as literature continues to question the efficacy of commercial disinfections in waterline cleansing protocols (Dillon *et al.*, 2014^a; Costa *et al.*, 2016). Local audits demonstrate that a high level of contamination is present despite disinfection protocols being used (Pearce *et al.*, 2013).

The biofilm under investigation was initiated by *A. facilis*, formerly known as *Pseudomonas facilis* (Rittenhouse *et al.*, 1973), on the lumen of the waterline. Morphological features documented previously by Kilb *et al.*, (2003) suggests that *A. facilis* is a common water contaminant. However, Kilb *et al.*, (2003) appear not to have definitively identified the bacterium. *A. facilis* has been described as a 'hydrogen Pseudomonad' due its ability to swarm (spread), colonise, and flourish rapidly in a mineral medium that contains hydrogen, oxygen and carbon dioxide with/without any organic matter (Palleroni, 1989). This suggests either *A. facilis* is able to grow chemolithotrophically, or the polyurethane plastic tubing components were leaching out as a supply of nutrients supporting its survival and proliferation (Nakajima- Kambe *et al.*, 1995).

The next early colonizer and culturable organism was *L. cholodnii* with Gram negative characteristics. This is a filamentous, sheathed, bacterium that has the tendency to form globules of poly-hydroxybutyrate in their cytoplasm as a food reserve, which enables them to survive in nutrient-poor environments (Furutani *et al.*, 2012). Thus this bacterium appears highly adaptable to conditions conducive to maintaining the progression in biofilms.

The *M. chelonae* strain B14 was the third successive early coloniser of the sDUWL tubing with Gram variable characteristics and is one of the faster growing *Mycobacterium* species that form biofilms under low (filtered water) and high nutrient conditions (Hall-Stoodley *et al.*, 1998). The genus *Herminiimonas* on the other hand is a relatively new group (Fernandes *et al.*, 2005) to which *H. saxobsidens* has been included a member since 2007 (Lang *et al.*, 2007). This bacterium was the fourth successive coloniser of the sDUWL tubing. There is a paucity of information about this bacterium in relation to its role in biofilm consortia, but this genus of bacteria do appear to survive on rocks alongside of lichens (Lang *et al.*, 2007).

Pathogenic *L. pneumophila* was also isolated from the polyurethane sDUWL tubing, when the biofilm had become fully established and matured over time.

This study reveals that not only bacteria, but fungi and amoebae which are also present in clinical DUWL biofilm community were part of the natural ecology of the experimental biofilm microorganisms that developed within the sDUWL system. This study confirms and supports the finding of Dillon *et al.*, (2014^a) that *V. vermiformis* is part of the natural ecology of biofilm microorganisms that develop within the DUWL systems. *V. vermiformis* has been reported to be the second most commonly found protozoan within DUWLs (Barbeau and Buhler, 2001) and is prevalent in freshwater and artificial water systems (Wadowsky *et al.*, 1988; Nazar *et al.*, 2012). *V. vermiformis* feeds mainly on bacteria with preference for some species over others (Pickup *et al.*,

2007^a). It is reported that *V. vermiformis* can also act as a vector to proliferate *L. pneumophila* (Rowbotham, 1980; Fields *et al.*, 1990; 1993). The benefit to *Legionellae* of using an amoeba host is the protection it affords from adverse environmental conditions and thus an enhanced capacity for distribution to new ecological niches including infecting humans via aerosolised DUWL discharged water (Gross *et al.*, 1992; Bently *et al.*, 1994; Szymanska, 2007).

The early colonisation of the polyurethane tubing by Gram negative and Gram variable phylotypes is interesting and may provide a plausible explanation for their sensitivity to quorum sensing molecules (signalling oligopeptides), or auto inducers (AI) (Pomianek and Semmelhack, 2007). Although, the release of such molecules was not tested, it is generally accepted that through quorum sensing, bacteria can influence the population density and the type of organisms that co-inhabit the same niche by switching on/off their genes accordingly (Reading and Sperandio, 2006; Scutera *et al.*, 2014). The role of sigmoid growth factors may also be involved. Autoinducer type 1 (AI-1) systems are employed by Gram negative bacteria in which the signalling molecule is an acyl-homoserine lactone (AHL) (Reading and Sperandio, 2006; Scutera *et al.*, 2014). Whilst AI-1 allows considerable “cross-talk” between Gram-negative species, it appears that the autoinducer type 2 (AI-2), released by Gram positive bacteria, can act as a common link across many more species (Reading and Sperandio, 2006). The AI-1 signals of various types for intra-species communication can be interpreted by other closely related species (Reading and Sperandio, 2006). The “other closely related species” also appear to be able to detect as well as produce AI-2 signals (Reading and Sperandio, 2006). Thus, *A. facilis* could be expected to respond to both AI-1 and AI-2 by increasing biofilm formation. In the sDUWL biofilm ecology, the Gram positive bacteria observed, such as *M. luteus* may also be capable of detecting and

producing AI-2 allowing precise communication with other bacteria of the same strain using oligopeptides.

The fluctuations in the biofilm monitored for 180 days demonstrates the possibility of a common molecule in the synthesis pathways of AI-1 and AI-2, that is S-adenosylmethionine (SAM), and it is plausible to postulate that changes in the AI-1 pathway causing this resource to be channelled into over production of AI-1 could reduce production of AI-2 in bacteria capable of both, and result in an imbalance in the signalling systems. This imbalance then leads to shedding of biofilm periodically resulting in the negative correlation between control and biofilm contaminated sDUWL output water.

Previous studies indicate that biofilms are a stable point in a biological cycle that includes initiation, maturation, maintenance, and detachment (Kokare *et al.*, 2009; Gupta *et al.*, 2016). Bacteria seem to initiate biofilm formation in response to specific environmental signals, such as nutrient availability. Biofilm continues to develop as long as fresh nutrients are available, but when it is deprived of nutrients, microorganism from the biofilm surface detach and return to a planktonic mode of growth. Apparently, this starvation response allows the cells to search for a fresh source of nutrients and is driven by well-studied adaptations that bacteria undergo when nutrients become limited (Kolter *et al.*, 1993). Very little is known about the self-cleansing step in biofilm development and maturation pathways. One possible signal for detachment of microbial cells from biofilm could be starvation.

Some studies suggest that the enzymes secreted by bacteria themselves promote biofilm dispersion by acting synergically with antibiotics. For example, alginate lyase enzyme has previously been shown to enhance amikacin antibiotic mediated killing of *P. aeruginosa* 144MR (a serum-resistant derivative of *P. aeruginosa* 144M) on the endocardium of live rabbits (Bayer *et al.*, 1992). Boyd and Chakrabarty, (1994) reported

that the alginate lyase enzyme secreted by *P. aeruginosa* may have a role in the detachment phase of biofilm and subsequent cell sloughing. Allison *et al.*, (1998) showed a decrease in *P. fluorescens* biofilm after its extended incubation in alginate lyase enzyme, a result that was partly attributed to the loss of extracellular polysaccharide matrix. Further investigations have highlighted that *Streptococcus equi* secretes hyaluronidase; *P. aeruginosa* and *E. coli* secretes N-acetyl-heparosan lyase to breakdown different types of biofilm matrices (Sutherland, 1999). Alginate produced by *P. aeruginosa* is acetylated and alginate lyase produced by the same bacterium appears less effective on acetylated alginates and more effective on non-acetylated matrices (Wong *et al.*, 2000). In 2006, a study conducted by Alkawash *et al.*, (2006) reported that alginate lyase produced by *P. aeruginosa* acts synergically with gentamycin for successful elimination of mucoid strains of *P. aeruginosa* established in the respiratory tracts of cystic fibrosis patients. However, Lamppa and Griswold, (2013) dispute this effect being attributed to the catalytic activity of the enzyme as use of bovine serum albumin or simple amino acids also leads to the same results. Although none of the above mentioned bacteria were isolated in this study, previous studies suggest that detachment of biofilm is caused by enzymes secreted by bacteria. In this study *V. vermiformis* was isolated from biofilm developed on sDUWL which may have decreased the load of bacteria by feeding on them.

Based on this and earlier studies, biofilm formation can be considered as a well-regulated developmental process that results in the formation of a complex community of organisms. Multispecies biofilm formation in DUWLs suggests the possibility of particular organisms performing specialized roles in the community. Further understanding of bacterial proteases and other enzymes together with a greater understanding of the molecular interactions between different species within biofilms,

will add to our general understanding of the diverse strategies for survival in the microbial world and their control in biofilm formation.

The polyurethane waterline tubing biofilm was predominantly made up of Gram negative rod shaped bacteria. The biofilm was of heterogeneous consortia of microorganisms including bacteria (cocci and rods), fungi and protozoa. Due to *Legionella* species and amoebae residing side by side, it is advised that dentists should perform *Legionella* testing as a precautionary measure and water companies should do more to make sure that the water they supply is free of opportunistic human pathogens such as *Legionella* and *Mycobacterium* species that propagate and become virulent via their amoebal host especially of the *Acanthamoeba* species. The simulated model described here is an excellent model for biocide efficacy testing as it closely replicates the conventional clinical set up.

In this study, the biofilm model was not tested for antimicrobial cleansing because understanding the biofilm diversity and how closely it represented the heterogeneous biofilm of the clinical DUWL over much longer time span (three years) was considered more important due to the current gap in such knowledge. Biocide testing using the simulated DUWL is part of a different project involving manufacture of new nanocomposites in collaboration with Nanotechnology Institute at UCLan. Currently the new antimicrobials are being tested in the laboratory for their minimal inhibitory concentration and/or minimal bactericidal concentration. Once data from preliminary concentrations are made available, they will be applied to the *in-vitro* DUWL model for biofilm cleansing.

Further understanding the morphological features of the species identified in this chapter will add to the general characteristics of these organisms and are described in the next chapter.

Chapter 7. Morphological analysis of the early/late microbial community from the *in-vitro* simulated dental-unit waterline biofilm

7.1. Introduction

The early colonisers develop a number of strategies that allow them to adhere, and spread onto the polyurethane tubing of DUWLs. These strategies or adaptations include the presence of fimbriae for attachment to surfaces (Bullitt and Makowski, 1995), adaptation for greater adhesion onto hydrophobic surfaces (Bendinger *et al.*, 1993), motility for rapid colonization (Korber *et al.*, 1989), and the secretion of an extracellular matrix for protection against desiccation. Colonisers which arrive later in the sequence may possess or develop a different set of strategies (Costerton *et al.*, 1995) which help them to compete in the biofilm. For example, the development of biocide resistance, a feature adopted by nosocomial bacteria such as *S. marcescens* (Maseda *et al.*, 2009). This may enable these bacteria to out-compete the early colonisers and to establish themselves (Donlan, 2002). There is also a possibility that late colonisers have ability to bind with already-adherent cells but do not have ability to attach with the tubing surfaces (Periasamy and Kolenbrander, 2010). This chapter explored the possibility of identifying some of these factors, on the basis of biofilm formation and the characteristics of the resident microbiota using high resolution electron microscopy.

7.2. Materials and methods

7.2.1. Source of reagents

As per chapters 3, 5 and 6.

7.2.2. Electron microscopy

7.2.2.1. SEM for tubing

SEM of simulated *in-vitro* dental-unit waterline (sDUWL) tubing from chapter 6 was performed as described in chapter 5, section 5.2.2.4.1. This was to visualize formation of a biofilm within a section of tubing lumen taken from the sDUWL initially from start of the set up to the end of the study spanning 3 years. The earliest examination was from day 1 to day 14 and then intermittently once a month for up to 1 year and finally once every three months to just over 2 year period in total.

7.2.2.2. SEM of the pure bacterial early colonisers

Bacteria with their known molecular identity (from chapter 6) were also prepared for SEM examination (for methodology see chapter 5, section 5.2.2.4.1).

All specimens were examined and imaged using the FEI Quanta 200 SEM as per Chapter 5.

7.2.2.3. TEM of early/late bacterial colonizers and the mature biofilm from the simulated in-vitro dental-unit waterline

Bacteria of known molecular identity (from chapter 6) were also prepared for TEM examination as described in chapter 3, section 3.2.4.1. TEM images of amoebae were taken directly from the biofilm, 2+ years post its initiation in the tubing used to study SEM morphology.

(Following expiry date of the Don Claugher bursary prize duration (Jan, 2015), specimen grids were sent to Dr Nicola J. Mordan at UCL Eastman Dental Institute for Oral Health Care Sciences, London, UK for analysis. The images were recorded on a Philips CM 120 BioTwin TEM with a Hamamatsu C8484-05G digital camera with AMT V601 software).

7.3. Results

7.3.1. SEM evidence of early microbial biofilm colonisers within the sDUWL tubing

Following examination of the new unused A-dec tubing, a rough luminal surface was observed but this did not equate to any microbial growth. The rough luminal surface of the tubing was taken to have been pre-coated with an unknown antimicrobial agent (Fig. 7.1), a conclusion, subsequently confirmed by the tubing supplier (A-dec, UK) in favour of the antimicrobial coating.

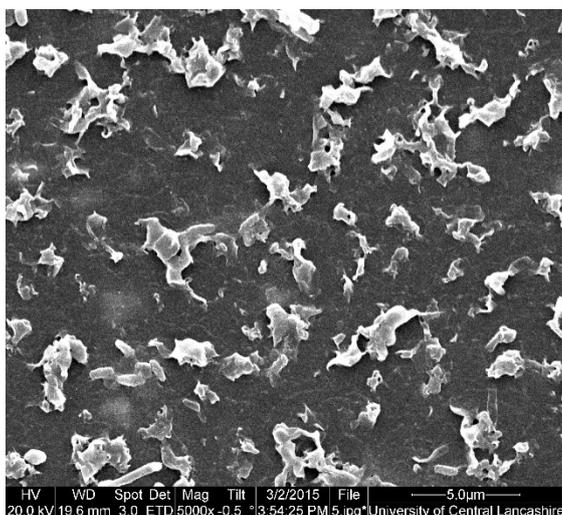


Figure 7.1: SEM micrograph demonstrating rough luminal surface of an antimicrobial coating on the brand new, unused tubing.

SEM examination of the section of experimental tubing from day 1 demonstrated no growth except for the rough antimicrobial coated luminal surface. Only a few bacterial cells adhered to the rough luminal coating from days 2 to 5 and their presence was difficult to demonstrate on the captured images. After 5 days of the biofilm having been initiated, capturing images of the biofilm flora became easier (Fig. 7.2) and overtime, a fully established biofilm could be easily identified.

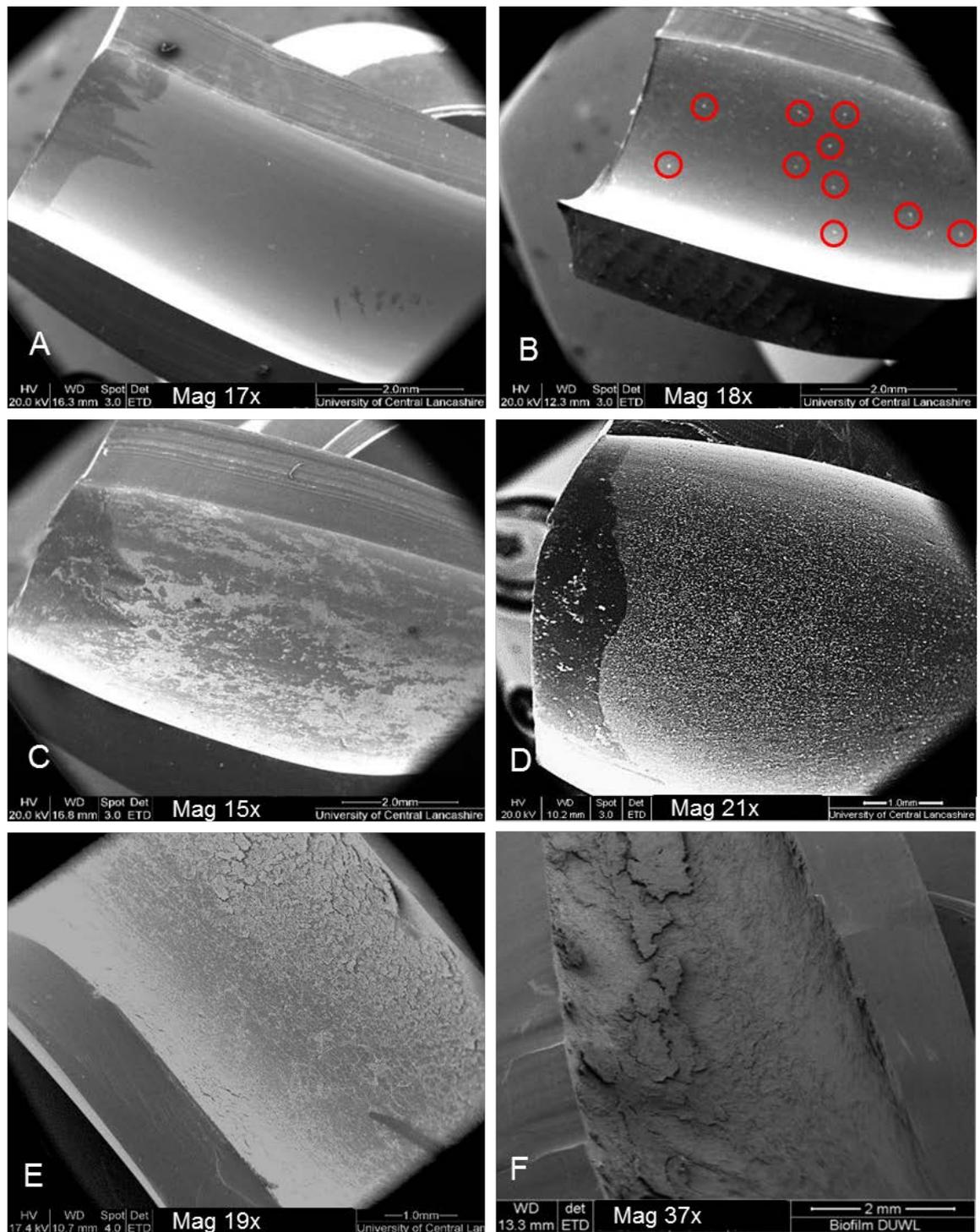
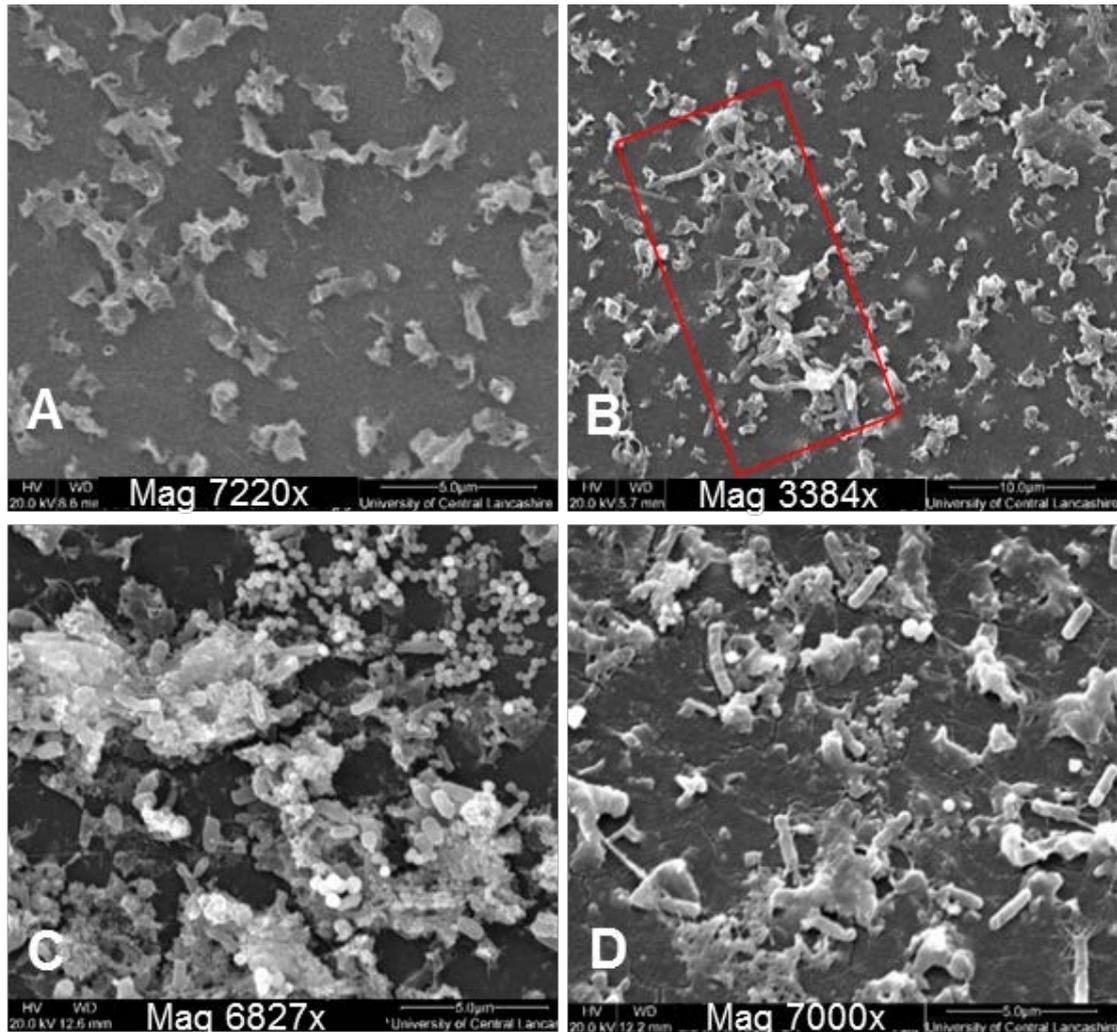


Figure 7.2: SEM of the biofilm within the tubing lumen. **A)** No microbial growth was observed on new tubing before installation, **B)** Few colonies observed after 5 days (circles), **C)** higher density of microbial growth after 14 days, **D)** abundant growth after 3 months, **E)** thick layer of biofilm after 1+ year, **F)** multilayers of biofilm after 2+ years. These images were captured by the investigator at UCLan.

7.3.2. Microbial succession

SEM images confirmed the eventual succession of microbial species in the sDUWL biofilm in the order of bacteria, fungi and amoeba (Fig. 7.3 A-I). Various bacterial morphotypes were also observed such as rods, cocci and spiral shaped organisms (Fig. 7.3 A-I).



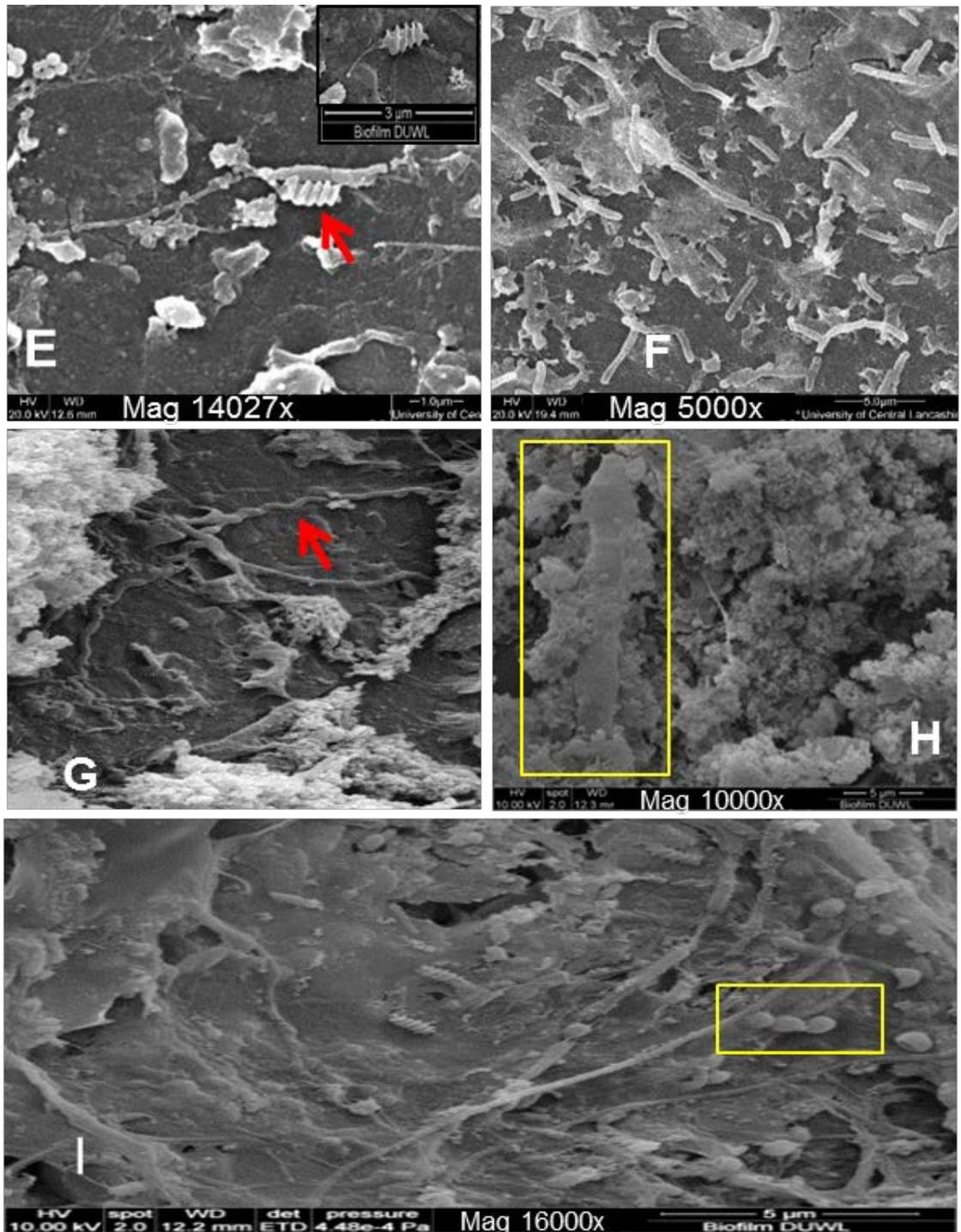


Figure 7.3: SEM micrographs of sequence of biofilm flora colonisation on lumen of waterline tubing, **A**) no bacterial growth after 1st day, **B**) rod shaped bacteria from 2nd to 5th day (Box), **C**) cocci and rod shaped bacteria after 6 days, **D**) cocci and rods with exopolysaccharide matrix after 6 days, **E**) spiral shaped bacteria (possibly an actinomycete spore) after 8 days (arrow), **E1** insert) showing image of spiral shaped bacterium, **F**) long curved rod shaped bacteria after 9 days, **G**) long spiral shaped bacteria noted after 3 months (arrow), **H**) fungal hypha imaged after 1+ years (box), **I**) fully established biofilm with bacteria, fungi and amoeba (box) after 2+ years. These images were captured by the investigator at UCLan.

7.3.3. SEM of early bacterial colonizers

SEM confirmed the morphology of early bacterial colonizers following Gram staining undertaken in chapter 6. *A. facilis* (Fig. 7.4A) and *L. cholodnii* SP-6, strain were small rods (Fig. 7.4B). *M. chelonae* strain B14 bacteria (Fig. 7.4C) were long rods which were slightly curved. *H. saxobsidens* bacteria (Fig. 7.4D) were also long rods but smaller than *M. chelonae* strain B14 but more curved than *M. chelonae* strain B14 with a maggot-like appearance. *M. luteus* bacteria were cocci with groups of four cells (tetrads) (Fig. 7.4E). SEM revealed no obvious surface membrane features such as flagella and/or fimbriae.

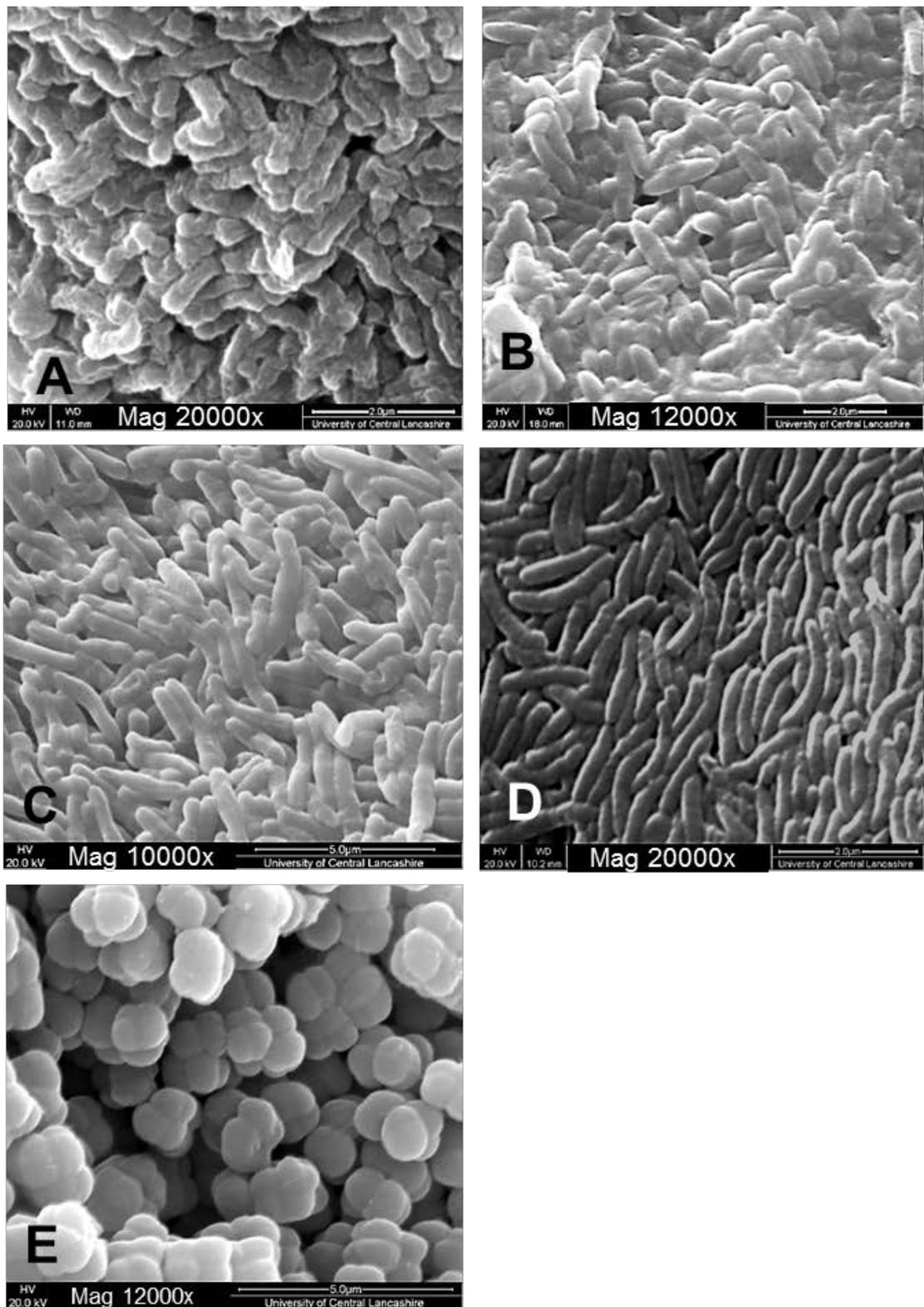


Figure 7.4: SEM of early bacterial colonizers, **A)** *A. facilis*, **B)** *L. cholodnii* SP-6 strain, **C)** *M. chelonae* strain B14, **D)** *H. saxobsidens*, **E)** *M. luteus*. These images were captured by the investigator at UCLan.

7.3.4. TEM of early/late bacterial colonizers

The ultrastructure of *A. facilis* confirmed these bacteria to be rod shaped with approximately 0.74 μm in length (Fig. 7.5A) and an abundance of hair-like projections that resembled fimbriae (Fig. 7.5A, insert A1). *L. cholodnii* bacteria were also rod shaped approximately 1.4 μm in length, and were observed as single cells and not as chains located within a sheath (Fig. 7.5B). One hair like projection (possibly a pilus) was observed connecting two bacterial cells (Fig. 7.5B, small arrow). All cells clearly revealed centrally located nucleoid DNA (Fig. 7.5B, long arrow). The grainy background seen in images (Fig. 7.5B, D) suggested these bacteria were possible active secretors of an extracellular polysaccharide matrix. *M. chelonae* strain B14 were long and rod shaped with uneven ends of approximately 0.8 μm length (Fig. 7.5C). Distinct cell walls and membranes were intact occasionally associated with a hair-like projection (Fig. 7.5C, arrow) suggestive of a possible pilus. *H. saxobidens* was a slow swarmer, and a rod with an ovoid shape (Fig. 7.5D) approximately 1.4 μm in length, which appeared to actively secrete extracellular matrix. Two possible appendages representative of pili were observed on one side of this bacterium (Fig. 7.5D, arrow). *M. luteus* were generally observed as groups of three (triads) (Fig. 7.5E arrow head), four (tetrads) (Fig. 7.5E arrow) and six cells (Fig. 7.5E and insert E1). Two distinct layers on the surface of this bacterium are present in which the outer thicker layer corresponds to peptidoglycan (Fig. 7.5E, insert E1 short arrow) and the inner (thinner) layer as the plasma membrane (Fig. 7.5E, insert E1 long arrow). *L. pneumophila* were predominantly long, rod shaped with a size of approx. 2.9 μm in length. These bacteria clearly exhibited centrally located nucleoid DNA (Fig. 7.5F, short arrows). The actual size of the isolated bacteria and their expected size from literature is given in Table 7.1.

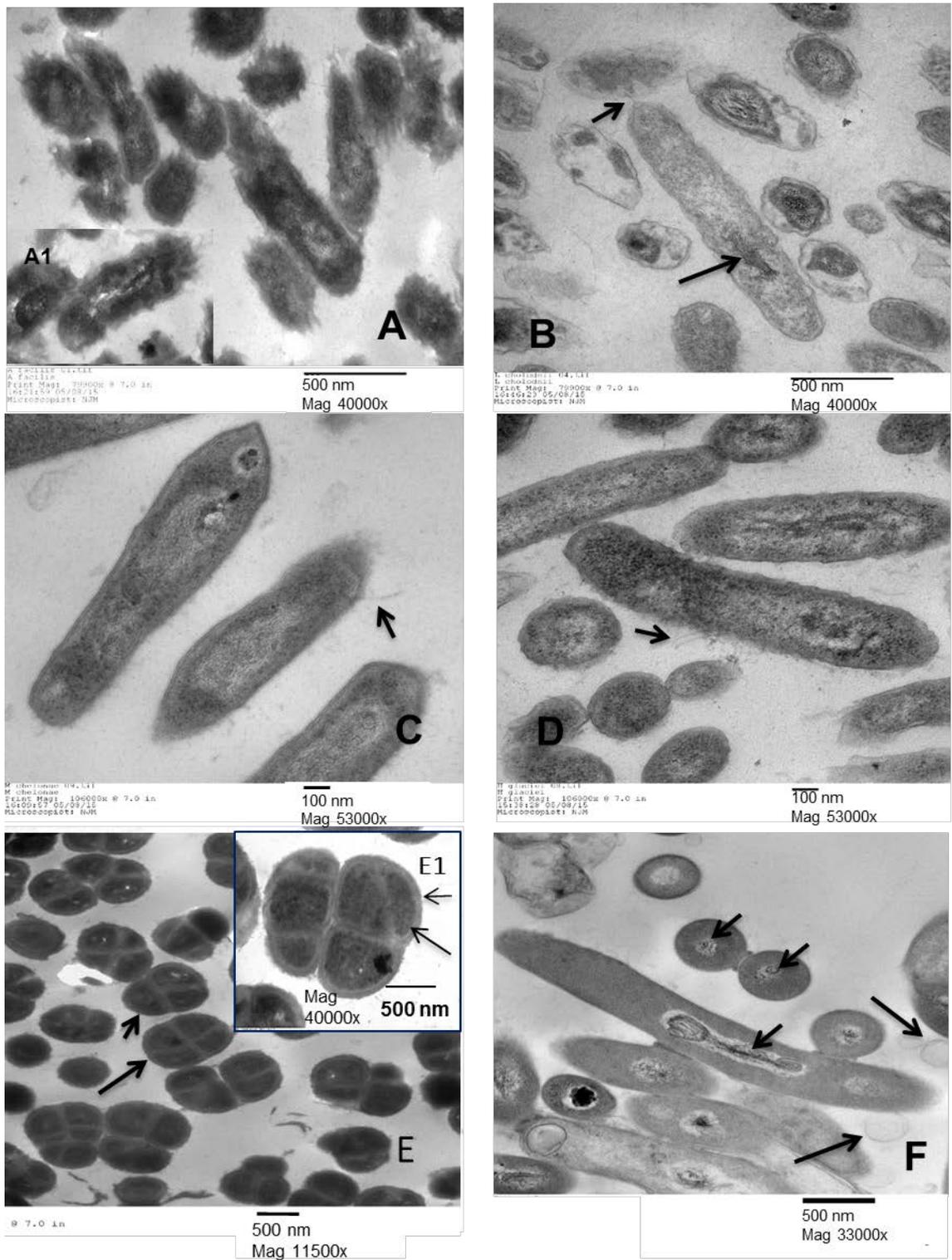


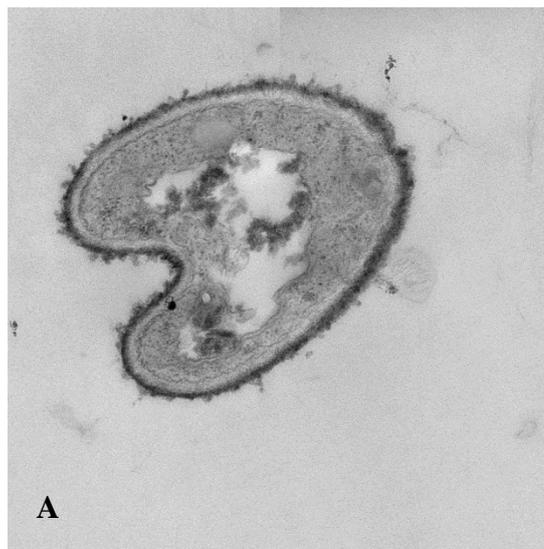
Figure 7.5: TEM of early and late bacterial colonizers, A) *A. facilis*, B) *L. cholodnii* SP-6 strain, C) *M. chelonae* strain B14, D) *H. saxobsidens*, E) *M. luteus*, F) *L. pneumophila*.

Table 7.1: Size of bacteria isolated from sDUWL in this study.

Bacterial specie	Size (µm in length)) (This study)	Size (µm in length) (From literature)
<i>A. facilis</i>	0.74	0.5-4 (Palleroni, 1989)
<i>L. cholodnii</i> SP-6 strain	1.4	1-12 (John <i>et al.</i> , 1993)
<i>M. chelonae</i> strain B14	0.8	1-10 (John <i>et al.</i> , 1993)
<i>H. saxobsidens</i>	1.4	0.8 (Lang <i>et al.</i> , 2007)
<i>M. luteus</i>	0.7	0.5-2.0 (John <i>et al.</i> , 1993)
<i>L. pneumophila</i>	2.9	2.0-20 or more (John <i>et al.</i> , 1993)

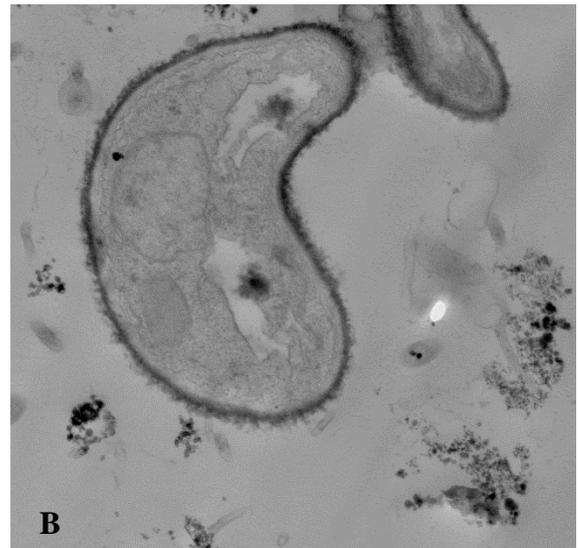
7.3.5. TEM of amoebae isolated from the simulated dental-unit waterline biofilm at 2+ year age

Having isolated a potential human pathogen such as *L. pneumophila* in the sDUWL, it was appropriate to check whether *V. vermiformis*, also sharing the same niche, were supporting their life cycle. In order to do this, a small piece of the biofilm rich in amoebae was examined. TEM demonstrated the typical trophozoite form of amoeba (*V. vermiformis*) without any evidence of bacteria in the food vacuoles and/or in the cytoplasm. The trophozoite form of the amoeba (*V. vermiformis*) displayed finger like projections, pseudopodia and hyaloplasm around the periphery of the cell (Fig. 7.6A). Nucleus and food vacuoles were also apparent (Fig. 7.6B).



A

500 nm
HV=120.0kV
Direct Mag: 33000x



B

500 nm
HV=120.0kV
Direct Mag: 24500x

Figure 7.6: TEM micrographs showing an amoebal trophozoite of *V. vermiformis* with pseudopodia and the hyaloplasm around the periphery of the cell (A), a nucleus and food vacuole visible in some trophozoites (B).

7.4. Summary

Following SEM examination of the test sDUWL tubing, the biofilm developed rapidly from day 2 onwards with several different phylotypes of bacteria (rods, cocci, spiral shaped bacteria, fungi and protozoa) all residing side by side. Presence of fimbriae and pili were noted, confirming some of the early colonising bacteria with strategic factors for biofilm formation. Defective cell division and altered phenotype of specific bacterial species, suggested unusual local environmental condition possibly due the antimicrobial coating on the tubing.

7.5 Discussion

This chapter explored the possibility of identifying the strategies possessed by microorganisms which allow them to attach on tubing of DUWLs. This was achieved by observing biofilm formation and the nature of the resident microbiota of sDUWL tubing.

Results from culture methods in chapter 6 showed fluctuations in CFU counts after some time. However, SEM micrographs in this chapter showed the biofilm becoming thicker and fully established as time progressed. Such an observation had been made by Tall *et al.*, (1995) following their clinical DUWL biofilm formation. A plausible explanation for discrepancies between planktonic bacterial count and observation by SEM of a thicker biofilm suggested a contribution to the low CFU counts from aggregates and microcolonies of bacteria that resist disruption results in colony formation from clumps rather than from single cells and this would result in decreased bacterial count. Another reason for some microbes to be detected by SEM, and not by culture systems could be that those planktonic microbes were non-viable and un-culturable in the laboratory (Stewart, 2012). For example, spiral shaped bacteria were detected by SEM exclusively whereas culturing and molecular analysis in chapter 6 by 16S rRNA universal bacterial gene primers failed their detection.

The early colonisers contributed to the laying down of the polysaccharide extracellular matrix which allows bacterial cells to become scattered within its channels thereby providing extra surface area to volume ratio for biofilm growth, and cell protection against desiccation and toxic substances (Donlan and Costerton, 2002). This could be one reason why the dental biocides fail to control the DUWL biofilm (Costa *et al.*, 2016).

Embedded within the polysaccharide extracellular matrix were spiral shaped *Actinomycetes* species of bacteria known for their earthy-musty smell (geosmin) and taste in drinking water (Wood *et al.*, 1983) and the filamentous fungus *C. cladosporioides*. The presence of *C. cladosporioides* in clinical DUWLs has been previously reported by Kadaifciler and Cotuk, (2014). A plausible explanation for the bacterial-fungal co-existence and interaction could be a provision of biotic support for the longer term establishment of a bacterial biofilm (Hogan *et al.*, 2007; Seneviratne *et al.*, 2008). Their contact and adhesion are said to be important during formation of mixed bacterial-fungal biofilms. Electron microscopy demonstrated bacteria remained outside of *C. cladosporioides* suggesting an ectosymbiotic relationship of these two phylotypes in the sDUWL biofilm community. The biofilm bacterial isolates identified appeared to be on the larger size and an explanation for this phenotypic observation may lie in bacterial-fungal interactions reducing stressors in their environment and bacterial-amoebic interactions in which “if the size fits” result in them being engulfed. Thus bacteria exhibiting physiological differences in size compared to their smaller free-living infective counterparts may be a survival strategy.

The presence of fimbriae and/or pili in some isolated species revealed that these special appendages help them to attach to the substratum so that the bacteria can withstand shear forces and obtain nutrients (Proft and Baker, 2009). Flagella appeared to be missing on the isolates. The reasons for the lack of flagella are unknown, but whether this is a reflection of their habitat in the laboratory maintained biofilm, or that this appendage becomes redundant once the biofilm becomes established, remains to be investigated.

It was alarming to note that *L. pneumophila* and *V. vermiformis* were co-habiting in the same niche where the free living amoebae were grazing on the same biofilm. To confirm whether *V. vermiformis* had ingested *L. pneumophila* and supported their

multiplication, the amoebae directly from the biofilm were examined under the electron microscope for the presence of any metabolically active bacterial cells. The results indicated the presence of healthy amoebae with completely digested debris in their vacuoles without internalised viable bacteria. This suggested that *V. vermiformis* did not act as a vector for *L. pneumophila* despite sharing the same niche of the simulated DUWL. The large size of the *L. pneumophila* seen under the electron microscope also supports this conclusion that *V. vermiformis* did not act a vector for *L. pneumophila*, as it was out-with the size-range of bacteria, amoebae choose to feed upon (Dillon *et al.* 2014^b).

Conclusions

It is important to monitor the DUWL output water on regular basis as their status can change from being clean to being dirty within short periods (4 months in this study). Failure of the units can be prevented by delegating duties concerning the maintenance of DUWLs to responsible staff to ensure that protocols are followed correctly. Staff should keep a log of the date of purging and how long the DUWL has been exposed to disinfectant. Staff should use biocides within their use-by-date. Staff may continue to use the Dip Slide™ test (Accepta Ltd., UK) as it will indicate gross planktonic contamination and will be of use for some dental practices. The 3M™ Petrifilm™ Aerobic Count Plates (3M Food Safety) can be used if the Dip Slide™ test indicates contamination at the 1000 CFU/mL threshold (as this is where most surgeries demonstrated their contamination levels) for a more accurate assessment of the quality of their treatment water. The conventional laboratory tests for monitoring the quality of DUWL output water should be performed once a week initially and then every 3 months, although this timing needs to be determined experimentally.

The relevance of the R2A agar conventional testing protocol may need revision to provide a method which achieves better values for specificity and sensitivity for in-office tests in the clinical setting. The in-office tests currently available are showing that clinically significant contaminants are present in the water discharged from the DUWL. *Legionella* testing should be performed as precautionary measures and water companies should do more to make sure that the water they supply is free of opportunistic human pathogens. This study demonstrates *V. vermiformis* is not a vector for *L. pneumophila* and *P. aeruginosa*. *V. vermiformis* should be considered as an organism which selectively grazes biofilm bacteria especially *E. coli* and *S. marcescens*. However, more research needs to be conducted in order to understand the link between protozoa and

potential pathogens in DUWLs. The simulated DUWL described here is an excellent model for such investigations.

Manufacturers of the dental waterlines should do more to develop antimicrobial coatings on tubing. The coating should possess a broad spectrum of antimicrobial activity to make them resistant to colonization by microbial biofilms. The activity should endure for the life time of the device and should not be reduced by oral fluids. In addition, it should not select for and spread resistance to antibiotics and other antimicrobials.

Researchers need to establish and understand why disinfection protocols may work satisfactorily in some practices and some dental-units but fail, unpredictably in other locations. There is also a need for further in-office testing and to determine whether more rigorous cleansing methods are required. Furthermore, investigation into how the cleansing protocol may be modified should be considered. There is a need to ensure that any protocol is safe for the patient and dental team and does not interfere with clinical treatment. This work also needs to be carried out in close cooperation with the manufacturers of dental-units to ensure that dental-units are not damaged by the biocides.

Although the current microbiological culture in-office tests appear less than adequate, other ways of measuring contamination should also be explored. One culturable method would be to use the R2A medium in conjunction with commercial test such as the Petrifilm™ AC Plate. However, practical limitations in the design and hydration of the medium and shelf-life of such tests may have hindered their progress. Alternatively, non-culturable methods such as measuring a protein component of peptidoglycan/and or DNA concentration from water-borne bacteria by a colorimetric/spectrophotometric/fluorometric analysis of in-surgery test could be explored.

**Guidelines for Infection Control in Dental Health-Care Settings and
Key recommendations resulting from this research project**

Guidelines for Dentists	
Centres for Disease Control (CDC)	
1.	Use water that meets EPA regulatory standards for drinking water (i.e., <500 CFU/mL of heterotrophic water bacteria) for routine dental treatment output water.
2.	Consult with the dental unit manufacturer for appropriate equipment and methods to maintain the recommended quality of dental output water.
3.	Follow manufacturer recommendations for waterline treatment product and monitoring water quality.
4.	Minimize the potential impact of anti-retraction device failure. For this discharge water and air for a minimum of 20-30 seconds after each patient use of devices connected to the dental water system that enter the patient's mouth (e.g., air/water syringes, hand pieces and ultrasonic scalers). This procedure will physically flush out patient material that might have entered the turbine, air, or waterlines.
5.	Consult dental unit manufacturer on the need for periodic maintenance of anti-retraction valves.
6.	Use sterile single use solutions (e.g., sterile saline or sterile water) for surgical procedures.
7.	Use oral surgery and implant hand pieces as well as ultrasonic scalers that bypass the dental-unit to deliver sterile water or other solutions by using single use disposable or sterilisable tubing.
8.	After each patient clean, lubricate and sterilize all dental hand pieces connected to DUWLs.
Department of Health (DoH), UK	
1.	Use sterile single use solutions (e.g., sterile saline or sterile water) for surgical procedures.
2.	Remove self-contained water bottles, flush with distilled or reverse osmosis water and leave open to the air for drying overnight and thereafter store inverted.

3.	Disinfect DUWLs periodically. Consult manufacturer for use of disinfectants. If disinfectants are used, take care to ensure that DUWLs are thoroughly flushed after disinfection and before being returned to clinical use.
4.	Flush DUWLs for at least two minutes at the beginning and end of the day and after any significant downtime (for example, after lunch break). In addition, flush for at least 20-30 seconds between patients.
5.	Make sure that all dental hand pieces, ultrasonic scalers and/or waterlines are equipped with anti-retraction valves.
6.	Sterilise dental hand pieces, ultrasonic scalers in-between patients.
7.	If in-line filters are being used, clean them with appropriate cleansing solution recommended by manufacturer – but always at the end of each session. If the DUWL has disposable filters, replace them daily.
8.	Where monitoring is undertaken, the total viable cell count (TVC) should be expected to lie in the range 100 to 200 colony forming units per millilitre (CFU/ml). In general, incubation should be at around 22 °C.
9.	Test for <i>Legionella</i> species once a year.
Conclusions from this study	
1.	Consistently achieving clean treatment water in the range of 100 - 200 CFU/mL is difficult. If possible, replace older dental-unit waterlines with new ones as the quality of clinical treatment water often fails to meet the standards required. This can be expensive! Use automated disinfection devices as these can reduce the handling of chemicals, increase uniformity of disinfection protocols and decrease the incidence of human error.
2.	Strictly adhere to biocide and maintenance protocols for dental-units.
3.	Retraction valves on the whole appeared to working.
4.	Avoid operator failure, make sure the responsible staff understand the treatment regime of the biocide used for their DUWLs (when to purge and working concentrations for purging and for daily treatment use). Maintain a log counter signed by manager as proof of compliance.
5.	Use biocides within their use-by-date.
6.	Wear protective face clothing e.g. splash guard, face mask. (For staff and patients)

7.	Monitor the quality of waterline output water by Dip Slide™ at least for gross planktonic contamination and by consulting commercial microbiological services for testing DUWL water.
8.	Test for planktonic <i>Legionella</i> species once a year.
Key message for manufacturers of the dental-unit waterlines from this study	
1.	Improve in-use life time of retraction valve. Suggest a test for likely failure.
2.	From an engineering perspective, design “semi-disposable”, but cheaper dental-units that can be replaced once a year with the existing chair.
3.	Design waterlines that can be detached and autoclaved.
4.	Develop antimicrobial coating on DUWL tubing that lasts for long time and possesses broad spectrum of antimicrobial activity.
5.	As a priority, collaborate with researchers to develop a reliable, cheap and user friendly in-office test specifically for dental use.
6.	Collaborate with researchers to develop a reliable modern day <i>in-situ</i> sterilising system that avoids chemical cleansing by operator.
Key message for water companies following this study	
1.	Supply water free of opportunistic human pathogens such as <i>Mycobacteria</i> , <i>Legionella</i> species.
2.	Screen for <i>Acanthamoeba</i> species and any others that promote virulence and multiplication of <i>Legionella</i> and <i>Mycobacteria</i> in waterlines. Provide water free of such microbes.

Future research work

Biofilms are difficult to control and eradicate and the hope for any effective control mechanisms rely upon the ability of scientists to explore multiple lines of enquiry. Thus it has come to light that aspects of research which are important to understanding the factors leading to contaminated dental treatment water, must be clearly understood at the outset of any investigation. These are discussed below.

There is a need to establish how often the water from dental-unit waterlines (DUWLs) should be monitored. Currently there are no guidelines from the Department of Health (DoH), UK on the frequency of monitoring. However this study has highlighted that 4 months in between monitoring the DUWL output water is not long enough. This is because their status can change from being clean to being dirty during this period. An initial study monitoring water once a week for 3 months should perhaps be considered between those clinics that conform to a biocide cleansing regime supported by documented notes. This would allow cause of failure, whether due to a highly contaminated water source, lack of diligence by the operator, loss of biocide effectiveness over time or the build-up of a resistant bacterial population within the system.

There is a high likelihood of retraction-valve failure taking place, as reported by recent study performed in China (Ji *et al.*, 2016). Although retraction valve failure is interpreted as a direct mode of potential human pathogenic bacterial cross contamination of the DUWL tubing/biofilm and output water, this is difficult to demonstrate ‘in practice’ because hand-pieces and any retraction volume trapped inside the retraction valve is sterilised in between treatment. However, there is some controversy about how effective the degree of sterilisation is within the actual tubing. However, what is not clear is how much (if any) of the trapped retraction volume leaks

into the waterline tubing when the rotary action of the drill is interrupted. Further research into establishing potential oral fluid contamination from retraction volume is recommended.

There remains a need to develop a rapid, sensitive and reliable test for monitoring the quality of dental-unit treatment water. According to HTM 01-05: <http://www.dh.gov.uk>, at present, this is not a requirement. HTM 01-05 does not recommend the use of the Dip Slide™ test which dentists have adopted as an aid to monitoring DUWL output water, despite the requirement for maintaining low numbers of mesophilic, heterotrophic planktonic bacterial counts, currently in the 100-200 CFU/mL range, to meet the UK guidelines set by the DoH. It is plausible to suggest that development of a culture-based in-office test that equates to the sensitivity and specificity of the current benchmark R2A culture method would have to be considered. Thus a future in-office test would have to be based on viable counts. Having tested a range of commercial cultural in-office tests, they all appear less than adequate and further research and development is still needed. One possibility would be to combine the concept of the existing commercial test kits from Petrifilm™ AC Plate and Dip slide™, specifically for dental needs. This investigation (Chapter 2) has shown that the design of the Dip slide™ commercial kit was unsuitable and the growth medium on Petrifilm™ commercial kit was more nutritious than R2A medium which is normally used for laboratory growth of waterborne, biocide stressed bacteria.

As to the control of biofilm formation, the currently employed chemical disinfectants seem unreliable. Although, manufacturers of the dental-units should invest in finding tubing that is not prone to biofilm formation, exploring alternative methodologies to eradicate biofilm formed on DUWLs is also essential. Since a biofilm is composed primarily of microbial cells and EPS (extracellular polymeric substances) which immobilises bacteria, their enzymes are capable of breaking down EPS thus

allowing new species to fill the vacant niche as the biofilm matures. Very little is known about the maturation pathways and for periodic renewal of microbial species in biofilms but alginate lyase enzyme secreted by *P. aeruginosa* has been suggested to have a role in cell detachment phase of biofilm (Boyd and Chakrabarty, 1994) and another study reported that *Streptococcus equi* secretes hyaluronidase; *P. aeruginosa* and *P. fluorescens* secrete alginate lyase and *E. coli* secretes N-acetyl-heparosan lyase to breakdown the biofilm matrix (Sutherland, 1999). Some authors have reported that alginate lyase produced by *P. aeruginosa* acts synergically with antibiotics for successful elimination of mucoid strains of *P. aeruginosa* (Bayer *et al.*, 1992; Alkawash *et al.*, (2006). Whether such bacterial enzymes would provide universal application to disrupt biofilms is not clear; although knowledge of such enzymes may lead to their laboratory synthesis and testing to control the biofilm in *in-vitro*. Toxicity studies of these enzymes would first have to be carried out, but when given intravenously to rabbits alginate lyase did not reveal toxicity (Bayer *et al.*, 1992).

In this study it was noted that only when the biofilm had become fully established and matured over time (2+ years), *Legionella* spp. were detected. This indicates that the organisms forming biofilm are likely producing chemicals favoring the growth of *Legionella* spp. establishing later on. Since monitoring of dental treatment water for *Legionella* spp. is a requirement (UK guidelines set by the DoH), it may be of value to investigate if *Legionella* spp. having interdependency with other early colonisers of the biofilm. Controlling less bacteria that *Legionella* spp. may depend on for their colonisation, may be another way of delivering safe treatment water. Growth conditions and the choice of media are the current barriers preventing progress in the understanding of *Legionella* spp. interdependency with other mesophilic, heterotrophic bacteria. Understanding of the virulence factors of early and late colonisers of waterlines is important as inhibitors may be found to control their initial attachment.

V. vermiformis should be considered as an organism which selectively grazes biofilm bacteria especially *P. aeruginosa*. However, more research is needed in order to understand the link between protozoa and potential pathogens in DUWLs and for their beneficial influence on the control of bacterial numbers in DUWL biofilm populations. Thus, ways of genetically manipulating amoebae to feed on bacteria more actively and keep DUWL tubing free from biofilm formation in the future should be explored. The simulated DUWL described here is an excellent model for such investigations and for biocide efficacy.

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Appendices

Document	Title
A1	BSO ethical approval (UCLan)
A2	STEM Ethics Committee approval (UCLan)
A3	NHS ethical approval
A4	Consent letter sent to dental surgeries
A5	Information sheet sent to dental surgeries
A6	UCLan Annual research conference 2014-Abstract
A7	UCLan Annual research conference 2014-Poster
A8	UCLan Annual research conference 2014-Poster nomination letter
A9	Don Claughier bursary prize 2013-Letter
A10	SEMT one day meeting, UCL School of Pharmacy 2014- Abstract
A11	Composition of bacteriological media and reagents
A12	Lal, S. <i>et al.</i> , 2014 Current Microbiology-Manuscript
A13	Lal, S. <i>et al.</i> , 2015 Current Microbiology-Manuscript

A1 - BSO ethical approval (UCLan)

**For Internal Use
Only**

BSO Ref. No.: 1112-02

Date Received: 15th November 2012

**University of Central Lancashire
Biological Safety (Microbes & Genetically Modified Organisms)
– Application for Project Approval**

RISK ASSESSMENT – for the use of micro-organisms, genetic modification or the use of genetically modified micro-organisms (GMMs).

Please note, this document deals with the health and safety issues for projects using micro-organisms or genetically modified micro-organisms (viruses, bacteria, algae, yeasts, protozoans, fungi and moulds). This does not constitute ethical approval which must be obtained from your school Ethics Committee.

For projects using other biological material, animals or animal tissue, health and safety issues should be integral to your project design in accordance with University policy. Ethical approval must be obtained from either your school Ethics Committee or the Animal Projects Committee.

If you have any questions please see the Biological Safety Officer (Judith Smith; email JASmith@uclan.ac.uk)

Please refer to the Safe Code of Working Practice: Genetic Modification - SCWP29.

Please complete ALL sections (failure to do so may delay the approval process):

SECTION I - PROJECT DETAILS.

Project Type:

Staff research	Commercial Project	PhD research	MPhil research	MSc-by research	Taught MSc research	BSc research
		X				

Applicant(s):

Name	Title/Position	Email
Mr Sham Lal	Student	

If student, Name of Supervisor:

Name	Title/Position	Email
Prof. StJohn Crean	(Dean of School) Postgraduate Medical & Dental Education	screan@uclan.ac.uk
Prof. Waqar Ahmed	Professor in Postgraduate Medical & Dental Education	WAhmed4@uclan.ac.uk
Dr Sim Singhrao	Senior Research fellow	sksinghrao@uclan.ac.uk

Project Title:

Developing tests and monitoring the quality of DUWL output water and the clinical environment in dental practices of the North West of UK

Anticipated Start Date:

Month	Year
ASAP	2012-2015

Anticipated Duration of Project:

3 years.

Location of work to be carried out: Note, genetic modification can only be carried out in MB336

Maudland building: room 335 and microbiology classroom

Brief Project Description (in layman’s terms) including the aim of the project (ca. 250 words): Sufficient detail and background should be included to enable the Biological Safety Committee to understand clearly both the nature of the proposed experimentation and methodology (host organism, vector, target DNA/genes etc)

Aim of the project:

Aim of the project is to develop rapid means of testing the quality of water specimens that will be obtained from consenting dental practices from the north west of England and from an experimental *in-vitro* model in the laboratory. The gold standard R2A agar culture method for enumerating bacterial growth, serial dilutions of the same sample will be prepared down to 10^{-5} using sterile water. A fixed volume (100 μ L) of the serially diluted suspension will be inoculated (in triplicate), onto fresh R2A agar plates. All plates will be incubated at 22 °C for five days and the CFU/mL was calculated according to the appropriate dilution factor. In parallel the same samples will be inoculated on a commercial agar based rapid test known as the Pertifilm™ test.

SECTION II – HAZARD IDENTIFICATION: in respect of human health and environmental safety, your proposed work needs to be assigned an activity classification and appropriate control measures.

Does the activity involve micro-organisms or genetic modification?

Yes	No
x	

If NO, you are applying to the wrong committee, please see your school Health, Safety and Ethics committee or Animal Committee for project approval.

If YES, please consider the following:

For the micro-organism/GMO in question, consider if there are any potential mechanisms by which it could represent a hazard to human health and how severe those consequences might be (identify the hazard). Microorganisms of interest are culture-able, aerobic heterotrophic biofilm bacteria. We have not identified the specific species of bacteria that are to be expected from our previous related studies. However, literature suggests *Pseudomonas* spp., *Methylobacterium* spp., *Sphingomonas* spp., *Acinetobacter* spp., and *Legionella* spp. and even free living *amoebae* which could in principle, be present. Although, direct exposure may pose a low level biological hazard to the experimenter, but we have successfully managed 3 related projects by being health and safety conscious at all times and by working in an enclosed cabinet (class II safety air flow chamber) to ensure the exposure is controlled and is limited to the hands only, which should be protected by wearing surgical gloves in the first instance.

Consider the likelihood that, in the event of exposure the micro-organism/GMO could actually cause harm to human health (identify the risk).

The anticipated organisms (listed above) for culture are classified as class II pathogens by the Advisory Committee on Dangerous Pathogens (Health and safety Executive document, <http://www.dh.gov.uk/ab/ACDP/index.htm>). It is our intension to use the class II hood when handling plates and cultures. UCLan has approved facilities (Maudland building) for all microbiology related cultures within the remit of class 2 category of pathogens. We have cultured avirulent form of *L. Pneumophila* in a previous project for which approval was obtained **BSO ref no 0112-01**. In this project, we adhered to good microbiology practice (GMP) which involved wearing protective clothing (lab coat, gloves) and training was given to cover the hazards of the work and the practical use of special procedures, techniques and equipment that are needed to minimise the risks. The organisms to be cultivated grow within bio-films taking root in tubing used by dental practitioners for oral therapy. When their numbers increase, they may cause flu-like symptoms, difficulty in breathing and stomach upsets.

For the micro-organism/GMO in question, consider if it could represent a hazard to the other life forms and the environment and how severe those consequences might be (identify the hazard).

The plates cultivated with the said microbes will be destroyed by autoclaving. This will minimise any potential risks to the environment.

Consider the likelihood that, in the event of exposure the micro-organism/GMO could actually cause harm to other life forms and the environment (identify the risk).

The organisms that are going to be cultured are water bourn and they also live in the soil where they live as a community and there are benefits to the environment. Adverse

effects of their discharge into the environment are very low. The plates cultivated with the said microbes will be destroyed by autoclaving. The waste bags for autoclaving will be clearly labelled for the type of waste and the responsible experimenter who originated it. Waste made harmless by autoclaving will be disposed of via the appropriate route as per UCLan's Health and Safety regulations.

SECTION III – CONTROL MEASURES: safeguards for human health, the environment and the storage and disposal of microbes and GMOs.

Consider the nature of the work to be undertaken and provide details of the controls necessary to safeguard human health during this project.

By working in an enclosed cabinet (class II safety air flow chamber) ensures the exposure becomes controlled and is limited to the hands only, which should be protected by wearing surgical gloves in the first instance. Wearing the laboratory coat and gloves will be mandatory. Training will be provided to cover the hazards of work and in the initial setting up of the experiment and any special procedures to minimise any risks.

Consider the nature of the work to be undertaken and provide details of the controls necessary to safeguard other life forms and the environment during this project.

Bench tops and equipment will be disinfected using Virkon. All loops, spreaders and tips etc will be placed into Virkon disinfection solution which is active against microorganisms. All biohazard/clinical waste disinfected in Virkon will be rendered safe by autoclaving prior to disposal.

Please provide details as to the storage and protection for any microbes or GMOs generated during this project.

The organisms will be cultivated on R2A agar plates as this is a universal solid medium for use with both bacteria and amoebae. The plates with amoebae will be sealed with parafilm and placed collectively into plastic bags. These will be stored at room temperature in a labelled cardboard box.

The bacterial cultures on plates will also be sealed with parafilm and collectively placed into plastic bags and stored at 4 °C.

--

Please provide details as to the disposal of any microbes or GMOs generated during this project.

The plates cultivated with the said microbes will be destroyed by autoclaving. The waste bags for autoclaving will be clearly labelled for the type of waste and the responsible experimenter who originated it. Waste made harmless by autoclaving will be disposed of via the appropriate route as per UCLan's Health and Safety regulations.

Given the control measures in place, assign a Biosafety Level (Class 1- Class 4) and overall level of risk (Low, Medium, High).

Class	Risk
Class II	Low

SECTION IV - HAZARDOUS SUBSTANCES.

Does the activity involve substances hazardous to health?

Yes	no
	x

If YES, please attach relevant COSHH and Risk Assessment documents (please address issues of quantity involved, disposal, and potential interactions as well as a thorough evaluation of minimisation of risk.)

If NO, please continue.

SECTION V - OTHER RISKS.

Does the activity involve lone working or activities or equipment requiring Personal Protective Equipment?

Yes	no
	x

If YES, please attach relevant Risk Assessments.

If NO, please continue.

SECTION VI – DECLARATION.

I certify that this information is correct at the time of submission and I agree to inform the committee of any substantive changes.

Applicant Signature ___Sham Lal___ date__16th Nov, 2012__

Temporary approval granted:

BSO Signature

date

Full approval granted:

_____JASmith_____ _____15th December
2012_____

BSO Signature

date

REVIEW DATE:

The safety considerations and implications for any project of this nature are a continuous and ongoing activity which should be constantly monitored and reviewed by all personnel. This risk assessment must undergo review on an annual basis and any significant alterations to procedures reported to the Biological Safety Officer.

PLEASE KEEP A COPY OF YOUR SUBMISSION FOR YOUR RECORDS.

SECTION VII – DECISION.

The decision regarding the proposal was:

[256]

[Appendices]

	Approved	You may proceed with the research project
X	Approved by Chair's Action	You may now proceed with the research project
	Approved Pending Minor Revisions (see comments below)	You must re-submit the proposal according to the specifications below; once you have done this, the committee chairperson will review the revision and notify you that you may proceed
	Requires Major Revision (see comments below)	The proposal must be revised extensively and resubmitted to the committee as a whole
	Rejected (see comments below)	The research proposal is not acceptable
Comments:		
Project approval granted by chairs action. As the microbes that will be isolated during this project have not fully been characterised project safety must be reviewed on a regular basis and any significant changes reported to the BSO.		

A2 - STEM Ethics Committee approval (UCLan)



31 January 2013

StJohn Crean / Sham Lal
School of Postgraduate Medicine and Dental Education
University of Central Lancashire

Dear StJohn / Sham

Re: STEM Ethics Committee Application
Unique reference Number: STEM100

The STEM ethics committee has granted approval of your proposal application '**Developing tests and monitoring the quality of DUWL output water and the clinical environment in dental practices of the North West of UK**'. Ethical approval is contingent on any health and safety checklists (e.g. lone working risk assessment) having been completed, and necessary approvals as a result of gained.

Please note that approval is granted up to the end of project date or for 5 years, whichever is the longer. This is on the assumption that the project does not significantly change in which case, you should check whether further ethical clearance is required.

We shall e-mail you a copy of the end-of-project report form to complete within a month of the anticipated date of project completion you specified on your application form. This should be completed, within 3 months, to complete the ethics governance procedures or, alternatively, an amended end-of-project date forwarded to roffice@uclan.ac.uk together with reason for the extension.

Please also note that it is the responsibility of the applicant to ensure that the ethics committee that has already approved this application is either run under the auspices of the National Research Ethics Service or is a fully constituted ethics committee, including at least one member independent of the organisation or professional group.

Yours sincerely

Paola Dey
Deputy Vice Chair
STEM Ethics Committee

A3 - NHS ethical approval



Direct Dial: 01282 644812
Email: paula.cooper@eastlancspct.nhs.uk
Our Ref: SE22/SL/MP
Date: 18th January 2013

Walshaw House
Regent Street
Nelson
BB9 8AS

Mr Sham Lal (University of Central Lancashire)
Dr Mark Pearce
Barrowford Dental Practice,
59 Gisburn Road,
Barrowford,
Nelson,
Lancashire
BB9 8ND

Dear Mr Lal/Mark,

Thank you for providing information regarding your project '*Developing a novel test for monitoring and analysis of the quality of DUWL output water and in clinical environments in dental practices of the North West of UK*', undertaken by the University of Central Lancashire. I have considered the information you have provided and can confirm that this project meets the criteria set by the National Research Ethics Service indicating that the work should not be managed as research under the Research Governance Framework for Health and Social Care 2005.

In this instance the work is to be managed as service evaluation and does not require NHS research ethics approval or formal research governance approval. In this regard the project can be undertaken within NHS Lancashire and any contracted sites therein with permission from each dental practice.

Details of your project will be retained by R&D personnel and I would be grateful to receive notification of any outputs as well as when the active participation in the project has ended. If you have any queries regarding compliance with the conditions set out herein, please do not hesitate to ask. Similarly if there are any access issues that require assistance relating to the completion of the project please contact me.

Good luck with the project

Yours sincerely

A handwritten signature in purple ink that reads "P. Cooper".

Paula Cooper
RESEARCH AND DEVELOPMENT MANAGER

Chairman: Peter Kenyon
Chief Executive: Janet Soo-Chung

A4 - Consent letter sent to dental surgeries



**UNIVERSITY OF CENTRAL LANCASHIRE (UCLan)
Consent form**

Dean, of School: Professor St John Crean

(BDS, MBBS, FDSRCS, FFGDP(UK),

FRCS FRCS(OMFS), PhD, FHEA)

Mr Sham Lal (PhD student, Oct 2012-Sept-2015) slal1@uclan.ac.uk

Practice Name _____

1. I agree to take part in the above service evaluation.

Please initial boxes

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason.

Practice owner/manager _____

Date _____

Project leader ___ Prof. St J Crean _____

A5 - Information sheet sent to dental surgeries



**UNIVERSITY OF CENTRAL LANCASHIRE (UCLan)
Information sheet**

Dean, of School: Professor St John Crean

(BDS, MBBS, FDSRCS, FFGDP(UK),
FRCS FRCS(OMFS), PhD, FHEA)

Mr Sham Lal (PhD student, Oct 2012-Sept-2015) slal1@uclan.ac.uk

Prof. St John Crean (Director of Studies and project leader) screan@uclan.ac.uk

Mr Mark Pearce BDS, MCDH, DDPH, Lead GDP for R&D, NHS East Lancashire,
(Advisor of the research project), oliver.pearce@tiscali.co.uk , Mobile:
07826040959

Dr Sim Singhrao C Biol, MI Biol, M Phil, PhD, AFHEA.,FHEA., PGCert (supervisor
and Senior Research Fellow) sksinghrao@uclan.ac.uk, 01772 895137

26th January, 2013

Dear Practice Owner,

**RE: A service evaluation of the Petrifilm™ Test for its potential use in
monitoring DUWL output water and for assessing the clinical environment**

As part of my PhD degree I wish to invite you to taking part in a service
evaluation about testing water discharged from dental units and take swabs
from the clinical environment to assess the occupational health risk from
water-borne bacteria directly related to the water from dental units.

A recent local (2012) service evaluation using the Dip test alongside of
conventional means of testing water demonstrated that 78% of water
discharged from dental units had more than the suggested maximum measure
for bacteria contamination as advised by the Department of Health (DoH) 200
CFU/mL despite having protocols in place to maintain clean waterlines. This

state of affairs poses a health risk to patients and the dental team while at the same time indicating that time and money is being wasted. A quality control system is needed to show if protocols are being effective. While regular testing of water samples in a laboratory is one way of ensuring clean water, it is inconvenient and expensive.

This recent study highlighted that the Dip Slide™ test, was not sensitive enough at the 200 CFU/mL level advised for dentistry. Another commercial test called the 3M™ Petrifilm™ aerobic count plates (3M Healthcare Ltd UK/Ireland) method is suggested to “fit the dental in-office needs” - as its range of sensitivity lies between 0 - 1,000 CFU/mL and it is very cost effective (around 50p a test). This project as a whole sets out to discover if the simple Petrifilm™ test is a valid quality control tool. As a first step we want to see if the Petrifilm™ test is sensitive enough to detect if the water discharged from dental waterlines is clean as defined by HTM01-05. To this end I would like to ask the advisor of my studies Mark Pearce to visit the practice and collect 50 mL of water from the units. I would also like to see if the disease causing bacteria from the discharged water lingers on clinical surfaces for which there are a need to take swabs. Mark Pearce has kindly offered to do this on my behalf as that will provide complete confidentiality and anonymity of the consenting dental practices. The visits would not take long and there is no financial implication to any of the participating practices. The collection of water samples and swabs could easily be accomplished between patients to minimize any inconvenience for the practice and at a time to suit you.

All samples will be made anonymous by designating a code number by the time they reach the microbiology lab at UCLAN for analysis using the Petrifilm™ test alongside the standard test for water-borne bacteria. Though using a key, we plan to inform individual practices of their results and if any units are contaminated offer a retest (free of charge) once changes in protocol have been made, to see if they are effective. We plan to ask about 10 local practices in East Lancashire to take part and these practices will be informed about the overall findings.

I hope you are able to help and, if you are, I would be very grateful if you would sign the consent below. If you have any questions please feel free to contact the following supervisory team members:

Yours sincerely,

Mr Sham Lal

(PhD student at UCLan)

A6 - UCLan Annual research conference 2014-Abstract

RESEARCH & INNOVATION OFFICE ANNUAL RESEARCH CONFERENCE 2014

Surname:	LAL
First name:	SHAM
Email address:	slal1@uclan.ac.uk
Award	PhD
Mode of Study:	FT
Year of Study:	4

Poster Presentation

Title
DOES <i>VERMAMOEBEA VERMIFORMIS</i> SUPPORT THE PROLIFERATION OF NOSOCOMIAL BACTERIAL PATHOGENS IN THE ECOLOGICAL NICHE OF DENTAL-UNIT WATERLINE?
Author(s)
Sham Lal ¹ , Sim K. Singhrao ¹ , Undine E.M. Achilles-Day ² , L. H. Glyn Morton ¹ , Mark Pearce ¹ , StJohn Crean ¹ ¹ Oral & Dental Sciences Research Group, School of Medicine and Dentistry, University of Central Lancashire, ² Culture Collection of Algae and Protozoa, Scottish Association for Marine Science (SAMS), Scotland.
School/Centre
School of Medicine and Dentistry
Abstract
Introduction: The free-living amoeba <i>Vermamoeba vermiformis</i> has been found in dental-unit waterline (DUWL) biofilm ecology ¹ . Amoebae can act as "breeding grounds" for some bacteria. This study set out to ascertain if <i>V. vermiformis</i> supported the multiplication of freshly isolated bacteria from clinical dental water. Methods: The DUWL output water was tested on <i>Legionella</i> selective-medium plates. The emerging bacterial colonies from three dental-water units, DUWLs 9, 10, and 21 were sub-cultured and subsequently identified using molecular methods. Colonies isolated from DUWLs 9 and 10 together with <i>P. aeruginosa</i> were tested for their sensitivity to standard dental biocides at the recommended concentrations. In addition, <i>V. vermiformis</i> isolated previously from a DUWL ¹ was cultured for eight weeks on bacteria from DUWL 9 as well as laboratory strains of <i>P. aeruginosa</i> and <i>Escherichia coli</i> (XL blue). To determine if <i>V. vermiformis</i> supported growth of these bacteria, presence of bacteria inside the amoeba and/or within their encysted form was examined using electron microscopy as described elsewhere ² . Results: A clinical isolate of <i>Serratia marcesens</i> was identified from DUWL 9, <i>Phyllobacterium myrsinacearum</i> from DUWL 10, and <i>Mycobacterium llatzerense</i> that failed to grow from DUWL 21. All biocides, tested under laboratory conditions, were able to control <i>S. marcesens</i> , and <i>P. myrsinacearum</i> alongside of <i>P. aeruginosa</i> . <i>V. vermiformis</i> grew well on all these bacteria with an equal preference for feeding on <i>S. marcesens</i> ($p = 0.0001$), <i>P. aeruginosa</i> ($p = 0.0001$), and <i>E. coli</i> ($p = 0.0001$). No evidence for bacterial cells within the encysted amoebae was observed by ultrastructure. Conclusions: <i>V. vermiformis</i> are not acting as a vector to support the proliferation of the nosocomial pathogen <i>S. marcesens</i> and the laboratory strain of <i>P. aeruginosa</i> . References: 1. Dillon A et al., (2014 ^a) International Biodeterioration & Biodegradation 88:97-105. 2. Dillon A, et al., (2014 ^b). International Biodeterioration & Biodegradation 90:1-7.

Does *Vermamoeba vermiformis* support the proliferation of nosocomial bacterial pathogens in the ecological niche of dental-unit waterlines?



Sham Lal*, Sim K. Singhrao, Undine E.M. Achilles-Day¹, L. H. Glyn Morton, Mark Pearce, StJohn Crean
 Oral & Dental Sciences Research Group, School of Medicine and Dentistry, University of Central Lancashire, Preston, PR1 2HE.
¹Culture Collection of Algae and Protozoa, Scottish Association for Marine Science (SAMS), U.K. *Email: slal1@uclan.ac.uk

I N T R O D U C T I O N

Infection control measures are important in dentistry. A biofilm will invariably form in Dental-Unit Waterlines (DUWLs) which supply water to the instruments required during clinical treatment (Fig. 1). This water enters the mouth of patients and in the process aerosols are generated, these can spread opportunistic respiratory pathogens to patients and dental health workers alike.

The Department of Health, in the UK is aware of this problem and recommends that water discharged from DUWLs should be of the same quality as domestic drinking water (between 100-200 cfu/mL).

This study focused on a free-living amoeba *Vermamoeba vermiformis* which dominates the DUWL niche¹. Due to its non-selective feeding habits within the biofilm community *V. vermiformis* may inadvertently contribute to transmission of disease.

T h e A I M

The aim of this study is to provide recommendations on safe water in clinical services and prevent potential occupational health and public outbreaks of disease.

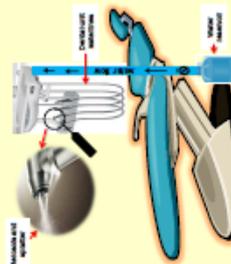


Fig. 1 A typical dental chair with waterlines 100-200 cfu/mL.

O B J E C T I V E S

- Test water samples from dental practices (N=31).
- Isolate and determine identity of any pathogenic bacteria.
- Test for biocide resistance.
- Cultivate *V. vermiformis* and determine their growth characteristics with appropriate bacterial cultures.
- Examine *V. vermiformis* for presence of bacteria in their cell bodies.

M E T H O D O L O G Y

The methodology involved the culturing of microorganisms, molecular biology to identify the isolates and electron microscopy to evaluate the potential of *V. vermiformis* to disseminate bacteria by internalisation as described previously².

R E S U L T S

Molecular identity of isolated bacteria

This study identified *Serratia marcescens*, *Phytobacterium myrsinacearum*, and *Mycobacterium fortuitense*³.

¹Not nosocomial pathogen therefore further studies were not conducted.
²“Screw for up to 2 weeks only under laboratory conditions and further studies were not possible”

Morphology of the isolated *S. marcescens*

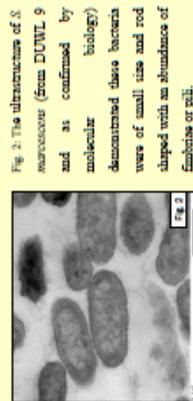


Fig. 2 The ultrastructure of *S. marcescens* (from DUWL 9) and as confirmed by molecular biology demonstrated these bacteria were of small size and rod shaped with an abundance of fimbriae or pili.

The effect of dental biocides on *S. marcescens* isolated from DUWL water

S. marcescens was non-pigmented and was not a biocide resistant strain as all the dental biocides tested were highly effective at killing this bacterium at 1x10¹⁰ cfu/mL.

The growth characteristics of *V. vermiformis*



Fig. 3a and b: *V. vermiformis* cultured in the laboratory
 a) Phase contrast image and b) scanning electron microscope image

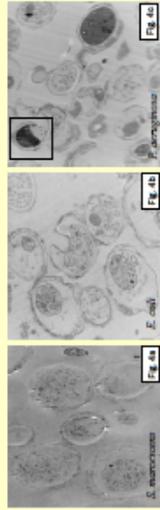


Fig. 4: The vegetative and the encysted forms of *V. vermiformis* following feeding with *S. marcescens* (Fig. 4a), *Escherichia coli* (Fig. 4b) and *Pseudomonas aeruginosa* (Fig. 4c).

Healthy bacterial cells were not observed within the trophozoital amoebae and/or encysted amoebae with one exception of *V. vermiformis* feeding on *P. aeruginosa* (Fig. 4c, box).

C O N C L U S I O N S

- *V. vermiformis* prefers to feed on *S. marcescens* rather than acting as a vector to support its life cycle and/or transmit associated disease.
- This study shows *V. vermiformis* appears to feed on *P. aeruginosa* but not propagate it as suggested elsewhere³.
- *V. vermiformis* may beneficially influence/control biofilm populations in DUWLs.
- This is a promising outcome for infection control in the dental treatment.

R E F E R E N C E S

1. Oller A, et al., (2014) International Biodeterioration & Biodegradation 96:97-105.
2. Oller A, et al., (2014) International Biodeterioration & Biodegradation 96: 1-7.
3. Calvert E, et al., (2008) Letters in Applied Microbiology 47: 405-417.

A8 - UCLan Annual research conference 2014-Poster nomination letter



Preston PR1 2HE
Telephone 01772 201201
www.uclan.ac.uk

UCLan
Research Development & Support
Preston
PR1 2HE

13th December 2014

Dear Sham Lal

RE – Research Student Conference 2014

We are delighted to inform you that your Poster competition entry came in our top 3 of the judge's panel. Huge congratulations for this as the competition was of a high calibre with some interesting and educational posters.

We hope you will enter next year's conference and produce more excellent work.

Yours sincerely

A handwritten signature in blue ink, consisting of the initials "P.P." followed by a stylized, scribbled signature.

Emma Sandon-Hesketh
Head of Research Development & Support



A9 - Don Claugher bursary prize 2013-Letter



Society of Electron Microscope Technology

Hon. Secretary:
Mr. D.E. McCarthy
The School of Pharmacy
29-39 Brunswick Square
London WC1N 1AX
Tel : 020 7753 5806

Email: d.mccarthy@ucl.ac.uk

Date: 18/12/2013

Don Claugher bursary prize 2013

Recipient: **Sham Lal**

Please find the enclosed cheque for **£160.00** which is a bursary prize awarded by the Society of Electron Microscope Technology (SEM).

Yours sincerely

A handwritten signature in black ink, appearing to read 'D E McCarthy', with a horizontal line extending from the end of the name.

David E McCarthy
Hon. Secretary SEMT

The Don Claugher Bursary Winners

Microbial analysis of dental-unit waterline biofilm ecology

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All surgeries depend on dental-unit waterlines (DUWLs) to supply water to dental drills and 3 in 1 syringes during clinical treatment. A DUWL is essentially a complex of tubes and valves that supplies clean water from a reservoir at one end to a dental drill or syringe at the other end, on demand.

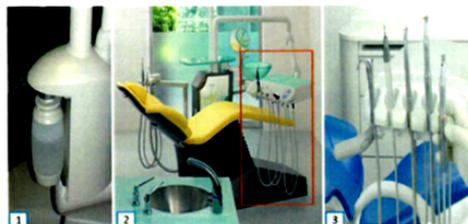


Fig. 1 Dental chair and its parts (1: water reservoir; 2: A typical dental chair, red box denotes dental-unit waterlines or tubing (DUWLs); 3: Various high speed dental hand-pieces attached to DUWLs for dental treatment. (Images are taken from www.google.com)

In-between treatments, the water within the tubing remains stagnant and this allows the development of a biofilm that can survive recommended biocidal disinfection regimes used to clean the lines. Biofilms consist of a consortium of microorganisms, some of which can detach from the biofilm during the hydrodynamic conditions and shearing forces occurring within a water distribution system. Clumps of microorganisms that slough off from the biofilm become re-suspended in the water and thereby decrease the quality of output water from the DUWL. The Department of Health (UK) takes this problem very seriously and expects water from the DUWL to be of the same quality as that of drinking water.

When following the biocide manufacturer's recommendations, the bacterial contamination of the water samples when measured using the culture method on R2A agar medium can vary significantly, ranging from clean (≤ 200 cfu/mL) to heavy contamination (1×10^6 cfu/mL). Out of 31 DUWL output water samples tested 16 (52%) exceeded the Department of Health, UK recommended threshold of ≤ 200 cfu/ml. The remaining 15 samples (48%) met these standards. These results were reported to the participating dental practices with an additional re-test offered following cleansing procedures for their DUWL systems. The re-test results demonstrated that despite

the additional efforts to clean the waterlines 10 (32%) of the water samples still exceeded the UK recommended threshold and two samples from surgeries that originally met the UK's standards now exceeded the recommended threshold. These findings indicate that it is important to regularly monitor for the quality of DUWL output water.

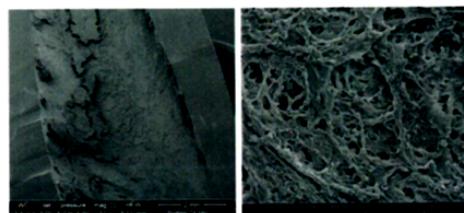


Fig. 2 SEM image of dental-unit water line tube.

Fig. 3 SEM image of biofilm within the lumen of the dental-unit waterline

The research next explored ways to develop more sensitive and rapid methods of testing the water from dental units. More recent research work has explored the potential of retraction valve failure to cause cross-contamination of DUWLs with human pathogens. The results indicated the retraction valves were not failing.

The amoeba, *Vermamoeba vermiformis* is associated with DUWL biofilm ecology where they feed on mixed communities of bacteria. During grazing, they may come into contact with, and feed upon pathogenic bacteria such as, species of *Legionella* and *Mycobacteria*. Once engulfed, these pathogens can survive within the amoebal cell vacuoles and are given sanctuary from unfavourable environmental conditions and a place to multiply. The contaminated output water might be introduced into the oral cavity of immunocompromised patients or inhaled by dental professionals from the resultant aerosol. This constitutes a serious concern in the healthcare system and represents the greatest challenge to health care providers who are responsible for maintaining the quality of water and thus, for preventing potential risks in public outbreak of disease.

Work from our laboratory previously reported amoeba, *V. vermiformis*, having been isolated from a clinical and a simulated experimental model of DUWL. Currently experiments are providing information as to the diversity of these protozoa contributing to the incidence, propagation and protection from biocides of pathogens that share the same ecological niche in the DUWL. The significance of this research lies in providing safe water in clinical services and the prevention of potential occupational health and public outbreaks.

A11 - Composition of bacteriological media and reagents

Table 1: Composition of R2ATM Medium (Lab M) Catalogue No. LAB163.

Ingredients	Grams/Litre	Ingredients	Grams/Litre
Yeast extract	0.5	Meat peptone	0.5
Casamino acids	0.5	Glucose	0.5
Starch	0.5	Dipotassium hydrogen phosphate	0.3
Magnesium sulphate	0.05	Sodium pyruvate	0.3
Agar	15		

pH: 7.2 ± 0.2

Directions for R2ATM Medium preparation: Weigh 18 g of powder and dispense in 1 Litre of deionized water. Allow to soak for 10 min. Swirl to mix and sterilise by autoclaving at 121 °C for 15min. (If required bring to the boil to dissolve the agar, and pour into smaller volume before sterilising). Cool to 47 °C and pour into sterile petri dishes. Do not leave this medium molten at 47 °C for more than 3h before use.

Table 2: Composition of *Pseudomonas* Isolation AgarTM (sigma/Fluka) Catalogue No.17208.

Ingredients	Grams/Litre	Ingredients	Grams/Litre
Peptic digest of animal tissue	20	Magnesium chloride	1.4
Potassium sulphate	10	Triclosan (Irgasan)	0.025
Agar	13.6		

pH: 7.0 ± 0.2 at 37 °C

Directions for preparation of *Pseudomonas* Isolation AgarTM: Suspend 45.03 g in 1 Litre of distilled water containing 20 mL glycerol (Fluka 49767). Boil to dissolve the medium completely. Sterilise by autoclaving at 121 °C for 15 min.

Table 3: Composition of HiCrome™ *Klebsiella* Selective Agar Base™ (Sigma/Fluka) Catalogue No. 90925.

Ingredients	Grams/Litre	Ingredients	Grams/Litre
Peptone, special	12	Yeast extract	7
Sodium chloride	5	Bile salts mixture	1.5
Chromogenic mixture	0.2	Sodium lauryl sulphate	0.1
Agar	15.0		

pH: 7.1 ± 0.2 at 25 °C

Table 4: Composition of *Klebsiella* Selective Supplement™ (Sigma/Fluka) Catalogue No. 15821.

Ingredients	Per Vial (For 500 mL medium)
Carbenicillin	25 mg

Directions for preparation of HiCrome™ *Klebsiella* Selective™ medium: Suspend 20 g in 500 mL of distilled water. Heat to boiling to dissolve the medium completely. Do not autoclave. Cool to 45-50 °C and aseptically add the rehydrated contents of 1 vial of *Klebsiella* Selective Supplement (Fluka 15821). Mix well and pour into sterile petri plates.

Table 5: Composition of Sabouraud dextrose agar™ (Oxoid) Catalogue No. CM0041.

Ingredients	Grams/Litre	Ingredients	Grams/Litre
Mycological peptone	10	Glucose	40
Agar	15		

pH: 5.6 ± 0.2 at 25 °C

Directions for preparation of Sabouraud dextrose agar™: Suspend 65 g in 1 Litre of distilled water. Bring to the boil to dissolve completely. Sterilise by autoclaving at 121 °C for 15 min.

Table 6: Composition of Nutrient agar™ (Oxoid) Catalogue No. CM0003.

Ingredients	Grams/Litre	Ingredients	Grams/Litre
Lab-Lemco powder	1	Yeast extract	2
Peptone	5	Sodium chloride	5
Agar	15		

pH: 7.4 ± 0.2 at 25 °C

Directions for preparation of Nutrient agar™: Suspend 28 g in 1 Litre of distilled water. Bring to the boil to dissolve completely. Sterilise by autoclaving at 121 °C for 15 min.

Table 7: Composition of Nutrient broth™ (Oxoid) Catalogue No. CM0001.

Ingredients	Grams/Litre	Ingredients	Grams/Litre
Lab-Lemco powder	1	Yeast extract	2
Peptone	5	Sodium chloride	5

pH: 7.4 ± 0.2 at 25 °C

Directions for preparation of Nutrient broth™: Add 13 g in 1 Litre of distilled water. Mix well and distribute into final containers. Sterilise by autoclaving at 121 °C for 15 min.

Table 8: Composition of Malt extract agar™ (Lab M) Catalogue No. LAB37.

Ingredients	Grams/Litre	Ingredients	Grams/Litre
Malt extract	30	Mycological peptone	5
Agar No. 2	15		

pH: 5.4 ± 0.2 (if XO37 is added pH 3.5-4.0)

Directions for preparation of Malt extract agar™: Weigh 50 g of powder, disperse in 1 Litre of deionised water, allow to soak for 10 minutes, swirl to mix then sterilise at 115 °C for 10 minutes. If the addition of XO37 Lactic Acid is required this should be done after sterilisation. One 5 mL vial of XO37 will lower the pH of 250 mL of medium to 3.5-4.0. Cool to 47 °C before making additions and pouring plates.

Table 9: Composition of Malt extract brothTM (Lab M) Catalogue No. LAB159.

Ingredients	Grams/Litre	Ingredients	Grams/Litre
Malt extract	17	Mycological peptone	3
Agar No. 2	15		

pH: 5.4 ± 0.2

Directions for preparation of Malt extract brothTM: Weigh 20 g of powder and disperse in 1 litre of deionised water. Allow to soak for 10 minutes, swirl to dissolve and dispense into final containers. Sterilise by autoclaving at 115 °C for 10 minutes.

Table 10: Composition of LB agarTM, Miller (Fluka) Catalogue No. BP1425-500.

Ingredients	Grams/Litre	Ingredients	Grams/Litre
Tryptone	10	Yeast extract	5
Sodium chloride	10	Agar	15

Adjust pH to 7.2

Directions for preparation of LB agarTM: Suspend 40 g in 1 Litre of purified water.

Table 11: Composition of LB brothTM, Lennox (Fluka) Catalogue No. 1288-1650.

Ingredients	Grams/Litre	Ingredients	Grams/Litre
Tryptone	10	Yeast extract	5
Sodium chloride	10		

Adjust pH to 7.2

Directions for preparation of LB brothTM: Suspend 20 g in 1 Litre of distilled water.

Directions for preparation of Reynolds lead citrate TEM stain

Add 2.66 g of lead citrate and 3.52 g of trisodium citrate in distilled water up to 60 mL. Stand for 30 min at room temp with occasional shaking in between. Add 16 mL of N-NaOH and wait for solution to become clear. Make final volume = 100 mL with distilled water. Store the solution at 4 °C in a dark glass bottle. Filter before use. This solution can be used for up to 3-6 months.

Monitoring Dental-Unit-Water-Line Output Water by Current In-office Test Kits

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Abstract The importance of monitoring contamination levels in the output water of dental-unit-water-lines (DUWLs) is essential as they are prone to developing biofilms that may contaminate water that is used to treat patients, with opportunistic pathogens such as species of *Legionella*, *Pseudomonas* and others. Dentists and practice staff are also at risk of being infected by means of cross-infection due to aerosols generated from DUWL water. The unit of measurement for the microbial contamination of water by aerobic mesophilic heterotrophic bacteria is the colony-forming unit per millilitre (cfu/ml) of water. The UK has its own guidelines set by the Department of Health for water discharged from DUWL to be between 100 and 200 cfu/ml of water. The benchmark or accepted standard laboratory test is by microbiological culture on R2A agar plates. However, this is costly and not convenient for routine testing in dental practices. A number of commercial indicator tests are used in dental surgeries, but they were not developed for the dental market and serve only to indicate gross levels of contamination when used outside of the manufacturer's recommended incubation period. The aim of this article is to briefly review the universal problem of

DUWL contamination with microbial biofilms and to update dental professionals on the availability of currently available commercial in-office monitoring systems for aerobic mesophilic heterotrophic bacteria and to discuss their limitations for testing water samples in assuring compliance with recommended guidelines.

The Formation of Biofilms in Dental-Unit-Water-Lines or (DUWLs) and the Need to Monitor Water Used for Treatment

During the past few decades, infection control practices in dentistry have changed significantly. The basis of dental infection control is to create, and maintain, a safe clinical environment and to remove, or reduce, as much as possible, the risk of disease transmission between patients and dental healthcare workers. It has been recognised for some time that dental treatment water delivered by DUWLs can be contaminated by microorganisms originating from the water supply and the human oral fluids [1, 4, 5, 42, 44, 45, 72].

DUWLs provide water via a network of small-bore tubing to the high-speed dental hand-pieces, three-way air and water syringes, and the ultrasonic scaler [11, 35]. The water is used to cool the tooth surfaces, for rinsing debris from teeth, and for oral rinsing by patients [48].

The Centers for Disease Control and Prevention (CDC)—a US federal agency—and the American Dental Association—the largest dental association in the US—have recommended that the output water from DUWLs should contain <500 aerobic mesophilic heterotrophic bacteria/ml [29]. This number is based on recommendations for levels of heterotrophic bacteria in potable water [1, 2]. They also recommend that the DUWL water should

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be monitored routinely to maintain bacterial counts within a safe range and to assess the effectiveness of DUWL. At present, this can only be reliably performed by conventional microbiological techniques. However, in-office monitoring systems would provide a cheaper practical option as a preliminary aid to monitoring DUWL output water in the dental practice premises.

The aim of this article is, therefore, to provide an overview of the universal problem of DUWL output water contamination and update dental professionals to the various currently available commercial in-office monitoring systems. Discussing their limitations and usefulness will provide choices for the dental practitioner while selecting the best test to adopt with the ultimate aim of keeping the planktonic bacterial load down.

What is Special About Dental Unit Water Biofilm Organisms When Testing for Water Contamination?

The problem of bacterial contamination of DUWL output water was first recognised in the early 1960s [9] and is supported to date by others [12, 13, 35, 51, 62, 65, 68]. The consequence of a high colony-forming unit per millilitre (cfu/ml) count of heterotrophic bacteria in water is considered as a significantly increased risk of legionellosis [45, 57].

Typical dental units are equipped with different types of plastic tubing that can extend for up to 10 m. The internal diameter of this tubing is usually approximately 0.5 mm, and inside the small lumen of such narrow bore tubing, water flows freely at the centre leaving a thin layer of undisturbed water around the walls [1, 8]. This allows for the formation of a conditioning pellicle of chemicals on the inner walls of plastic tubing which, over a very short time (days), promotes the attachment of microorganisms. In addition, high surface area/volume conditions, suitable temperatures and long-term stagnation of water (night and at weekends) in tubing provide an active planktonic population of bacteria, which together with microorganisms from the oral cavity, results in a significant microbial population with the consequences of a rapid biofilm formation along the entire tubing [1, 8].

Biofilms are made up of a sessile, heterogeneous consortium of microbial cells that are irreversibly attached to a substratum or interface or to each other [17]. These microflora become embedded in a self-secreted complex exopolysaccharides [17, 70] containing dead microorganisms, and inorganic materials derived from the supply water and oral fluids [1, 31]. With the passage of time, planktonic microorganisms and sections of biofilm continuously detach, and are discharged in the DUWL output water during dental treatment [37].

The CDC recommended [2] that infection control measures should be included in dental practices to keep the bacterial load down [2, 29, 71]. Several proprietary biocides are commonly used to maintain clean DUWLs: a few of them appear insufficient in targeting the entire biofilm microflora [16], whilst others are partially effective in controlling bacterial numbers [30, 55] or when the biocide is unable to act on the biofilm matrix [61]. This can happen if protocols for disinfection are not adhered to [41, 71], if the biofilm organisms develop natural resistance to biocides used [38, 41], or when disinfection protocols are not working (i.e. expired) [10]. Inadequate dosing of water systems with biocides may provide time for organisms to adapt to the biocide by phenotypic/genetic changes and/or form a more complex biofilm in which to protect themselves via a mechanism known as quorum sensing [48].

Quorum sensors, within a biofilm population, play a role in stress-tolerating factors, such as control of cell division, growth rate and metabolic activity [17], as well as in the development of multiple drug resistance [17, 31]. For example, acyl homoserine lactones, a class of autoinducer-signalling molecules used by Gram-negative proteobacteria, are responsible for intra-species communication [17]. They are also thought to up-regulate efflux pump genes which enable cells to pump out antimicrobials from the cell, further contributing to antibiotic resistance [17]. Biofilms are tolerant to phagocytosis through the ability of members of its biological community to produce toxins that rapidly kill incoming immune cells. For example, *Pseudomonas aeruginosa* has been shown to produce glycolipid rhamnolipid which lyses phagocytic cells attacking the biofilm [48].

Microbes Frequently Found in DUWL-Associated Biofilm

A diverse range of organisms have been isolated from DUWL output water [1, 16, 23, 43, 57, 60]. Respiratory diseases that are of concern include *Legionella* spp., *P. aeruginosa* and non-tuberculosis *Mycobacterium* species [5, 18, 49, 52].

Legionella Species

Legionella pneumophila are the aetiological agents of legionellosis [34] of which serotypes I and II are highly virulent forms for humans [5, 52] (Table 1). It is reported that the droplet sizes of 5 µm and smaller, which are generated by the aerosol, carry around 1,000 cfu of *L. pneumophila* which have the capacity to penetrate deeply into the lungs via inhalation [20, 53]. Aerosolisation of water [24, 25] is an inevitable part of dental treatment and

Table 1 Summary of health risks associated with dental-unit-water-lines

Organisms	Medical causes	References
<i>Pseudomonas and Proteus</i> species	Rhinitis	[11]
<i>Pseudomonas aeruginosa</i>	Oral abscesses	[33]
<i>Legionella pneumophila</i>	Humoral responses initiated	[51]
<i>Legionella dumoffi</i>	Pneumonia/legionellosis	[5]
<i>Mycobacterium gordonae</i>	Endocarditis	[45]
Non tuberculosis Mycobacterium	Cervical lymphadenitis	[32, 72]
Bacterial endotoxins	Asthma, inflammation due to acute phase cytokine release, hypersensitivity pneumonitis	[43, 46, 50]
<i>Acanthamoeba</i>	Ocular keratitis	[6]
<i>P. aeruginosa</i>	Acute purulent maxillary sinusitis	[14]
<i>Legionella pneumophila</i>	Legionellosis	[52]

is also a recognised mode of exposure of dental professionals to pathogenic bacteria [59].

Domestic hot water systems harbour 6–30 % *Legionella* [58] suggesting mains water supply may be a typical source of *Legionella* within DUWL's. Legionellae require a temperature range of 20–45 °C to multiply in the DUWL environment, and the CDC advises ambient temperature to be maintained in dentistry, whereas previously it was quite common for the water supply to 3 in 1 syringes to be heated to make its use more comfortable for the patient. The incidence of *Legionella* is increased by the presence of a host usually amoebae which browse on microbial biofilms containing Legionellae as a source of nutrients [64].

Pseudomonas Species

Amongst the Pseudomonads, *P. aeruginosa* is the most frequently found bacterium in DUWL water. This is because it has the ability to survive and grow in a low nutrient environment such as water. In addition, this organism is resistant to a wide range of biocides and antibiotics [5, 7]. It can cause pneumonia-like disease in elderly or immunocompromised individuals. The infective dose of this bacterium for colonisation in a healthy individual is $>1.5 \times 10^6$ cfu/ml [39]. Such high numbers of these bacteria are rarely found in DUWL water [54]; however, due to antibiotic resistance, patients become more sensitive to this opportunistic pathogen. The true impact of infection by *P. aeruginosa* from DUWL output water was demonstrated by Martin [33] following dental treatment and subsequent hospitalisation of the infected

patients. This serious outcome enabled the CDC to enforce effective cleaning measures in dentistry where the use of biocides has become essential [71].

Non-tuberculosis Species of *Mycobacteria*

These are opportunistic pathogens causing pneumonia, cutaneous and systematic infections. These organisms are frequently transmitted through environmental sources such as the ingestion or inhalation of water, particulate matter via aerosols, or through trauma [19]. The presence of these organisms in biofilms and in DUWL water reflects the original source of mains water supply [42, 65, 67]. It has been reported that a number of non-tuberculosis *Mycobacteria* in DUWL water exceeds that of drinking water by a factor of 400 [56]. The matter of concern is that a large number of non-tuberculosis *Mycobacteria* present in DUWL water may be inhaled and contaminate oral wounds of immunosuppressed patients due to therapy and/or are HIV-positive during dental treatment and cause colonisation and infection [19]. Two cases of cervical lymphadenitis following dental extraction and prosthetic heart valve infection with *M. gordonae* have been reported [32, 69].

Bacterial Endotoxin Levels in DUWL Water

Bacterial endotoxin made up of lipopolysaccharide released from the cell walls of live and dead Gram-negative bacteria have also been found from DUWL water at levels ranging from 500 to 2,560 endotoxin units/ml [22, 43]. The generally accepted range for irrigation devices in the USA is 0.06–0.5 endotoxin units/ml and is regulated by the US federal government [29, 63]. Bacterial endotoxin can cause local inflammation, high grade fever and shock in sensitive individuals. Hypersensitivity pneumonitis has been documented in patients following exposure of DUWL water contaminated with bacterial endotoxin [43]. According to Michel et al. [36], severity of asthma in patients is directly correlated with the concentration of endotoxin. Moreover, bacterial endotoxin found in DUWL water can encourage the release of pro-inflammatory cytokines in gingival tissue during dental surgery and adversely affect the healing process [50].

Occupational Health Risk

During dental treatment microbial aerosols are regularly generated [24, 25]. Particles (less than 5 µm) can be inhaled by dental staff, whilst large particles settle easily onto working surfaces [15, 25]. In the dental practice, surfaces, such as dental unit switches, drawer knobs, and light handles, which are most frequently touched, can act as

reservoirs of microorganisms. Researchers have demonstrated a high titre of antibodies against *Legionella* in the serum of dentists compared to non-dental practitioners [51]. In another study [21], a group of dental staff with more than 2 years clinical experience revealed significant neutralising antibodies IgM (20 %) and IgG (16 %) for *L. pneumophila*, compared with a lower 8 % (IgM) to 10 % (IgG) titres in individuals who had no clinical experience. Another study demonstrated significant difference in nasal flora of 50 % of dentists when compared with the nasal flora of the dental surgery assistants [11]. This indicated a positive correlation between bacteria present in the nasal sinuses of dentists to that of the control group. The nature of the altered nasal flora in 14 out of 30 dentists was largely Pseudomonads and/or *Proteus* species as well as water-borne bacteria whereas 3 out of 29 dental surgery assistants (control group) had altered flora consisting of *Proteus*, *Aeromonas* and *Klebsiella* species [11].

Monitoring the Quality of DUWL Output Water

It has been demonstrated that a newly commissioned dental unit will rapidly become grossly contaminated if the waterlines are not treated with biocides within a few weeks [42], and our audits (unpublished data) show that as many as half of DUWLs, treated with biocides but unmonitored, can exceed the guidelines for contamination. However, a clear understanding of how frequently and why biocides fail in a dental practice setting requires further investigation. Pragmatically DUWL could be monitored monthly, with more frequent checks, if unacceptable contamination is detected.

Conventional Microbiological Tests

In order to count bacterial colonies in potable water samples, the conventional laboratory microbiological test is the use of R2A agar plates. R2A agar medium is preferred as it is of a lower nutrient formulation that enhances the recovery of stressed organisms and those organisms with a low nutrient requirement that would otherwise not grow on higher nutrient concentrations [3]. When water samples are placed on R2A agar plates, the larger volume of the solid medium allows these biocides to permeate away from the already stressed bacteria. This contributes to an enhanced overall recovery of viable bacterial cells being tested by the conventional method. The limitations of this method are that a standardised set of conditions and apparatus are required that are costly, and not suitable, or convenient for use in an in-office setting. It would be more convenient and practical to monitor the quality of DUWL water using a rapid test method in the dental practice premises so that, if

Table 2 Summary of tests available for monitoring quality of water

Tests	Optimal counting range/sensitivity	Sensitivity/specificity	Suppliers
R2A conventional microbiology lab test	30–300 for enumerating (0–10 ⁹ cfu/ml)	N/A	Lab M Ltd.
Heterotrophic Plate Count Sampler: cooling towers, renal dialysis units and food industry. It is also recommended for use to monitor DUWL output water	0–200 cfu/ml	98.3/77.3 % [26] 54/95 % [40] 50/100 % [39]	Millipore
Aquasafe™ water test	Unknown	21/100 % [39]	Pall Corporation
For dental use 3M™ Petrifilm™ aerobic count plates: developed for the food industry	25–250 cfu/ml	79/98 % [40], 7 days incubation 57/100 % [39]	3M Food Safety
Dip Slide™ test: developed for testing water contamination in cooling towers. Commonly used by dentists in the UK	10 ³ –10 ⁴ cfu/ml	66/83 % at 2 days and 95/85 % 5 days incubation [49]	Dimanco Ltd. Distributed by Accepta Ltd., UK and 3M Food Safety

gross contamination is apparent, then remedial action can be taken immediately.

Commercial In-Office Rapid Methods for Testing DUWL Output Water

Since there is no rapid, in-office test developed specifically for the dental market, dentists have adopted the use of various existing in-office rapid testing systems for monitoring contaminated DUWL-discharged water (Table 2). The relevant test kits that have been used for the in-office testing of DUWL output water include the Heterotrophic Plate Count Sampler (Millipore); or Millipore HPCS; Aquasafe™ water test (Pall Corporation); the 3M™ Petrifilm™ aerobic count plates (3M Food Safety); and the Dip Slide™ test (Accepta Ltd., UK).

The Dip slide[®] test is marketed in the UK as an aid to monitor the DUWL output water. All of the above mentioned tests have been evaluated for use with DUWL contaminated water [26, 39, 40, 47] and send out a universal message that discrepancies in bacterial recovery between R2A agar medium, and medium based rapid in-office test kit remain [26, 39, 40, 47]. These discrepancies may be due to differences in the nutrient media, counting areas and preference of certain types of bacterial colonies to grow on some media over others. When water samples are transferred to R2A agar plates, the larger volume of agar present allows any residual biocides to permeate away from the already stressed bacteria and reduces the on-going toxic effect on growing organisms. With the in-office test kits such as the Millipore HPCS, the 3M[™] Petrifilm[™] and the Dip slide[®] test, this effect is reduced due to the smaller volume of agar and the larger inoculum size, ultimately increasing the concentration of biocides in contact with bacteria compared to R2A plates. Thus, the acceptability of an in-office test must be confirmed against R2A for subsequent confirmation of specificity and sensitivity. Conventional methodology (R2A plating) remains the important “gold standard” when determining the contamination levels within and/or above the threshold for sensitivity/specificity of a specific in-office test.

Heterotrophic Plate Count Sampler (HPCS)

The Millipore HPCS is a rapid method for the microbiological analysis of water in the environment. This includes the water used in cooling towers and waste water from a range of industries, including the electronics industry and processed water from the food and beverage industry and waste, laboratory grade water and dialysis water. The Millipore HPCS is also used in dental surgeries as it has a threshold of less than 200 cfu. It consists of a plastic paddle, a Millipore membrane filter with a pore size of 0.45 μm which is in close contact with a nutrient pad, an air-vent on the back of paddle and a plastic case for sampling and incubating. Total volume of inoculum is 1 ml/test [26]. The Millipore HPCS test has been evaluated for DUWL output water but with differing sensitivity and specificity values from each investigator’s laboratory [26, 39, 40] (Table 2).

Aquasafe[™] Water Test

The Aquasafe[™] water test kit is a ready to use disposable, filtration monitor system for the microbial analysis of heterotrophic bacteria in water from DUWLs. Each individual Aquasafe[™] water test device consists of a 0.45- μm membrane overlying a media-pad impregnated with a dehydrated growth medium. 1 ml of water sample from

DUWLs is allowed to pass through the grid-marked membrane. Medium becomes hydrated and supports the growth of microorganisms on the surface of membrane. Microbes are counted at room temperature after 72 h of incubation period [39].

Petrifilm[™]

The 3M[™] Petrifilm[™] is a rapid test for the quantitative microbial analysis of water from DUWLs. Manufacturers of the 3M[™] Petrifilm[™] test (3M Food Safety) maintain that the optimal counting range lies between 25 and 250 cfu and therefore makes it more suitable for adopting it for dental use. The 3M[™] Petrifilm[™] is a ready-to-use thin paper and plastic film which has dehydrated culture medium bound to it. Ingredients of culture medium vary from plate to plate depending upon the microorganisms to be cultured. Generally, 3M[™] Petrifilm[™] contains a cold-water-soluble gelling agent, nutrients and indicators to show the activity of microorganisms. The 3M[™] Petrifilm[™] is inoculated with 1 ml water sample and incubated to permit the growth of microorganisms present in water sample [39]. Both Morris et al. [40] and Momeni et al. [39] evaluated the 3M[™] Petrifilm[™] aerobic count plate method and concluded that it was unsuitable for the required threshold for dental needs due to its poor sensitivity/specificity values. Testing of contaminated water serially diluted, and plating on multiple plates may help us clarify its optimal evaluation further.

The Dip Slide[™] Test

The Dip Slide[™] similar to the 3M[™] Petrifilm[™] test is a qualitative indicator test for aerobic water borne organisms as an aid to testing the quality of water in cooling towers, but is marketed in the UK as an aid to monitoring DUWL output water. Manufacturers of the Dip Slide[™] test (Table 2) maintain that the range of sensitivity lies between 1,000 and 100,000 cfu and from the outset suggests that it is unsuitable for estimating the required threshold for dentistry, but does suffice a much higher threshold set for cooling towers. We have evaluated the applicability of the Dip Slides[™] to use in the dental premises and found longer incubation time increased the sensitivity without compromising the specificity. At 2 days, the incubation period specified by the manufacturers, sensitivity was 66 % and specificity 83 %, whilst after 5 days of incubation, sensitivity increased to 95 % and specificity to 85 % at the 1,000 cfu/ml threshold. The test is applicable as a practical means of monitoring general levels of planktonic bacteria in water systems and can be used to screen for gross contamination of dental waterlines if used for more than 5 days. It is not sufficiently sensitive to meet the threshold set

by the Department of Health, in the UK [47]. It is important to be aware of false negative Dip Slide™ test results since it lacks the sensitivity required to meet the required standards in dentistry. The Department of Health (UK) does not recommend it for use in monitoring DUWLs.

Would Improving Sensitivity of Existing Tests Help?

Due to the low threshold for detection of heterotrophic bacterial counts using the 3M™ Petrifilm™ test., Morris et al. [40] suggested that a longer incubation period was the best way to improve the results for the DUWL output water testing. Water-borne organisms are difficult to culture due to the changes in their metabolism when they move from an environment with a low level of nutrients to a nutrient-richer one. This is likely to cause the organism to become shocked/stressed [3] and needing time to adapt. As mentioned earlier, the biofilm organisms from the DUWL output water are from a habitat in which they are in contact with biocides [10, 16, 27, 30, 38, 41, 55, 66, 71, 72], and this can retard their growth when they are directly placed onto a growth medium. There is also the possibility that biocides with longer residual effectiveness can be transferred with the organisms to the growth medium. They may then continue to act upon the organism albeit, at reduced concentrations from those present in its original environment.

Traditionally sodium hypochlorite-based biocides were favoured because of their effectiveness on both bacteria and the biofilm matrix [27, 61]. When determining the heterotrophic bacterial counts using R2A, a neutralising chemical (0.1 % sodium thiosulphate) should be used to halt the antimicrobial action of chlorine-containing biocides [28] in the water from DUWLs and also if the dental units are directly drawing municipal water which may contain chlorine. Hence, initial treatment of DUWL output water samples in 0.1 % sodium thiosulphate is necessitated to neutralise residual chlorine from chlorine-containing biocides as well as municipal water. Failure to neutralise the chlorine from DUWL water samples as in the case of the Dip Slide™ test can result in bactericidal action prior to sample processing, resulting in lower bacterial counts. Hence, a longer incubation of the commercial in-office tests may be useful for monitoring DUWLs when the same tests are used for dental needs.

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Conflict of interest All named authors declare that there is no conflict of interest despite one co-author (Matt Bricknell) being employed by a manufacturing organisation that supplied the in-office test kits. However, this co-author (Matt Bricknell) has not inappropriately influenced or biased the content of the manuscript.

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Risk Assessment for the Spread of *Serratia marcescens* Within Dental-Unit Waterline Systems Using *Vermamoeba vermiformis*

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Abstract *Vermamoeba vermiformis* is associated with the biofilm ecology of dental-unit waterlines (DUWLs). This study investigated whether *V. vermiformis* is able to act as a vector for potentially pathogenic bacteria and so aid their dispersal within DUWL systems. Clinical dental water was initially examined for *Legionella* species by inoculating it onto *Legionella* selective-medium plates. The molecular identity/profile of the glassy colonies obtained indicated none of these isolates were *Legionella* species. During this work bacterial colonies were identified as a non-pigmented *Serratia marcescens*. As the water was from a clinical DUWL which had been treated with AlpronTM, this prompted the question as to whether *S. marcescens* had developed resistance to the biocide. Exposure to AlpronTM indicated that this dental biocide was effective, under

laboratory conditions, against *S. marcescens* at up to 1×10^8 colony forming units/millilitre (cfu/ml). *V. vermiformis* was cultured for 8 weeks on cells of *S. marcescens* and *Escherichia coli*. Subsequent electron microscopy showed that *V. vermiformis* grew equally well on *S. marcescens* and *E. coli* ($P = 0.0001$). Failure to detect the presence of *S. marcescens* within the encysted amoebae suggests that *V. vermiformis* is unlikely to act as a vector supporting the growth of this newly isolated, nosocomial bacterium.

Introduction

Dental-unit waterlines (DUWLs) consist of fine narrow bore tubing that extends to approximately 6 m in length [9]. The DUWL is an essential component of the modern day dental treatment unit that supplies water to cool the dental drill-tip and avoids causing heat-related damage to the soft, pulpal nerve tissues of healthy teeth [25, 49, 52]. It has long been recognised that the DUWL readily harbours a microbial biofilm [11, 30] and that the discharged water can contain very high planktonic bacterial and protozoan loads, which could lead to the exposure of patients and health care workers to an increased risk of infection [27]. The Centers for Disease Control and Prevention (CDC) in the USA, made recommendations to the manufacturers of the dental units that they should have a separate reservoir, typically a container of about a litre, attached to the dental unit from which tap water, deionised water and/or distilled water can be fed to the drill to cool it. Such external reservoirs are also better suited for the introduction of biocides. This measure has been introduced by the CDC in dentistry for delivery of safe treatment water [23], although there are working dental units that are fed directly from

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municipal water. Where biocides are used to control the contamination levels of the DUWL output water, their use is questioned for a number of reasons; it is considered that as a result of their activity, biocides may encourage biofilm formation, introduce bacterial resistance and, furthermore, are limited in their ability to control the diverse range of microbes associated with the DUWL heterogeneous biofilm community [14, 15].

It has now become apparent that there is a problem, common to all forms of man-made tubing, which attracts microbes from the water that flows through them and this is the phenomenon of biofilm formation. Even a newly commissioned DUWL with antimicrobial coating on its internal surfaces, which is maintained according to the recommended daily decontamination protocols [12, 13], will rapidly develop a microbial biofilm which is then sustained throughout the entire life of this clinical device. Health care providers accept that bacteria will always persist in the dental treatment water, but the emphasis now lies on reducing the microbial loading of the discharged water to meet the CDC recommend level of ≤ 500 colony forming units (cfu) of aerobic mesophilic heterotrophic bacteria/millilitre, for infection control in dental health care system [23]. Although the American Dental Association (ADA) has set its own heterotrophic bacterial load of ≤ 200 cfu/ml for water delivered from DUWLs [2], ADA also endorses the CDC recommendation that patient treatment water should be the same quality as the Environmental Protection Agency (EPA) standard of ≤ 500 cfu/ml [2, 23]. The recommended standards for dentistry set by the Department of Health, in the UK, are of ≤ 200 cfu/ml [1, 3, 23]. These reports highlight a risk to patients and stress the importance of maintaining and delivering clean water during dental treatment; hence, it is important to assess the risk factor of microbial propagation by amoebae.

A variety of human pathogenic bacteria including *Legionella* have been isolated from DUWLs, by various researchers [5, 37, 43, 51, 59]. Furthermore, non-tuberculosis *Mycobacterium* species [42, 48], *Klebsiella pneumoniae* [38, 58], *P. aeruginosa* [17, 27] and *S. marcescens* [31, 45, 58] have also been identified. Despite tight controls to make sure the treatment water is safe, an elderly patient died from legionellosis following dental treatment in which *L. pneumophila* serogroup I was identified using molecular profiling from isolates taken both from the patient and from the clinical environment of the dental practice where treatment was performed, confirming the source of infection [44]. Circumstantial evidence also surrounds the death of a dental practitioner due to the same cause in the early nineties [5]. A report by Oppenheim et al. [36] describes a near-miss of a clinical infection on a larger scale with *L. pneumophila* exposure from aerosols generated from dental drills within a teaching institute.

Although the majority of waterborne bacteria pose no risk of infection, guarding against the risk to health of opportunistic

nosocomial pathogens, including *Legionella*, non-tuberculosis *Mycobacterium* species, *K. pneumoniae*, *P. aeruginosa* and *S. marcescens*, to an ever growing list of people with immunocompromised status remains a cause for concern.

Amoebae feed on mixed communities of bacteria within biofilms, including pathogenic bacteria such as species of *Legionella*, *Mycobacterium*, *P. aeruginosa*, *Vibrio cholerae* and *Helicobacter pylori* [6, 12, 13, 46, 47, 60]. Once inside the amoebal cell, some bacteria will survive or escape the adverse conditions presented by digestive vacuoles, but can also find sanctuary from unfavourable environmental conditions and can multiply [26]. Furthermore, bacteria such as *S. aureus* may be ingested, but released undigested and intact [40]. This implies that amoebae can transport certain species of nosocomial bacteria within their ecological niches [57].

Free living amoebae, *Legionella*, *Mycobacterium* and yeast species, found to reside within DUWLs are sourced from the tap water shared by domestic users and the healthcare providers alike [10, 26]. In the context of healthcare, the greatest challenge to overcome is when pathogenic nosocomial bacteria use free living amoebae to support their growth [7, 8, 32, 38, 44]. Lawsuits can be brought against the dental practitioner if causal links are confirmed between an infection and the use of dental treatment water [8].

Serratia marcescens is known to be a nosocomial pathogen which can acquire antimicrobial resistance [28]. This bacterium can cause a variety of infections in the susceptible host including septicaemia, meningitis, endocarditis and blindness [18, 21, 53]. Previous work from our laboratory on grazing habits of *V. vermiformis* [15] indicated that small-sized bacteria were favoured as a food source. The small size of *S. marcescens* makes it an ideal target for protozoa to graze on. Furthermore, it is known that protozoa can support bacterial growth in aquatic ecosystems and a prior study by Cateau et al. [12] implied that *P. aeruginosa* can be propagated through *V. vermiformis* and this could also be true for *S. marcescens*.

This study therefore tested the hypothesis that by grazing on *P. aeruginosa*, and other potential human pathogens *V. vermiformis*, (CCAP 1534/16) could promote the growth of these bacteria within the DUWL aquatic ecosystem. Thus, highlighting the risk factor associated with pathogenic bacteria commonly found in DUWL systems grazing alongside other free living amoebae.

Materials and Methods

Sample Collection

The proposed study was a service evaluation. Approval from the relevant NHS authorities concerning research governance (R & D North West) was obtained (proposal

No 310), and general dental practices in the North West of UK were, subsequently, approached and asked for their willingness to participate in the study. In all, 31 practices consented and all of them conformed to a biocide water treatment recommended by the manufacturer of their dental chairs. The consenting practices were visited on mutually agreed days between 10.00 am and 12.00 pm, and DUWL water (100 ml) from the air/water syringe was sampled. A number code was assigned to each sample from which output water was taken (DUWL 1 to 31) for traceability. Samples were transported in a cool box at 4 °C to the laboratory at the Biomedical Research Facility, at our academic institute for further analysis.

Isolation of Bacteria from Clinical DUWL Water Including Testing for Legionella Species

In addition to performing conventional viable cell counts of aerobic mesophilic bacteria on R2A agar plates, three (100 µl) replicate samples of the water were also inoculated onto commercially prepared GVPC medium (Glycine–Vancomycin–Polymyxin–Cycloheximide) plates (Fisher Scientific) to test for the presence of *Legionella* spp. The GVPC plates were incubated at 30 °C in a humid environment for up to 7 days.

Maintenance of Newly Isolated Individual Bacterial Colonies

Discrete bacterial glassy colonies growing on GVPC plates (suggestive of possible *Legionella* species), designated DUWL 9, 10 and 21, were picked and sub-cultured onto fresh GVPC medium and incubated at 30 °C as described previously. Following incubation, colonies were tested for their Gram reaction and molecular identity. Subsequently, they were inoculated onto R2A medium and nutrient agar at temperatures between 15 and 37 °C, for maintenance and to assess the incidence of pigmentation.

Molecular Identification of Bacterial Colonies

Following sub-culture, some colonies grew well under laboratory conditions on GVPC plates. Genomic DNA was isolated from 10 different colonies from each of the three DUWLs chosen for likely *Legionella* species of bacteria (DUWL 9, 10 and 21) and analysed for molecular identity using the method described previously by Dillon et al. [15].

The Effect of a Dental Biocide on *S. marcescens* Isolated from Clinical DUWL 9 Output Water

The efficacy of Alpron™ was tested on pure laboratory cultures of *S. marcescens*, (the bacterium isolated from

DUWL 9) together with cultures of *P. aeruginosa* (NCTC 10662) as control organisms.

Culture Maintenance

Escherichia coli (XL blue), *P. aeruginosa* (NCTC 10662) and *S. marcescens* from DUWL 9 were maintained by aseptically transferring cultures onto R2A plates and were incubated at 30 °C for 2 days.

Bacterial Liquid Cultures

The antimicrobial testing was undertaken using 18 h culture in nutrient broth at 30 °C in a shaker set at 200 rpm.

Dilution Profiles/Regimes

The log-phase bacterial cultures were centrifuged using a Sigma 3-16PK bench top centrifuge at 4000×g for 20 min at 4 °C (Sigma-Aldrich Ltd., Dorset, UK). The resulting pellets were washed and re-suspended three times in 10 ml of sterile Ringer's solution made from 1/4 strength Ringer's solution tablets (Lab M Ltd., Bury, UK) and the final suspension was held on ice until needed.

Assessment of Resistance to Alpron™ Dental Biocide

A 1 in 10 dilution of commercial Alpron™ is recommended by the manufacturer as the daily working dilution for use within the device/system. The active ingredients in Alpron™ are sodium hypochlorite and a mixture of citric acid anhydrite with non-ionogenic tensides and dye.

The dilution in this study was prepared within 1/4 strength Ringer's solution (Lab M Ltd., UK) containing 100 µl of each bacterial suspension (final concentration of bacteria at 1×10^8 cfu/ml) for laboratory use. The controls consisted of 100 µl of bacterial suspension added to Ringer's solution (900 µl). After approximately 12 h contact time with the biocide at room temperature, each suspension was serially diluted and inoculated on R2A agar plates using the Miles and Misra [33] method. The plates were incubated at 30 °C for up to 7 days and examined after 24 h, 2 days and 7 days using a colony counter.

Phase-Contrast and Differential Interference Contrast Microscopy

Vermamoeba vermiformis (CCAP 1534/16) isolated and maintained in the laboratory as described by Dillon et al. [14] were placed onto a glass slide containing sterile isotonic saline solution and examined directly under a Zeiss Axio Imager A2 microscope. Images were taken using a Zeiss AxioCam HRc digital camera. For the image

acquisition, phase-contrast and differential interference contrast microscopy methods were employed.

Maintenance of *V. vermiformis*

At the start of the experiment *V. vermiformis* was maintained on *E. coli* following the procedure described previously [14].

Preparation of Fresh, Live Bacterial Feed

Strains of *E. coli*, *P. aeruginosa* and the newly isolated *S. marcescens* from DUWL 9 were maintained on R2A plates at 30 °C for 3 days. On the 3rd day, each bacterium was taken and placed as food lines onto R2A plates to feed *V. vermiformis* at 22 °C for 7 days as described previously [14].

Vermamoeba vermiformis Feeding on *E. coli*, *P. aeruginosa*, and *S. marcescens*

The density of cells growing on a 1 cm² plug of agar was calculated by detaching amoebae and suspending them in 2 ml of 1× PBS. A 10-μl aliquot was taken and used to count cells. This was carried out using a standard cell counter (haemocytometer) [15]. Plugs of agar with equivalent numbers of *V. vermiformis* on their respective bacterial feeds were taken weekly for up to 8 weeks. This procedure was carried out in triplicates. The plates were incubated at 22 °C for 5 days. Following incubation, the plates were examined for growth and the area onto which amoeba had migrated over the R2A agar plates was measured to calculate the total number of cells/unit area as described previously [15].

Transmission Electron Microscopy (TEM)

Specimen Preparation

In order to establish whether *V. vermiformis* was a carrier for *P. aeruginosa* and the newly isolated *S. marcescens* from DUWLs 9, three plates with amoebae grown on their respective feed (*P. aeruginosa* or *S. marcescens*) were incubated at 22 °C for 5 days. Amoebae were subsequently transferred from the plate by gentle re-suspension in a small volume of neutral pH phosphate-buffered saline (PBS). The cells were collected into a Falcon™ 15-ml conical centrifuge tube (BD Biosciences) and pelleted by centrifugation at 1500 rpm for 30 min with further washings in between in PBS prior to fixation in neutral buffered glutaraldehyde (2.5%) for 3 h at 4 °C. Following fixation, the pellets were processed for embedding in Araldite as described previously [15].

Sectioning, Examination and Image Capture

Thin sections of each specimen were cut using glass knives at 80–100 nm thickness using the Leica Ultracut E microtome and examined under an electron microscope as described elsewhere [15].

Statistical Analysis

Where appropriate, data are presented as the mean ± SD ($N = 3$), tested for normality and equal variances, and analysed by one-way ANOVA (Minitab 16 statistical software and the IBM SPSS statistics 20). Differences were considered significant at $P \leq 0.05$.

Results

Phase-Contrast and Differential Interference Contrast Microscopy

The *V. vermiformis* (CCAP 1534/16) under phase-contrast and differential interference contrast microscopy demonstrated their limax (trophozoite) morphology and round cysts (Fig. 1).

Molecular Identification of Bacteria from GVPC Plates

Molecular sequencing of the 16S rDNA gene and subsequent Nucleotide BLAST search (ebi.ac.uk) identified the newly isolated bacterium from DUWL 9 as a non-pigmented *Serratia marcescens*, *Phyllobacterium myrsinacearum* from DUWL 10 and *Mycobacterium llatzerense* from DUWL 21; all with sequence similarities of 98–100%

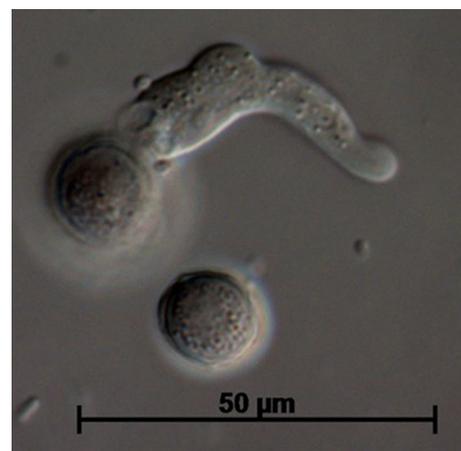


Fig. 1 Phase-contrast image of *Vermamoeba vermiformis*. Small newly emerging trophozoite and smooth spherical cysts

encompassing >200 bases [15]. *S. marcescens* gave a 98% nucleotide similarity, whilst *P. myrsinacearum* gave 100% and *Mycobacterium llutzerense* gave 99% nucleotide similarity. No *Legionella* species were identified from any of the colonies using molecular identification.

Longer Term Maintenance of the Newly Isolated Bacterium from DUWL 9

Pure cultures of *S. marcescens* from DUWL 9 were maintained on R2A and Nutrient agar. This bacterium produced white-coloured colonies on both R2A and Nutrient agar medium at all temperatures tested when sub-cultured from GVPC medium. No pigmented colonies were observed. A unique identification code (UL 234 14) has been assigned to *S. marcescens* and freeze-dried stocks are stored at 4 °C at our academic institute.

The Effect of Alpron™ on *S. marcescens* from Clinical DUWL Output Water

Pure cultures of *S. marcescens* and the accompanying *P. aeruginosa* at 1×10^8 cfu/ml showed that these bacteria were killed by Alpron™ at the manufacturers' recommended treatment levels as there was no recovery after 7 days of incubation under laboratory conditions ($P = 0.0001$).

Growth Statistics for *V. vermiformis*

As anticipated, *V. vermiformis* grew well on all freshly prepared, live bacterial feeds: *E. coli* ($P = 0.0001$), *S. marcescens* ($P = 0.0001$), *P. aeruginosa* ($P = 0.0001$) using one-way ANOVA.

Transmission Electron Microscopy (TEM)

vermiformis Grown on Pure Freshly Grown Live Bacteria

To determine whether *V. vermiformis* supported growth of the Gram negative *S. marcescens* (from DUWL 9, Fig. 2a) and two laboratory strains *E. coli* (XL blue, Fig. 2b) and *P. aeruginosa* (Fig. 2c), samples of *V. vermiformis* cells were examined for internalised bacterial cells within the cytoplasm and/or within their encysted form, using high-resolution electron microscopy. Neither the trophozooidal amoebae nor their encysted forms produced metabolically active bacterial cells within their cell bodies with the exception of the occasional *V. vermiformis* cell that fed on *P. aeruginosa* (Fig. 2c, box).

Discussion

Water supports all forms of life within complex biomes in which the established biofilm microbes vary considerably, in number and range. The physiological condition of the planktonic consortium is also constantly changing within the biofilm. Many bacteria possess a variety of virulence factors which, upon entry to a human host, can challenge the health of that individual. Those most at risk of infection are patients and practitioners with immunocompromised status such as the elderly, HIV patients, smokers, alcohol/drug addicts, diabetics and sufferers of chronic lung diseases, heart disease and renal disease. This study set out to characterise a risk factor associated with pathogenic bacteria commonly found in DUWL systems alongside free living amoebae that graze on these bacteria. In some instances, ingested pathogenic bacteria manipulate the amoebal host for their own survival and multiplication, potentially leading to the death of their infected human host [44]. Lawsuits can be brought against the dental practitioner if causal links between an infection and the dental treatment water are confirmed [8]. Thus, the importance of improving the quality of dental treatment water is essential, clinically, ethically and financially, to halt spread of disease from DUWL water to humans. Although infection rates in humans are generally minimal [24], continued preventative measures must be taken to decrease the possibility of contracting disease from contaminated DUWL output water.

Hartmannella vermiformis (now called *Vermamoeba vermiformis*) dominates the DUWL environment [7] and their initial introduction into this interventional device is likely to come from fresh water supplies used for the reservoir. The same source of water, which may supply domestic and clinical service providing premises, will also have nosocomial pathogens and amoebae prevalent within them [19, 22, 26, 35, 54]. However, *Vermamoeba* species are also reported to have been isolated from the throat of humans from as long ago as 1967 [55] implying that the high prevalence of *V. vermiformis* in the DUWL could also come from humans. Although this study reports of one clinical isolate of *S. marcescens*, it was nevertheless, a serendipitous find, given that the many glassy colonies analysed by sequencing were taken from *Legionella* selective growth medium plates.

As *V. vermiformis* is a much more cosmopolitan feeder than many other free living amoebae [56], this means that there is a greater likelihood of a pathogenic bacterium utilising this taxon of amoeba as an effective means of transport and dispersal in this environment [41]. Previous work on grazing habits of *V. vermiformis* [15] indicated

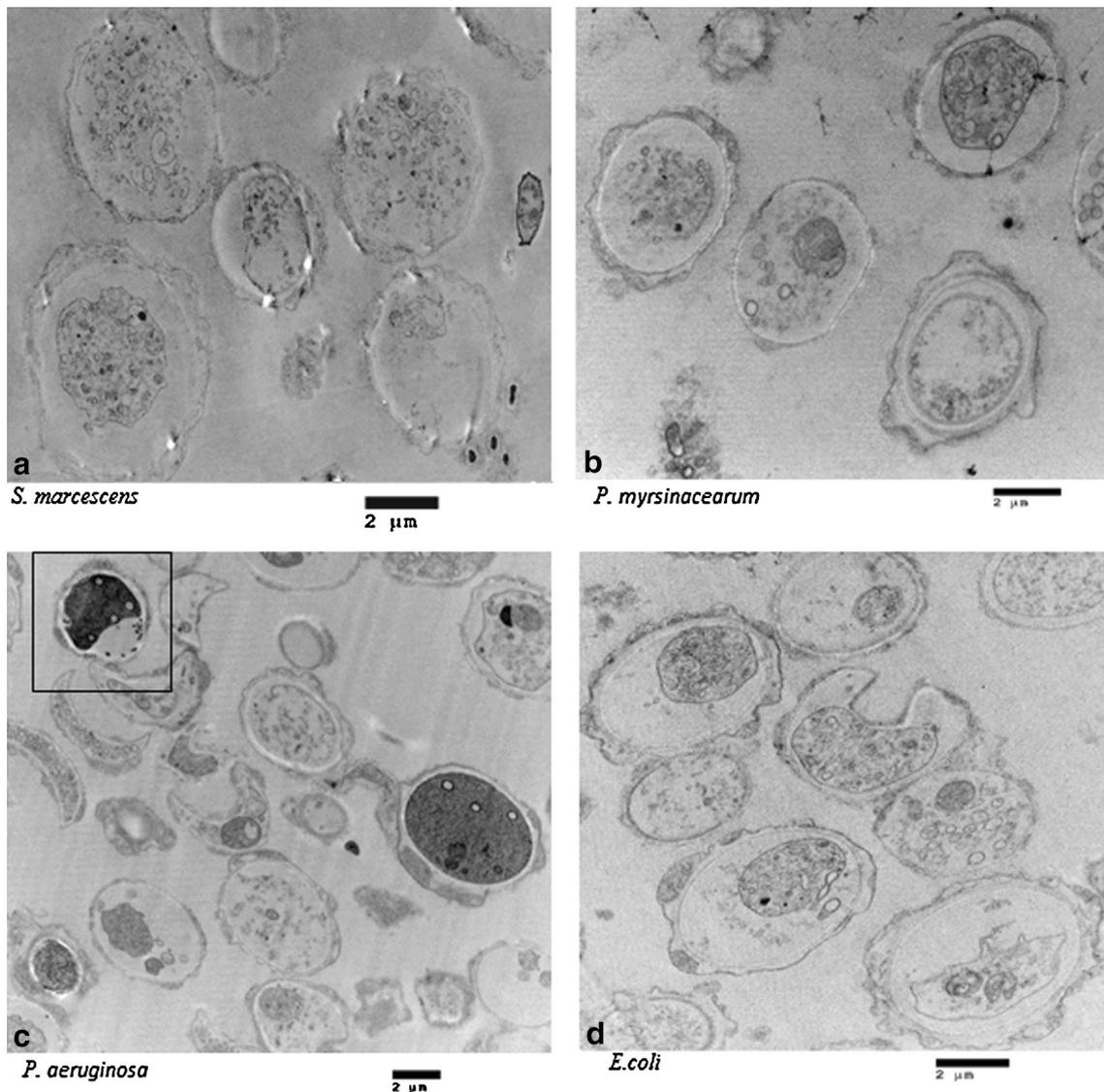


Fig. 2 Transmission electron micrographs of encysted *V. vermiformis* after feeding on *S. marcescens* (Fig. 2a), *E. coli* (XL blue) (Fig. 2b) and *P. aeruginosa* (Fig. 2c). No bacterial cells were

observed within the amoebae or their encysted forms except, for one amoebal cell fed on *P. aeruginosa* (Fig. 2c, box)

that small-sized bacteria, from the simulated DUWL biofilm, were favoured as a food source.

Since the molecular identity of the bacterium from DUWL 9 was identified as *S. marcescens*, which is a typical nosocomial bacterium, considered to be a clinical isolate, its likely survival in the clinical DUWL may have been related to the development of biocide resistance [28, 29]. Since the dental biocide used to treat the DUWL from which the water sample was taken was AlpronTM, its efficacy was tested in the laboratory on the isolated strain of *S. marcescens*. The results demonstrated that this dental biocide was effective on pure cultures of *S. marcescens* in the laboratory up to 1×10^8 cfu/ml. In a previous feasibility study [39], it was found that despite

being treated with the same biocide, the planktonic bacterial counts of aerobic mesophilic bacteria were significantly higher in some DUWLs than those set for dentistry by government authorities. Other studies have demonstrated that a consortium of biofilm organisms is capable of surviving antibacterial agents at higher inoculum levels [4, 16, 34]. However, *S. marcescens* is prone to developing multidrug resistance in the presence of inappropriate concentrations of biocide and antibiotic usage [28, 29]. Our results failed to confirm biocide resistance in *S. marcescens* under laboratory conditions suggesting the extracellular polymeric matrix environment of the biofilm provided protection from AlpronTM within the DUWL.

An earlier investigation suggested a permissive role of *V. vermiformis* for *P. aeruginosa* [12, 13]. *P. aeruginosa* was the organism responsible for the introduction of control measures in dentistry after reports that it caused serious health problems to patients following dental treatment [27]. This study also explored the likelihood of *V. vermiformis* supporting the life cycle of *P. aeruginosa* under laboratory conditions.

The results of this study demonstrated that *V. vermiformis*, which was fed on *E. coli* and *P. aeruginosa* ($P = 0.0001$), grew to the same extent as it did on the non-pigmented *S. marcescens* isolated from clinical DUWL water ($P = 0.0001$). These results agree with those of Singh [50] in which free living amoebae were fed only on a non-pigmented *S. marcescens*. However, in this investigation, *V. vermiformis* also fed on *P. aeruginosa* ($P < 0.05$). These results strongly agree with the study conducted by Pickup et al. [40], but disagree with those of Grosop and Brent [20] who suggested that *P. aeruginosa* was toxic to an unknown species of the genus *Vermamoeba*.

Serratia marcescens is a known nosocomial pathogen and can cause a variety of infections in humans including blindness in the susceptible host [18, 21, 53]. The presence of even one isolate of a pathogenic bacterium such as *S. marcescens* warrants research on understanding its proliferative mechanisms in relation to its existence in the DUWL environment and to inform the future development of disinfection regimes. Since no evidence for bacterial cells within the encysted amoebae was observed by ultrastructure, this suggests that *V. vermiformis* is not acting as a vector to support the proliferation of the nosocomial pathogen *S. marcescens* and disagrees with Cateau et al. [12] for *P. aeruginosa*, although strain differences may apply.

During the past few decades, infection control procedures in dentistry have changed significantly. The basis of dental infection control is to create and maintain a safe clinical environment and to remove, or reduce, the risk of disease transmission as much as possible to patients and dental health care workers. This study confirms that, despite the recommended and appropriate control measures being employed, bacteria such as *S. marcescens* can still be isolated in the laboratory from clinical DUWL water. Care must be taken to use biocides according to manufacturer's instructions to avoid multidrug resistance taking place. In addition, it is also important to adhere to the regular purging protocols recommended by the manufacturers' of the biocide.

Our investigation confirms that *V. vermiformis* can actively feed on fresh *P. aeruginosa* and *S. marcescens*, both are small-sized bacteria of which the latter was isolated in this study. This is in agreement with the description of an ideal food source for amoebae suggested by Pickup

et al. [40], i.e. ease of intake during phagocytosis/ingestion. Since amoebae appear to be genetically programmed to eat bacteria, it is plausible to suggest that *V. vermiformis* may be able to control bacterial populations by feeding on newly dividing *S. marcescens* providing a promising outcome for infection control in dental treatment.

Recommendations

- To avoid operator failure, make sure the responsible staff knows the treatment regime of the biocide used for their DUWLs (when to purge and working concentrations for purging and for daily treatment use).
- Keep log of date of purging and how long the DUWL has been exposed to disinfectant.
- Use biocides within their use-by-date.

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Compliance with Ethical Standards

Conflict of interest All named authors declare that there is no conflict of interest.

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