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An investigation into the biofilm-associated bacterial contamination of dental chair units and dental unit output water supplies and its control

A thesis submitted to the University of Dublin in fulfilment of the requirements for the degree Doctor of Philosophy by

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December 2004

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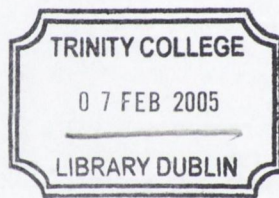
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This thesis is dedicated to the One who loved me and gave Himself for me

Summary

Many modern medical devices, including dental chair units (DCUs), are potential reservoirs of infection as they can harbour biofilm-associated microorganisms. This study investigated several aspects of DCUs related to cross-infection, particularly microbial contamination of dental unit waterline (DUW) output water and DCU suction systems. The first part of this study evaluated the efficacy of the novel Planmeca Waterline Cleaning System, the first integrated disinfection system specifically designed for the disinfection of DUWs, with the two hydrogen peroxide-based disinfectants Sterilex Ultra and Sanosil at reducing bacterial contamination of DUW output water in the Dublin Dental Hospital to safe levels (≤ 200 cfu/ml). Ten DCUs were retrofitted with the novel system: Sterilex Ultra and Sanosil were used weekly to disinfect six and four of these DCUs, respectively, over a 20-week and eight-week period, respectively. Both disinfectants were equally effective at reducing the bacterial density of DUW output water to < 200 cfu/ml and maintaining this water quality for at least a week. Following cessation of once-weekly disinfection with either disinfectant, DUW output water quality deteriorated within three weeks. Electron and confocal microscopy of the internal lumens of DUWs demonstrated that the improved water quality correlated with biofilm removal. Sanosil was found to be more effective, as the use of Sterilex Ultra was associated with clogging of DUWs in some DCUs. Following completion of this study all DCUs in the Hospital were retrofitted with the system. Two years after the implementation of weekly disinfection with Sanosil throughout the Hospital, it was noted that bacterial densities rose to significant levels five days following disinfection, which correlated with a significant increase in the prevalence of catalase-positive organisms in the DUW output water, probably due to the consistent use of the hydrogen-peroxide based disinfectant Sanosil.

Within six months of the opening of the new Dublin Dental Hospital, corrosion, dampness and bacterial contamination were found on the baseplates of the DCUs where the suction hoses attach. Isolates of *P. aeruginosa* (n=41) recovered from the baseplates and multiple sites within the suction system of 19 DCUs were used as marker organisms to trace the source of contamination. These isolates were serotyped and subjected to computer-assisted analysis of *Spe*I-generated DNA fingerprint patterns, which revealed that the isolates belonged to seven distinct strains, one of which predominated (serotype O:10, *Spe*I-fingerprint group II). The presence of identical strains on DCU baseplates and internal suction system sites in 10/13 of the DCUs tested demonstrated that baseplate contamination was caused by leakage of material from within the suction system hoses at the point of connection to the DCU baseplates. As a consequence of this study, a new type of suction hose connector was developed and fitted to all DCUs following collaboration between the clinical microbiology team and the DCU manufacturer, Planmeca. No further bacterial contamination has since been observed.

The next part of this study investigated biofilm contamination of DCU suction system hoses. The suction system hoses of DCUs in the Hospital are subject to twice-daily disinfection with the phenolic disinfectant Puli-Jet, yet the hoses were found to harbour extensive biofilm and yielded high densities of predominantly Gram-negative bacteria. This phenomenon is of concern as previous studies showed that backflow from suction hoses can result in fluid from suction hoses entering the oral cavities of patients during use. The bacterial diversity of biofilm within the high volume suction system hoses of Planmeca Prostyle Compact DCUs was investigated using 16S rDNA sequence analysis and culture methods as very few studies have investigated this phenomenon thus far. *Stenotrophomonas maltophilia* and *Pseudomonas* species were the most prevalent organisms detected by both methods, with prevalence rates of 27.2% and 10.2%, respectively, for 16S sequence analysis and 37.1% and 12.9%, respectively, for culture. Anaerobic bacteria associated with oral infections were detected by 16S sequence analysis, but predominantly at the proximal end ("patient end") of suction hoses.

A new *real-time* PCR assay for faster and more sensitive detection of *Legionella* in DUW output water was developed using the TaqMan Sequence Detection System. Culture techniques and *real-time* PCR were used to quantify numbers of *Legionella* species in DUW output water, particularly *L. pneumophila*. To validate the system water samples from healthcare institutions in the Mid-Western Health Board region were investigated for *Legionella* by culture and PCR. *Legionella pneumophila* was cultured from 18/77 water samples received from healthcare institutions in the Mid-Western Health Board region, while *L. pneumophila* DNA was detected in 64/77 samples by *real-time* PCR. The strain most often associated with Legionnaires' disease, *L. pneumophila* serogroup 1, was cultured from 3/77 healthcare institution water samples. In contrast, *Legionella* was cultured from the DUW output water of only 1/19 (5%) DCUs tested but *Legionella* DNA was detected in the DUW output water of 15/19 (79%) DCUs by *real-time* PCR, suggesting that the presence of *Legionella* in DUW output water may be a more significant problem than has been hitherto appreciated.

The results of this study have highlighted DCU waterlines and suction systems as potentially significant sources of cross-contamination and cross-infection. While the use of Planmeca Waterline Cleaning System with the disinfectant Sanosil proved to be very effective at maintaining DUW output water quality at safe levels, the results of this study demonstrate that the DCU suction system is an important reservoir of both human-derived and environmental bacteria. The presence of extensive biofilm within suction hoses despite disinfection is of concern and requires urgent investigation. This study has provided practical solutions to waterline contamination and DCU baseplate contamination. It is anticipated that further research on DCU-associated biofilms will significantly reduce the potential for cross-infection associated with these medical devices.

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Abbreviations

A_{260} and A_{600}	absorption at 260 nm and 600 nm
ADA	American Dental Association
AIDS	Acquired Immune Deficiency Syndrome
bp	base pairs
BSA	bovine serum albumin
BCYE	buffered charcoal yeast extract
CDC	Center for Disease Control
cfu	colony forming units
cm	centimetre(s)
DCU	dental chair unit
Δ	delta
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	dideoxynucleoside
DUW	dental unit waterline
EC	European Commission
e.g.	for example
et al.	and others
EU	European Union
g	gram
g	gravity
GVPC	glycine, vancomycin, polymyxin B sulphate and cycloheximide
h	hour(s)
HIV	Human Immunodeficiency Virus
i.e.	that is
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilo base pairs
kg	kilogram(s)
l	litre
M	molar
ml	millilitre
mm	millimetre
μm	micrometre
μl	microlitre
min	minute(s)
NA	nutrient agar
NB	nutrient broth
ng	nanogram
No.	number

PBA	Pseudomonas base agar
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
ppm	parts per million
rDNA	ribosomal DNA
RNA	ribonucleic acid
Rnase	ribonuclease
rpm	revolutions per minute
spp.	species
TBE	tris-borate EDTA
Tris	tris (hydroxymethyl) aminoethane
U	unit(s)
UK	United Kingdom
USA	United States of America
UV	ultra violet
v/v	% "volume in volume" expresses the number of ml of an active constituent in 100 ml of solution.
w/v	% "weight in volume" expresses the number of g of an active constituent in 100 ml of solution
X-Gal	5-bromo-4-chloro-3-indolyl-D-galactopyranoside
°C	degree centigrade
<	less than
>	greater than
≤	less than or equal to

Publications

Some of the original work presented in this thesis has been published in refereed international publications as listed below. Offprints of the published manuscripts are included at the end of the thesis.

Tuttlebee, C. M., O'Donnell, M. J., Keane, C. T., Russell, R. J., Sullivan, D. J., Falkiner, F., & Coleman, D. C. (2002) Effective control of dental chair unit waterline biofilm and marked reduction of bacterial contamination of output water using two peroxide-based disinfectants. *J. Hosp. Infect.* **52,** 192-205.

M.J. O' Donnell, C. M. Tuttleebee, F.R. Falkiner & D.C. Coleman. (2004) Bacterial contamination of dental chair units in a modern dental hospital caused by leakage from suction system hoses containing extensive biofilm. *J. Hosp. Infect. In press.*

Chapter 1

General Introduction

1.1 Introduction

Many medical devices are known to be reservoirs of infection due to the growth of biofilm-associated microorganisms on them. Well-characterised examples of such devices are endo-tracheal tubing, prosthetic heart valves, central venous catheters, urinary catheters and even contact lenses (DePaola *et al.*, 2002; Donlan & Costerton, 2002). For example, bacterial biofilms on prosthetic valves are the leading cause of endocarditis in patients who have had heart valves replaced- with a mortality rate as high as 70% (Davey & O'Toole, 2000; Donlan & Costerton, 2002). Another example of a medical device that can become a reservoir of infection is the urinary catheter. Between 10 and 50% of patients undergoing short-term urinary catheterisation develop infections, while almost all patients undergoing long-term catheterisation develop infections, due to microorganisms attaching to the catheter surface and forming biofilms. Urinary catheter biofilms are quite unique in that some of the microorganisms within the biofilm may alter the local pH so that minerals contained in the urine, such as calcium phosphate, can precipitate out of solution, forming a deposit in the catheter lumen, which can then lead to complete blockage of the catheter (Donlan & Costerton, 2002).

While the dental chair unit (DCU) would not automatically spring to mind as an example of a medical device, as it is "used for human beings for the purpose of diagnosis, prevention, monitoring, treatment or alleviation of disease" it should therefore be considered as a medical device according to the EU Medical Devices Directive (Anonymous, 1993). It is a modular complex piece of equipment that is central to the practice of modern dentistry. Unlike the medical devices described above, however, DCUs are obviously not indwelling devices, but there is still potential for human infection because dental procedures expose staff and patients to pathogens and potential pathogens originating from biofilms within the dental unit waterlines (DUWs) and suction systems of DCUs.

Many studies published over the last four decades have investigated the contamination of DUWs and have shown that it is widespread with almost all standard DUWs providing contaminated water to dental patients. Bacterial levels in DUW output water have been shown to vary from approximately 100 to 1,000,000 colony forming units (cfu) per ml of output water (Abel *et al.*, 1971; Furuhashi & Miyamae, 1985; Whitehouse *et al.*, 1991; Douglas & van Noort, 1993; Pankhurst & Philpott-Howard, 1993; Williams *et al.*, 1993; Williams *et al.*, 1994; Williams *et al.*, 1995; Pankhurst *et al.*, 1998; Walker *et al.*, 2000; Putnins *et al.*, 2001; DePaola *et al.*, 2002; Tuttlebee *et al.*, 2002).

However, while there are many published studies investigating DUWs, there are very few publications investigating the extent of microbial contamination of DCU suction systems. The DCU suction system is designed to reduce aerosols and remove excess fluids that have been generated in the oral cavity during dental work, e.g. DUW output water, saliva and blood. As such, it quickly becomes contaminated with aerial, oral and DUW-derived microorganisms, which can lead to build-up of biofilm as microorganisms aspirated through the suction system adhere to the lumen of the suction system hosing and multiply. A number of studies that have investigated contamination of DCU suction systems have shown that liquid from the suction system hoses can enter a patient's mouth during use, posing a risk of cross-infection to patients during treatment (Watson & Whitehouse, 1993; Mann *et al.*, 1996; Barbeau *et al.*, 1998).

This study investigated the problem of bacterial contamination of DUWs and suction systems of DCUs that were located, in the main, in the Dublin Dental Hospital (Lincoln Place, Dublin 2).

1.2 Description of Dublin Dental Hospital

The new Dublin Dental Hospital was opened in 1998 and handles over 2,000 patient visits per week (over 100,000 per year), including some immunocompromised and medically compromised patients (Tuttlebee *et al.*, 2002). It is equipped with 103 Planmeca Prostyle Compact DCUs (Planmeca Oy, Asentajankatu 6, Helsinki, Finland), the dental unit investigated in this study, located on three different floors within the Hospital.

The Accident and Emergency clinic, located on the ground floor, contains 11 DCUs, while the West clinic, also located on the ground floor, contains eight units. There are 40 DCUs located in Clinic 1, located on the first floor, with two separate surgeries located adjacent to it, containing one DCU each. Similarly, Clinic 2, located on the second floor, contains 40 DCUs with two separate surgeries adjacent to it each containing one DCU.

1.3 Dental Chair Units

1.3.1 Function of dental chair units

The DCU is central to the practice of modern dentistry and is an integral part of all dental surgeries and clinics. It is required for the comfortable positioning of patients during dental treatment. The DCU is also the most important piece of equipment involved in the examination and subsequent treatment of patients, as it not only comprises the

patient's chair, but also the auxiliary attachments that provide and control the handpieces, air/water syringes and the suction system. DCUs vary in style, depending on the space available in the clinic or surgery, whether the dentist is left or right-handed and whether the dentist works alone or with assistance. The DCU (usually) consists of an adjustable patient chair, an instrument console to which handpieces are attached, the suction system hoses and the operating light. In some DCUs, there may also be a cupfiller and spittoon for patient rinsing following dental treatment; however, this is not always the case.

1.3.2 Description of Planmeca Prostyle Compact DCU

The Planmeca Prostyle Compact DCU consists of an adjustable patient chair that is connected to a pedestal unit to which the suction system hoses and the nurse's three-in-one air/water syringe are attached. The pedestal unit also houses the spittoon, cupfiller and it supports the dentist's operating light and the instrument console arm (see Figure 1.1).

In this model, the instrument console arm is on a moveable arm, positioned so that the attached instruments used by the operator are across the chest of the patient (Figure 1.2). There are five positions available on the console for the attachment of instruments, the most usual instruments used being the three-in-one air/water syringe, the turbine and conventional (low-speed) handpieces, the ultrasonic scaler and the light-curing unit.

1.3.2.1 Three-in-one air/water syringe

The three-in-one air/water syringe is used for the washing and drying of teeth during oral examination and tooth preparation. It is designed to deliver either a stream of water, a stream of air, or a combination of the two.

1.3.2.2 Turbine and low-speed handpieces

The handpieces include the high-speed turbine, used for gross removal of tooth structure during cavity preparation and polishing of teeth, and the conventional low-speed motor attachment, used for the removal of soft caries (O'Donnell, 2002). They are hand-held connecting devices placed between the handpiece hose and the rotary cutting instrument, referred to as a bur. This bur can rotate at speeds of up to 500,000 rpm. Sterile handpieces and burs are used for each patient to reduce the likelihood of cross-infection. Anti-retraction valves are positioned where the waterline connects to the handpiece to prevent the backflow of water and debris from the patient's mouth flowing back into the airlines and waterlines, also reducing cross-infection.

4
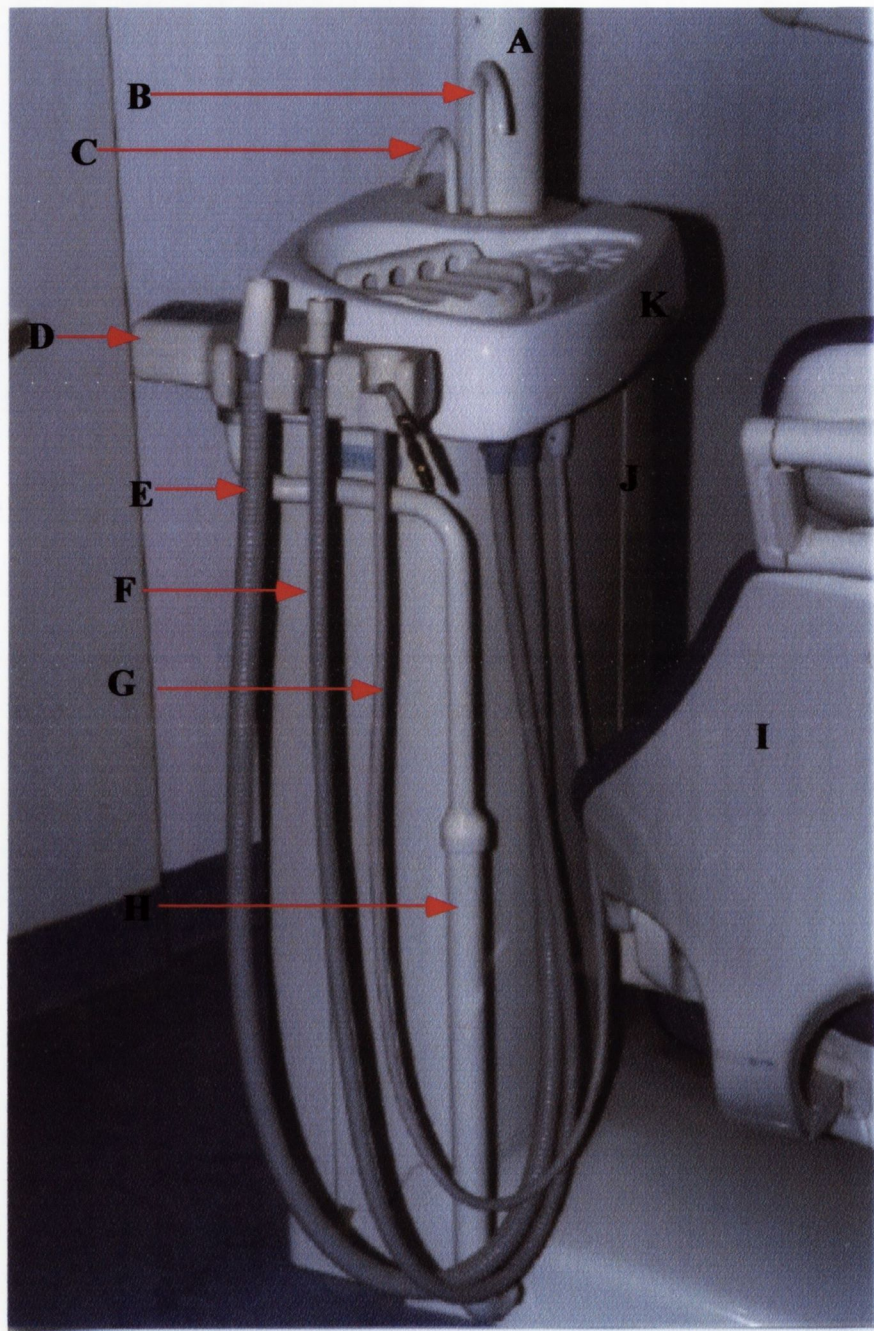
Figure 1.1. General overview of the spittoon and rear of a Planmeca Prostyle Compact dental chair unit. Labelled components are as follows: A, instrument console arm; B, cupfiller; C, spittoon rinse; D, nurse's control panel; E, high-volume suction hose; F, low-volume suction hose; G, nurse's 3-in-1 air/ water syringe hose; H, suction holder arm; I, patient chair; J, pedestal unit; K, ceramic spittoon

Operating light

Console arm

Glass fill tube

Bowl rinse tube

Control panel

Instrument (doriot) arms

Bowl

Suction holder

Instrument console

Optional
suction arm

Patient chair

Foot control
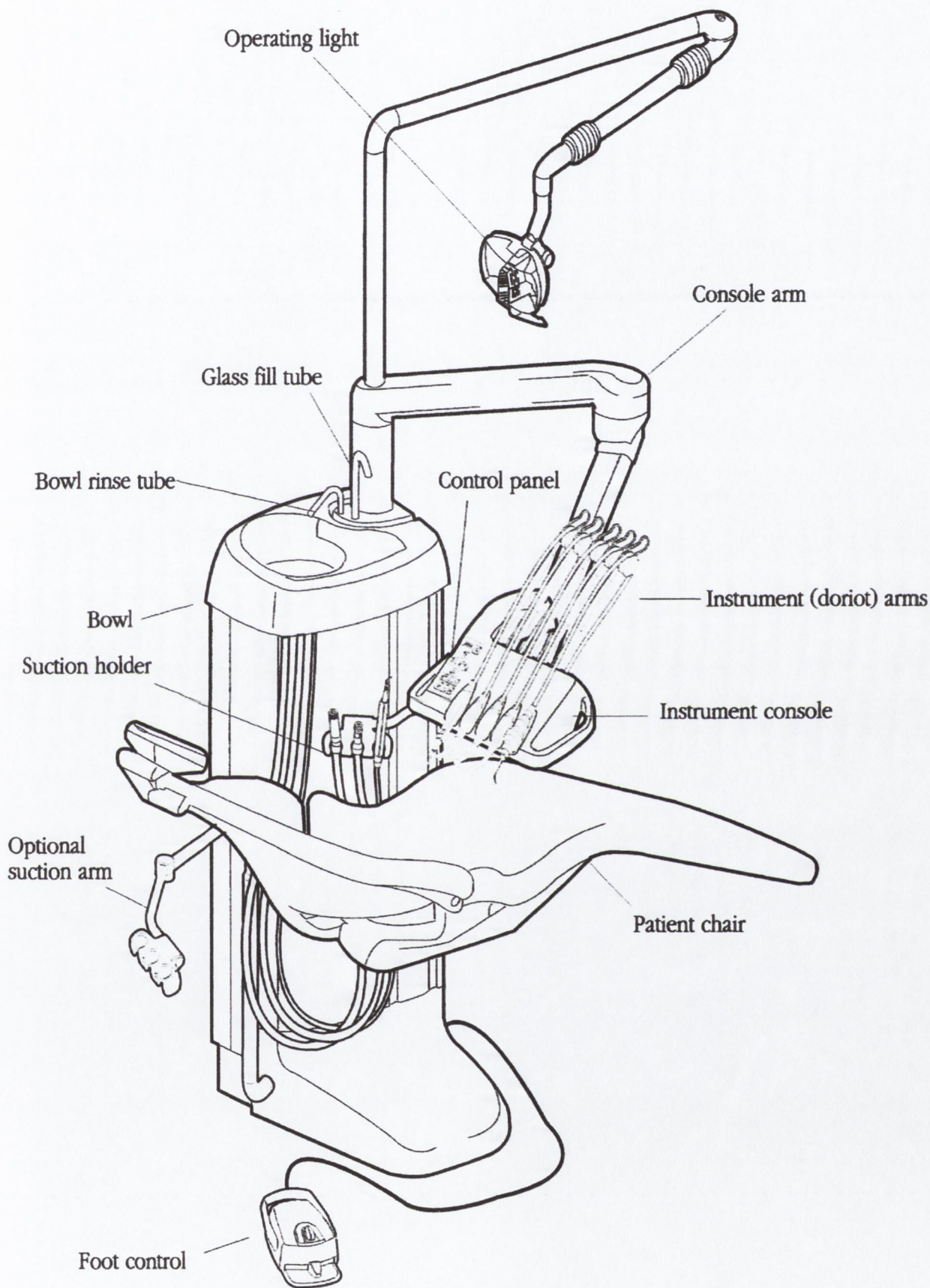
Figure 1.2. Schematic representation of a Planmeca Prostyle Compact dental chair unit.

The turbine handpiece can be used with a diamond bur for grinding tooth structure, or a tungsten carbide bur for cutting tooth structure (O'Donnell, 2002). It is driven by filtered, clean and dry compressed air and its free running speed varies between 250,000 rpm and 500,000 rpm. However, the bur slows down to rotational speeds of approximately 200,000 rpm, ideal for cutting, when the operator applies slight pressure to the tooth. Because the use of this instrument can generate frictional heat up to 1200°C, and studies have shown that an increase in heat of only 6°C within the pulp of teeth can lead to necrosis (Stanley, 1971), air and water, mixed at the head of the handpiece, produce a cooling aerosol spray of water while the instrument is operating, which enters the patient's mouth during treatment.

1.3.3 DCU water system

The water for the cooling aerosol spray is supplied by the DUWs of the DCU. In the Planmeca Prostyle Compact model, DUWs supply water to each of the turbine handpiece, ultrasonic scaler, three-in-one air/water syringes, patient rinse cup filler and the spittoon (Prostyle Compact Dental Unit Users Manual, 1996-03). The DUWs of DCUs may be supplied with water via individual bottle-fed systems, a water holding tank or the municipal water supply. In the Dublin Dental Hospital, water destined for the DUWs is transferred initially from the public water mains to a 12,000-l cold-water storage tank before undergoing filtration and UV treatment as described in Section 2.5.1.

1.3.4 DCU suction system

The DCU is also fitted with a suction system, which is designed to remove water, blood, saliva and debris from patients' mouths and to reduce aerosols generated during dental treatment. This is accomplished by means of suction tips that are connected to suction hoses, which are themselves connected to a vacuum source (O'Donnell, 2002). In the Planmeca Prostyle Compact DCUs, the suction system is a fixed and integral part of the DCU, comprising the high volume suction, the saliva ejector and the spittoon. The flexible hoses of the high volume and low volume suction system are attached to receivers within the DCU through a steel baseplate by heavy-duty plastic connectors (Figures 1.3 and 1.4) (O'Donnell, 2002). The high volume suction system is used for removal of water, blood, saliva and debris from the patient's mouth, while the low volume suction system is used for removal of excess fluids from the patient's mouth generated during some dental procedures, and is also referred to as the saliva ejector. Liquid aspirant from the patient's mouth is drawn through the suction system hosing and then passes through a removable
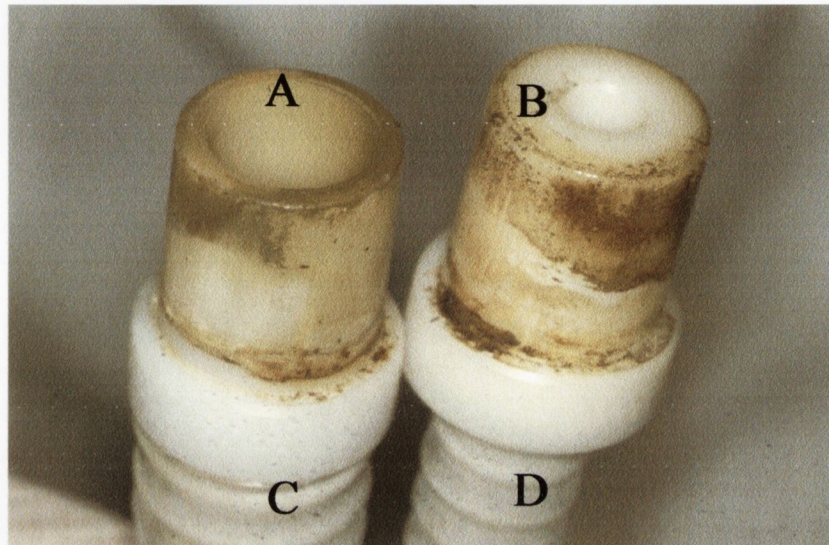
Figure 1.3. Suction connectors and suction hoses from a Planmeca Prostyle Compact dental chair unit. Items labelled as follows: A, high-volume suction hose connecting collar; B, low-volume suction hose connecting collar; C, high-volume suction hose; D, low-volume suction hose. The green/brown staining evident on both connectors was due to biofilm formation by *Pseudomonas* and related species.
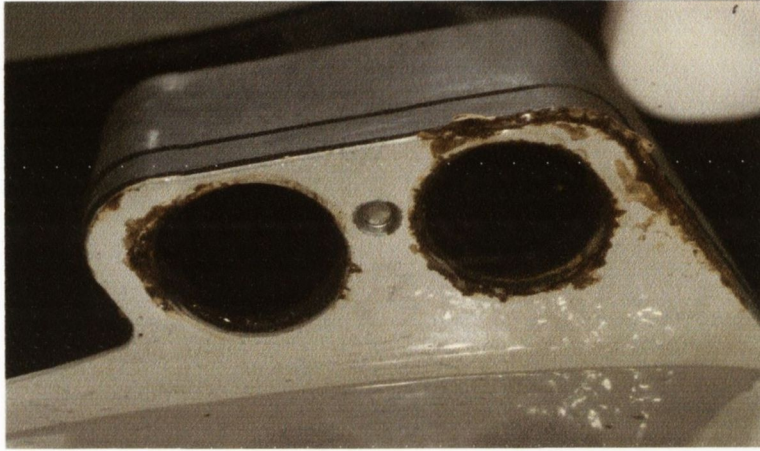
Figure 1.4. View of DCU baseplate *in situ* photographed from underneath showing the orifices into which the suction hose connectors are inserted. Evidence of corrosion is visible around the orifices and adjacent areas.

filter within the DCU pedestal unit that collects particles greater than 1 mm in diameter. This filter is housed in a filter cage that is not removable. Following filtration, the aspirant passes into the central suction system. The part of the suction system hosing that is closest to the patient will be referred to henceforth as the proximal end. Removable sterile tips are attached to this end of the hosing before insertion into the patient's mouth and are removed and disposed of or sterilised following treatment. For the purpose of this thesis, the distal end of the high volume suction system hosing is that part of the tube to which a rigid plastic connecting collar is attached, by which means it can then be inserted into the suction hose receiver orifices in the DCU baseplate located beneath the ceramic spittoon (see Figures 1.1, 1.3 and 1.4).

1.4 Biofilms

1.4.1 Definition of biofilm

Although the usual perception of bacteria is as unicellular planktonic organisms, it is now widely accepted that the most typical lifestyle of bacteria in the environment is as part of a wider population or community (Davey & O'Toole, 2000). Indeed, the vast majority of bacteria (up to 99.9%) have been shown to grow in matrix-enclosed structures called biofilms that are attached to surfaces in almost all aquatic systems, including DUWs and dental unit suction systems, and these static bacterial communities differ greatly from planktonic bacteria (Donlan & Costerton, 2002). Within the biofilm community, bacteria have additional protection from antibacterial chemicals, environmental bacteriophages and amoebae (Donlan & Costerton, 2002).

A biofilm can be defined as "a microbially derived sessile community characterised by cells that are attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription" (Donlan & Costerton, 2002).

Biofilms consist of a collection of microcolonies complete with water channels and an assortment of extracellular polymers such as polysaccharides, glycoproteins and proteins (Johansen *et al.*, 1997). This matrix of extracellular polymers is highly hydrated and predominantly polysaccharide in nature and also mediates adhesion (Donlan & Costerton, 2002). Other physical features of biofilms include spatial and temporal heterogeneity and the involvement of inorganic substances all held together within the biofilm matrix (Donlan & Costerton, 2002). Usually, biofilms formed on hard surfaces are

several cell layers thick and may reach up to several hundred micrometres in thickness if left undisturbed (Donlan & Costerton, 2002).

1.4.2 Advantages of biofilm formation

There are many advantages to community living within this biofilm matrix for bacteria, such as protection from antimicrobial agents and the general environment, greater nutrient availability and the acquisition of new genetic traits (Stewart *et al*, 1998; Davey & O'Toole, 2000; Donlan & Costerton, 2002). Protection from antimicrobial agents and the environment is the advantage of greatest relevance to this project and will therefore be discussed in greatest detail.

1.4.2.1 Protection from antimicrobial agents

The biofilm matrix provides protection from antimicrobial agents to the bacteria within its matrix by a number of different mechanisms, such as preventing or delaying access of antimicrobial agents into the biofilm matrix and altered growth rate of organisms within the biofilm matrix.

1.4.2.1.1 Prevention or delay of access of antimicrobial agents

By simply presenting an additional barrier for antimicrobial agents to diffuse through, the exopolysaccharide (EPS) matrix provides considerable protection for the bacteria located within the biofilm (Dunne, 2002). The access of many antimicrobial agents past the EPS matrix and into the biofilm has been shown to be delayed. For example, the penetration of ciprofloxacin into surfaces lined with *Pseudomonas aeruginosa* biofilm was shown to take 21 min compared to 40 s for sterile surfaces (Donlan & Costerton, 2002) and Hoyle *et al.* found that dispersed bacterial cells were 15 times more susceptible to tobramycin than cells in intact biofilms (Hoyle *et al.*, 1992).

The EPS matrix can also prevent access of certain antimicrobial agents into the biofilm by acting as an ion exchanger, thus restricting diffusion of compounds from the environment into the biofilm by binding of the antimicrobials (Davey & O'Toole, 2000). In fact Hatch and Schiller showed that a suspension of EPS isolated from *P. aeruginosa* inhibited diffusion of both gentamicin and tobramycin, but that this effect could be reversed by using EPS lyase (Hatch & Schiller, 1998). Another study demonstrated the ability of EPS produced by *Staphylococcus epidermidis* to hinder the antimicrobial effect of a large number of agents against *Bacillus subtilis* (Donlan & Costerton, 2002). However, not all antimicrobial agents are equally affected by this property; while

hydrophilic positively charged antimicrobial agents such as the aminoglycoside antibiotics are most affected, other agents such as the macrolides and β-lactam antibiotics are either minimally affected or totally unaffected (Davey & O'Toole, 2000; Donlan & Costerton, 2002). Studies have also shown that the EPS of biofilm has sequestered metals, cations and toxins, thus preventing their access into and possible damage to the biofilm; one study discovered that a highly purified EPS preparation from an unidentified bacterium isolated from metal-laden sediments was found to be capable of binding copper (Davey & O'Toole, 2000). This metal-binding property of biofilms is a key link in the transfer of metals through the ecosystem, as some invertebrates that feed on these biofilms have been shown to accumulate high amounts of metal (Davey & O'Toole, 2000).

1.4.2.1.2 Altered growth rate of biofilm organisms

A second reason why bacteria within biofilms are more protected against antimicrobials than their planktonic counterparts is that these sessile bacteria grow more slowly and, consequently, absorb antimicrobial agents more slowly (Dunne, 2002). For example, studies have shown that the cell counts of older *P. aeruginosa* biofilms were only minimally reduced following exposure to piperacillin and tobramycin, whereas these antimicrobials completely inactivated both planktonic bacteria and younger biofilms (Donlan & Costerton, 2002). Similarly, separate studies investigating varying growth rates of both *Escherichia coli* and *S. epidermidis* found that the slowest growing bacterial biofilms were the most resistant to antimicrobial agents (Donlan & Costerton, 2002).

1.4.2.2 Protection from the general environment

Another advantage that biofilm formation conveys on the resident microbes is the presence of EPS, which has been reported to provide protection from many environmental stresses, such as UV radiation, changes in pH, osmotic shock, and desiccation (Davey & O'Toole, 2000). For example, although the *recA* gene of *P. aeruginosa* is normally induced by DNA-damaging agents, *P. aeruginosa* located within the EPS matrix were protected from damage following exposure to UV radiation, demonstrated by the fact that the *recA* gene was not induced (Davey & O'Toole, 2000). Another study investigating the protection of biofilm-associated bacteria showed that mucoid strains of *E. coli*, *Acinetobacter calcoaceticus*, and *Erwinia stewartii* survived conditions of dehydration better than their nonmucoid counterparts (Ophir & Gutnick, 1994).

1.4.2.3 Greater nutrient availability and metabolic co-operation

A further advantage to life within the biofilm matrix is that the water channels interspersed throughout the biofilm are highly permeable and are similar to very primitive circulatory systems. They enhance nutrient availability to and the removal of toxic metabolites from individual microcolonies within the biofilm matrix by providing an efficient means of exchange with the main aqueous phase. They also provide the opportunity for metabolic co-operation between microcolonies and the formation of different environmental niches within the biofilm with several species occupying the same microcolony (Davey & O'Toole, 2000). Very efficient co-operations and symbiotic relationships can thus readily develop within biofilms, enabling the maximum utilisation of available nutrients (Davey & O'Toole, 2000).

1.4.2.4 Acquisition of new genetic traits

As most bacteria in the natural environment live within biofilms and therefore in close proximity to one another, it is natural that conjugation and the consequent transfer of plasmids among bacteria within the community should occur. Plasmids conferring resistance to mercury have been isolated from bacteria residing within biofilms in the marine environment and it was also found that gene transfer occurred between the bacteria isolated from the marine biofilm and laboratory strains. Gene transfer has also been demonstrated in artificial dental plaque where a *Streptococcus* species acquired a conjugative transposon conferring resistance to tetracycline from *B. subtilis*. While the above examples indicate horizontal transfer of genes, studies have also shown that vertical transfer of plasmids can occur when a recipient bacterial cell grows to form a microcolony, thus establishing the plasmid within the biofilm (Davey & O'Toole, 2000). This transfer of genes within the biofilm confers significant advantage to bacteria residing therein in comparison to their planktonic counterparts and is important for the genetic development and diversity of natural bacterial communities.

1.5 Microbial contamination of DCU waterline output water

Most dental procedures use relatively large quantities of water for irrigation, cooling of instruments and for oral rinsing. This water invariably enters the oral cavities and the upper respiratory tracts (the latter as an aerosol) of individuals receiving dental treatment. Studies over the last three decades have shown that the problem of bacterial contamination of DUWs is widespread and that virtually all standard dental units provide contaminated water, with bacterial levels varying from approximately 100 to 1,000,000

colony forming units (cfu) per ml of output water (Abel *et al.*, 1971; Furuhashi & Miyamae, 1985; Douglas & van Noort, 1993; Pankhurst & Philpott-Howard, 1993; Williams *et al.*, 1993; Williams *et al.*, 1994; Williams *et al.*, 1995; Pankhurst *et al.*, 1998; Putnins *et al.*, 2001; DePaola *et al.*, 2002).

The majority of microorganisms isolated from the DUW output water are Gram-negative bacilli now believed to originate from incoming water supplies, although organisms normally resident in the oral cavity such as streptococci and *Candida* have also been recovered (Abel *et al.*, 1971; Pankhurst & Philpott-Howard, 1993; Pankhurst *et al.*, 1998; Mills, 2000; Walker *et al.*, 2000). The recovery of oral microorganisms from DUWs indicates that there may be some back siphonage of microorganisms from the patient's mouth back into the DUWs; indeed, one study estimated that each time a handpiece is stopped while the bur is still in the mouth of the patient, almost 1 ml of oral fluids can be aspirated back into the DUW (Bagga *et al.*, 1984; Al Shorman *et al.*, 2002). However, the introduction of anti-retraction valves in the manufacture of modern dental handpieces has virtually eliminated this problem and the resulting aspiration of microorganisms from the patient's mouth (Al Shorman *et al.*, 2002).

No significant differences in microbial contamination between different DUW systems, whether mains, bottle or storage tank fed, have been observed (Walker *et al.*, 2000). The chemical quality of the water supplied to the DUWs, whether hard, soft, deionised or distilled, also has no significant effect on microbial load of the output water (Walker *et al.*, 2000).

The presence of large numbers of microorganisms in DUW output water poses an increased risk of infection for dental patients and staff and is incompatible with good hygiene and cross-infection control practices. DUW contamination is of particular concern with respect to dental treatment provided to immunocompromised individuals. Over the last two decades there have been many advances in medicine and technology that have led to the continued survival of immunocompromised individuals, including the elderly, organ transplant recipients, cancer patients, cystic fibrosis patients and those on immunosuppressive therapy etc. (Shearer, 1996; Pankhurst *et al.*, 1998; Pontón *et al.*, 2000; Al Shorman *et al.*, 2002). This cohort of individuals is much more susceptible to cross-infection and rigorous cross-infection control procedures should be followed when treating such patients.

1.5.1 Biofilm formation within dental unit waterlines

DUW contamination is caused predominantly by bacteria from the incoming water supply, which form multi-species adherent biofilms on the inside of the waterlines. These biofilms provide a reservoir for ongoing contamination of dental unit water as water passing through the lumen sloughs off bacteria into the DUW output water (Whitehouse *et al.*, 1991; Shearer, 1996; Pankhurst *et al.*, 1998; Meiller *et al.*, 2000; Putnins *et al.*, 2001). Bacteria have a great tendency to attach to surfaces in nutrient-limited ecosystems with adverse environmental conditions, such as the aquatic environment within the DUWs (Johansen *et al.*, 1997; Pankhurst *et al.* 1998). As described above, sessile microbes within a biofilm have many survival advantages over planktonic microbes in this situation, such as increased resistance to antimicrobial substances and retention on surfaces in a co-operative ecosystem (Davey & O'Toole, 2000; Shearer, 1996; Stewart *et al.*, 1998). Nutritional advantage is also conferred on sessile bacteria within a biofilm because organic and inorganic nutrients are bound by the biofilm matrix (Shearer, 1996; Donlan & Costerton, 2002).

A wide variety of environmental and human-derived potential pathogens have been recovered from biofilms in DUWs, especially aerobic heterotrophic Gram-negative bacteria, but fungi and protozoa have also been identified (Furuhashi & Miyamae, 1985; Whitehouse *et al.*, 1991; Williams *et al.*, 1993; Pankhurst *et al.*, 1998). Most of the bacterial populations found in DUWs resemble those in mains water, where they are present in lower numbers (Pankhurst & Philpott-Howard, 1993; Pankhurst *et al.*, 1998; Mills, 2000). The design of the DUWs themselves lends itself to biofilm formation, with the narrow microbore of the tubing, approximately 1 mm in diameter, providing a high ratio of lumen surface area to water volume (Pankhurst *et al.*, 1998; Al Shorman *et al.*, 2002; DePaola *et al.*, 2002; Spratt *et al.*, 2004). Bacteria also adhere more readily to the hydrophobic polymeric plastic material used in DUWs (e.g. polyvinyl chloride, polyurethane) than to glass or steel tubing (Pankhurst *et al.*, 1998).

Surprisingly, biofilms form preferentially in very high shear environments, i.e., environments through which liquid is rapidly flowing. It is believed that perhaps the turbulent flow impinges planktonic cells onto the surface, thus enhancing bacterial adhesion and consequent biofilm formation. Furthermore, while biofilms formed at high shear are remarkably strong and resistant to mechanical breakage, those formed in low-shear environments have a low tensile strength and break easily (Donlan & Costerton, 2002). The flow of water passing through the DUWs, which results in maximum flow at the centre of the lumen and minimum flow at the periphery, encourages deposition of

organisms onto the surface of the tubing. Furthermore, the stagnation of water within DUWs in between patient treatments and for extended periods overnight and at weekends promotes unchecked proliferation of the bacteria within the biofilm (Pankhurst *et al.*, 1998).

It is now widely believed that bacteria form biofilms in roughly the same manner, regardless of which substrate they are attached to. This process involves two stages, namely, (i) initial surface attachment, and (ii) formation of microcolonies and their development into mature biofilms within EPS matrix (Donlan & Costerton, 2002). However, different species of bacteria are known to use varying means to achieve these different stages.

1.5.1.1 Initial surface attachment

The initiation of bacterial biofilm formation on a surface is influenced by a number of factors, including the nature of the substrate to which it attaches, and other environmental factors such as pH, oxygen tension, iron availability and temperature (Davey & O'Toole, 2000). Within DUWs, biofilm formation begins initially when hydrophobic molecules in the water adsorb to the surface, forming conditioning films that alter the characteristics of the surface, perhaps enhancing the efficiency of bacterial adhesion to the internal wall of the lumen (Shearer, 1996). Microorganisms that already possess attachment structures, e.g. fimbriae, immediately attach firmly to the surface of the lumen (Shearer, 1996). In *P. aeruginosa*, flagellum-mediated motility is essential for initial interaction with the surface (Davey & O'Toole, 2000). *Escherichia coli* requires flagella and pili to initiate attachment processes (Davey & O'Toole, 2000). One component of the outer bacterial membrane, lipopolysaccharide (LPS), also seems to be essential for initial surface attachment in both *P. aeruginosa* and *E. coli*. Other bacteria attach in a time-dependent process following prolonged exposure to the surface that results in a reversible association between the bacteria and the surface.

It has been shown that some bacteria, including *P. fluorescens* and *Vibrio cholerae*, use different genetic pathways for initial surface attachment depending on the environmental conditions. For example, *P. fluorescens* mutants that were defective for biofilm formation when grown on glucose recovered their ability to form biofilms following growth on citrate, suggesting an alternative citrate-dependent pathway for biofilm formation. In fact, *P. fluorescens* has been shown to form a biofilm under every condition tested (Davey & O'Toole, 2000). Furthermore, adhesion of bacteria to surfaces triggers expression of genes controlling production of bacterial components necessary for

biofilm formation. For example, the *P. aeruginosa* gene *algC* that controls phosphomannomutase production, an enzyme involved in exopolysaccharide (EPS) synthesis, is upregulated within minutes of adhesion to a solid surface (Donlan & Costerton, 2002). Thus, bacteria involved in biofilms exhibit differing phenotypes to their planktonic counterparts.

1.5.1.2 Microcolony development and biofilm maturation

Following exopolysaccharide (EPS) secretion and microbial multiplication, irreversible attachment and colonisation are achieved (Shearer, 1996). This biofilm will then develop to a mature community of micro colonies with water channels in between and an assortment of extracellular polymers (polysaccharides, glycoproteins and proteins) forming an extracellular amorphous matrix within six days (Johansen *et al.*, 1997; Pankhurst *et al.*, 1998) (see Figure 1.5).

1.5.2 Current potable water standards

In recent years there has been increased media and public concern about deficiencies in infection control within the health care system in general. The United States and the European Commission have set national standards of 500 and 100 cfu/ml, respectively, for aerobic heterotrophic bacteria in drinking water (Anonymous, 1998; Anonymous, 2002a). Similarly, the Japanese Waterworks Act set a standard for potable water of 100 cfu/ml (Furuhashi & Miyamae, 1985). Clearly the quality of DUW output water should be at least as good as that recommended for human consumption, as DUW output water is going directly into patients' mouths and also into their upper respiratory tracts as aerosols. Currently, there are no standards for the microbiological quality of dental unit water set by the European Commission, nor is it a parameter subject to regular monitoring. However, the American Dental Association (ADA) set a goal for the year 2000 of no more than 200 cfu of heterotrophic bacteria per ml of dental unit output water (Anonymous, 1996), although this has not yet been achieved in practice (Mills, 2000). Furthermore, approved codes of practice for controlling *Legionella* in water systems, including dental units, have recently been implemented in the UK and in Ireland (Anonymous, 2001; Anonymous, 2002b) in order to ensure that the water supply does not contain high levels of these potential pathogens.
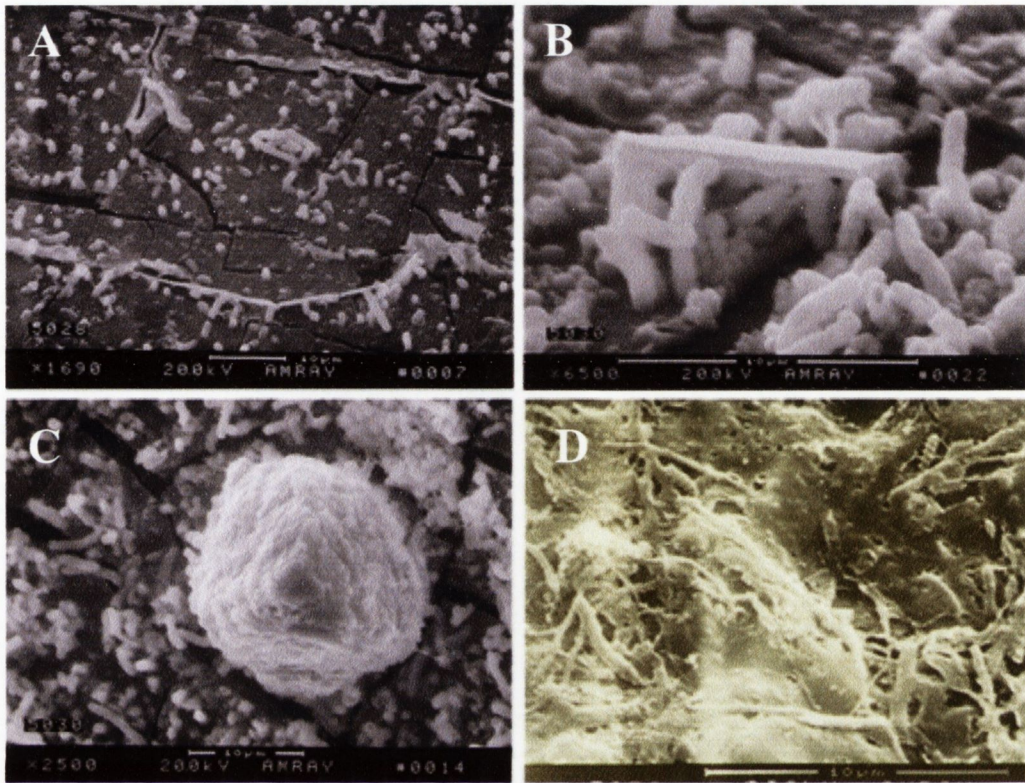
Figure 1.5.   Electron micrographs showing biofilm formation on dental unit waterline surface (taken from Mills, 2000). **A.** Carbonate deposits (which resemble ice floes); **B.** Initial attachment of bacteria to DUW surface; **C.** Division of cells into microcolonies; **D.** Formation of mature biofilm

1.5.3 Bacteria in DUW output water that can cause disease

Some of the bacteria found in dental unit water are known to cause disease in humans; of particular concern are *Pseudomonas*, *Legionella* and non-tuberculosis *Mycobacterium* species (Pankhurst & Philpott-Howard, 1993; Barbeau *et al.*, 1996; Pankhurst *et al.*, 1998, Walker *et al.*, 2000). In addition a range of toxic by-products (e.g. endotoxin) could also have clinical consequences (Putnins *et al.*, 2001). *Burkholderia* (formerly *Pseudomonas*) *cepacia*, an organism frequently responsible for pulmonary disease in cystic fibrosis patients (Pankhurst *et al.*, 1995; Pankhurst & Philpott-Howard, 1996), and *Stenotrophomonas* (formerly *Xanthomonas*) *maltophilia*, an organism with pathogenic potential in debilitated patients (Denton *et al.*, 1998), have also been isolated from dental unit water, as well as more innocuous organisms such as *Alcaligenes faecalis* and a variety of other *Pseudomonas* species (Furuhashi & Miyamae, 1985; Barbeau *et al.*, 1996; Pankhurst *et al.*, 1998; Walker *et al.*, 2000).

1.5.3.1 Pseudomonas aeruginosa

Pseudomonas species, especially *P. aeruginosa*, are well-documented opportunistic pathogens that can survive on a meagre supply of nutrients and often exhibit resistance to antibiotics and disinfectants (Michel-Briand *et al.*, 1981; Wilson & Dowling, 1998). *Pseudomonas aeruginosa* is a rod-shaped, polarly flagellated Gram-negative bacterium that produces two pigments, pyocyanin and fluorescein, which colour *P. aeruginosa* colonies green (Lyczak *et al.*, 2000). While *P. aeruginosa* is ubiquitous in nature, it is found especially in soil and water, which are considered to be its natural habitat (Mushin & Ziv, 1973; Wilson & Dowling, 1998). Its ubiquity is due to several factors, such as its abilities to colonise many environmental niches and to utilise many environmental compounds as sources of energy (Lyczak *et al.*, 2000).

According to Lyczak *et al.* (2000), *P. aeruginosa* was very likely first reported in human infections in 1862 when rod-shaped particles in blue-green pus were observed by Luke, although similar colouration had been previously observed by Sedillot on surgical dressings. This unusual colour is now known to be caused by pyocyanin, a pigment produced by *P. aeruginosa*. The microbe itself was first isolated from infections by Gessard in 1882, who named it *Bacillus pyocyaneus* (Lyczak *et al.*, 2000).

1.5.3.1.1 Infections caused by *Pseudomonas aeruginosa*

Pseudomonas aeruginosa usually only causes infections in people who are compromised in some way, particularly burns victims and those suffering from the genetic

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disorder, cystic fibrosis (CF), although it also causes significant morbidity and mortality in AIDS patients and neutropenic patients undergoing chemotherapy (Govan & Deretic, 1996; Lyczak *et al.*, 2000; Maschmeyer & Braveny, 2000).

1.5.3.1.1.1 Pseudomonas aeruginosa *infections in burns patients*

Patients who have been badly burned are extremely susceptible to infection with this organism due to the breach in the skin barrier. As *P. aeruginosa* occurs so widely in the environment, it is very likely that burns victims will be exposed to this micro-organism before the burns have time to heal. The pathogenesis of *P. aeruginosa* infections involves a variety of virulence factors produced by the organism itself, such as pili, fimbriae and elastase. The pili and fimbriae enable the organism to persist at the wound site and disseminate throughout the host organism. The production of elastase and other proteases enables degradation of collagen and other host proteins, and are also used to disrupt the integrity of the host basement membrane (Lyczak *et al.*, 2000).

1.5.3.1.1.2 Pseudomonas aeruginosa *infections in cystic fibrosis patients*

In cystic fibrosis patients, the major cause of morbidity and mortality is the chronic microbial colonisation of the airways leading to debilitating exacerbations of pulmonary infection (Govan & Deretic, 1996). The most common pathogen infecting these patients is mucoid *P. aeruginosa*, although a closely related organism, *Burkholderia cepacia*, is also emerging as a significant pathogen in this regard (Govan & Deretic, 1996). However, *P. aeruginosa* only colonises the CF lung after a succession of other more easily eradicated pathogens, such as *Staphylococcus aureus* and *Haemophilus influenzae* (Govan & Deretic, 1996). Mucoid *P. aeruginosa* is not currently known to have any natural ecological niche in the environment; however, all non-mucoid wild-type *P. aeruginosa* strains appear to have the potential to give rise to spontaneous mucoid variants (Govan & Deretic, 1996). The non-mucoid variants initially colonise the lung but most patients eventually succumb to lung infection with multiresistant mucoid *P. aeruginosa*, following transition within the lung to the mucoid phenotype and selection of resistant strains following antibiotic treatment (Govan & Deretic, 1996; Lyczak et al., 2002).

1.5.3.1.1.3 Pseudomonas aeruginosa *infections in contact lens wearers*

While *P. aeruginosa* is not usually associated with infection of immunocompetent individuals, normally healthy individuals who are users of extended-wear soft contact lenses are also susceptible to infection with this organism in the cornea (Lyczak *et al.*,

2000). This leads to ulcerative keratitis, a rapidly progressing inflammatory response to bacterial infection of the cornea, which is extremely destructive to the corneal tissue. This condition was historically associated with prior injury or trauma to the cornea and the correlation of *P. aeruginosa* corneal infection with the wearing of contact lenses is not yet fully understood but some explanations include the possibility of abrasion of the cornea by the contact lens, trapping of the bacteria at the corneal surface or biofilm formation of *P. aeruginosa* on the lens surface (Lyczak *et al.*, 2000; Donlan & Costerton, 2002).

1.5.3.1.1.4 Community-acquired pneumonia caused by Pseudomonas aeruginosa

Although rare, there have also been case reports of previously healthy individuals with community-acquired pneumonia (CAP) caused by *P. aeruginosa*. These cases may be fatal and are often rapidly progressive and have been associated with exposure to contaminated aerosolised water (Hatchette *et al.*, 2000). For example, a previously healthy patient was exposed for 90 min to aerosolised mist while in a whirlpool spa and consequently developed *P. aeruginosa* pneumonia. *Pseudomonas aeruginosa* was subsequently isolated from both the water in the whirlpool and the mist. Another patient contracted *P. aeruginosa* CAP following exposure to a home humidifying device that contained water contaminated with *P. aeruginosa* (Hatchette *et al.*, 2000). This indicates that exposure to aerosols containing *P. aeruginosa* poses a potential risk to both healthy and immunocompromised individuals.

1.5.3.1.2 *Pseudomonas aeruginosa* and DUWs

While *P. aeruginosa* is known to be naturally antibiotic resistant, it can also form biofilms embedded in an EPS matrix, which enhance its natural antibiotic resistance. In fact, it has recently been shown that antibiotic-resistant phenotypic variants of this species with enhanced ability to form biofilms arise frequently both *in vitro* and *in vivo* (Drenkard & Ausubel, 2002).

The plastic tubing within dental units is ideal for formation of biofilm by *P. aeruginosa*. A number of previous studies have reported the predominance of *Pseudomonas* species, often including *P. aeruginosa*, in DUW output water (Furuhashi & Miyamae, 1985; Martin, 1987; Williams *et al.*, 1993; Barbeau *et al.*, 1996; Jensen *et al.*, 1997). One such study reported the presence of *P. aeruginosa* in the DUWs of 24% of DCUs investigated (Barbeau *et al.*, 1996). The authors also reported that when *P. aeruginosa* was isolated from a given DCU, it was repeatedly recovered from all its waterlines over the year-long study period and, furthermore, *P. aeruginosa* accounted for

75-100% of the cultivable flora of these DCUs, even when using a non-selective medium such as R2A (Reasoner & Geldreich, 1979; Barbeau *et al.*, 1996). The authors speculated that the observed predominance of *P. aeruginosa* in the DUWs of these DCUs could be the result of the production of bacteriocins by *P. aeruginosa* that can inhibit growth of other microorganisms, thus conferring a competitive advantage to *P. aeruginosa* in colonisation of the DUWs (Barbeau *et al.*, 1996). *Pseudomonas aeruginosa* could of course be predominant in the supply water, which would also account for its predominance in the DUW output water.

1.5.3.1.3 *Pseudomonas aeruginosa* in dental unit waterline output water causing infection

In 1987, Martin reported that, following routine dental treatment, two immunocompromised patients developed oral abscesses caused by *P. aeruginosa*. Further investigation revealed that isolates of *P. aeruginosa* with the same pyocin types as those in the patients' abscesses were recovered from the DUW output water of the DCUs with which these patients had been treated, suggesting that the dental unit water was the source of these infections (Martin, 1987). Martin also isolated *P. aeruginosa* from the oral cavities of 78 healthy patients for three to five weeks following exposure to dental unit water contaminated with *P. aeruginosa*, even though *P. aeruginosa* is not normally resident within the oral cavity. In fact, one study recovered the organism from the oral cavities of only 4% of those investigated (Botzenhardt *et al.*, 1987; Martin, 1987; Barbeau *et al.*, 1996). These findings suggest that the patients investigated in this study must have been colonised by *P. aeruginosa* that was present in the DUW output water.

1.5.3.2 Legionella pneumophila *and other* Legionella *species*

Legionella are Gram-negative, aerobic, monopolarly flagellated rod-shaped bacteria, approximately 0.5 μm in width and 2 μm in length that are found in freshwater environments (Fields *et al.*, 2002; Steinert *et al.*, 2002). They are intracellular parasites of freshwater protozoa and amoebae and appear to use a similar mechanism for multiplication within mammalian cells such as macrophages (Fields *et al.*, 2002). More than 42 *Legionella* species have been described since the first reported outbreak of legionellosis in 1977 (Fraser *et al.*, 1977). They cause respiratory disease in humans when a susceptible host aspirates aerosols or water that contain *Legionella* species (Fields *et al.*, 2002).

1.5.3.2.1 Legionellosis

Legionellosis is the collective term for diseases caused by *Legionella* species. The pneumonic form of legionellosis, called Legionnaires' disease, is a severe multisystem disease that gives rise to non-specific clinical manifestations such as mild cough, malaise, muscle aches and low fever in the early stages after an incubation period of two to ten days (Fields *et al.*, 2002; Steinert *et al.*, 2002). Diarrhoea, neurological symptoms such as confusion, a fever greater than 39°C and hepatic dysfunction have also been prominent in several studies (Sabria & Yu, 2002). The mild flu-like form of legionellosis is a self-limiting illness called Pontiac fever (Fields *et al.*, 2002; Steinert *et al.*, 2002). *Legionella* species rarely cause infections in organs other than the lung; however, a case of recurrent soft tissue abscesses caused by *L. cincinnatiensis* has recently been reported (Gubler *et al.*, 2001). Because of the non-specific nature of legionellosis it is believed that many cases go unreported (Waterer *et al.*, 2001; Sabria and Yu, 2002; Steinert *et al.*, 2002), as pneumonia caused by legionella cannot be differentiated from other pneumoniae by clinical, radiographic or non-specific laboratory testing (Helbig *et al.*, 1999). However, the case-mortality rate of Legionnaires' disease, ranging from 7% to 24%, is so high that accurate early diagnosis is critical for effective treatment (Steinert *et al.*, 2002).

1.5.3.2.2 Contraction of legionellosis by humans from water sources

While *Legionella* species do occur naturally in lakes and rivers, their concentration in these habitats is usually quite low (Steinert *et al.*, 2002). Conditions such as elevated temperature (optimum growth temperatures are 25°C-42°C), inorganic and organic water contents and the presence of host protozoa, in which *Legionella* species can survive and replicate, play significant roles in the growth and spread of these bacteria (Steinert *et al.*, 2002). It is for reasons such as these that most cases of legionellosis can be traced back to man-made aquatic environments where the water temperature is usually higher than ambient temperature and where there are more opportunities for biofilm formation, e.g. on the lumen of water pipes (Fields *et al.*, 2002; Steinert *et al.*, 2002).

Humans contract legionellosis by aspiration and/ or inhalation of contaminated aerosols (Steinert *et al.*, 2002). These aerosols can be produced by water-cooling towers, air-conditioning systems, whirlpools, spas, fountains, ice machines, vegetable misters and DCU handpieces (Steinert *et al.*, 2002). *Legionella* species also survive within biofilms of building water systems. The material of the piping system has been shown to influence its concentration: plastic materials such as that used in DUWs actually support higher concentrations of the bacteria, whereas copper plumbing material seems to reduce

numbers, probably because the copper ions are toxic to bacteria (Fields *et al.*, 2002; Steinert *et al.*, 2002). In hospitals, cooling towers were originally implicated as the main source of legionella, however, between 1982 and 1985, it was discovered that the potable water supply was the actual source of the vast majority of cases of nosocomial legionellosis (Sabria & Yu, 2002).

1.5.3.2.3 *Legionella* species isolated from DUW output water

There have been varying reports regarding the prevalence of *Legionella* species in DUW output water. Several studies from the US, Italy and England have demonstrated the presence of *Legionella* species in as many as 62% of DUWs investigated, often in high numbers, i.e. > 1,000 cfu/ml (Atlas *et al.*, 1995; Challacombe & Fernandes, 1995; Williams *et al.*, 1996; Pankhurst *et al.*, 2003).

Another recent paper (Zanetti *et al.*, 2000) reported that 61% of 101 water samples examined from various DUWs of 23 dental units were contaminated with *Legionella* species, with a very high prevalence (52%) of *L. pneumophila*. This is of considerable concern because *L. pneumophila*, particularly serogroup 1 (the most commonly isolated serogroup in the study), is the most pathogenic *Legionella* species and most often linked with disease. There is also evidence that occupational exposure to aerosols of waterborne bacteria, generated by dental unit handpieces, can lead to colonisation of dental staff and a higher prevalence of antibodies to *Legionella*. However, the health consequences to these individuals are unclear, not least because non-pneumonic legionellosis of the Pontiac fever type may also occur in dental staff or patients and cause symptoms clinically indistinguishable from other flu-like episodes (Fotos *et al.*, 1985; Atlas *et al.*, 1995; Tolentino *et al.*, 1996). A study just published found no correlation between exposure to DUW output water and respiratory illness in healthy dental students and suggested that the microbes present in the DUWs are not pathogenic for healthy individuals, despite their abundance in DUW aerosols (Scannapieco *et al.*, 2004). However, this study did not investigate any correlation between specific microorganisms present in the DUW output water, e.g. *Legionella* species, and consequent respiratory illnesses in the students, e.g. Legionnaires' disease. In fact, this study did not even attempt to characterise the microorganisms present within the DUW output water of the dental hospitals investigated, as the results were based solely on questionnaires completed by the dental students.

In contrast to the above studies reporting relatively high numbers of *Legionella* species in DUW output water, two more recent papers from England and Northern Ireland have reported a low prevalence of *Legionella* species in DUW output water with both

studies reporting the presence of *Legionella* species in less than 2% of DCUs investigated (Walker *et al.*, 2000; Pankhurst *et al.*, 2003). The authors postulated that this could be due to the majority of samples being taken during the autumn and winter months when the temperature of municipal waters held in reservoirs and lakes, from which the DUWs of DCUs are fed, would be cooler, thus limiting the presence of *Legionella* species (Pankhurst *et al.*, 2003).

1.5.3.2.4 *Legionella* from DUW output water associated with infection

In 1995, Atlas *et al.* reported the death of a Californian dentist resulting from Legionnaires' disease, probably due to exposure to dental unit water. Prior to his death, the clinical laboratory team investigating the case used anti-*L. dumoffii* monovalent fluorescent-antibody staining to identify the culture obtained from the patient. *Legionella* cells that stained using the same monovalent anti-*L. dumoffii* fluorescent antibodies were also detected in the DUWs of his dental office, particularly within protozoa in the biofilms of the DUWs, and also in the lung tissue collected during the dentist's autopsy. Fluorescent antibody staining also showed the presence of *L. pneumophila* and *L. longbeachae* in the *post mortem* lung tissue samples. Subsequent to his death, water samples from both his home and the DUWs of the DCU located in his dental office were examined for the presence of *Legionella* species using PCR and fluorescent-antibody methods. Both methods detected high levels of *Legionella* species (>10,000 organisms per ml) in the DUW output water, while low levels (<100 organisms per ml) were detected in his domestic water. However, only low levels of *Legionella* species were detected in the DUW output water and domestic water by culture. Atlas *et al.* could not definitively ascribe this case of Legionnaires' disease to exposure to *Legionella* species originating from within DUWs because the *Legionella* species cultivated from the DUW output water were not subsequently identified by the clinical team. However, the high levels of *Legionella* species detected in the DUW output water by PCR and fluorescent-antibody methods make it likely that aerosols from these dental units were indeed the source of the fatal infection (Atlas *et al.*, 1995).

1.5.4 Previous approaches at improvement of dental unit output water quality

Numerous suggestions for reducing the bacterial density in dental unit water have been proposed but none has been universally adopted that is both efficient at eliminating biofilm, as well as being safe for patients (Pankhurst & Philpott-Howard, 1993; Mills, 2000).

1.5.4.1 Flushing of DUWs before use

One widely used practice for reducing the bacterial density in dental unit water involves flushing DUWs with water (Fiehn & Henriksen, 1988). While this can result in a reduction in microbial density by several orders of magnitude, many studies reported that microbial densities after flushing were still unacceptably high (Furuhashi & Miyamae, 1985; Pankhurst & Philpott-Howard, 1993; Mills, 2000). Clearly, while flushing water through waterlines may reduce bacterial counts somewhat, it is not sufficient to deal with the problem and another solution must be found.

1.5.4.2 Use of biocides

Many biocides have been examined for their efficacy in controlling microbial contamination of DUWs. Strategies involving elution of ethanol or chlorine through waterlines have been devised to reduce microbial density, however these methods have been found to be unsuitable for routine use in dental clinics (Furuhashi & Miyamae, 1985; Pankhurst & Philpott-Howard, 1993; Meiller, *et al.*, 2000). Chlorine is known to be corrosive and the apparatus used in one study for the elution of ethanol through the DUWs was found to be quite cumbersome (Pankhurst & Philpott-Howard, 1993). A built-in decontamination system which involved the flushing of a glutaraldehyde solution through the DUWs of a DCU, followed by rinsing with water was also examined but found to be unsuitable, as the disinfection and rinsing cycle took seven minutes and was undertaken between each patient visit, which obviously resulted in a great loss of working time for dental practitioners (Douglas & Rothwell, 1991). Furthermore, glutaraldehyde is a tissue fixing agent and could be very detrimental to patients' health if not properly flushed out of the DUWs. Another study that examined the efficacy of Listerine Antiseptic for disinfecting of DUWs found that immediately following overnight treatment with the antiseptic, the DUW output water was free from recoverable bacteria. However, by day 7, the density of bacteria in DUW output water had increased to pre-treatment levels, thus indicating that Listerine Antiseptic alone was not altogether a suitable disinfectant for DUWs, as the investigators also recommended a periodic use of a second antimicrobial to supplement the action of Listerine (Meiller *et al.*, 2000). A recent study of the use of ozone in the treatment of DUWs has shown promising results and further investigations are being conducted (Al Shorman *et al.*, 2002).

1.5.4.3 Other alternatives

Other options discussed in a recent review (DePaola *et al.*, 2002) include using independent reservoirs that isolate the DUWs from municipal water so that water of known microbial quality can be introduced, however, unless the biofilm within the DUWs is removed, the DUW output water will still be contaminated. Microfiltration and ultraviolet irradiation of incoming water have also been suggested, however, these options are expensive and, as above, unless the biofilm lining the DUWs is removed, the output water will still be contaminated. Filtration of DUW output water may be a viable option, however, filters are prone to rapid clogging with bacteria and need to be changed frequently, leading to a recurring expense (DePaola *et al.*, 2002).

1.6 Bacterial contamination of the DCU suction system

1.6.1 Introduction

While many previous papers (Abel *et al.*, 1971; Williams *et al.*, 1993; Walker *et al.*, 2000; Putnins *et al.*, 2001; Tuttlebee *et al.*, 2002) have investigated the contamination of DUWs, there are very few publications investigating the extent of microbial contamination of DCU suction systems. The suction system is designed to remove excess fluids that have been generated during dental work, e.g. dental unit water, saliva and blood, from the patient's mouth. As such, the suction system quickly becomes contaminated with both oral and dental unit waterline-derived microorganisms. Biofilm may build up as microorganisms contained in the dental unit water, saliva and other fluids aspirated from the patient's mouth adhere to the lumen of the hosing and proliferate.

1.6.2 Bacteria associated with suction systems

Barbeau *et al.* (1998) recently characterised the bacterial flora associated with suction systems using microbiological cultures and transmission electron microscopy. This study showed that the lumen of suction system hosing was coated with microbial biofilms where most bacteria were metabolically active and in which microcolonies of Gram-positive cocci and Gram-negative bacilli were visible, embedded in an EPS matrix. Most bacteria isolated were staphylococci, micrococci and non-fermentative Gram-negative rods. *Pseudomonas aeruginosa* and *S. aureus*, both known pathogens, were also isolated, indicating a risk of cross-infection (Barbeau *et al.*, 1998).

A previous study from this laboratory also investigated contamination of Planmeca Prostyle Compact DCU suction systems in the Dublin Dental Hospital using culture methods because areas of dampness and corrosion were observed on many of the

baseplates of the hospital's DCUs at the site of attachment of the suction hoses (Figures 1.3 and 1.4). This previous study showed that the corroded areas were heavily contaminated with *Pseudomonas* species and related genera posing a risk of cross-contamination and cross-infection, particularly for immunocompromised patients (O'Donnell, 2002). These species were then used as marker organisms to investigate the source of the contamination. Four separate sites within the suction systems of 41 separate DCUs were sampled: the proximal and distal ends of the high volume suction hosing, the baseplates to which the distal end of the suction hosing was attached to the DCU pedestal unit and the coarse filter cage housing in which the removable coarse filter is housed. The predominant microorganisms were found to be non-fermentative Gram-negative bacteria with *P. aeruginosa*, *P. putida* and *S. maltophilia* being the most prevalent. Other microorganisms isolated from the suction system included *P. fluorescens*, *B. cepacia* and *Comamonas acidovorans* (O'Donnell, 2002). This is of concern because these organisms are known opportunistic pathogens.

1.6.3 Cross-contamination potential of DCU suction systems

The fact that opportunistic pathogens are present within the biofilm lining the suction system is of concern not least because a number of studies that have investigated suction systems have also shown that liquid from the suction system hosing can enter a patient's mouth during use (Watson & Whitehouse, 1993; Mann *et al.*, 1996; Barbeau *et al.*, 1998). Backflow from the suction system back into patients' mouths sometimes occurs if patients form a seal with their lips around the tip of the saliva ejector, following the removal of the sealed lips from the tip (Watson & Whitehouse, 1993). A similar study published in 1998 also found that bacteria associated with backflow were sometimes aspirated back into the patient's mouth following treatment (Barbeau *et al.*, 1998). This indicates that retraction of oral fluids and biofilm-derived microorganisms from contaminated suction lines could be a source of cross-infection for patients. Furthermore, maintenance and dental staff are regularly exposed to the potential risk of infection from suction system biofilm at the attachment end of the suction hose during routine maintenance and disinfection of the suction system and also at the coarse filter cage housing following the removal of the coarse filter cage after each clinical session.

1.6.4 Current guidelines regarding disinfection of suction system hosing

While a new or sterile suction tip is placed on the proximal end of the suction system hosing during patient treatment and is removed after use, it is not practical to

replace or sterilise the hosing itself between patients. The United States Center for Disease Control (CDC) recommend that any dental unit surface that may have become contaminated with patient material, which includes suction system hosing, should be disinfected after treatment of each patient and at the end of each working day, using a chemical germicide with mycobactericidal activity, such as a phenolic, iodophor or chlorine-containing compound (Centers for Disease Control and Prevention, 1993). The mycobactericidal activity is specified because mycobacteria are such a resistant group of organisms that germicides effective against these will undoubtedly also be effective against many other pathogens too (Centers for Disease Control and Prevention, 1993).

1.6.5 Current practices regarding disinfection of suction system hosing

Despite these recommendations, a study undertaken in Canada by Watson and Whitehouse (1993) indicated that only 23% of surgeries surveyed disinfected the suction hosing after each patient. While 9% of the surgeries disinfected the suction hosing twice-daily, a further 41% disinfected or rinsed the suction hosing only once daily and the remaining 27% disinfected or rinsed the suction system hosing only once a week (Watson & Whitehouse, 1993).

Because the DCU suction system operates in a manner analogous to a household vacuum cleaner, all liquids, including disinfectants, are aspirated rapidly through the hosing. Thus, any disinfectant used to disinfect the hosing would have a very limited contact time with the internal lumen of the hosing and would presumably also have limited efficacy against biofilm lining the suction system hosing. In this regard, it is important to note that it is well documented that microorganisms within biofilm are much more resistant to biocides than planktonic organisms, even those of the same species (Michel-Briand, 1981; Wilson & Dowling, 1998). For these reasons it is very likely that bacteria within biofilm lining the suction system hosing may survive despite regular disinfection.

1.6.5.1 Disinfection of the Planmeca Prostyle Compact DCU suction system

In the Dublin Dental Hospital, the phenolic disinfectant, Puli-Jet (Cattani, Parma, Italy), is used to disinfect the DCU suction system hosing after each clinical session (i.e. twice daily, Monday-Friday). This disinfectant is recommended for use in the suction system hoses by the manufacturers of the central suction plant that provides the vacuum for each DCU suction system. During each disinfection cycle, one litre of disinfectant diluted to the appropriate working concentration is aspirated through both the low-volume and

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high-volume suction hoses by activating the suction system. The estimated contact time of the disinfectant with the internal surfaces of the suction hosing is only 90 s.

1.7 Non-culture methods for assessing microbial diversity

1.7.1 Difficulties associated with cultivation of environmental microorganisms

It is being increasingly recognised that the vast majority of environmental microbes cannot yet be cultured in the laboratory. One recent study has estimated that only 20% of environmental bacteria are cultivable (Rolph *et al.*, 2001). Another study brought this estimate to as low as 1% (Schmeisser *et al.*, 2003). If these estimates are true, this means that the majority of bacteria are not accessible for basic research and may never be unless new culture methods are developed and applied (Schmeisser *et al.*, 2003). Furthermore, while many environmental bacteria are cultivable, they may have fastidious growth requirements and give unreliable results with commercial identification systems, leading to misidentification (Rolph *et al.*, 2001). Thus, current culture-dependent investigations of various environments cannot accurately determine the diversity of microorganisms present. Some culture-independent approaches employing molecular techniques have been used to detect specific bacteria within microbial communities such as the use of oligonucleotide probes (Rolph *et al.*, 2001). Again, however, the use of specific DNA probes is biased, as it presupposes which microbes are of importance within the community (Rolph *et al.*, 2001).

1.7.1.1 Determination of microbial diversity using 16S rRNA sequence analysis

A recent approach to assessing the microbial diversity of environments is the use of 16S rDNA amplification and sequence analysis. This technique has been employed for use in microbial ecology for evaluation of the members of many diverse microbial communities, including biofilms lining gas industry pipelines (Zhu *et al.*, 2003), endodontic infections (Rolph *et al.*, 2001), tongues of halitosis patients (Kazor *et al.*, 2003) and DUW biofilms (Singh *et al.*, 2003). The advantage of this method is that it can detect bacteria that are currently uncultivable (Rolph *et al.*, 2001), as well as anaerobes and other fastidious organisms that may not be as easily cultured as fast-growing aerobes. Using 16S sequence analysis, the relative abundance of the different micro-organisms identified can be compared; however, this approach cannot be definitively described as quantitative because of the present lack of knowledge regarding the effect each stage of the protocol has on the final analysis. In this regard it is important to note that this culture-independent method is open to bias at various stages including cell lysis, DNA extraction and

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purification, primer choice, PCR parameters and cloning (Farrelly *et al.*, 1995). Of course, many of these parameters can be overcome with improved techniques and with standardisation of procedure. Other factors that may leave this method open to bias are the various genomic properties of each bacterial cell, such as genome size, genome copy number and the number of genes coding for 16S rRNA (Farrelly *et al.*, 1995). However, this approach is still currently the least biased method for investigating the diversity of microbial communities and reveals much more diverse ranges of microorganisms than culture methods (Singh *et al.*, 2003).

1.7.1.2 Use of real-time PCR in quantitating numbers of bacteria in environmental samples

As discussed above, culture-dependent methods for enumerating bacteria are sometimes biased as bacteria can only be cultivated if all their metabolic requirements are reproducible *in vitro* and enumeration of fastidious microbes may yield misleading results. Also, culture-dependent techniques may take several days to yield results (Nadkarni *et al.*, 2002). Molecular methods for detection of bacteria in samples have been developed that apply PCR techniques to overcome the limitations of culture (Wellinghausen *et al.*, 2001). PCR techniques have advantages, such as detection of non-culturable bacteria, fast acquisition of results and easier handling of large sample numbers (Wellinghausen *et al.*, 2001).

Real-time PCR is a new technology that amplifies a target sequence using oligonucleotide primers that are homologous to a region within the target sequence. However, unlike conventional PCR, this technology can also be used to quantify the number of copies of the gene in the original sample. When using the ABI Prism 7700 Sequence Detection System (TaqMan) (which was used in this study) for *real-time* PCR, a fluorescent sequence-specific hybridisation probe is also included within each sample mixture. This probe is designed so that it is homologous to a region within the target sequence amplified by the primers. A fluorescent signal, such as 6-carboxyfluorescein (6-FAM), the fluorescent signal used in this study, is attached to the probe (Nadkarni *et al.*, 2002). While the probe is in the intact state and bound to the homologous region of the amplimer, the fluorescent signal is quenched by the close proximity on the probe of a second dye, 6-carboxy-tetramethylrhodamine (TAMRA) (Figure 1.6). However, following cleavage of the fluorescent dye by the 5′-exonuclease activity of *Taq* polymerase during each round of amplification, fluorescence can be detected by the TaqMan Sequence Detection System and the amount of fluorescence detected is directly proportional to the amount of accumulated PCR product (Nadkarni *et al.*, 2002). A minor-groove DNA
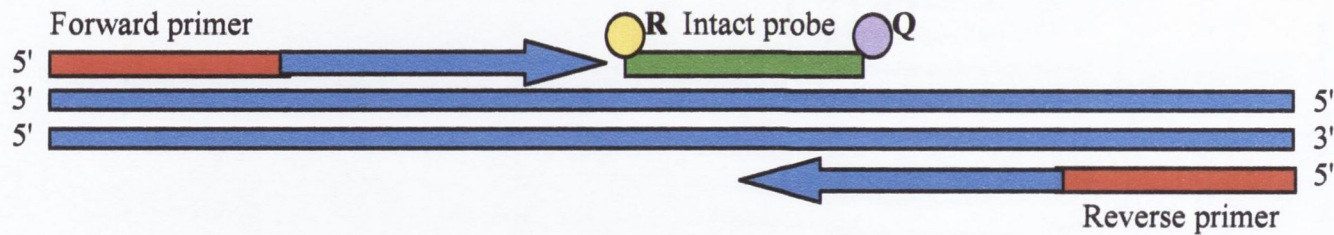
Figure 1.6. Schematic diagram of principle of TaqMan *real-time* PCR. During synthesis of new 5' and 3' strands, primers and probe are bound to the template DNA. While the probe is intact, the 3' quencher dye (Q) (TAMRA) quenches expression of the 5' fluorescent reporter (R) (FAM). As the new strand is synthesised the probe is displaced and the fluorescent reporter is released, enabling fluorescence which is detected by the ABI Prism 7700 Sequence Detection System.

binding dye known as SYBR Green with a high affinity for double-stranded DNA can also be used instead of the sequence-specific hybridisation probe as it exhibits enhanced fluorescence upon binding to the DNA (Dhar *et al.*, 2001). However, as SYBR Green binds indiscriminately to all double-stranded DNA, it should only be used once it has been established that the primers are specific for the sequence being amplified and that there is no primer-dimer formation or cross-reaction of any sort. This is in order to avoid misleading results that could arise should SYBR Green bind to double-stranded DNA other than the target sequence.

A number of samples containing known amounts of the target sequence are amplified simultaneously with the unknown samples so that the amount of target DNA in the unknown samples can be interpolated. This allows the quantification of DNA without any need for post-PCR processing, which eliminates the risk of potential contamination and also reduces the handling time (Desjardin *et al.*, 1998). Also, the 96-well format of the TaqMan system allows a high number of samples to be analysed simultaneously (Nadkarni *et al.*, 2002).

1.8 Aims and objectives

The aims and objectives for the present study were as follows:

1. To investigate levels of microbial contamination in dental unit waterline (DUW) output water in the Dublin Dental Hospital and other dental clinics and to identify the bacterial species present because, to date, no other published studies from the Republic of Ireland have investigated DUW contamination.

2. The density of microbes in DUW output water needs to be reduced and controlled because of the increased numbers of medically and immunocompromised patients (e.g. the elderly, cancer patients and HIV-positive individuals) attending dental clinics for treatment. Therefore, this study aimed to test the efficacy of the novel Planmeca Waterline Cleaning System, the first custom-designed disinfection module for the disinfection of DUWs, in a prospective controlled study. Two peroxide-based disinfectants, one of which has been approved by the American Dental Association for use in DUWs (Kramer, 2000), were used with the Planmeca Waterline Cleaning System and were tested for their efficacy at reducing aerobic heterotrophic bacterial contamination of DUW output water to less than 200 cfu/ml. This study also aimed to determine the extent of biofilm formation within DUWs using scanning electron microscopy and to note if biofilm was removed after disinfection.

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3. A further aim of this study was to use *real-time* PCR for the rapid quantitative detection of numbers of *Legionella* species in DUW output water, particularly *L. pneumophila*, because it can take up to ten days to detect *Legionella* by culture methods. No previous study has used *real-time* PCR techniques for the detection of *Legionella* in DUW output water.

4. This study also aimed to further investigate the source of the bacterial contamination of dental chair unit (DCU) baseplates in the Dublin Dental Hospital by serotyping and DNA fingerprinting isolates of *P. aeruginosa* recovered from the DCU baseplates and various sites within the suction system of the same DCUs.

5. Finally, this study aimed to investigate the microbial diversity of biofilm within the high volume suction system hoses of Planmeca Prostyle Compact DCUs using 16S rDNA sequence analysis and culture methods in order to assess the potential risk of cross-infection. Very few studies have investigated biofilm within suction systems thus far and, therefore, very little is known about the potential risk of cross-infection of patients and staff from pathogens within the biofilm.

Chapter 2

General Material and Methods

2.1 General Microbiological Methods

2.1.1 Chemicals, enzymes and oligonucleotides

Analytical-grade or molecular biology grade chemicals were purchased from Sigma-Aldrich Ireland Ltd. (Tallaght, Dublin, Ireland) or Roche Diagnostics Ltd. (Lewes, East Sussex, UK). Custom-synthesised oligonucleotides were purchased from Sigma-Genosys Biotechnologies Europe Ltd. (Pampisford, Cambridgeshire, UK) and stored at a stock concentration of 1 mM in sterile water at $-20°C$. Lambda (λ) ladder molecular weight markers were purchased from New England Biolabs (Beverly, Massachusetts, USA). Restriction enzymes were purchased from Promega Corporation (Madison, WI, USA).

2.1.2 Culture media and growth conditions

Sterile 90 mm diameter single-vented Petri dishes were used for growth of microorganisms and contained 25 ml agar media. Unless otherwise specified, incubation was carried out in a static Gallenkamp incubator, Model IEF097-XX2.5 (Gallenkamp, Leicester, England).

2.1.2.1 Total aerobic heterotrophic bacteria

Water samples used for total aerobic heterotrophic bacterial density determinations were routinely cultured on R2A medium (Difco, Becton Dickinson, Le Pont de Claix, France), at room temperature for six to seven days. Dental chair unit (DCU) suction system samples were routinely cultured on nutrient agar (NA) medium (Oxoid Ltd, Hampshire, England) for up to five days at 30°C. Following incubation, the number of cfu on each plate was recorded, as well as a brief description of the phenotypic appearance of different colony types present, including colour, shape and size.

2.1.2.2 Legionella *isolates and strains*

Legionella reference strains and water samples tested for the presence of *Legionella* species were routinely cultured on Buffered Charcoal Yeast Extract (BCYE) agar (Oxoid) or BCYE agar supplemented with glycine, vancomycin, polymyxin B sulphate and cycloheximide (GVPC) (Oxoid), prepared according to the manufacturer's instructions. Plates were incubated for up to ten days at 37°C in an atmosphere of 5% (v/v) CO_2 in a Sanyo incubator, model MCO-17A1.

For liquid culture and rehydration of *Legionella* reference strains purchased from the American Type Culture Collection (ATCC) and the UK National Collection of Type

Cultures (NCTC), BCYE broth was prepared according to the protocol (ATCC medium: 1099) described on the ATCC website (www.atcc.org). Briefly, 10.0 g yeast extract (Sigma-Aldrich) and 2.0 g charcoal (Sigma-Aldrich) were suspended in 730 ml sterile distilled water. The pH was adjusted to 6.90 +/- 0.05 using KOH. Following sterilisation by autoclaving, 10 ml of 0.04% (w/v) L-cysteine.HCl solution (Sigma-Aldrich), 10 ml of 0.025% (w/v) ferric pyrophosphate (Sigma-Aldrich) solution and 250 ml of 4% (w/v) n-2-acetamido-2-aminoethane sulfonic acid (ACES) (Sigma-Aldrich) buffer were added to the agar suspension. KOH was again used to adjust the pH to 6.90 +/- 0.05 and the resulting solution was then filter-sterilised using sterile Minisart non-pyrogenic, hydrophilic 0.2 μm syringe filters (Sartorius AG, Göttingen, Germany).

2.1.2.3 Pseudomonas *isolates and strains*

Environmental samples investigated for the presence of *Pseudomonas* species and isolates of *Pseudomonas* and related genera were cultured on Pseudomonas Base Agar (PBA) (Oxoid) or PBA supplemented with cetrimide (10 μg/ml), fusidic acid (10 μg/ml), and cephaloridine (50 μg/ml) (Oxoid), prepared according to manufacturer's instructions.

2.1.2.4 Escherichia coli *strains*

Escherichia coli strains were routinely cultured on Luria-Bertani agar (L agar) (Sambrook *et al.*, 1989) at pH 7.4 at 37°C or, for liquid cultures, in Luria-Bertani broth (L broth), (pH 7.4) (Sambrook *et al.*, 1989) at 37°C in an orbital incubator, model 10x400.xx2.c (Gallenkamp), at 200 rpm.

The *E. coli* strain JM109 (Promega; genotype *endA*1, *recA*1, *gyrA*96, *thi*, *hsi*17 (r_k, m_k^+), relA1, *supE*44, Δ (*lac-proAB*), [F', *traD*36, *proAB*, *lacI*$_q$Z Δ M15]) was used as the host strain for the plasmid pBluescript II KS (-) (Stratagene, La Jolla, California, USA) and the pGEM® T-Easy Vector (Promega) was cultured on L agar containing ampicillin, X-Gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) (Sigma-Aldrich) and IPTG (isopropyl-β-D-thiogalactopyranoside) (Roche) at concentrations of 100 μg/ml, 40μg/ml and 40 μg/ml, respectively.

2.1.3 Storage of bacterial isolates

2.1.3.1 Storage of reference strains at −70°C

Following culture of all reference strains received from the NCTC and the ATCC, five to six colonies were removed and suspended in individual cryo-vials (Protect, Protect-Technical Service Consultants, Lancashire, UK) for storage at −70°C.

2.1.3.2 Storage of Legionella *reference strains on agar slopes*

Legionella reference strains were also streaked onto BCYE agar (Oxoid) that had been poured as slopes in sterile five-ml glass vials. Following inoculation of slopes, the *Legionella* reference strains were incubated at 37°C in 5% CO_2 (v/v) for five to seven days and then removed and stored in the dark at room temperature.

2.1.3.3 Storage of other bacteria on slopes

Non-*Legionella* species reference strains and bacteria isolated from DCU suction system and dental unit waterline (DUW) output water were inoculated onto NA slopes (Cruinn Technologies Ltd, Knockmitten Lane, Dublin, Ireland) and incubated for 48 h at 30°C before storage in the dark at room temperature.

2.1.4 Buffers and solutions

TBE buffer was prepared at 10X concentration and consisted of 0.9 M Trizma base, 0.45 M boric acid and 0.01 M EDTA. Following dilution in distilled water to 0.5X TBE buffer was used as the buffer for agarose gel electrophoresis and pulsed field gel electrophoresis (PFGE).

Tris-EDTA (TE) buffer was used to rinse agarose plugs and consisted of 10 mM Tris-HCl and 1 mM EDTA, pH 6.6.

DNA loading dye was prepared at 10X concentration and consisted of 30% (v/v) glycerol, 0.25% (w/v) bromophenol blue and 3.8% (w/v) EDTA.

SE buffer consisted of 75 mM NaCl and 25 mM EDTA (pH 7.4) and was used for resuspension of bacterial cells for incorporation into agarose gel plugs for use in PFGE.

For digestion of *P. aeruginosa* chromosomes within agarose plugs, 2.5 mg Proteinase K (Roche) per ml was suspended in ES buffer (1% (w/v) N-lauroylsarcosine and 0.5 M EDTA).

For digestion of *Legionella* chromosomes within agarose plugs, 2 mg Proteinase K (Roche) per ml was suspended in Tris-ES buffer (1% (w/v) N-lauroylsarcosine, 0.5 M EDTA and 10 mM Tris-HCl [pH 8.0]).

2.1.5 Identification of bacteria

2.1.5.1 Identification of bacteria isolated using R2A, PBA or NA media

A description of each colony phenotype (i.e. colour, size, mucoid, filament formation etc) present on each type of culture media was recorded and selected examples of the colony types present were purified by subculture for subsequent identification.

2.1.5.1.1 Catalase test

Microorganisms isolated from DCUs were routinely tested for their ability to break down hydrogen peroxide by catalase production. First, a 30% hydrogen peroxide stock solution (Sigma-Aldrich) was diluted to 10% with sterile ultra-pure water. One colony of each isolate was then suspended in a drop of 10% hydrogen peroxide solution on a glass slide. If the drop of hydrogen peroxide then became rapidly effervescent, indicating its reduction to water and oxygen, the isolate was considered strongly catalase-positive. Colonies that produced minor effervescence following suspension in hydrogen peroxide were considered weakly positive, while those that yielded no effervescence after one min were considered to be catalase negative.

2.1.5.1.2 Oxidase test

Bacteria were tested for oxidase production using the BBL™ DrySlide™ (Becton Dickinson & Co., Maryland, USA) system, according to the manufacturer's instructions. Briefly, a single colony was removed from a fresh PBA plate using a sterile disposable plastic inoculating loop (Sigma-Aldrich) and rubbed on to the surface of a DrySlide™. A positive reaction is indicated by the development of a purple colour on the DrySlide™ surface within 20 s. This test indicates the presence of cytochrome oxidase which oxidises cytochrome c, which in turn oxidises reagents like N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (present in the DrySlide™) to form a purple-coloured compound. Bacteria that contain cytochrome c as part of their respiratory chain are oxidase-positive and turn the reagent purple; oxidase-negative bacteria lack cytochrome c and therefore have no reaction with reagents within the DrySlide™.

2.1.5.1.3 Biotyping using API 20NE system

As the vast majority of isolates tested were Gram-negative rods, formal identification was undertaken using the API 20 NE system (bioMérieux, Marcy-l'Etoile) for identifying non-enteric Gram-negative rods, according to manufacturer's instructions. The API 20 NE system can distinguish between and identify sixty-one bacterial species. It

is presented in a plastic strip format containing 20 tubules of dehydrated media or substrate (Table 2.1). A standardised inoculum of each isolate for identification is inoculated into each tubule and the strip incubated at 30°C for 24-48 h, following which time the isolate can be identified on the basis of the pattern of growth or reaction in the individual tubules.

Each 20 NE strip was supplied with a disposable plastic incubation box consisting of a ribbed base tray and a lid. Five-ml aliquots of sterile distilled water were placed into each tray to provide humidity during incubation and prevent the 20 NE strips from drying out. For each isolate to be tested, one 20 NE test strip was removed from its packaging and immediately placed into an incubation box tray.

One to four fresh colonies of each purified isolate were picked from an agar plate using a sterile disposable inoculating loop (Sigma-Aldrich) and placed into individual ampoules containing 2 ml of sterile NaCl 0.85% (w/v) solution (bioMérieux). The suspension was homogenised using the loop to achieve turbidity equivalent to the 0.5 McFarland turbidity standard (bioMérieux).

For each 20 NE strip, the tubules containing potassium nitrate, tryptophane, glucose, arginine, urea, esculin, gelatine (with Indian ink) and p-nitrophenyl-β-D-galactopyranoside (Table 2.1) were inoculated with 200 µl of the bacterial suspension. Mineral oil was added to the tubules containing glucose, arginine and urea. The inoculum for the remaining tubules was prepared by adding 200 µl of the bacterial suspension described above to a seven-ml ampoule of AUX medium (per litre; ammonium sulphate 2 g, agar 1.5 g, mineral base 82.8 mg, amino acids 250 mg, vitamin and nutritional supplements 35.9 mg in 0.04 M phosphate buffer at a final pH of 7.0-7.2) supplied with the 20 NE system. The contents were mixed gently by aspiration with a pipette. Aliquots containing 350 µl of this suspension were then used to fill the entire microtube of those containing glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine, maltose, gluconate, caprate, adipate, malate, citrate, or phenyl-acetate (Table 2.1). Following inoculation, the lid was replaced on each incubation box tray and the boxes were incubated at 30°C. Strips were read at 24 and 48 h.

Following 24 h incubation, each strip was removed from the incubator. One drop each of reagents NIT 1 (bioMérieux; sulfanilic acid 0.4 g, acetic acid 30 g, and H_2O 70 ml) and NIT 2 (bioMérieux; N,N-dimethyl-1-naphthylamine 0.6 g, acetic acid 30 g, H_2O 70 ml) were added to the NO_3 tubule. If a red colour appeared within 5 min, a positive reaction was recorded. If no colour change was observed 2-3 mg zinc dust was added to the tubule in case the bacteria had further reduced the nitrates to nitrogen gas. If, after a further 5 min, no colour change was observed, a positive reaction was recorded. However, if a red

Table 2.1 Tests included in the API 20NE strip (bioMérieux).

Tests	Substrates	Reactions/ Enzymes	Results Positive	Results Negative
NO3	Potassium nitrate	Reduction of nitrates to nitrites	***NIT 1 + NIT 2 for 5 min*** Pink	Colourless
		Reduction of nitrates to nitrogen	***Zinc powder for 5 min*** Colourless	Pink
TRP	Tryptophane	Indole production	***James reagent*** Pink	Yellow/ green
[a] GLU	Glucose	Acidification	Yellow	Blue/ green
[a] ADH	Arginine	Arginine dihydrolase	Orange/ pink/ red	Yellow
[a] URE	Urea	Urease	Orange/ pink/ red	Yellow
ESC	Esculin	Hydrolysis (β-glucosidase)	Grey/ brown/ black	Yellow
GEL	Gelatine	Hydrolysis (protease)	Black	No change
PNPG	*p*-nitrophenyl β-D-galactopyranoside	β-galactosidase	Yellow	Colourless
[b] GLU	Glucose	Assimilation	Opaque[c]	Transparent[c]
ARA	Arabinose	Assimilation	Opaque	Transparent
MNE	Mannose	Assimilation	Opaque	Transparent
MAN	Mannitol	Assimilation	Opaque	Transparent
NAG	*N*-acetyl glucosamine	Assimilation	Opaque	Transparent
MAL	Maltose	Assimilation	Opaque	Transparent
GNT	Gluconate	Assimilation	Opaque	Transparent
CAP	Caprate	Assimilation	Opaque	Transparent
ADI	Adipate	Assimilation	Opaque	Transparent
MLT	Malate	Assimilation	Opaque	Transparent
CIT	Citrate	Assimilation	Opaque	Transparent
PAC	Phenyl-acetate	Assimilation	Opaque	Transparent

[a] Mineral oil was added to tubules containing glucose, arginine and urea before incubation

[b] Tests positioned above the dashed line were inoculated with 200 μl bacterial suspension in 0.85% (w/v) NaCl, while tests positioned below the dashed line were inoculated with 350 μl bacterial suspension prepared by addition of 200 μl bacterial suspension in 0.85% NaCl into 7 ml AUX medium (bioMérieux)

[c] An opaque appearance in the microtubule indicates growth of test isolate, while a transparent appearance indicates absence of growth

colour appeared this time, the reaction was recorded as negative because the zinc reduced the nitrates to nitrogen within the tubule (Table 2.1).

In the case of the indole production test, one drop of James reagent (bioMérieux; compound J 2183 [composition confidential-not disclosed by bioMérieux] 0.5 g and HCL 1 N per 100 ml) was added to the TRP tubule. If a pink colour developed immediately the reaction was recorded as positive; otherwise, a negative reaction was recorded.

The reactions in the remaining 17 tubules were then recorded and the strip re-incubated for a further 24 h, after which time these 17 tests were reread and the results recorded. Having recorded all of the test results as positive or negative, a substrate assimilation profile of each isolate was converted into a seven-digit numerical profile. These numerical profiles were then cross-referenced in the 20 NE (Version 6) database of the APILAB software package (bioMérieux) to obtain identification of each test isolate.

In the database, each numerical profile is listed with a percentage of identification, which is an estimate of how closely the profile corresponds to that of a particular taxon, relative to all other taxa in the database and the T index, which is an estimate of how closely the profile corresponds to the most typical set of reactions for a particular taxon. Based on these parameters, a profile that closely resembles those of a particular taxon will be classed as "excellent" or "good" identification, yielding identification to the genus or species level, whereas atypical results are classed as having low discriminatory powers and are usually unable to yield good identification.

2.1.5.2 Identification of Legionella species

BCYE and GVPC plates inoculated with test water samples and incubated as described above were examined every three to four days for up to ten days; any colonies exhibiting typical legionella morphology (bluish-white round colonies with translucent edges; see Figure 2.1) were subcultured onto another GVPC plate and also onto a Colombia blood or NA plate, as recommended in the ISO guidelines (Bartie *et al*., 2003). Any colonies displaying the typical legionella morphology on GVPC agar that could not grow on agar lacking cysteine (i.e. Colombia blood or NA plates) were presumed to be legionellae.

2.2 Analysis of genomic DNA

2.2.1 Extraction of genomic DNA from bacterial species

Genomic DNA was prepared from bacterial cells grown overnight (16 h) in individual sterile 13-ml plastic test tubes (Sarstedt, Nümbrecht, Germany) containing 2 ml

Figure 2.1. Appearance of *Legionella* colonies on GVPC agar following incubation in a Sanyo CO_2 incubator at 37°C for up to 10 days.

nutrient broth (NB; Oxoid) at 30°C rotating at 200 rpm in an orbital incubator, model 10x400.xx2.c (Gallenkamp). Following overnight growth, the bacterial suspension was removed using a pipette and placed in 1.5 ml Eppendorf microfuge tubes (Eppendorf AG, Hamburg, Germany). Cells were then harvested by centrifugation in an Eppendorf microfuge (Model 5417C) at 7,500 x g for 1 min. The supernatant was removed and DNA was extracted following treatment with lysozyme and Proteinase K, using the DNeasy tissue kit (Qiagen), according to the manufacturer's instructions as described briefly below.

Following removal of the supernatant, the pellet was resuspended in 180 µl lysis buffer containing 20 mg of lysozyme per ml (Sigma-Aldrich), as previously described (Singh *et al.*, 2003). The resuspended sample was incubated in a static incubator (Gallenkamp) at 37°C for 30 min. Nucleic acids were then extracted from the sample using the DNeasy® Tissue Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions for the extraction of DNA from Gram-positive and Gram-negative bacteria. Briefly, following lysozyme treatment as described above, cells were further digested with Proteinase K (Qiagen) at 70°C. A 200-µl aliquot of ethanol (Sigma-Aldrich) was added and the suspension was then pipetted into clean one-ml columns placed in 1.5 ml collection tubes, both of which were supplied with the kit (Qiagen). Following centrifugation of the tubes, the cells were washed twice using the wash buffers supplied with the DNeasy kit (Qiagen). The columns were then placed in sterile 1.5 ml microfuge tubes (Eppendorf) and DNA was collected following addition of 150 µl elution buffer to the column by centrifugation. The concentration of each resulting DNA solution was determined by measuring its absorbance at 260 nm using a Genesys 2 spectrophotometer (ThermoSpectronic, Thermo Electron Group, Waltham, MA, USA) and calculating the concentration using the formula: 1 unit of A_{260} = 50 µg DNA. Samples were then stored at -20°C until required.

2.2.2 Macrorestriction fragment analysis of bacterial species

2.2.2.1 Harvesting of bacteria for incorporation into agarose plugs

2.2.2.1.1 Harvesting of *P. aeruginosa* for incorporation into agarose plugs

For preparation of *P. aeruginosa* chromosomes in agarose plugs, 45-ml aliquots of tryptone-broth (1% (w/v) casein hydrolysate, 0.5% (w/v) NaCl, (pH 7.5)) in sterile 50-ml Falcon tubes (Becton Dickinson) were inoculated with *P. aeruginosa* and incubated in an orbital incubator (Gallenkamp) at 37°C at 220 rpm overnight (15 h). Following incubation, each 45-ml sample was centrifuged in an Eppendorf Model 5804 centrifuge at

3,000 x *g* for 10 min. The supernatant was discarded and the remaining pellet was resuspended in 900 µL SE buffer.

2.2.2.1.2 Harvesting of *Legionella* species for incorporation into agarose plugs

For preparation of *Legionella* chromosomes in agarose plugs, the legionellae of interest were inoculated onto BCYE agar (Oxoid) and incubated for four days at 37°C in 5% CO_2 (v/v). Following incubation, three colonies of each isolate were suspended in separate 1.5 ml microfuge tubes (Eppendorf) containing 0.5-ml aliquots of SE buffer.

2.2.2.2 Preparation of agarose plugs

For bacterial suspensions of either *P. aeruginosa* or *Legionella* species one 500-µl aliquot of the resulting bacterial suspension was mixed gently with an equal volume of 2% (w/v) low melting point Genetic Technology Grade™ InCert® agarose (BioWhittaker Molecular Applications, Rockland, ME, USA) at 45°C. Each mixture was then dispensed in triplicate into moulds (10 mm × 6 mm × 1 mm) (Bio-Rad Laboratories, Hercules, CA, USA) and was allowed to solidify for 30 min at 4°C. After this time, each agarose plug was removed from the mould and placed in a 1.5 ml Eppendorf microfuge tube.

Plugs containing *P. aeruginosa* cells were incubated for 72 h at 55° C in ES buffer with the buffer being changed daily, after which time plugs were removed from the buffer, washed three times in TE buffer and stored at 4°C in TE buffer until used.

For plugs containing *Legionella* species, lysis of the bacteria within the plugs was carried out at 37°C for 1 h in lysis buffer (6 mM Tris-HCl; 1 M NaCl, 100 mM EDTA; 1 mg/ ml lysozyme; 0.06 mg/ ml DNase-free RNaseA; 0.5% Brij 58; 0.2% deoxycholate; 0.5% N-lauroylsarcosine). Plugs were then removed from the lysis buffer and incubated in a 1.5 ml Eppendorf microfuge tube containing Proteinase K buffer (10 mM Tris-HCl; 0.5 M EDTA; 2 mg/ml Proteinase K; 1% N-lauroylsarcosine) overnight in a water bath at 55°C, as previously described (Jonas *et al.*, 2000).

2.2.2.3 Restriction endonuclease digestion of chromosomal DNA in agarose plugs

For restriction enzyme digestion, a three-mm sample of each plug was cut and placed in a clean 1.5 ml Eppendorf microfuge tube for digestion by the appropriate restriction enzyme as discussed below in Sections 4.2.9 and 5.2.3.1. Following restriction enzyme digestion, plugs were removed from the buffer for separation of the digested high molecular weight DNA fragments within the plugs on 1.1% (w/v) ultra-pure DNA grade agarose (Bio-Rad) gels that had been cast in 210 x 127 mm trays.

2.2.2.4 Pulsed-field gel electrophoresis (PFGE)

Clamped homogeneous electrical field (CHEF) electrophoresis was used in this project to separate high molecular weight restriction fragments and was performed using the CHEF mapper PFGE apparatus (Bio-Rad) with 0.5X TBE as the electrophoresis buffer. The buffer was maintained at 14°C during electrophoresis by recirculation through the Bio-Rad minichiller (model 1000). The gels containing the plugs were placed securely into the gel tank and gels were subject to electrophoresis as described in sections 4.2 and 5.2.

Following electrophoresis, gels were stained with ethidium bromide (0.5 μg/ml) for 45 min, de-stained in water for 30 min and examined under UV light using a High Performance Ultraviolet Transilluminator (UVP, Ultraviolet Products, California, USA). Stained gels were photographed using Polaroid 667 film and PFGE DNA fingerprint profiles in the gels were also captured using the UVP Products Gel Documentation System ImageStore 7500 version 7.22 (UVP) and saved on diskette. DNA fingerprint profiles were analysed using the Dendron fingerprint profile analysis software package version 2.0 (Solltech, Iowa City, Iowa, USA) (Soll, 2000).

2.3 Recombinant DNA techniques

2.3.1 Small scale isolation of plasmid DNA from *E. coli*

Small scale preparations of plasmid DNA from *E. coli* cells were used for sequencing and were prepared using the Gen-Elute miniprep kit according to manufacturer's instructions (Sigma-Aldrich). Briefly, individual *E. coli* colonies thought to contain the plasmid of interest were inoculated into sterile plastic 13-ml test-tubes (Sarstedt) containing 2 ml L broth supplemented with ampicillin (100 μg/ml) and grown overnight at 37°C in a shaking incubator (Gallenkamp) rotating at 200 rpm. A 1.5-ml aliquot of the resulting bacterial suspension was placed in a sterile 1.5 ml Eppendorf microfuge tube and pelleted at 12,200 x *g* in an Eppendorf microfuge Model 5417C.

Following removal of the supernatant, plasmids were harvested using the Gen Elute Mini Prep kit according to the manufacturer's instructions (Sigma-Aldrich) with the sole exception of using only 60 μl sterile distilled water for elution of plasmid DNA in order to have a more concentrated sample for sequence analysis.

2.3.2 Polymerase chain reaction (PCR)

Bacterial rDNAs were amplified by PCR from 30 ng of DNA extracted from bacteria isolated from DUW output water or suction system biofilm and were cloned into the pGEM® T-Easy vector (Promega). A portion of the 16S rDNA gene was amplified

using the previously described primers 533F and 1492R (Singh *et al.*, 2003), which were synthesised by Sigma-Genosys (see Table 2.2). The specificity of the primers for all bacteria was confirmed by searching the GenBank databases for nucleotide sequence similarities using BLAST, which is run through the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/; Altschul *et al.*, 1990). These primers, 533F and 1492R, target regions of the *E. coli* 16S rDNA gene that have universal identity with all bacteria and may amplify regions of between 950 bp and 1.5 kb in length, depending on the bacterial species. Each reaction mixture was made up on ice and contained 1X Mg-free reaction buffer (Promega); 2.5 mM $MgCl_2$ (Promega); 200 µM of each dNTP (Promega); 300 nM of each primer and 2.5 U *Taq* DNA polymerase (Promega) made up to a total volume of 50 µl per reaction with sterile distilled water. Each reaction mixture was vortexed thoroughly and then transferred into separate wells on a Micro-Amp Optical 96 well reaction plate (Applied Biosystems, Foster City, California, USA), which was then placed in an Applied Biosystems 7700 thermal cycler. Reaction mixtures were initially denatured for 2 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s; annealing at 60°C for 30 s and extension at 72°C for 10 s. This was followed by a final extension step at 72°C for 2 min.

2.3.3 Purification of PCR amplicons from agarose gels

PCR products were resolved in 0.8% (w/v) agarose gels containing ethidium bromide (0.5 µg/ml). All PCR products with molecular weights between 1 kb and 1.5 kb were used to create clone libraries. Amplified PCR products were excised from the gels using a clean scalpel and purified using the Wizard® SV Gel and PCR Clean-up system (Promega) according to the manufacturer's instructions.

2.3.4 Ligation of DNA fragments

Clone libraries of purified PCR products were generated using the pGem® T-Easy Vector System I (Promega). The T-Easy vectors used in this system are prepared by the manufacturers by digestion with *Eco*RV followed by the addition of a 3' terminal thymidine (T) to both ends. The 3'-T overhangs at the vector insertion sites prevent recircularisation of the vector, thus improving the efficiency of ligation reactions with PCR products. Furthermore, because *Taq* DNA Polymerase adds a single deoxyadenosine to the 3'-ends of PCR products, the 3'-T overhangs at the insertion site of the vector also make for easier insertion of amplicons. The multiple cloning region of the vector is located within the α-peptide coding region of β-galactosidase; therefore, following insertion of the PCR

Table 2.2. Primers used in this study

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[a] 533F   5'-AGA GTT TGA TYM TGG CTC AG-3'   (Singh *et al.*, 2003)

[a] 1492R   5'-CGG YTA CCT TGT TAC GAC-3'   (Singh *et al.*, 2003)

[b] pUC/M13F   5'-CGC CAG GGT TTT CCC AGT CAC GAC-3' (Singh *et al.*, 2003)

[b] pUC/M13R   5'-TCA CAC AGG AAA CAG CTA TGA C-3'   (Singh *et al.*, 2003)

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[a] Primers used for amplification of a 1-1.5 kb region of the 16S rRNA gene
[b] Primers used for DNA sequencing following subcloning of PCR products
   into the pGEM® T-Easy Vector

product, recombinant clones can be directly identified by colour screening on appropriate media.

Ligation reactions were carried out according to manufacturer's instructions (Promega). Briefly, a 10 μl-reaction mixture containing 5 μl 2X rapid ligation buffer (Promega), 1 μl pGEM® T-Easy vector (Promega), 1 μl T4 DNA ligase and 3 μl purified PCR product was made up on ice and incubated at room temperature for 1 h to allow ligation to occur. One positive control (as above, but with 2 μl control insert DNA (Promega) and 1 μl sterile distilled water instead of PCR product) and one negative control (as above, but with 3 μl sterile distilled water instead of PCR product) were also made up and incubated for 1 h during initial experiments.

2.3.5 Transformation of competent *E. coli* prepared using CaCl$_2$

Preparation of competent *E. coli* cells was carried out according to the method of Sambrook *et al.* (Sambrook *et al.*, 1989). A two-ml overnight culture of *E. coli* JM109 (Promega) was used to inoculate 400 ml L broth in a two-l sterile glass conical flask. The inoculum was incubated at 200 rpm in an orbital incubator at 37°C for 2.5-3 h to a cell density equivalent to A_{600} 0.4-0.5, following which time the culture was chilled by placing the flask in ice for 1 h. The culture was then decanted into two ice-cold 250-ml centrifuge tubes (Sorvall, Dupont Co., Denver, Colorado, USA) and cells were pelleted by centrifugation at 4,000 rpm in a Sorvall GSA rotor at 4°C for 10 min. The supernatant was removed and each pellet was resuspended separately in 30 ml ice-cold 100 m M MgCl$_2$ and recentrifuged. Following centrifugation, pellets were resuspended separately in 100 ml ice-cold 100 mM CaCl$_2$ and placed on ice for 1 h, following which time the suspensions were recentrifuged. Following removal of the supernatant, each pellet was then resuspended once again in 20 ml ice-cold 100 mM CaCl$_2$. A three-ml aliquot of ice-cold 80% (v/v) glycerol was then added to each suspension to ensure a 10% concentration of glycerol. A chilled 10-ml disposable plastic pipette was then used to aliquot 0.5 ml of this final bacterial suspension into 1.5 ml Eppendorf microfuge tubes, which were kept on ice. The tubes were then placed in dry ice in order to snap-freeze the cells and from there stored in a freezer at −70°C.

For each transformation experiment one tube of frozen cells was removed from the freezer and thawed on ice. Fifty-μl aliquots of the thawed cells were pipetted into individual sterile 1.5 ml Eppendorf microfuge tubes. To each tube a 2.5-μl aliquot of ligated DNA was added and the suspension was mixed by aspiration with a pipette and

incubated on ice for 20 min. The tubes were then heat shocked at 42°C for 45-50 s and rapidly transferred to an ice bath for a further 2 min. The cells were then aspirated into sterile 50-ml Falcon flasks containing 950 µl L broth and incubated at 37°C for 90 min in a shaking incubator (Gallenkamp) at 200 rpm to allow the cells to recover and also to allow transformation cells to express the ampicillin resistance marker. Following this short incubation, cells were pelleted by centrifugation. Half the supernatant was removed and the cells were resuspended in the remainder. Following resuspension, 200-µl aliquots were spread on L agar plates containing ampicillin (100 µg/ml), IPTG (40 µg/ml) (Roche) and X-Gal (40 µg/ml) (Sigma-Aldrich).

Transformant colonies harbouring recombinant plasmids were selected using blue/white selection as described by Sambrook *et al.* (Sambrook *et al.*, 1989). Only white colonies were suspected of containing plasmids with cloned DNA inserts. Plasmids were harvested from these bacterial cells using the Sigma-Aldrich Gen-Elute miniprep kit, as described in Section 2.3.1.

2.3.6 Confirmation of the presence of cloned inserts in recombinant plasmids

The presence of cloned DNA inserts in recombinant plasmids was confirmed by restriction enzyme digestion with *Eco*R1 (New England Biolabs). The reaction mixture consisted of 0.5 µl plasmid, 1X Buffer H (Promega) and 3 U *Eco*R1 restriction enzyme made up to 5 µl with sterile distilled water, which was incubated at 37°C for 30 min in a static incubator (Gallenkamp). DNA fragments were resolved in 0.8% (w/v) agarose gels containing ethidium bromide (0.5 µg/ml). Plasmids that contained a DNA insert between 1 and 1.5 kb in length were stored at –20°C for further analysis.

2.4 DNA sequence analysis

DNA sequencing was undertaken on a commercial basis by Lark Technologies (Takeley, Essex, UK) using the dideoxy chain termination method described by Sanger *et al.*, (Sanger *et al.*, 1977), an automated Applied Biosystems 373A DNA sequencer and dye-labelled terminators. The sequencing of all cloned inserts was primed using the standard pUC/M13R primer (see Table 2.2). Some inserts were also sequenced using the pUC/M13F primer in order to confirm results (see Table 2.2). Chromatograms were analysed using the 373A Data Analysis programme, version 1.2.0 (Applied Biosystems). Sequences were then compared with published 16S rRNA gene sequences using the BLAST programme, which is run through the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/) for the determination of

closest matches (Altschul *et al.*, 1990). If the highest scoring sequence match (as determined by BLAST) had greater than 95% sequence similarity to the clone sequence they were considered to be of the same species.

2.5 Dublin Dental Hospital water supply

2.5.1 Treatment of mains water and supply to dental unit waterlines (DUWs)

Water from the Dental Hospital mains water supply destined for the dental chair units is transferred initially from the public water mains to a 12,000-l cold water storage tank (Figure 2.2). After leaving this tank, the water passes through two consecutive particle filters, which remove particles larger than 30 μm and 10 μm, respectively. Following particle filtration, the magnesium and calcium ions in the water are removed by ion exchange using a WSX-A Automatic Water Softener (Waterman, Dublin, Ireland). Following ion exchange, the water passes through an IH-1 SS Ultraviolet Disinfection Unit (Ideal Horizons, VT), which emits UV at 254 nm. After this initial UV treatment, the water then enters a 6,000-l recovery tank. This tank supplies a ring-main that provides water for the Dental Hospital's dental chair units. On leaving the 6,000-l holding tank, water passes through a second IH-1 SS Ultraviolet Disinfection Unit (Ideal Horizons), and is then pumped to the dental chair units or around the ring-main circuit back to the recovery vessel as depicted in Figure 2.2.

2.5.2 Annual treatment of mains water supply pipes

As part of routine maintenance, mains water supply pipes and dental unit waterlines are treated annually with Oxonia Active (Henkel-Ecolab Ltd, Bray, Co. Wicklow, Ireland), which is composed of 20-60% hydrogen peroxide and <10% peracetic acid. The primary aim of this procedure was to disinfect the wide-bore ring-main pipes that bring the water to the dental chair units. Prior to use the Oxonia Active preparation was diluted to 2% (v/v) by adding 2 l Oxonia Active to every 98 l in the 6,000-l holding tank. This was then distributed to all the DUWs of each dental chair unit in the hospital by flushing the handpieces to aspirate the disinfectant through all the mains water supply pipes (Figure 2.2). When the water being eluted from the waterlines contained 25 μg/ml H_2O_2, which was measured using Merckoquant® Peroxid-Test analytical test strips (Merck KgaA, 64271 Darmstadt, Germany), flushing was stopped and the solution was left in the mains water supply pipes and DUWs for 12 h to act on biofilm. After this time, the 6,000-l holding tank was flushed out with water to remove the solution. When the amount of H_2O_2
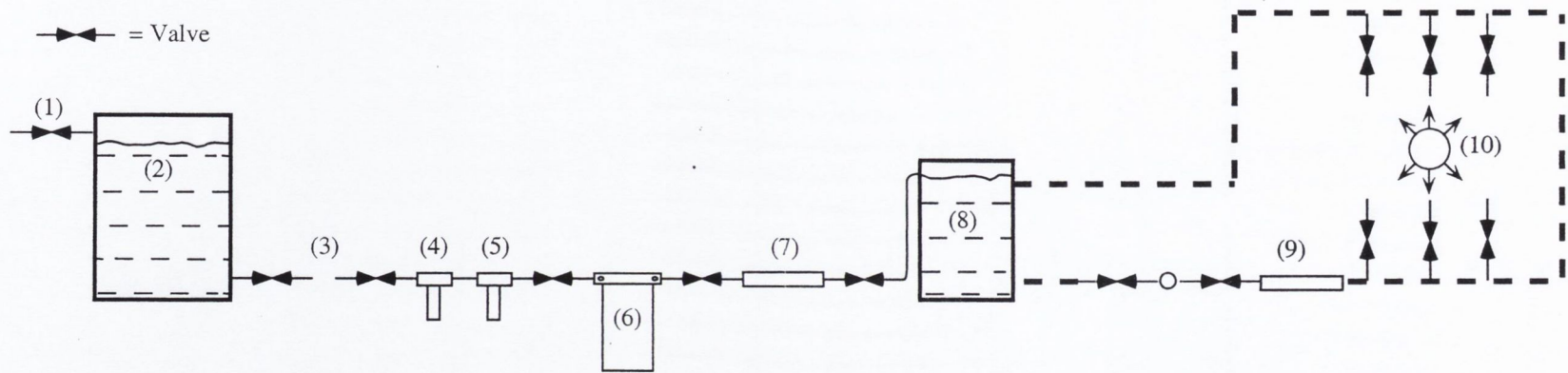
Figure 2.2 Schematic diagram of treatment system for input water destined for dental chair units in the Dublin Dental Hospital. Numbers in paretheses refer to specific components of the system as follows: (1) mains water intake, (2) 12,000-l water tank, (3) transfer pump, (4) 30μm particle filter, (5) 10μm particle filter, (6) ion exchange cartridge, (7) 254 nm UV source, (8) 6,000-l holding tank, (9) 254 nm UV source (10) dental chair unit outlets. The ring main providing water to the dental unit outlets is highlighted as a series of thick dashed lines on the right hand side of the figure.

was < 0.5 µg/ml, determined as above, using Merckoquant® Peroxid-Test strips (Merck), the water was considered safe for contact with patients.

Chapter 3

Effective control of dental chair unit waterline biofilm and marked reduction of bacterial contamination of output water using two peroxide-based disinfectants

3.1 Introduction

Large quantities of water are used during most dental procedures for irrigation, cooling of instruments and for oral rinsing. Inevitably, this water enters the oral cavities and the upper respiratory tracts (the latter as an aerosol) of patients receiving treatment. The presence of microbial biofilms in dental unit waterlines (DUWs) with a corresponding high level of microbial contamination of the output water was first reported over four decades ago (Blake, 1963) and studies over the ensuing forty years have shown that the problem of bacterial contamination of DUWs is widespread with virtually all standard DUWs providing contaminated water, ranging between 100 to 1,000,000 cfu/ ml, as discussed in Section 1.4 (Abel *et al.*, 1971; Furuhashi & Miyamae, 1985; Douglas & van Noort, 1993; Pankhurst & Philpott-Howard, 1993; Williams *et al.*, 1993; Williams *et al.*, 1994; Williams *et al.*, 1995; Pankhurst *et al.*, 1998; Putnins *et al.*, 2001; DePaola *et al.*, 2002).

The presence of large numbers of microorganisms in DUW output water is of concern as it poses an increased risk of infection for dental patients and staff and is incompatible with good hygiene and cross-infection control practices, particularly with regard to treatment provided to immunocompromised individuals. This group of individuals is much more susceptible to cross-infection and rigorous cross-infection control procedures should be followed during their treatment.

While most microorganisms isolated from the DUW output water are Gram-negative bacilli, such as those found in low numbers in mains water, where they are present in lower numbers, microorganisms normally resident in the oral cavity such as streptococci and *Candida* have also been recovered (Abel *et al.*, 1971; Furuhashi & Miyamae, 1985; Pankhurst & Philpott-Howard, 1993; Whitehouse *et al.*, 1991; Williams *et al.*, 1993; Pankhurst *et al.*, 1998; Mills, 2000; Walker *et al.*, 2000).

The design of the DUWs themselves lends itself to biofilm formation, with the narrow microbore of the tubing, approximately 1 mm in diameter, providing a high ratio of lumen surface area to water volume (Pankhurst *et al.*, 1998; Al Shorman *et al.*, 2002; DePaola *et al.*, 2002; Spratt *et al.*, 2004). Bacteria also adhere more readily to the hydrophobic polymeric plastic material used in DUWs (e.g. polyvinyl chloride, polyurethane) than to glass or steel tubing (Pankhurst *et al.*, 1998).

In recent years there has been increased media and public concern about deficiencies in infection control within the health care system in general. The United States, Japan and the European Commission have set national standards of 500, 100 and 100 cfu/ml, respectively, for aerobic heterotrophic bacteria in drinking water (Furuhashi &

Miyamae, 1985; Anonymous, 1998; Anonymous, 2002a). However, there are currently no standards for the microbiological quality of DUW output water set by the European Commission, nor is it a parameter subject to regular monitoring. However, the American Dental Association (ADA) set a goal for the year 2000 of no more than 200 cfu of heterotrophic bacteria per ml of DUW output water (Anonymous, 1996), although this has not yet been achieved in practice (Mills, 2000).

Many solutions have been proposed to reduce the numbers of bacteria and other microorganisms in DUW output water. These include flushing of DUWs between patients, and the use of biocides, such as ethanol (Furuhashi & Miyamae, 1985), chlorine (Meiller *et al.*, 2000), glutaraldehyde (Douglas & Rothwell, 1991) and ozone (Al Shorman *et al.*, 2002) to treat the biofilm within the DUWs.

3.1.1. Aims and objectives

This part of the present study aimed to examine the levels of microbial contamination in DUW output water of dental chair units (DCUs) located both in the Dublin Dental Hospital and in other Dublin dental clinics. This study also aimed to assess the efficacy of two peroxide-based disinfectants, Sterilex Ultra and Sanosil, at eliminating biofilm within DUWs and reducing microbial contamination of DUW output water using the novel Planmeca Waterline Cleaning system, which was designed by the DCU manufacturer Planmeca, and is the first DCU disinfection system custom-made for the disinfection of DUWs.

3.2 Materials and Methods

3.2.1 DCUs included in the study

Twenty DCUs from the hospital were included in this study; ten were not subjected to waterline disinfection and served as control units. Of the remaining ten dental units, the waterlines of six were disinfected weekly with Sterilex Ultra disinfectant (Sterilex® Corp., Baltimore, Maryland, USA), an alkaline peroxide-based powder, while the other four had their DUWs disinfected weekly with Sanosil® HWP- Blue (Sanosil) (Sanosil Ltd, Feldmeilen, Switzerland), a ready-to-use liquid disinfectant containing 1% (v/v) hydrogen peroxide and 0.001% (w/v) silver ions. The manufacturers of the DCUs included in this study, Planmeca, recommended the use of these disinfectants as they were compatible with the materials used in the DUWs of the DCUs.

3.2.2 Chemical analysis of water samples

Water samples from the processed mains supply and samples from air/water syringe waterlines of two DCUs were subjected to chemical analysis using standard methods (Clesceri *et al.*, 1998). The levels of calcium, magnesium and other metals in the water were determined by inductively coupled plasma-mass spectrometry, as shown in Table 3.1. Bicarbonate levels were determined by titration and the presence of ions such as sulfate, nitrate and ammonium were determined by colorimetric assay (see Table 3.1). The results obtained were then compared to the maximum admissible concentrations in water allowed by the European Commission, which are also shown in Table 3.1.

3.2.3 Annual treatment of mains water supply pipes

As part of routine maintenance, mains water supply pipes and dental unit waterlines are treated annually with Oxonia Active (Henkel-Ecolab Ltd), composed of 20-60% hydrogen peroxide and <10% peracetic acid, as described in Section 2.5.2. The aim of this procedure is to disinfect the wide-bore ring-main pipes that bring the water to the DCUs, and remove all biofilm from these ring-main pipes (see Figure 3.1). By the design of the water distribution system, however, the disinfectant material is also flushed into the DUWs of the DCUs. The disinfectant is left in the Hospital's ring-main supply pipes and the DUWs for 12 hours, thus cleaning the entire system. In order to determine if this Hospital wide disinfection procedure could have any additional benefit in reducing bacterial densities of DUW output water, the bacterial density of output water from the air/water syringe and cupfiller of one DCU was determined before, immediately after and once-weekly following the annual disinfection.

Table 3.1. Chemical analysis of treated mains water destined for the dental chair units and output water from an air/ water syringe from one Planmeca Prostyle Compaq dental chair unit

Test	Air/water syringe	Mains water	E.C.M.A.C.[a]	Method[b] of analysis
	mg/L			
Calcium	0.075	0.018	N/A	ICP-MS[d]
Magnesium	0.014	0.003	50.0	ICP-MS[d]
Sodium	56.65	57.19	150.0	ICP-MS[d]
Potassium	0.043	0.138	12.0	ICP-MS[d]
Iron	0.015	0.022	0.20	ICP-MS[d]
Manganese	<0.001	<0.001	0.05	ICP-MS[d]
Copper	0.041	0.008	3.00[b]	ICP-MS[d]
Lead	0.001	<0.001	0.05	ICP-MS[d]
Zinc	1.12	0.004	5.00	ICP-MS[d]
Bicarbonate[c]	64.05	61.0	N/A	Titration
Chloride	14.2	13.9	200.0	Colorimetric assay
Sulfate	<40.0	<40.0	250.0	Turbidimetric assay
Nitrate as NO_3	4.72	5.14	50.0	Colorimetric assay
Nitrite as NO_2	<0.05	<0.05	0.1	Colorimetric assay
Ammonia as NH_4	0.04	0.02	0.5	Colorimetric assay
Fluoride	0.91	0.89	1.50	Colorimetric assay
Phosphate as PO_4	<0.15	<0.15	6.7	Colorimetric assay
pH	7.37	7.79	6.5-9.5	Meter
Conductivity (µS/cm)	152.3	142.9	N/A	Meter

[a] European Commission maximum admissible concentration in water for human consumption (Anonymous, 1998)

[b] Based on standard methods as described previously (Clesceri, *et al.*, 1998).

[c] Carbonate is present in water as bicarbonate at pH values > 8.3.

[d] ICP-MS, inductively coupled plasma-mass spectrometry.
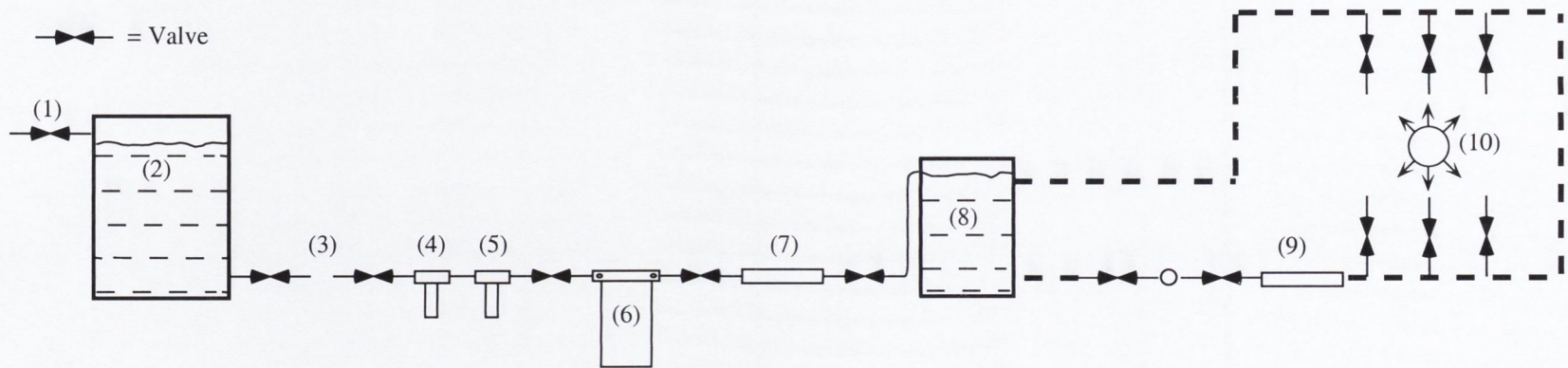
Figure 3.1 Schematic diagram of treatment system for input water destined for dental chair units in the Dublin Dental Hospital. Numbers in paretheses refer to specific components of the system as follows: (1) mains water intake, (2) 12,000-l water tank, (3) transfer pump, (4) 30μm particle filter, (5) 10μm particle filter, (6) ion exchange cartridge, (7) 254 nm UV source, (8) 6,000-l holding tank, (9) 254 nm UV source (10) dental chair unit outlets. The ring main providing water to the dental unit outlets is highlighted as a series of thick dashed lines on the right hand side of the figure.

3.2.4 Disinfection of DUWs in Dublin Dental Hospital

3.2.4.1 DCUs included in the Sterilex Ultra study

The efficacy of the alkaline-peroxide disinfectant Sterilex Ultra was assessed over a 20-week period. Initially, four Planmeca Waterline Cleaning System modules (Figure 3.2) designed and manufactured by Planmeca (the manufacturers of the DCUs used in this study) were available for testing and were fitted to four DCUs. Two of these DCUs were disinfected weekly for a 20-week period (January to May 2001). The other two DCUs were disinfected weekly for the first seven weeks (January to mid-February 2001), after which disinfection was then discontinued to investigate the length of time taken for the bacterial density to return to pre-disinfection levels. Eight weeks after the study began, two more Planmeca Waterline Cleaning System disinfection modules became available and were fitted to two additional DCUs, which were then disinfected weekly for a twelve-week period (March to May 2001).

3.2.4.2 DCUs included in the Sanosil study

The efficacy of the hydrogen peroxide- and silver ion-containing Sanosil disinfectant was assessed over an eight-week period (November 2001 to January 2002) with four additional DCUs fitted with the Planmeca Waterline Cleaning System. The waterlines of two of these DCUs were disinfected weekly using Sanosil for the entire eight-week period, while the remaining two DCUs had their waterlines disinfected weekly for the first five weeks, after which time disinfection was discontinued to determine how rapidly the bacterial density returned to pre-disinfection levels.

3.2.4.3 Planmeca Waterline Cleaning system

The Planmeca Waterline Cleaning system disinfection module consists of two parts; the disinfection module receiver (Figure 3.2A), which is fitted permanently to the dental unit, provides a mechanism whereby the internal DUWs can be isolated from the ring-main water supply by closing a valve (Figure 3.2A). Once the DUWs are isolated, the disinfection module (Figure 3.2B) is attached. This consists of an aluminium block which clips on to the module receiver attached to the dental unit and is connected to a 200-ml water bottle via an aluminium collar (Figure 3.2B). A plastic tube runs from inside the water bottle into the block, which in turn is connected directly to the DUWs. This enables the aspiration of the contents of the bottle into the DUWs. During disinfection, the DUWs in each test unit were isolated from the ring-main water supply, as described above. Then,
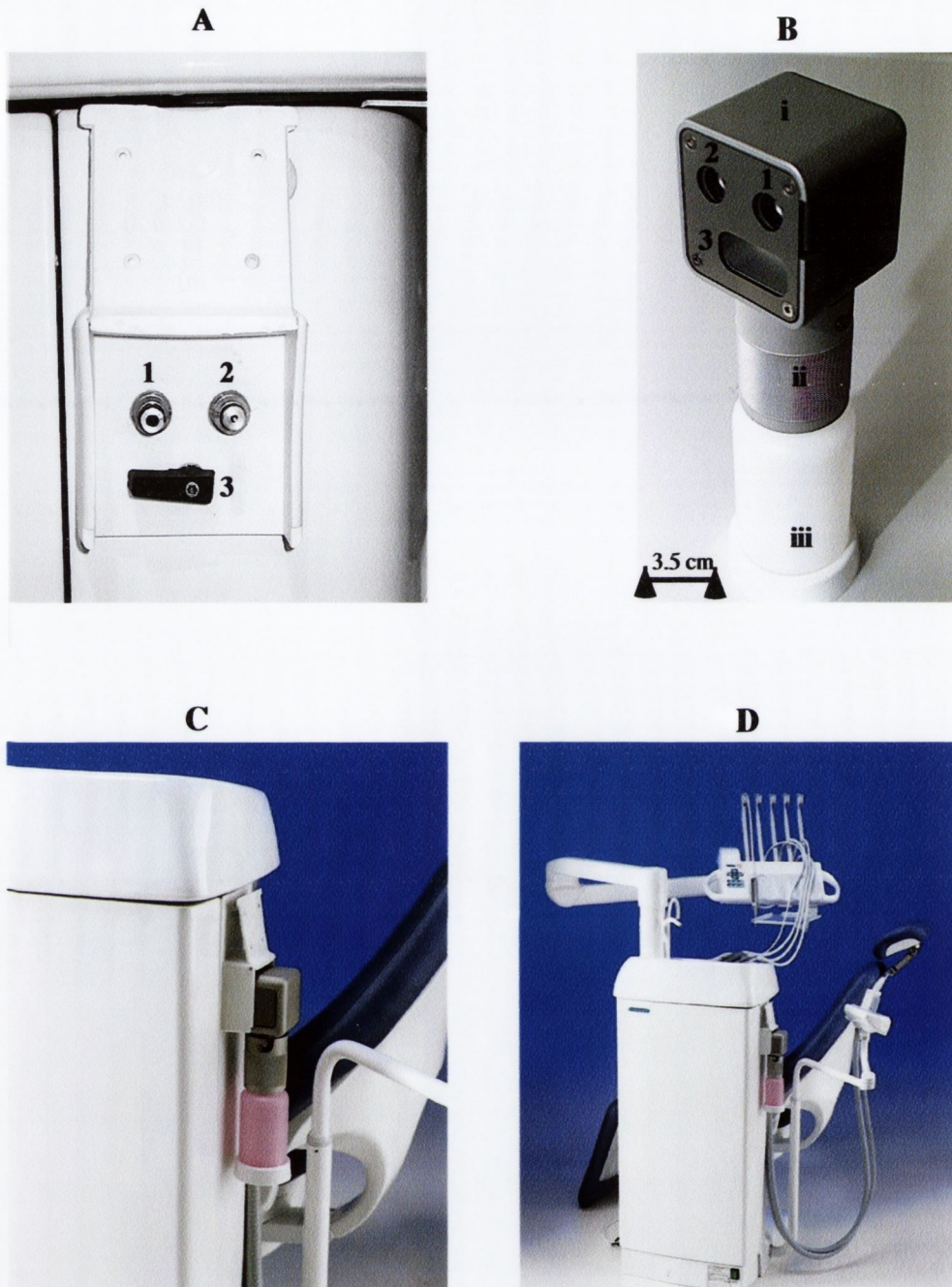
Figure 3.2. Components of the Planmeca Waterline Cleaning System used to disinfect DUWs in Planmeca Prostyle Compact dental chair units. The DUWs in each dental chair unit were plumbed directly to a mains water supply. (A) Disinfection module receiver permanently integrated into a dental chair unit showing (1) disinfectant/water inlet, linked directly to the DUWs, (2) air return outlet and (3) valve used to isolate the dental unit waterlines from the ring-main water supply. (B) Removable disinfection module showing (i) the aluminium block, the numbered components of which interface with the corresponding numbered components of the fixed module receiver shown in panel A, (ii) the central aluminium collar section which joins the aluminum interface block shown in (i) to the disinfectant container bottle shown in (iii). The block shown in (i) is designed so that it can only be attached to the receiver shown in panel A when the valve used to isolate the DUWs from the ring-main water supply is in the off position (see components labeled with a 3 in panels A and B). (C) Close-up view of removable disinfection module attached to a fixed receiver on a Prostyle Compact dental chair unit. (D) View of a Prostyle Compact dental chair unit with the removable disinfection module attached.

any residual water in the DUWs was drained by running the ultra-sonic scaler, three-in-one air/water syringes, conventional and turbine handpieces and cup filler and bowl rinse tubes for approximately 20 s each until no more water issued from any of the instruments.

3.2.4.4 Overnight disinfection and flushing

For disinfection with Sterilex Ultra, the disinfection module water bottle was filled with 200 ml of water at 50°C and one sachet (12.5 g) of disinfectant powder was added. The mixture was swirled thoroughly to dissolve the powder, forming a pink-coloured 6% (w/v) Sterilex Ultra solution. For disinfection using Sanosil, 200 ml of the 3 % (v/v) disinfectant was poured directly into the disinfection module water bottle.

Bottles containing disinfectant were then attached to the modules on the dental units via the aluminium block as described above (Figure 3.2). Disinfectant was aspirated through each internal DUW for approximately 15 s until the disinfectant solution emerged (Sterilex Ultra and Sanosil contain a pink dye and blue dye, respectively, for this purpose), after which time each waterline was turned off.

Initially, Sterilex Ultra solution was left in the DUWs of the test units for a 64–h period each weekend because the DUWs had not previously been subjected to regular disinfection and would have contained a very heavy build-up of biofilm. However, subsequent tests showed that 15-h (overnight) disinfection was as effective as 64-h disinfection in reducing bacterial density to acceptable levels, thus, from mid-February onwards (week 6), each test unit was disinfected once weekly for a 15-h period. Similarly, in the case of Sanosil, the disinfectant solution was initially left in the DUWs of the test units for a 64–h period each week-end for a two-week period and then once weekly for a 15-h period. For both disinfectants, following disinfection all the disinfectant was eluted from each unit by running each waterline for approximately 15 s until no more coloured solution was emitted and there was no liquid remaining in the disinfectant bottle. The bottle was then removed from the disinfection module, rinsed and filled with water at approximately 50°C and this hot water was aspirated through each internal DUW until, once again, no liquid was emitted from any of the instruments and the bottle had been emptied. The bottle was then removed and the water valve on the fixed module receiver was opened, reconnecting the unit waterlines to the ring-main water supply. Finally, each of the internal waterlines was rinsed again using the built-in flush function of the dental unit, which flushes water through the waterlines of the conventional handpiece, turbine handpiece and ultra-sonic scaler for 30 s each, and by running the remaining DUWs (cup

filler, air/water syringes and bowl rinse) for approximately 60 s, so that fresh water was also flushed through them.

3.2.5 Collection of water samples

Twenty-ml water samples were taken twice-weekly in sterile glass bottles from the air/water syringes and the cupfillers of the ten test DCUs and ten untreated DCUs included in the study, the morning prior to disinfection and immediately following disinfection. Samples were also taken from the water destined for the DCUs as it entered and left the 6,000-l cold water storage tank and after the first and second UV treatments, as described in Section 2.5.1 (see Figure 3.1). Additional water samples were taken from the air/water syringe waterlines of eighteen non-disinfected dental units from three other dental clinics in Dublin.

One hundred-ml samples of water from the air/water syringes of 18 dental units in three other Dublin dental clinics outside the Dublin Dental Hospital were taken early in the morning before clinics began. These dental units, which were all manufactured by Belmont (Takara Belmont Corporation, Osaka, Japan), ranged in age from less than one year old to ten years old and used bottle-fed reservoir systems with distilled water and did not have a cupfiller tube attached to the dental unit. Bacterial density was determined as for the output water from DUWs within the Dental Hospital.

3.2.6 pH of disinfectants and DUW output water after flushing

The pH of Sanosil and Sterilex Ultra disinfectants at the working concentration was measured using a pH meter (Model Φ 220, Beckman Instruments, Inc., Fullerton, California, USA). Samples of water from the air/water syringes and cupfillers of three dental units following disinfection with Sterilex Ultra and Sanosil were taken immediately following the flushing cycle and five days following disinfection and flushing. Output water from the DUWs of an adjacent non-disinfected dental unit was also taken as a control. The pH of each sample was then determined using a pH meter (Model Φ 220, Beckman).

3.2.7 Laboratory processing of water samples

3.2.7.1 Dilution of water samples

Following collection, water samples were transferred to the microbiology laboratory for analysis within 1.5 h. Initial tests showed that filtration of 5 ml neat water samples from non-disinfected DCUs resulted in semi-confluent growth of bacterial

colonies following culture and, therefore, samples were diluted in autoclaved ultra-pure water (18.2 MΩ-cm resistivity at 25˚C) from a Milli-Q® Biocel (Millipore, Massachusetts, USA) water purification system. Samples from control units were diluted by a factor of 1,000, while samples from test units were diluted by a factor of 10. Samples from the Hospital's water supply system (Figure 3.1) were filtered both neat and after dilution by a factor of 10.

3.2.7.2 Filtration of water samples and growth on R2A medium

Following dilution, five-ml aliquots of each water sample were vacuum filtered using separate commercially available disposable 100-ml analytical filtration funnels (Biopath, Inc., Florida, USA), using a 6-Branch Manifold Vacuum Filter System (Sartorius). Each funnel contained a sterile 47-mm diameter, black gridded cellulose nitrate removable membrane with a pore size of 0.45 μm. Following filtration, the membranes were removed from the funnels using sterile forceps. Duplicate membranes were placed on to two separate R2A agar plates (Difco). R2A agar was used to determine total aerobic heterotrophic bacterial density because it is particularly efficient for isolating aerobic heterotrophic bacteria from water (Reasoner & Geldreich, 1979). Negative controls consisted of five-ml samples of sterile ultra-pure water. The R2A plates were incubated at room temperature for six to seven days, after which time plates were examined and colonies were counted using a Stuart Scientific colony counter (Bibby Sterilin Ltd, Staffordshire, UK). The total number of colonies was recorded and the number of cfu in the original water samples was calculated.

3.2.7.3 Identification of bacteria isolated from dental unit output water

Following culture of microorganisms from the dental unit output water, selected colonies with differing morphologies e.g. different colours, shapes and sizes and mucoid or non-mucoid, were identified by biotyping using the API 20NE system, as described in Section 2.1.5.1.3. Further isolates that could not be identified by this system were identified by standard procedures (Murray et al., 1995) or by DNA sequence analysis following extraction of DNA from bacteria and subcloning of an amplified 1.5 kb region of the 16S rRNA gene using the pGEM® T-Easy vector system (Promega), as described in Sections 2.2.1, 2.3 and 2.4.

3.2.8 Electron and confocal microscopy of biofilm

For electron microscopy, two-cm-long samples of DUW tubing supplying water to the handpieces were removed from three non-disinfected DCUs, three DCUs immediately

following disinfection with Sterilex Ultra and a further three units immediately following disinfection with Sanosil. The samples were cut longitudinally through the lumen using a sterile scalpel. Then the internal wall of both halves was examined without prior fixation using a Hitachi S-3500N variable pressure scanning electron microscope (Hitachi Ltd, Tokyo, Japan).

For confocal microscopy, two-cm-long samples of DUW tubing were removed from one DCU prior to disinfection, immediately following disinfection with Sanosil and five days following disinfection. Samples were stained using the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes Europe BV, 2333 AA Leiden, Holland), according to the manufacturer's instructions, by technical staff at Queen's University Belfast (QUB). Using this stain, live bacteria are stained green while dead bacteria are stained red. Then the internal wall of DUW tubing was examined by technical staff at QUB using a confocal laser microscope (Model Olympus BX60) (Bio-Rad).

3.2.9 Disinfection of DUWs located outside Dublin Dental Hospital using Sanosil

In order to confirm the efficacy of Sanosil for disinfecting DUWs, a short additional study was undertaken in a local Dublin dental clinic. The DUWs included in this study were located separate from the DCU on microcarts and were bottle-fed with sterile distilled water. Three DCUs were included in the study: the DUWs of two DCUs in the clinic were disinfected weekly over a 60-h period (weekend) for the four-week study period and the disinfectant was then rinsed out using sterile distilled water; the third DCU was disinfected once for a 60-h period, and then weekly disinfection was stopped in order to observe how quickly microbial density rose again to pre-disinfection levels.

One-hundred ml aliquots were taken from the air/water syringe and high speed handpiece waterlines of each DCU included in the study both immediately before disinfection occurred, and immediately following disinfection and rinsing.

3.2.10 Ongoing disinfection of DUWs and monitoring of output water

Following completion of this project, all the remaining DCUs in the Dublin Dental Hospital were retrofitted with Planmeca Waterline Cleaning System modules and the DUWs are now disinfected once-weekly by dental personnel. Periodic monitoring of the bacterial density of the DCU output water is carried out by Ms Mary O'Donnell and Prof David Coleman to ensure continued efficacy of the disinfection procedure and to establish if any problems that arise are due to human error, engineering problems or development of resistance to the disinfectant by microorganisms within the DUWs.

3.3 Results

3.3.1 Chemical quality of water supplied to dental units

Water destined for the DCUs in the new Dublin Dental Hospital is processed by particle filtration (removal of particles greater than 10 μm), removal of calcium and magnesium ions by ion exchange and UV treatment on entering the hospital from the mains water supply before being distributed to the individual DCUs via a ring-main (Figure 3.1). The chemical quality of the water from the ring-main pipes following this treatment and of output water from the air/water syringe waterlines of two DCUs was determined and was consistently found to be well within the limits advocated by the European Commission Directive on the Quality of Water for Human Consumption (Clesceri *et al.*, 1998) and that advocated by the USA National Primary Drinking Water Regulations (Table 3.1) (Anonymous, 1998; Anonymous, 2002a).

3.3.2 Microbial quality of water supplied to dental units

Aerobic heterotrophic bacteria were chosen as marker organisms for monitoring levels of microbial contamination of the water as previous studies have shown that these are the predominant organisms present in dental unit waterlines (Furuhashi & Miyamae, 1985; Whitehouse *et al.*, 1991; Williams *et al.*, 1993; Barbeau *et al.*, 1996). At the outset of the study, water sampled from the mains water supply was found to contain relatively low levels of bacteria with a mean of 360 cfu/ml for the water as it enters the hospital and 184 cfu/ml after processing.

3.3.3 Annual treatments of mains water supply pipes

Although the main aim of this annual treatment is to treat the mains water supply pipes, the hydrogen peroxide disinfectant must be flushed out through the DUWs by design of the water distribution system (see Figure 3.1). Therefore, in order to determine if there was any additional benefit in reducing bacterial densities of DUW output water, the bacterial density of output water from the air/water syringe and cupfiller of one DCU was determined before, immediately after and for two weeks following annual disinfection as shown in Table 3.2. As discussed in Section 3.1, the ADA have set a goal of no more than 200 cfu of heterotrophic bacteria per ml of DUW output water (Anonymous, 1996). In the case of output water from the air/water syringe, bacterial density (13 cfu/ml) had been reduced to that recommended by the ADA, while the output water from the cupfiller had been reduced (860 cfu/ml), but still exceeded the ADA recommendation. However, one

Table 3.2. Density of aerobic heterotrophic bacteria in output water from one dental chair unit before and after annual disinfection with hydrogen peroxide

Sample time	Bacterial density[a] in cfu/ml	
	Air/ water syringe	Cupfiller
Before disinfection	130,000	3,200
One day post-disinfection	13	860
One week post-disinfection	320,000	130,000
Two weeks post-disinfection	720,000	44,000

[a]Density on R2A medium following 6-7 days incubation at 18-22°C

week following treatment, levels had increased again to pre-treatment levels that far exceeded the ADA's recommendation (Table 3.2).

3.3.4 Preliminary analysis of bacteriological quality of dental unit output water

3.3.4.1 Bacteriological quality of water from Dublin Dental Hospital DUWs

At the outset of the project, water samples were taken from the three-in-one air/water syringe waterlines and cupfillers of 12 of the 103 DCUs from the Dublin Dental Hospital. These samples were found to yield consistently high counts of bacteria, with an average bacterial density from the air/water syringe waterlines of 8,200 cfu/ml (range 900 to 20,000 cfu/ml). The average bacterial density from the cupfillers was 4,300 cfu/ml (range 390 to 12,000 cfu/ml). These findings indicated widespread contamination of the DUWs with bacterial counts well above recommended standards for drinking water.

3.3.4.2 Bacteriological quality of water from other Dublin dental clinics

The microbial quality of dental unit water from the air/water syringe waterlines of eighteen DCUs from four dental clinics outside the Dublin Dental Hospital was also determined. Results were similar to those obtained with the Dublin Dental Hospital DCUs, with a mean of 66,000 cfu/ml (range 8,600 to 280,000 cfu/ml) (Figure 3.3). These DCUs all used individual bottle-fed reservoir systems and distilled water.

3.3.5 Identification of microorganisms

R2A agar was used to determine total aerobic heterotrophic bacterial density and PBA was also used because previous studies had shown that DUWs often contain *Pseudomonas* and related species (Whitehouse *et al.*, 1991; Barbeau *et al.*, 1996; Shearer, 1996; Mills, 2000). Selected isolates of each colony type present on both types of media were Gram-stained and were found to be predominantly Gram-negative organisms (approximately 80%), the remainder consisting of Gram-positive bacilli. The most common bacterial isolates recovered from water samples from the ring-main supply to the dental units (Figure 3.1) and from output water from units disinfected with Sterilex Ultra were Gram-negative, oxidase-negative organisms that were identified as *Chryseomonas luteola* using the API 20 NE system (Table 3.3). No particular species seemed to predominate in output water from units that had been disinfected with Sanosil. *Chryseomonas luteola* was a minor constituent of the bacterial population recovered from output water from non-disinfected dental units. Other species identified from output water from both disinfected and non-disinfected dental units are shown in Table 3.3.

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Table 3.3. Aerobic heterotrophic bacterial species isolated from dental chair unit output water in the present study and references to previous studies implicating some of these species as agents of infection

Bacterial species	References[a]
Aeromonas salmonicida	
Agrobacterium radiobacter	(Edmond *et al.* 1993)
Burkholderia cepacia	(Pankhurst *et al.*, 1995)
CDC gr. IV c-2	(Osterhout *et al.*, 1998)
Flavobacterium indologenes	(Hsueh *et al.*, 1996)
Chryseomonas luteola	(von Graevenitz, 1995)
Comomonas acidovorans	
Ochrobactrum anthropi	(von Graevenitz, 1995)
Pseudomonas alcaligenes	
Pseudomonas chlororaphis	
Pseudomonas fluorescens	(Hsueh *et al.*, 1998)
Ralstonia picketti	

[a]Contaminated dental unit water was not implicated in any of these studies.
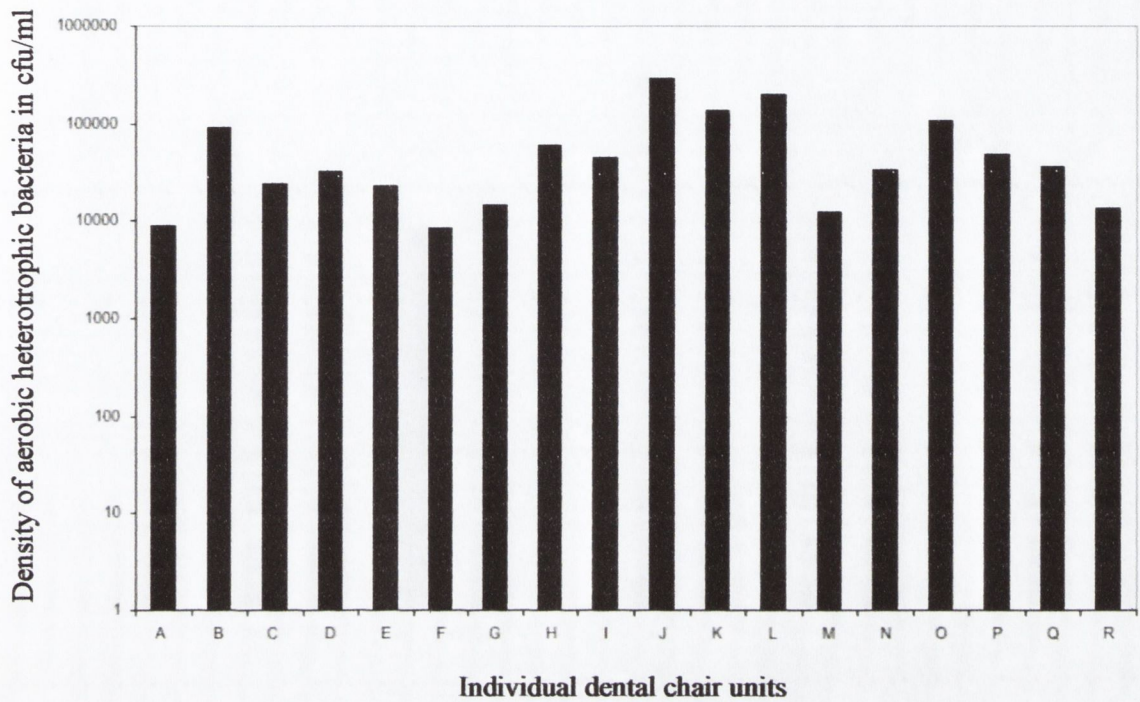
Figure 3.3. Density of aerobic heterotrophic bacteria in air/ water syrigne output water from 18 separate dental chair units in dental clinics in the greater Dublin area. Bacterial counts were determined after 6-7 days incubation at 20°C on R2A agar medium.

Approximately 15% of colony types isolated were identified by the API 20 NE system (bioMérieux) as CDC gr. IVc-2, which have been described as short to medium sized Gram-negative rods that stain irregularly (Osterhout *et al.*, 1998). A number of bacterial colony types that could not be identified by the API 20 NE system were identified as *P. alcaligenes* and *P. chlororaphis* by standard procedures (Murray *et al.*, 1995).

3.3.6 Disinfection of dental unit waterlines

3.3.6.1 Bacteriological quality of output water from untreated DUWs

Output water from the air/water syringe waterlines and cupfillers from the ten control units sampled twice-weekly consistently yielded a high bacterial cell density with mean bacterial counts of 57,000 cfu/ml (range 18,000 to 150,000 cfu/ml) and 7,100 cfu/ml (range 890 to 13,000 cfu/ml), respectively. Thus the bacterial cell density levels in output water from the air/water syringe waterlines and cupfillers of non-disinfected DCUs was 306-fold and 38-fold higher, respectively, compared to the water supplied to the units (mean average density of 184 cfu/ml; see above). Figures 3.4 and 3.5 show bacterial levels recorded for the output water from the air/water syringes and cupfillers of four non-disinfected control DCUs over the five-month study period.

3.3.6.2 Bacteriological quality of output water from disinfected DUWs

In contrast, immediately following disinfection and six days post-disinfection with both Sterilex Ultra and Sanosil, output water from the disinfected (test) DCUs had a greatly reduced bacterial density. The mean average bacterial densities of output water from air/water syringe waterlines and cupfillers of dental units disinfected with Sterilex Ultra were 35 cfu/ml (range 5 to 71 cfu/ml) and 29 cfu/ml (range 4 to 61 cfu/ml), respectively (Figure 3.4). The average bacterial densities of output water from the air/water syringe waterlines and cupfillers of dental units disinfected with Sanosil were 59 cfu/ml (range 2 to 119 cfu/ml) and 49 cfu/ml (range 12 to 92 cfu/ml), respectively (Figure 3.5).

3.3.6.3 Bacteriological quality of water from DUWs following cessation of weekly disinfection

In the case of two of the six test units disinfected with Sterilex Ultra and two of the four test units disinfected with Sanosil, weekly disinfection was discontinued after seven weeks and five weeks, respectively, to determine the length of time taken fof the bacterial density to return to pre-disinfection levels. Within three weeks of stopping weekly disinfection, the mean average bacterial density in water from the air/water syringe
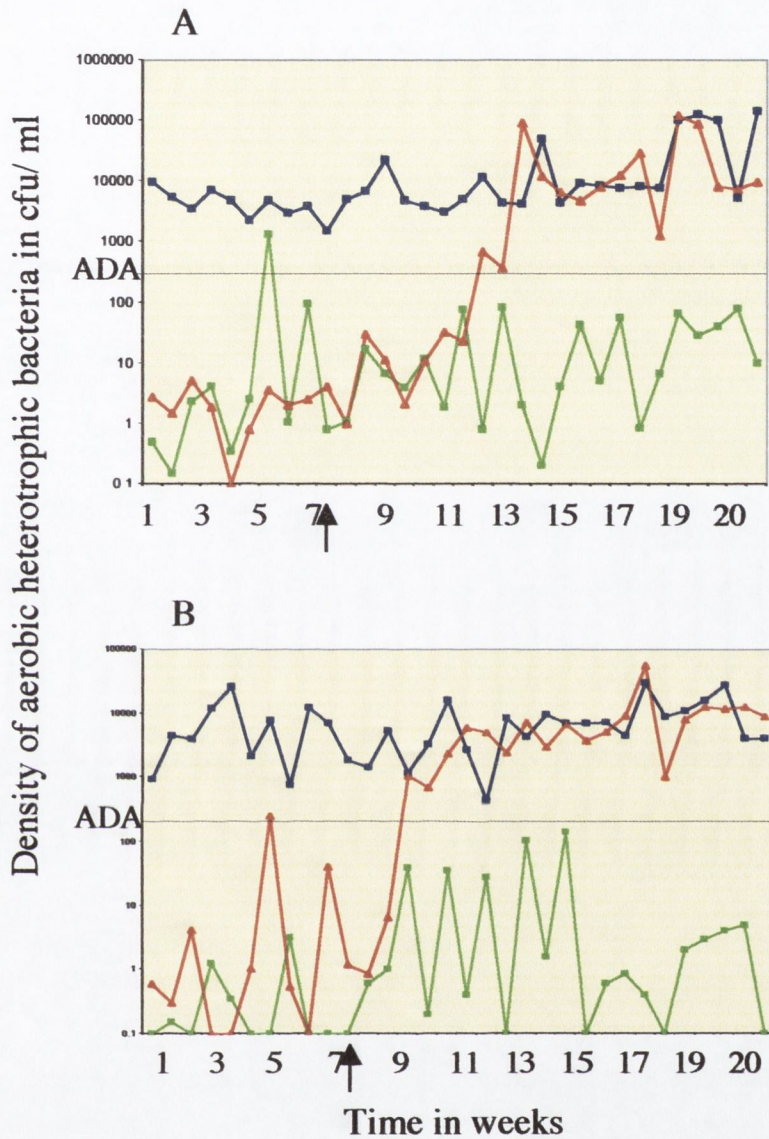
Figure 3.4 Density of aerobic heterotrophic bacteria in air/ water syringe (A) and cupfiller (B) output water from DUWs following disinfection with Sterilex Ultra and from non-disinfected dental chair units. Bacterial counts were determined after 6-7 days incubation at 20°C on R2A agar medium. Blue lines represent data from a non-disinfected unit; green lines represent data from a disinfected unit and red lines represent data from a unit disinfected for an initial seven-week period, after which time weekly disinfection ceased, as indicated by an arrow. The horizontal dashed line in the central part of the figure represents the maximum bacterial density ($\leq$ 200 cfu/ml) recommended by the ADA guidelines for DUW output water (Anonymous, 1996).
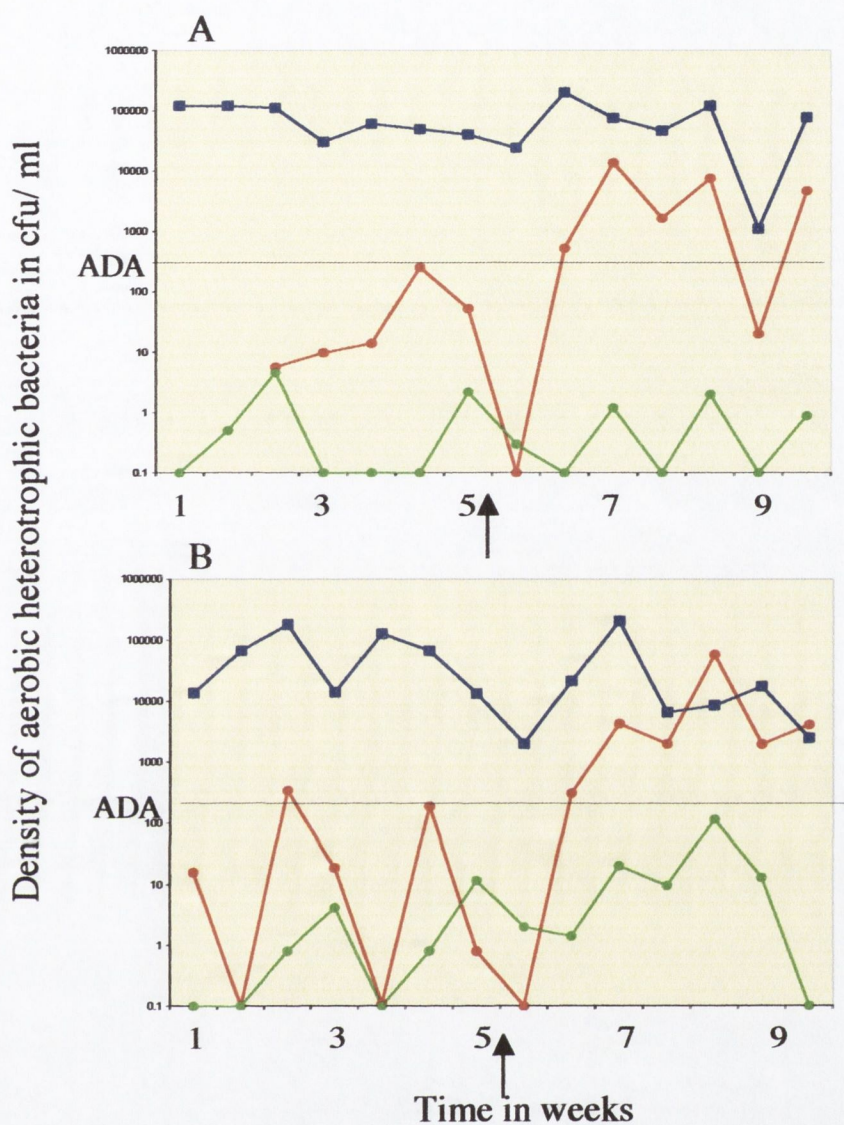
Figure 3.5. Density of aerobic heterotrophic bacteria in air/ water syringe (A) and cupfiller (B) output water from DUWs following disinfection with Sanosil and from non-disinfected dental chair units. Bacterial counts were determined after 6-7 days incubation at 20°C on R2A agar medium. Blue lines represent data from a non-disinfected unit; green lines represent data from a disinfected unit and red lines represent data from a unit disinfected for an initial five-week period, after which time weekly disinfection ceased, as indicated by an arrow. The horizontal dashed line in the central part of the figure represents the maximum bacterial density ($\leq$ 200 cfu/ml) recommended by the ADA guidelines for DUW output water (Anonymous, 1996).

waterlines and cupfillers of dental units disinfected with Sterilex Ultra increased to a mean density of 1,100 cfu/ml and 6,500 cfu/ml, respectively, (see Figure 3.4), while the corresponding bacterial densities in the DUWs of dental units disinfected with Sanosil increased to a mean density of 12,800 cfu/ml and 6,100 cfu/ml, respectively (Figure 3.5).

3.3.7 DUW output water that failed and passed ADA recommendations

These results demonstrated that during the study period, the bacterial density of output water sampled from all control units consistently failed the ADA recommendation of ≤ 200 cfu/ml (Figures 3.4 and 3.5). In contrast, the bacterial density of output water from test units undergoing weekly disinfection with either disinfectant was consistently within the ADA recommended limits (Figures 3.4 and 3.5). The bacteriological quality of output water from test units the morning following disinfection and six days following disinfection was within the ADA recommended limits (see Table 3.4).

3.3.8 Measurement of pH of output water following disinfection

The pH of Sanosil was found to be 3.7 and the pH of Sterilex Ultra at the recommended concentration (6.25% (w/v)) was 10.9. The output water from air/water syringe waterlines that had been disinfected overnight with Sterilex Ultra was found to have an average pH of 9.2 immediately after, and 7.8 five days after disinfection, while the output water from air/water syringes disinfected with Sanosil was found be 7.5 immediately after, and 7.2 five days after disinfection, as shown in Table 3.5. The average pH of the output water from cupfillers disinfected with Sterilex Ultra immediately following and five days after disinfection and flushing was 9.2 and 8.5, respectively, while the average pH of the output water from cupfillers disinfected with Sanosil was 7.3 on both occasions (see Table 3.5). The average pH of output water from the untreated air/water syringes and cupfillers was 7.4 and 7.0, respectively. These results indicate that while the disinfectant Sanosil seemed to be easily removed from the DUWs, Sterilex Ultra stayed in the DUWs for at least five days post-disinfection.

3.3.9 Waterline clogging following Sterilex Ultra disinfection

The use of Sterilex Ultra disinfectant was associated with clogging of DUWs in three of the six DCUs disinfected with this product. Clogging became apparent after the fourth consecutive week of disinfection in two of the units and after 14 weeks of disinfection in the third. In the latter case, it was impossible to aspirate water or disinfectant through the air/water syringe waterline, which had to be replaced with new

Table 3.4 Average bacterial density* of aerobic heterotrophic bacteria in DUW output water, immediately following disinfection (day 1) and six days after disinfection (day 7).

	Disinfectant			
	Sterilex Ultra (6 DCUs)		Sanosil (4 DCUs)	
Water Source	Bacterial density (cfu/mL)		Bacterial density (cfu/mL)	
	Day 1	**Day 7**	**Day 1**	**Day 7**
3-in-1 syringe	27.6 (4.4 - 97)	78.7 (8.9 - 175)	65 (3.6 - 160)	52 (0.8 - 130)
Cupfiller	12.81 (0.6 - 45)	68.5 (0.6 - 141)	21.5 (3.9 - 42)	77 (0.7 - 181)

*Ranges are shown in paretheses.

Table 3.5. pH of DUW output water immediately following disinfection and rinsing cycle with either Sanosil or Sterilex Ultra and five days post-disinfection and rinsing

Disinfectant	DCU No.	DUW	pH	
			Day 1	Day 5
Sterilex Ultra	1	air/water syringe	8.9	7.7
		cupfiller	10.2	8.9
	2	air/water syringe	9.2	7.8
		cupfiller	8.5	8.2
	3	air/water syringe	9.4	8.4
		cupfiller	8.9	8.4
Sanosil	4	air/water syringe	7.7	7.4
		cupfiller	7.5	7.3
	5	air/water syringe	7.7	7.3
		cupfiller	7.2	7.6
	6	air/water syringe	7.2	7.0
		cupfiller	7.6	7.2
Non-disinfected control	7	air/water syringe	7.4	7.5
		cupfiller	7.0	7.0

waterline tubing. No problems with DUW clogging were experienced in the four DCUs disinfected with Sanosil disinfectant during the eight-week period this product was investigated.

3.3.10 DUW disinfection of DCUs located outside of Dublin Dental Hospital with Sanosil

The mean average bacterial densities in water from the air/water syringe waterlines and high speed handpieces of dental units located in a local Dublin dental clinic before disinfection were 41,700 and 599,000 cfu/ml, respectively (see Figure 3.6). In contrast, immediately following disinfection and five days post-disinfection with Sanosil, output water from the DCUs had a greatly reduced bacterial density. The mean average densities of output water from air/water syringe waterlines and high speed handpieces of dental units disinfected with Sanosil were 14 cfu/ml and 143 cfu/ml, respectively. However, the bacterial densities in the air/water syringe and high speed handpiece of the DCU in which disinfection was stopped increased to 122,000 cfu/ml and 800,000 cfu/ml, respectively, within one week of stopping disinfection (see Figure 3.6).

3.3.11 Electron and confocal microscopy of biofilm

For electron microscopy, two-cm samples of waterline tubing from three DCUs, in each case, following disinfection with either Sterilex Ultra or Sanosil and from three separate non-disinfected DCUs were removed from the waterlines supplying the DCU handpiece instruments of the units. The samples of tubing were cut longitudinally through the lumen and examined by scanning electron microscopy without prior fixation. Abundant biofilm was evident on the internal surface of the waterline tubing from the non-disinfected dental units (Figure 3.7, Panels A and B), while biofilm had been virtually eliminated from the internal surface of the waterline tubing disinfected with both Sterilex Ultra (Figure 3.7, Panels C and D) and Sanosil (Figure 3.7, Panels E and F).

For confocal microscopy, two-cm samples of DUW tubing were removed from one further DCU prior to disinfection, immediately following disinfection and five days after disinfection with Sanosil. Samples were cut into thin sections through the lumen and examined by confocal microscopy following staining with the DEAD/ALIVE® BacLight™ Bacterial Viability kit (Molecular Probes). Abundant bacteria, both dead and alive, were evident on the DUW tubing prior to disinfection (Figure 3.8, Panel A; Figure 3.9). Immediately following disinfection, a reduced number of dead bacteria were evident, while the live bacteria had been virtually eliminated (Figure 3.8, Panel B). Five days following
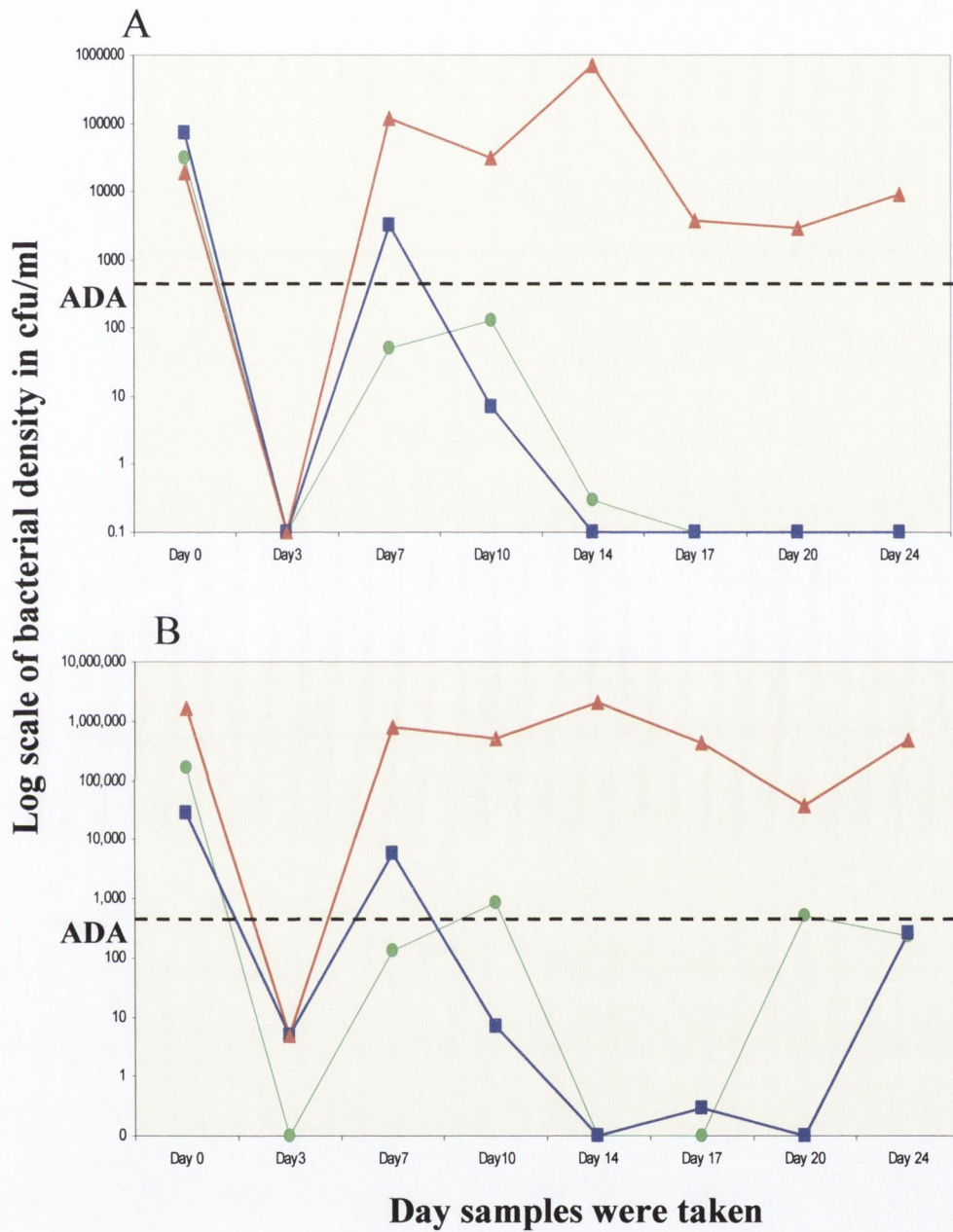
Figure 3.6. Density of aerobic heterotrophic bacteria in output water from air/ water syringes (A) and high speed handpieces (B) of three dental units in a Dublin clinic before and following weekly disinfection with Sanosil. The red lines represent the dental chair unit whose waterlines were only disinfected once on Day 1. The horizontal dashed line represents the maximum bacterial density in DUW output water (≤ 200 cfu/ml) recommended by the ADA.

0.5 mm   50 µm
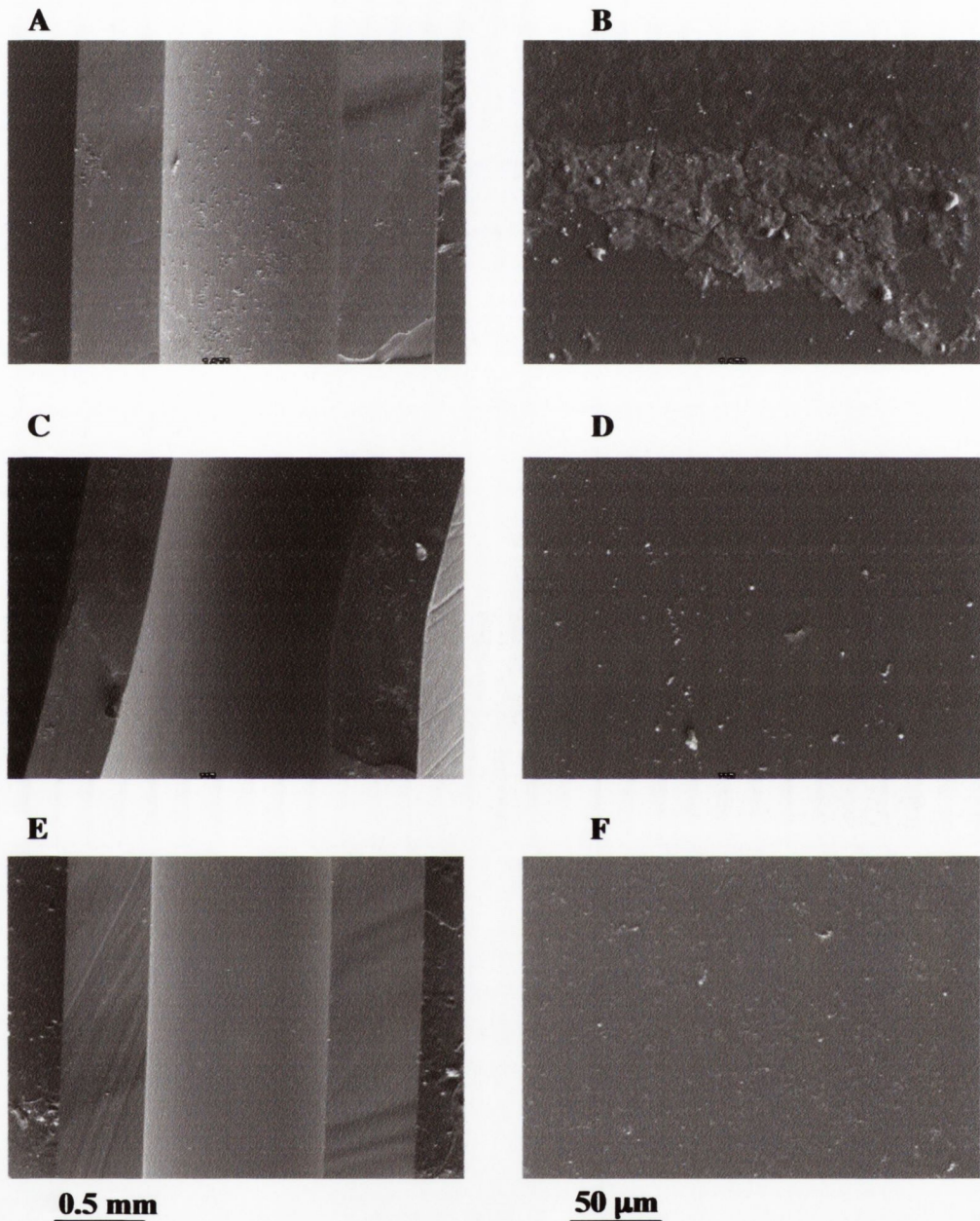
Figure 3.7. Electron micrographs of longitudinal sections of DUW tubing showing the internal surfaces from disinfected and non-disinfected dental chair units. Panels A and B are from a control non-disinfected unit; Panels C and D are from a dental chair unit disinfected with Sterilex Ultra and Panels E and F are from a dental chair unit disinfected with Sanosil.
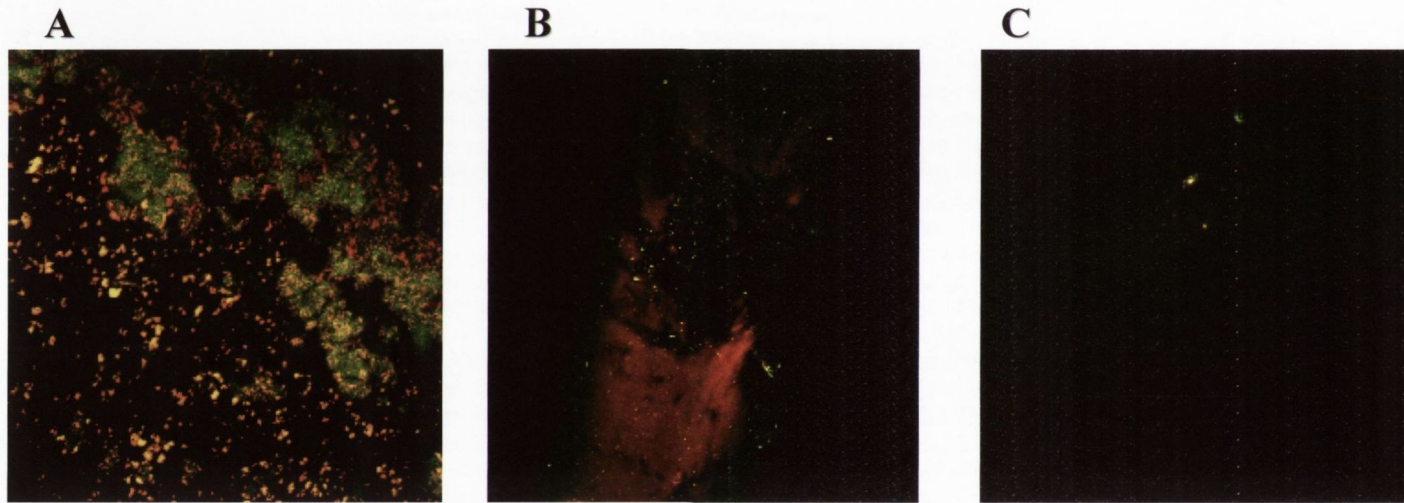
Figure 3.8. Confocal micrographs of sections of DUW tubing from a DCU showing the internal surfaces before and after disinfection. The internal surfaces of the DUW sections were stained using the LIVE/DEAD® *Bac*light™ Bacterial Viability Kit (Molecular Probes), which stains live bacteria green and dead bacteria red. Panel A shows the DUW before disinfection; Panel B shows the DUW immediately after disinfection, while Panel C shows the DUW five days after disinfection. Magnification in each case is X20.
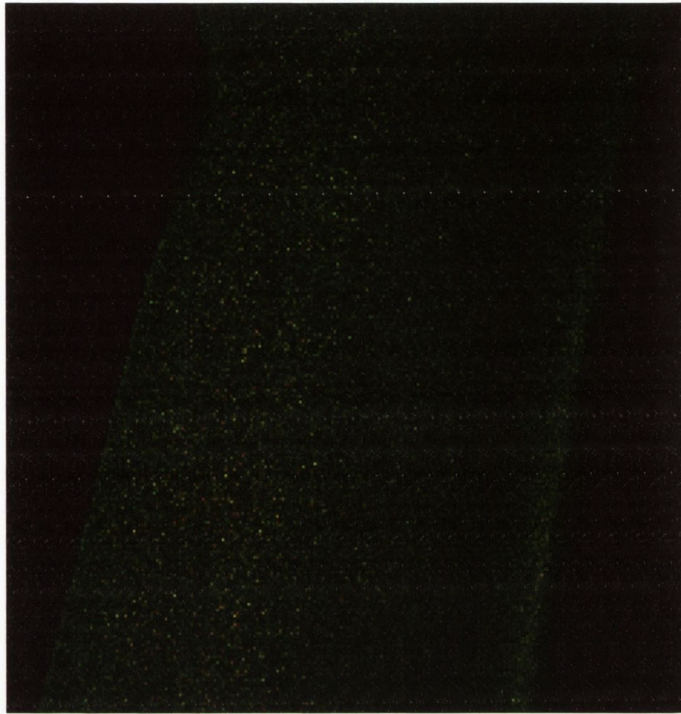
Figure 3.9. Confocal micrograph of a section of DUW tubing from a DCU that had never been previously disinfected. The internal surfaces of the DUW sections were stained using the LIVE/DEAD® *Bac*light™ Bacterial Viability Kit (Molecular Probes), which stains live bacteria green and dead bacteria red. Magnification is X10.

disinfection, most of the dead bacteria had also been eliminated from the DUW tubing (Figure 3.8, Panel C).

3.3.12 Ongoing disinfection of DUWs and monitoring of output water

Following the completion of this study in 2002, all remaining DCUs in the Hospital were fitted with Planmeca Waterline Cleaning System modules and continued weekly disinfection of DUWs with Sanosil is now undertaken by dental nurses. The efficacy of the disinfection regimen is monitored periodically in order to detect whether failures are occurring. Two years after regular weekly disinfection of DUWs began, it was noted that while immediately following disinfection bacterial densities of DUW output water met the ADA's recommendations of ≤ 200 cfu/ml, five days following disinfection bacterial densities rose to pre-treatment levels (Ms. M. O'Donnell, Personal Communication). A significant proportion (10%) of the bacterial population recovered from the DUW output water was found to be strongly catalase-positive with extensive effervescence produced immediately following testing with hydrogen peroxide (Prof. D. Coleman, Personal Communication). Ten of the most predominant catalase-positive colony types were identified by 16S rRNA sequence analysis. These were identified as *Sphingomonas subarctica*, *Bacillus pumilus*, *Micrococcus luteus*/ *Arthrobacter* sp., *Variovorax paradoxus* and *Flectobacillus speluncae*, as shown in Table 3.6.

Table 3.6 Identity of ten catalase-positive microorganisms recovered from DUW output water [a]

Name	Number
Sphingomonas subarctica	4
Bacillus pumilus	2
[b] *Micrococcus luteus/ Arthrobacter* sp.	2
Variovorax paradoxus	1
Flectobacillus speluncae	1

[a] Isolates were representative of the most predominant catalase-positive organisms, based on colony morphology
[b] The genera *Micrococcus* and *Arthrobacter* are closely related

3.4 Discussion

In recent years, numerous studies have shown that output water from DUWs contains high densities of bacteria, mainly Gram-negative aerobic heterotrophic bacterial species, many of which have been implicated in human infection (Martin, 1987; Atlas *et al.*, 1995; Mills, 2000; Putnins *et al.*, 2001). With the increasing numbers of immunocompromised and medically compromised individuals (some of whom cannot be always identified as such) receiving regular dental treatment, contaminated DUW output water poses a serious risk of infection. In this regard, effective practical methods for controlling microbial contamination of DUWs need to be developed. The purpose of this part of the present study was to determine the extent of bacterial contamination of DUWs in the new Dublin Dental Hospital and to test the efficacy of the first DCU disinfection system custom-made for the disinfection of DUWs, the Planmeca Waterline Cleaning System, designed by Planmeca for use with the Prostyle Compact DCUs with which the Dublin Dental Hospital is equipped. Furthermore, this study aimed to compare the efficacy of two hydrogen-peroxide based disinfectants at reducing the bacterial density to ≤ 200 cfu/ml, as recommended by the ADA. This study was not intended to be an investigation into the total microbial population of the DUW output water so, for the purpose of this study, only aerobic heterotrophic bacterial levels in DUW output water were determined, as previous studies have demonstrated that these organisms are the predominant cultivable species present in DUWs (Whitehouse *et al.*, 1991). The levels of aerobic heterotrophic bacteria were then used as marker organisms as a measure of contamination and disinfectant efficacy.

3.4.1 Microbial quality of untreated DUW output water in Dublin Dental Hospital and other Dublin clinics

Processed mains water (Figure 3.1) supplied to the DCUs in the hospital (mean average bacterial density 184 cfu/ml) passed the ADA recommendation for DUW water quality and the USA national primary drinking water regulations (≤ 500 aerobic heterotrophic bacteria/ml (Anonymous, 2002a). However, it failed EC potable-water guidelines on bacterial density (≤ 100 aerobic heterotrophic bacteria per/ml) (Anonymous, 1998). The chemical quality of the processed water supplied to the DCUs and of the output water from the dental units was well within the limits for water for human consumption established by the EC. However, the bacterial load in output water from the three-in-one air/water syringe waterlines (mean average bacterial density 8,200 cfu/ml) and the cupfillers (mean average bacterial density 4,300 cfu/ml) of the 12 DCUs from the hospital

included in the preliminary study failed both the USA and EC potable water guidelines and the ADA DUW water quality recommendations. Similar results were found with output water from all 18 separate reservoir-supplied dental units from other dental clinics in Dublin included in the study, where a mean average bacterial density of 66,000 cfu/ml was recorded, even though these latter units were supplied with sterile distilled water. These results demonstrated that high levels of bacteria in DUW output water were not restricted to the Dental Hospital but were widespread among the dental units tested. Furthermore, the density of bacteria in DUW output water in the non-disinfected units from the Dublin Dental Hospital and other Dublin dental clinics is in agreement with the range reported previously in many other studies (Furuhashi & Myamae, 1985; Whitehouse et al., 1991; Douglas & van Noort, 1993; Barbeau et al, 1996; Walker et al., 2000; Putnins et al., 2001).

3.4.2 Bacterial species cultured from DUW output water

The bacterial species cultured from the DUWs in the Dublin Dental Hospital were predominantly environmental bacteria (Table 3.3), the majority of which have been previously associated with nosocomial infection and infection of the immunocompromised, especially *B. cepacia* and *P. fluorescens* (Gilligan, 1995; Pankhurst et al., 1995; Pankhurst & Philpott-Howard, 1996; Hsueh et al., 1998). The same species of bacteria were cultured from the treated input water supplied to the dental units and from the DUW output water. These findings suggest that the bacteria present in the DUWs originated from the mains water supply, in agreement with previous studies (Pankhurst & Philpott-Howard, 1993; Shearer, 1996). While many other studies reported the isolation of a wide variety of *Pseudomonas* species from DUW output water, including *P. aeruginosa* (Whitehouse et al., 1991; Williams et al., 1993; Barbeau et al., 1996), *P. aeruginosa* was not isolated from any of the DUW output water sampled in the present study, nor did *Pseudomonas* species predominate, although some were isolated such as *P. fluorescens* (Table 3.3). Scanning electron microscopy and confocal microscopy of sections of waterline supplying the DCU handpieces in three non-disinfected dental units revealed the presence of compact biofilm covering most of the inner surface of the waterline tubing (Figure 3.7, Panels A and B). This is consistent with the continuous release of microorganisms from biofilm, the latter of which can act as a reservoir for ongoing contamination of DUW water.

3.4.3 Disinfection of ring-main water pipes and DUWs

Annual disinfection of the ring-main water pipes with hydrogen peroxide is effective at its purpose of keeping the wide-bore pipes that supply the DUWs clean, thus

limiting the numbers of aerobic heterotrophic bacteria in the dental unit input water to within ADA recommendations. However, it has no long-term effects on bacterial densities of DUW output water. Therefore, the hydrogen peroxide-based disinfectants Sterilex Ultra and Sanosil were selected for disinfecting DUWs as the manufacturers of the DCUs, Planmeca, found that these disinfectants were compatible with the materials used to construct the DUW tubing and valves of the DCUs used in the study (i.e. did not cause leakages etc). Furthermore, Sterilex Ultra has been approved by the ADA for DUW disinfection (Kramer, 2000). The bacterial density in output water from all ten non-disinfected control DCUs was consistently high during the entire study period with average bacterial counts of 82,000 cfu/ml and 43,000 cfu/ml for air/water syringe waterlines and cupfillers, respectively, (Figures 3.4 and 3.5). In contrast, the bacterial density in output water from the ten DCUs undergoing once-weekly overnight disinfection either with Sterilex Ultra or Sanosil consistently achieved the ADA recommendations of ≤ 200 cfu/ml and both the EC and USA potable water guidelines (Figures 3.4 and 3.5, Table 3.4). In the case of Sterilex Ultra, the present investigation confirmed the results of a recent study that showed that once-weekly disinfection with Sterilex Ultra maintained the bacterial density of DUW output water in bottle-fed dental units below 200 cfu/ml (Linger *et al.*, 2000). However, the present study also demonstrated that the output water quality deteriorated within three-to-four weeks and failed all three water quality guidelines referred to above (Figures 3.4 and 3.5) following cessation of once weekly disinfection with either Sterilex Ultra or Sanosil. All of these results indicate that once-a-week disinfection of DUWs with either Sterilex Ultra or Sanosil is sufficient to maintain good quality dental unit output water, but that this quality can only be maintained by continual once weekly disinfection.

The dramatic reduction in bacterial density observed in disinfected DCUs was associated with removal of biofilm from inside of the dental unit waterlines. Electron and confocal microscopy revealed that biofilm was almost absent from the inner surface of the waterline tubing from three DCUs in each case disinfected with either Sterilex Ultra or Sanosil (Figure 3.7, Panels C-F; Figure 3.8, Panels B-C).

3.4.4 Comparison of Sterilex Ultra and Sanosil

While both Sterilex Ultra and Sanosil were found to be equally effective at reducing the bacterial density of DUW output water to within acceptable limits during the study period, use of Sterilex Ultra with a once weekly disinfection regimen for four weeks resulted in clogging of some of the DUWs of three of the DCUs used in this study. One unit was so badly affected after 14 weeks of once weekly disinfection that neither

disinfectant nor water could be aspirated through the unit's air/water syringe waterline that had become completely blocked and had to be replaced with new waterline tubing. Clogging of DUWs following repeated once weekly disinfection with Sterilex Ultra may have been caused by the gradual accumulation of solid deposits in the waterlines. Sterilex Ultra is supplied as a dry powder that has to be dissolved in warm (50°C) water by thorough agitation prior to use. It is possible that some of the disinfectant precipitates within DUWs during overnight disinfection following cooling of the solution and that repeated precipitation eventually results in clogging of the DUWs. This suggestion is supported by the persistent alkaline nature of the output water from the air/water syringes and cupfillers (average pH 7.8 and 8.5, respectively) five days following overnight disinfection with Sterilex Ultra (Table 3.5). Apart from the obstruction of DUWs this accumulation of Sterilex Ultra causes, it is totally unacceptable to dispense output water contaminated with hydrogen peroxide into patients' mouths. Linger *et al.*, who also investigated the efficacy of Sterilex Ultra, did not report clogging of DUWs following once-weekly disinfection (Linger *et al.*, 2000). However, the investigators monitored the efficacy of Sterilex Ultra for only a five-week period, which may have been too short for the effects of accumulating deposits within the DUWs to become apparent. In the present study, the use of the liquid-based disinfectant Sanosil was not associated with clogging or blockage of DUWs during the eight-week period this disinfectant was used. Also, the average pH of the output water treated with this disinfectant was within 0.5-pH units of neutral the day after disinfection and 0.3-pH units five days following disinfection.

3.4.5 Ongoing disinfection of DUWs and monitoring of output water

Using the Planmeca Waterline Cleaning System with either Sterilex Ultra or Sanosil with a once-a-week overnight (15 h) disinfection regimen this study demonstrated that the bacterial quality of DUW output water could be managed so that it consistently passed the ADA recommendation for DUW output water quality as well as the USA and EC potable-water guidelines. The improved output water quality observed in disinfected units was directly associated with elimination of biofilm from inside DUWs. Due to the clogging of waterlines associated with the use of Sterilex Ultra in some DCUs, Sanosil was found to be the more practical solution for decreasing the bacterial density of DUW output water to recommended levels.

Following the results of this study and the retrofitting of all DCUs in the Dublin Dental Hospital with the Planmeca Waterline Cleaning System, Sanosil was used for weekly disinfection of the DUWs and the disinfection regimen was monitored periodically

to ensure its continued effectiveness. Two years after weekly disinfection began, it was found that the bacterial densities of DUW output water rose from acceptable levels immediately following overnight disinfection to unacceptable levels five days later (Prof. D. Coleman, Personal Communication). A significant proportion (10%) of bacteria recovered from the DUW output water were strongly catalase-positive microorganisms that had not been isolated from the DUW output water during the initial study period, e.g. *Sphingomonas subarctica*, *Bacillus pumilus*, and *Variovorax paradoxus* (see Tables 3.3 and 3.6). This suggests that prolonged use of the hydrogen peroxide-based disinfectant Sanosil selected for catalase-positive organisms within the DUWs. As the strongly catalase-positive microorganisms then reduced the potency of the disinfectant by degrading the hydrogen peroxide, they consequently allowed for the proliferation of other microorganisms within the DUWs, thus contributing even further to the increased bacterial density of the DUW output water. These results suggest that once-weekly disinfection with a single disinfectant is unwise as it is not suitable for maintaining long-term reduction of DUW output water contamination and should probably be interspaced with another disinfectant that has a different active ingredient. Therefore, in order to deal with this new phenomenon, other disinfectants that are not hydrogen peroxide-based are being tested for their suitability in supplementing the efficacy of Sanosil so as to maintain bacterial densities within the limits recommended by the ADA of no more than 200 aerobic heterotrophic bacteria per ml.

Chapter 4

Use of *real-time* PCR and culture methods for detection of *Legionella* species in dental unit waterline output water and healthcare institution water supplies

4.1 Introduction

4.1.1 *Legionella* in dental unit waterline (DUW) output water samples

Contamination of DUW output water with high numbers of *Legionella* bacteria has been reported in previous studies from England, the USA and Italy (Atlas *et al.*, 1995; Challacombe & Fernandes, 1995; Williams *et al.*, 1996; Pankhurst *et al.*, 1998; Zanetti *et al.*, 2000). One such study demonstrated that occupational exposure to aerosols of waterborne bacteria generated by DUWs could lead to colonisation of dental staff and a higher prevalence of antibodies to *Legionella* in dental staff compared to the general population. This study also found an association between the death of a Californian dentist from Legionnaires' disease and *Legionella* detected in the DUW output water in the dentist's surgery (Atlas *et al.*, 1995). However, in contrast, two more recent studies from the UK reported a low prevalence of *Legionella* in DUW output water with *Legionella* species being detected in the DUW output water of less than 2% of DCUs investigated (Walker *et al.*, 2000; Pankhurst *et al.*, 2003).

4.1.2 Prevalence of *Legionella pneumophila*, particularly serogroup 1

Of the many species within the genus *Legionella*, *L. pneumophila*, particularly serogroup 1, is the species most commonly associated with infection. *Legionella pneumophila* serogroup 1 accounted for 49% of cases reported to state health laboratories in the USA and 52% of cases reported to the Centre for Disease Control (CDC) between 1981 and 1982. Between 1980 and 1989, while 88.2% of the total cases proven by culture were shown to have been caused by *L. pneumophila*, 54.4% of the total cases were caused by *L. pneumophila* serogroup 1 (Waterer *et al.*, 2001). In 1998, *L. pneumophila* serogroup 1 accounted for 60% of the 1,442 cases of Legionnaires' disease reported from 28 European countries, while other or undetermined serogroups of *L. pneumophila* accounted for a further 34.4% of cases. The remaining 5.6% of cases were caused by *L. micdadei* and *L. bozemanii* (Rantakokko-Jalava & Jalava, 2001).

4.1.3 Detection of *Legionella* in clinical samples

Definite confirmation of legionellosis is achieved by isolation of *Legionella* species from lower respiratory tract specimens. However, this approach is quite slow and insensitive, as growth of legionellae, even on buffered charcoal yeast extract (BCYE)-α agar, can take up to ten days (Rantakokko-Jalava & Jalava, 2001). Direct fluorescent antibody screening is also available but is very technically demanding as well as being the method most prone to error if not performed by experienced staff (Waterer *et al.*, 2001).

Urine *Legionella* antigen detection is also available and is of some use, as approximately 80% of patients with *L. pneumophila* serogroup 1 infections excrete *Legionella* antigen in their urine at some stage of the illness. However, this is the only serogroup that is reliably detected and prolonged excretion of antigen in some patients may lead to false-positive results (Waterer *et al.*, 2001). Serological diagnosis is most commonly used, however the time required for seroconversion is variable and occasionally may not occur at all, particularly in immunocompromised patients (Rantakokko-Jalava & Jalava, 2001; Waterer *et al.*, 2001), which renders this method inadequate for initial management.

4.1.4 Quantitative detection of *Legionella* by *real-time* PCR

The polymerase chain reaction (PCR) is a sensitive method for detection of as little as one *Legionella* organism in a specimen. Although it can be more time-consuming than direct fluorescent antibody screening, results can still be obtained within a few hours, which is fast enough to affect empirical therapy (Rantakokko-Jalava & Jalava, 2001; Waterer *et al.*, 2001). A further advantage of PCR is that the same sample can sometimes be used for identification and typing of the agent by molecular methods, which is particularly useful if culture fails (Rantakokko-Jalava & Jalava, 2001). Some studies have recently described the use of *real-time* PCR to detect and enumerate legionellae and/ or *L. pneumophila* from both clinical and non-clinical samples (Ballard *et al.*, 2000; Hayden *et al.*, 2001; Rantakokko-Jalava & Jalava, 2001; Wellinghausen *et al.*, 2001; Raggam *et al.*, 2002; Reischl *et al.*, 2002; Herpers *et al.*, 2003; Welti *et al.*, 2003; Wilson *et al.*, 2003;). Only one study has used the TaqMan system (the system used in the present study) for amplification and detection of *Legionella* sequences, and only clinical samples were investigated with this system.

Real-time PCR has previously been used for detection of *Legionella* species within hospital water samples (Wellinghausen *et al.*, 2001; Hayden *et al.*, 2001), as nosocomial outbreaks caused by *Legionella* in hospital water supplies have been well documented (Berthelot *et al.*, 1998; Knirsch *et al.*, 2000; Oren *et al.*, 2002). However, these studies used the LightCycler system (Idaho Technology, Idaho Falls, Idaho), which is slightly different to the TaqMan system, used in the present study. Recent literature searches have shown that *real-time* PCR has not previously been used for quantification of *Legionella* species in DUW output water.

4.1.5 Aims and objectives

The purpose of this study was to design a faster, more sensitive system than culture for detecting *Legionella*, which could be used to screen DUW output water because *Legionella* screening of DUW output water has not previously been undertaken in the Republic of Ireland. This necessitated the design of appropriate primers and Fam-labelled probes, suitable for use with the TaqMan Sequence Detection System, which would enable the quantification of *Legionella* and *L. pneumophila* in water samples. The *real-time* PCR assays were used in conjunction with traditional culture methods to assess the extent of *Legionella* contamination of DUW output water samples and water samples from other selected healthcare institutions in Ireland.

4.2 Materials and Methods

4.2.1 Bacteria, culture and control DNA preparation

Legionella pneumophila serogroups 1-8 and *L. longbeachae* reference strains were obtained from the American Type Culture Collection (ATCC), while *L. pneumophila* serogroups 9-14 reference strains were obtained from the UK National Collection of Type Cultures (NCTC), along with other non-*L. pneumophila* legionellae used as reference strains. Other bacterial and yeast species used as negative controls were either obtained from DUW output water or were clinical isolates. The positive controls included *L. pneumophila* serogroups 1-14 and ten non-*L. pneumophila* legionellae. *Candida albicans* and *C. dubliniensis* clinical isolates recovered in the Dublin Dental Hospital were used as negative controls as these are eukaryotic species. *Pseudomonas* species, and other bacterial species isolated from DUW output water were also used as negative controls. Unless otherwise specified, all water samples investigated for the presence of *Legionella*, all *Legionella* isolates recovered from water samples and all *Legionella* reference strains obtained from the ATCC and NCTC were grown in a static Sanyo CO_2 incubator (Model MCO-17A1) at 37°C with 5% (v/v) CO_2 (Sanyo, Bensenville, Illinois, USA). *Legionella pneumophila* serogroup 2 (ATCC 33154) was used for the creation of a standard curve with the *real-time* PCR assays to enable quantification of *Legionella* in the test water samples. To create the standard curve, a suspension of four to five colonies (depending on size) was made in a 1.5 ml Eppendorf microfuge tube containing 1 ml sterile distilled water. DNA was extracted from the suspension in 150 µl elution buffer using the Qiagen DNeasy tissue kit according to the manufacturer's instructions, as described in Section 2.2.1. Ten-fold serial dilutions in sterile distilled water were made from the resulting DNA for the creation of the standard curve.

The *Legionella* strains used for specificity testing in *real-time* PCR assays were also grown on BCYE agar (Oxoid) and DNA was extracted from them as described for *L. pneumophila* serogroup 2 (ATCC 33154) above. The non-*Legionella* bacteria were grown on NA (Oxoid) for three days at 30°C in a static incubator (Gallenkamp) and DNA was subsequently extracted from these species following suspension of four colonies in 1 ml of sterile ultra-pure water as described above. *Candida* strains were grown on potato dextrose agar (PDA) for two days at 37°C in a static incubator (Gallenkamp). For DNA extraction from these yeasts, four colonies were suspended in a 1.5 ml microfuge tube (Eppendorf) containing 1 ml sterile distilled water and this suspension was boiled in a water bath for 10 min. The tubes were then placed in a FastPrep™ FP120 Bead Beater (Bio101, Savant, Holbrock, New York, USA) for 20 s at maximum speed (level 6.5) to break up cells. The

resulting suspensions were centrifuged at 12,200 x g in an Eppendorf microfuge (Model 5417C) for 3 min and the supernatant was removed and stored at –20°C until required.

4.2.2 Collection of water samples from DUWs, hospitals and other institutions

Water samples (500 ml) were collected in sterile bottles containing excess sodium thiosulphate (Plastiques Gosselin, Hazebrouck Cedex, France) from the DUWs of 15 DCUs (Planmeca) located in dental clinics in the Midland Health Board region and other dental clinics in Dublin serviced by Dublin Dental Hospital personnel (19 DUW output water samples in total). None of the DUWs included in this part of the study had ever been disinfected. DUW output water from DCUs in the Dublin Dental Hospital was not analysed in this part of the present study as once-weekly disinfection of DUWs with hydrogen peroxide had already been implemented (see Chapter 3). As this was a small sample number, 77 additional water samples were also taken from hospitals, health centres and other institutions located in the Mid-Western Health Board region in order to thoroughly assess the efficacy of culture and *real-time* PCR systems for detecting *Legionella*.

4.2.3 Determination of viable counts of *Legionella* species

For each water sample tested, one 100-μl aliquot of the undiluted water sample was spread onto a separate BCYE agar plate supplemented with glycine, vancomycin-hydrochloride, polymyxin B-sulphate and cycloheximide (GVPC) (Oxoid) and incubated in a 37°C Sanyo incubator with 5% (v/v) CO_2 for up to ten days. Following removal of this initial aliquot, each 500-ml water sample was vacuum filtered through separate commercially available disposable 100-ml analytical filtration funnels (Biopath), using a 6-Branch Manifold Vacuum Filter System (Sartorius). Each funnel contained a sterile 47-mm diameter, black gridded cellulose nitrate removable membrane with a pore size of 0.45 μm. The filtrate was discarded and the membrane was removed using a sterile forceps and placed in a sterile 2-ml microfuge tube (Sarstedt) containing approximately 0.3 ml of 450-600 μm acid-washed glass beads (Sigma-Aldrich) and 1.5 ml sterile distilled water. The tubes were then placed in a FastPrep™ FP120 Bead Beater for 5 s at level 6. This short time of bead beating broke up the filter, thus releasing the microorganisms into the liquid medium. Empirical studies using viable *Legionella* counts demonstrated that this short period of bead-beating had a negligible effect on cell viability. The microfuge tubes were then removed and vortexed thoroughly. Three 100-μl aliquots of this suspension were removed for further analysis, while a 750-μl aliquot of the suspension was used for

extraction of DNA for the *real-time* PCR analysis (see Section 4.2.4). The first 100-μl aliquot was used to inoculate a GVPC (Oxoid) agar plate and grown in a 37°C Sanyo incubator with 5% (v/v) CO_2 for up to ten days. The second aliquot was placed in a 1.5 ml tube (Eppendorf) and incubated in a water bath at 55°C for 30 min, after which time the aliquot was removed and spread onto a GVPC agar plate (Oxoid) for incubation as described above. The final aliquot was added to a 100-μl aliquot of HCl-KCl buffer (0.8 mM HCl; 5 mM KCl, pH 2.2) in a 1.5 ml Eppendorf microfuge tube and incubated at room temperature for 10 min, after which time the solution was removed and spread onto a GVPC agar plate (Oxoid) for incubation as described above. The latter two 100-μl aliquots were subject to heat and acid-treatment, respectively, to lyse amoebae in which *Legionella* may have been residing to release the bacteria into the suspensions. Each suspension was then removed and spread onto a GVPC agar plate and incubated as described above.

All agar plates were examined every three to four days for up to ten days and any colonies exhibiting typical *Legionella* morphology (bluish-white round colonies- see Figure 4.1) were subcultured onto another GVPC plate and also onto Colombia blood agar or NA. Any colonies displaying the typical *Legionella* morphology that could not grow on agar that lacked cysteine (i.e. Colombia blood agar or NA plates) were presumed to be *Legionella* species and stored for further analysis, as recommended by the ISO guidelines (Bartie *et al.*, 2003). Where *Legionella*-like colonies grew on two or more GVPC agar plates (i.e. on one or more of the agar plates used to grow the neat, concentrated, acid-treated or heat-treated samples) for the same sample, the agar plate with the most colonies was considered to reflect most accurately the number of *Legionella* in the sample and the number of colonies grown on this plate was recorded and used to determine the density of *Legionella* bacteria in the original sample.

4.2.4 Identification of *Legionella* cultured from water samples

Legionella species isolated from the water samples were identified as *L. pneumophila* or not by addition of one colony of each test isolate that had been subcultured on BCYE agar to a separate well in the *mip real-time* PCR assay described below in Sections 4.2.6 and 4.2.7. Where the *mip* assays detected fluorescence, indicating the presence of *L. pneumophila* DNA, the isolates were presumed to be *L. pneumophila*. *Legionella pneumophila* isolates subcultured on the BCYE agar were then subjected to serogrouping by use of the DrySpot *Legionella* serogroup 1 Latex Test kit (Oxoid), according to the manufacturer's instructions.

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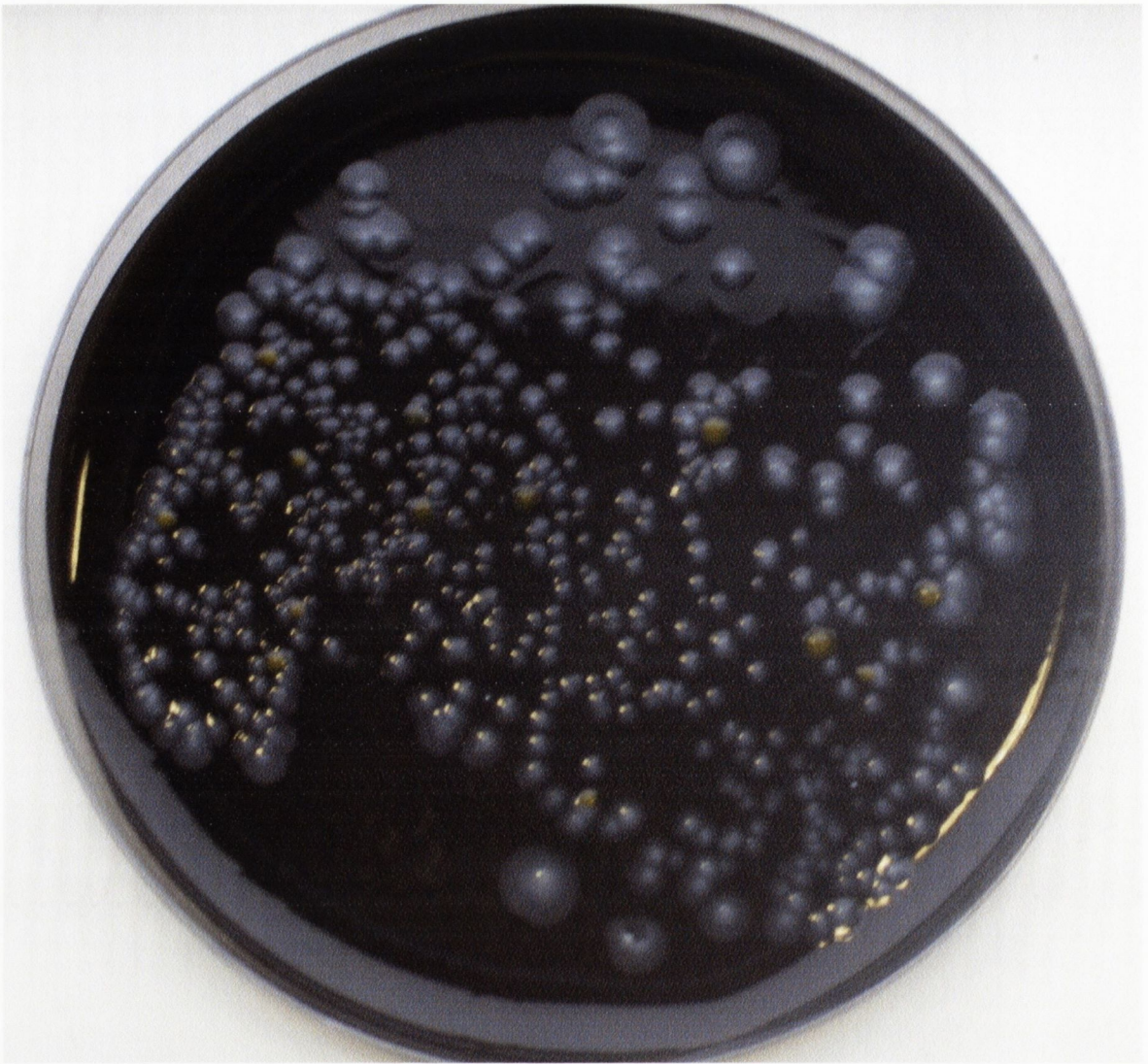
Figure 4.1. Appearance of *Legionella* colonies on GVPC agar following incubation for 10 days in a Sanyo CO_2 incubator at 37°C. The *Legionella* organisms were cultured from a water sample taken from a healthcare institution located in the Mid-Western Health Board region.

4.2.5 Preparation of water samples for *real-time* PCR

DNA was extracted from 750-μl aliquots (see Section 4.2.3) of the concentrated water samples using the DNeasy Tissue kit (Qiagen) and eluted in a final volume of 150 μl of elution buffer, as described in Section 2.2.

4.2.6 Design of primers and probes

Primers and probes were designed for the specific detection of *L. pneumophila* using the *mip* region of *L. pneumophila* (GenBank accession number AY676538). The *mip* (macrophage infectivity potentiator) gene, which encodes a 24-kDa protein virulence factor that facilitates the entry of *Legionella* into amoebae and macrophages, has sufficient sequence variability between the different *Legionella* species to permit the specific detection of *L. pneumophila* by *real-time* PCR (Wilson *et al.*, 2003). General primers (Table 4.1) that detected all *Legionella* species (pan-*Legionella* primers and probe) were also designed using the 16S ribosomal RNA gene of *L. pneumophila* strain CA1 (GenBank accession number AF129523). PCR primers were synthesised by Sigma-Genosys and HPLC purified 5' FAM-labelled and 3' TAMRA-labelled probes were synthesised by Applied Biosystems (Applied Biosystems, Cheshire, England). Both primers and probes were designed with the aid of the Primer Express software package (Applied Biosystems), a computer software programme specifically written for designing primers and probes for use with the TaqMan system. This programme takes into account various guidelines for primers and probes that are recommended by Applied Biosystems for optimal amplification and detection of target sequences, as detailed in Table 4.2. A BLAST search using the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) was then carried out to confirm if the primers and probe would detect the species for which they were designed. The resulting primers and probes are shown in Table 4.1.

The first set of primers and probes that were used (data not shown) for the general *Legionella* assay with the TaqMan Sequence Detection System were found to be ineffective as they amplified and detected many other species of bacteria. Amplification of the 'No Template Control' was also detected due to contaminating bacterial DNA within the TaqMan reagents supplied by Applied Biosystems. Therefore, a second set of primers and probe was designed using Primer Express (Applied Biosystems) and the BLAST search facility of the NCBI website to detect the specificity by searching for the closest 500 matches to each sequence. These *Legionella* primers and probe were found to be quite specific following the BLAST search. However, following testing of the primers and

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Table 4.1. Names and sequences of primers and probes designed for use in this study

Name	Comment	Sequence (5'-3')	Co-ordinates*	GenBank
LP forward	*mip L. pneumophila* forward primer	AAAGGCATGCAAGACGCTATG	*mip* gene, 62-82	AY676538
LP reverse	*mip L. pneumophila* reverse primer	GAAACTTGTTAAGAACGTCTTTCATTTG	*mip* gene, 113-140	AY676538
LP probe	*mip L. pneumophila* probe	TGGCGCTCAATTGGCTTTAACCGA	*mip* gene, 85-108	AY676538
Leg forward	pan-*Legionella* forward primer	CTTTCGTGCCTCAGTGTCAGTATTA	16S rRNA gene 705-729	AF129523
Leg reverse	pan-*Legionella* reverse primer	GGGAGAGGGTAGTGGAATTCC	16S rRNA gene 792-813	AF129523
Leg probe	pan-*Legionella* probe	AGGTAGCCGCTTCGCCACTGG	16S rRNA gene 734-754	AF129523

* +1 refers to the first base of the ATG start codon

Table 4.2. Characteristics of primers and probes for optimum target sequence amplification and detection for use with the TaqMan System

Primer	Melting temperature between 58°C and 60°C
	Less than 2°C difference between melting point of primers
	20 – 80% G C content
	9-40 bp long
	Maximum of 2/5 G or C at the 3' end
Probe	Melting temperature is to be 10°C higher than primers
	20-80% G C content
	9-40 bp long
	No G on the 5' end
	Must not have more G than C
	Located as close as possible to the primers without overlapping
Amplicon	Less than 150 bp long

probe with both positive and negative controls in the 7700 Sequence Detection System, it appeared that while the 'No template control' and some of the other negative controls were no longer amplified, these primers and probe (designed to amplify only *Legionella* species) amplified and detected *P. aeruginosa*, *P. fluorescens* and *P. putida* DNA. These *Pseudomonas* isolates had been recovered from DCUs and DUW output water and should have been negative controls. All *Legionella* species were also amplified.

A further BLAST search of each sequence used for the primers and probe was carried out, this time searching for the closest 1,000 matches (the maximum number this facility allows). It was found that the forward primer contained a large region of homology with *Pseudomonas* species (Table 4.3), which had not been evident when only the closest 500 matches were examined. In total, 167 *Pseudomonas* sequences were between 68% and 91% homologous to the *Legionella* forward primer. Furthermore, a high number of sequences from *Stenotrophomonas* species, which are common in DUW output water (Al Shorman *et al.*, 2002), were among the top 1,000 matches for the general *Legionella* forward primer. Therefore, a second forward primer was designed that was 11 base pairs upstream from and two base pairs longer than the first primer, as this was the region more specific to *Legionella* (see Table 4.3). A BLAST search showing the nearest 1,000 sequence matches was carried out and no *Pseudomonas* or *Stenotrophomonas* species were among these matches.

4.2.7 *Real-time* PCR assay for detection of *Legionella*

The ABI PRISM® 7700 Sequence Detection System (Applied Biosystems) was used for carrying out the PCR reactions and for subsequent analysis. MicroAmp Optical reaction plates (Applied Biosystems) contained 96 wells available for use in each experiment. Each sample well contained 2.5 µl of the test DNA solution, 900 µM each of the forward and reverse primers, 250 µM 5' Fam-labelled probe and 1X TaqMan Universal PCR Master Mix (Applied Biosystems) made up to a final volume of 25 µl with sterile distilled water. 'No template' and 'no amplification' controls were included in each assay, where sterile ultra-pure water was used instead of template DNA and primers, respectively. The Universal Master Mix provided a PCR mix that may be used with any appropriately designed primers and probe to detect any DNA sequence. Included in the Master Mix was AmpErase® uracil-N-glycosylase (UNG) that can prevent the re-amplification of carryover-PCR products by removing any uracil incorporated into single- or double-stranded DNA.

Table 4.3. **Region of homology between pan-*Legionella* forward primer (2F) and *Pseudomonas* species**

Pan-*Legionella* 2F	GGGAGAGGGTAGTGGAATTTCC
Sequence of homologous region	AGAGGGTGGTGGAATTTCC

Ten-fold serial dilutions of *L. pneumophila* DNA (see Section 4.2.1) were included in each assay as standards and these were used to construct a standard curve from which the estimated quantity of bacteria could be determined in the unknown samples. Each negative control, standard and unknown sample was tested in duplicate. After loading each well, the reaction plate was covered with an optical adhesive cover (Applied Biosystems) using the applicator supplied for this purpose. An optical cover compression pad (Applied Biosystems) was placed over this to protect the samples from the heat of the PCR machine and the plate was then placed inside the machine. Each well was labelled using the Sequence Detection System software loaded on an Apple Macintosh G4 computer (Apple Computer International, Holyhill Industrial Estate, Cork, Ireland) attached to the ABI PRISM® 7700 and the cycle was initiated. Running conditions were as shown in Table 4.4. The total reaction time was 1 h 56 min. After completion of the test, samples were analysed using the Applied Biosystems Sequence Detection System software.

It may be of interest to note that there were some technical difficulties associated with the use of the ABI PRISM® 7700 Sequence Detection System in this project. These included the fact that Applied Biosystems sent faulty probes for use with the Sequence Detection System, which led to wasted time and reagents, and the fact that the ABI PRISM® 7700 also broke down, leading to further wastage of time and reagents. These difficulties inevitably led to increased costs associated with this part of the present study.

4.2.8 Macrorestriction analysis of *L pneumophila* isolates recovered from water distribution system outlets of an acute hospital in the southeast

Isolates of *L. pneumophila* recovered from water outlets in Waterford Regional Hospital in 2003 following a case of nosocomial Legionnaires' disease were investigated by macrorestriction analysis of high molecular weight chromosomal DNA with *Sfi*I. Agarose plugs containing *L. pneumophila* were prepared as described in Section 2.2.2.1 and 2.2.2.2. For restriction enzyme digestion with *Sfi*I, a three-mm sample of each plug was cut using a clean scalpel and placed in a 1.5 ml Eppendorf microfuge tube with the *Sfi*I buffer. The buffer contained 1X NE Buffer 2 (New England Biolabs), 0.1 µg BSA (New England Biolabs) and 10 U *Sfi*I (New England Biolabs) made up to 100 µl with sterile distilled water. The *Legionella* DNA within the plugs was digested overnight for 16 h in a water bath at 50°C. After this period, plugs were removed from the buffer for separation of the DNA fragments contained within them by PFGE.

The *Sfi*I restriction-enzyme digested DNA fragments in agarose plugs were separated in 1% (w/v) agarose gels made with ultra-pure DNA grade agarose (Bio-Rad) in 0.5X TBE in one ramp for 19.3 h at 14°C. Pulse times were increased linearly in 1 s increments from 5.3 s to 49.9 s with field strength of 6 V/cm, as described by Jonas *et al.*, 2000. λ phage DNA oligomers (New England BioLabs) were used as size reference markers and were applied to the outermost lanes of each gel. Plugs containing *Sfi*I restriction-enzyme digested DNA fragments from *L. pneumophila* serogroup 2 (ATCC 33154) were applied to the left-hand lane of each gel adjacent to the lane containing the λ ladders and the resulting DNA fragment patterns following PFGE were used for gel-to-gel comparisons.

4.3 Results

4.3.1 Specificity of the primers and probes used in this study

As shown in Table 4.5, the *mip*-specific primers amplified all 14 serogroups of *L. pneumophila*. None of the ten non-*L. pneumophila* legionellae was detected by the *mip*-specific primers and probe. Furthermore, this primer and probe set did not detect the non-*Legionella* bacterial isolates tested or the *Candida* species tested (see Table 4.5). The pan-*Legionella* primers and probe also detected all ten non-*L. pneumophila Legionella* species tested, as well as the 14 serogroups of *L. pneumophila*, but *Pseudomonas* species were no longer amplified (see Table 4.5).

4.3.2 Determination of the external standard curve

In order to establish a quantitative *real-time* PCR assay, a standard curve of serial ten-fold dilutions of *L. pneumophila* serogroup 2 genomic DNA in sterile distilled water was developed from 150 ng to 150 fg per PCR test (Figure 4.2). The standard curve was created by the second-derivative maximum method of the Applied Biosystems software, which is a software algorithm that identifies the first turning point of the fluorescence curve (i.e., the first maximum of the second-derivative curve). This then serves as the crossing point in the calculation of the standard curve. The standard curve is the linear regression line through the data points on a plot of the crossing point versus the logarithm of the standard sample concentration (Figure 4.2). Because the crossing points are determined by a software algorithm, interpretation of the data values at different times and by different investigators will give identical results. With the aid of the standard curve, the concentrations of unknown samples could then be estimated.

4.3.3 Determination of viable counts of *Legionella* species in DUW output water and other water samples

Water samples were concentrated from 500 ml to 1.5 ml and a 100-µl aliquot was placed on GVPC agar plates. The minimum detection limit of the culture assay was one colony of *Legionella* per agar plate, which corresponds to a minimum detection limit of 15 viable *Legionella* cells in the original 500-ml water sample. Of the 19 DUW output water samples from separate DCUs included in the study, *Legionella* bacteria were cultured on GVPC from only one sample (5%). This water sample was taken from the air/water syringe waterline of a DCU that is located in a Dublin prison, which is used on a once-weekly basis. To confirm these results, a second sample was taken from the same DCU four weeks later and similar results were obtained. The average number of *Legionella*

Table 4.5. Reference standard strains, environmental isolates and clinical isolates used in this study to establish the specificity of the pan-*Legionella* and *L. pneumophila* specific *real-time* PCR assays

| Species | Reference no. | Results with *real-time* PCR assay | |
		pan-*Legionella*	*mip*
L. pneumophila serogroup 1	ATCC 33152	+	+
L. pneumophila serogroup 2	ATCC 33154	+	+
L. pneumophila serogroup 3	ATCC 33155	+	+
L. pneumophila serogroup 4	ATCC 33156	+	+
L. pneumophila serogroup 5	ATCC 33216	+	+
L. pneumophila serogroup 6	ATCC 33215	+	+
L. pneumophila serogroup 7	ATCC 33823	+	+
L. pneumophila serogroup 8	ATCC 35096	+	+
L. pneumophila serogroup 9	NCTC 11986	+	+
L. pneumophila serogroup 10	NCTC 12000	+	+
L. pneumophila serogroup 11	NCTC 12179	+	+
L. pneumophila serogroup 12	NCTC 12180	+	+
L. pneumophila serogroup 13	NCTC 12181	+	+
L. pneumophila serogroup 14	NCTC 12174	+	+
L. adelaidensis	NCTC 12735	+	-
L. anisa	NCTC 11974	+	-
L. birminghamensis	NCTC 12437	+	-
L. bozemanii	NCTC 11368	+	-
L. dumoffii	NCTC 11370	+	-
L. gormanii	NCTC 11401	+	-
L. israelensis	NCTC 12010	+	-
L. jamestowniensis	NCTC 11981	+	-
L. jordanis	NCTC 11533	+	-
L. longbeachae	ATCC 33462	+	-
[a]*Staphylococcus aureus*	Clinical isolate	-	-
[b]*Pseudomonas fluorescens*	DCU[c] isolate	-	-
[b]*Pseudomonas putida*	DCU isolate	-	-
[b]*Pseudomonas aeruginosa*	DCU isolate	-	-
[b]*Burkholderia cepacia*	DUW[d] isolate	-	-
[b]*Stenotrophomonas maltophilia*	DUW isolate	-	-
[b]*Agrobacterium radiobacter*	DUW isolate	-	-
[b]*Candida albicans*	Clinical isolate	-	-
[b]*Candida dubliniensis*	Clinical isolate	-	-

[a] (Coleman *et al.*, 1985)
[b] Clinical isolates recovered in the Dublin Dental Hospital
[c] DCU refers to dental chair unit
[d] DUW refers to DUW output water

Figure 4.2. Standard curve created by Sequence Detection System software (Applied Biosystems) showing standards in black and unknowns in red.

species cultivated from the air/water syringe waterline of this DCU was 1,000 cfu/l. One colony was subcultured for further analysis and subsequently identified as *L. anisa* by 16S sequence analysis using the pGEM T-Easy Vector System, as described in Sections 2.3 and 2.4.

Of the 77 water samples taken from the Mid-Western Health Board region, *Legionella* bacteria were cultured from 18 samples (23%), representing nine different healthcare institutions (see Table 4.6). These included water samples from three hospitals, one community care centre, one elderly care centre and a day care centre. The average number of *Legionella* bacteria present in these samples was found to be 7,840 cfu/l. One *Legionella* colony recovered from each of the culture-positive samples was used with the *L. pneumophila*-specific *mip* PCR assay to identify the species of the isolates. Each colony tested was found to be *L. pneumophila*. Because of this, two further *Legionella* colonies recovered from the water samples were subject to serogrouping by use of the DrySpot *Legionella* serogroup 1 Latex Test kit (Oxoid), according to the manufacturer's instructions. Isolates recovered from three of the water samples were found to be *L. pneumophila* serogroup 1, the serogroup most often associated with Legionnaires' disease. The numbers of *Legionella* bacteria isolated from two of these three water samples were relatively high: 30,000 cfu/l and 90,000 cfu/l (Table 4.6).

4.3.4 Determination of numbers of *Legionella* species in DUW output water and other water samples by the pan-*Legionella real-time* PCR assay

Legionella DNA in the water samples was quantified using an external standard curve, generated as described above in Section 4.3.2. The genome of one *Legionella* organism consists of approximately 4.3 fg of DNA (Bender *et al.*, 1990). Therefore, the quantity of *Legionella* species in the water samples could be estimated with reference to the DNA concentrations of the standards used for generation of the external standard curve. The lower quantification limit of this assay was found to be 80 fg per PCR test, which corresponds to approximately 19 *Legionella* bacteria per test. For the assay conditions used in this study (see Sections 4.2.3 and 4.2.5), the lower quantification limit corresponds to approximately five *Legionella* organisms per ml of the original sample.

In order to fully evaluate the general pan-*Legionella* and *L. pneumophila* specific *mip real-time* PCR assays for use with DUW output water samples, results obtained from the 77 water samples from healthcare institutions in the Mid-Western Health Board region were analysed first because *Legionella* had been cultured from 18 of these samples (23%). Wellinghausen *et al.* (2001) suggested that, despite the fact that exact quantities of

Table 4.6. Densities of *Legionella* bacteria recovered from various healthcare institutions in the Mid-Western Health Board regions. All *Legionella* isolates tested were *L. pneumophila*.

Sample	Institution	Density in cfu/l	Serogroup 1
10	Hospital L	210	+
31	Residential care centre L	60	-
33	Hospital NT-B	90	-
40	Hospital NT-B	120	-
42	Hospital NT-B	90	-
44	Hospital NT-B	300	-
36	Hospital C-A	120	-
76	Hospital C-A	510	-
51	Community care centre-C	390	-
56	Elderly care centre-C	1,800	-
77	Elderly care centre-C	4,500	-
61	Community care centre-NT	10,000	-
62	Hospital NT-A	150	-
63	Hospital NT-A	90,000	+
64	Hospital NT-A	30,000	+
68	Hospital NT-A	1,500	-
66	Day care centre-NT	390	-

Legionella detected in each water sample can be estimated from *real-time* PCR results, it may be more appropriate to show results of *real-time* PCR assays in categories rather than as exact values, due to the inevitable inaccuracies during sample concentration and DNA extraction. Therefore, this method of displaying data has been adopted in this study (Wellinghausen *et al.*, 2001). *Legionella* DNA was detected in 73/77 (96%) of these water samples by the pan-*Legionella real-time* PCR assay (Figure 4.3). The four water samples that were negative for the presence of *Legionella* by *real-time* PCR were also negative by culture. There was a further correlation between culture results and *real-time* PCR results in that, overall, the mean average *real-time* PCR results for the culture-negative samples (19,560 *Legionella* genome equivalents per l) were lower than those for the culture-positive samples (37,440 *Legionella* genome equivalents per l). The *Legionella* densities calculated from the PCR standard curve were higher than those detected by culture by a mean average factor of 108.

With regard to the DUW output water samples, *Legionella* bacteria were recovered by culture from only one sample. However, high levels of *Legionella* DNA (mean average 166,000 genome equivalents per l) were detected in 15/ 19 DUW output water samples (Figure 4.4). Of particular concern was the fact that the numbers of *Legionella* detected from one DCU located in the dental clinic of an acute hospital that is used on a once-weekly basis for the treatment of medically ill patients was 500,000 genome equivalents per l, although no viable *Legionella* were recovered from the DUW output water of this DCU by culture.

4.3.5 Specific detection of *L. pneumophila* in water samples by *mip* gene PCR

Since *L. pneumophila* is the most pathogenic *Legionella* species, a *L. pneumophila*-specific PCR hybridisation probe assay was also developed based on amplification of the *mip* gene. This *mip* gene PCR assay was specific for *L. pneumophila* in that it detected all serogroups 1-14 as listed in Table 4.5, but none of the non-*L. pneumophila* legionellae. As in the pan-*Legionella real-time* PCR assay, the *mip* gene was detected quantitatively by means of external standards of *L. pneumophila* serogroup 2 DNA. The *mip* gene *real-time* PCR assay had a sensitivity equal to that of the pan-*Legionella real-time* PCR assay and, therefore, a standard curve measuring a similar range (150 ng to 150 fg *L. pneumophila* DNA) was set up. Of the water samples from healthcare institutions located in the Mid-Western Health Board region tested, 64/77 (83%) gave positive PCR results, thus indicating the presence of *L. pneumophila* (Figure 4.3). Of the 13 *mip*-negative samples, 12 were from culture-negative water samples. Again, there was a correlation between the
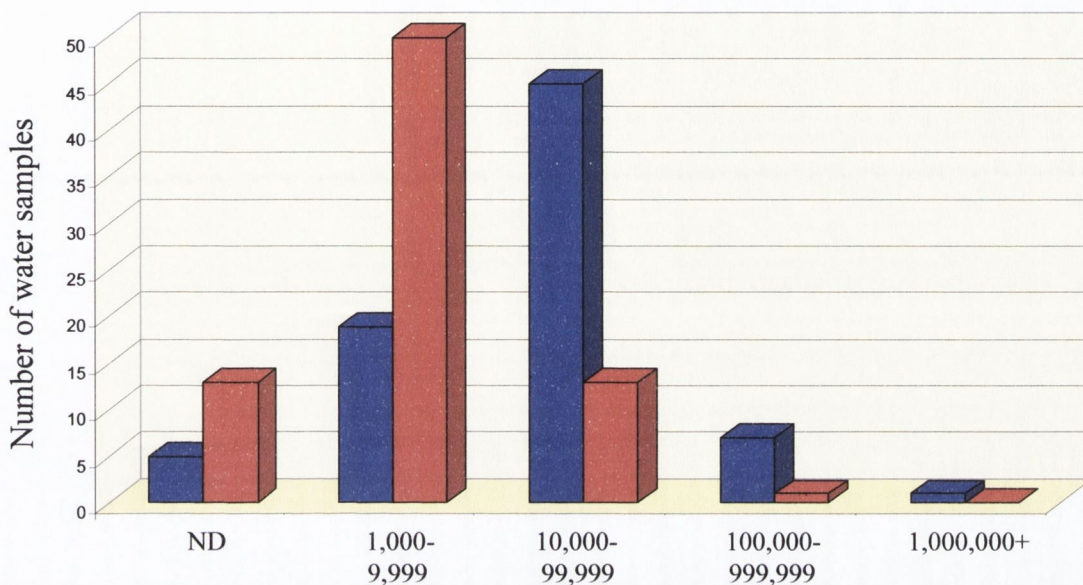
Legionella density in genome equivalents/ l as determined by *real-time* PCR

Figure 4.3. Density of *Legionella* bacteria in genome equivalents per l detected by *real-time* PCR in 77 water samples taken from healthcare institutions located in the Mid-Western Health Board region. The blue columns represent the total number of *Legionella* bacteria detected in the water samples using pan-*Legionella* primers and probe, while the red columns represent the number of *L. pneumophila bacteria* detected using the *L. pneumophila*-specific *mip* primers and probe. ND indicates that *Legionella* could not be detected in water samples.
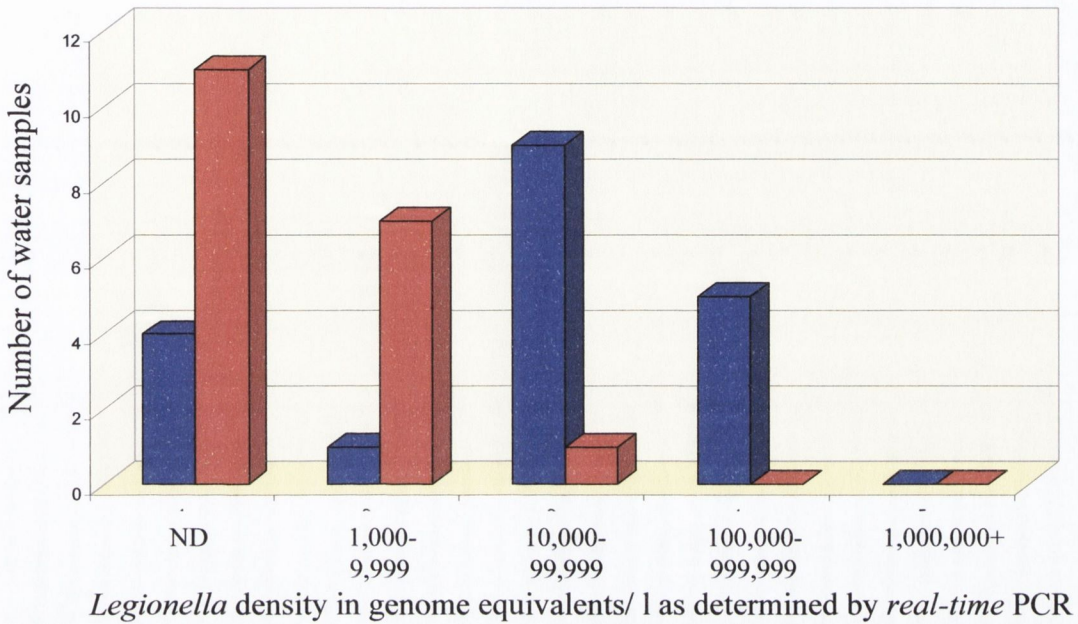
Figure 4.4. Density of *Legionella* in genome equivalents per l detected by *real- time* PCR in 19 DUW output water samples. The blue columns represent the total number of *Legionella* detected in the water samples using pan-*Legionella* primers and probe, while the red columns represent the number of *L. pneumophila* detected using the *L. pneumophila*-specific *mip* primers and probe. ND indicates that *Legionella* could not be detected in water samples.

detection of *L. pneumophila* by culture and by *real-time* PCR in that the mean average levels of *Legionella* detected by PCR in culture-negative water samples (5,400 genome equivalents per l) were considerably lower than in those samples from which *L. pneumophila* was isolated (17,400 genome equivalents per l). In comparison to the results obtained by 16S rRNA gene PCR, *mip* PCR results detected smaller amounts of DNA and thus lower estimated numbers of *Legionella* genome equivalents per l (see Figures 4.3 and 4.4).

Of the DUW output water samples, 8/19 (47%) gave positive results with the *mip* assay. The mean average number of *Legionella* detected in DUW output water samples by *real-time* PCR was 5,100 genome equivalents per l (Figure 4.4).

4.3.6 Macrorestriction fragment analysis of *L. pneumophila* samples obtained from an Irish hospital

Legionella pneumophila isolates recovered from water supplies obtained from various water outlet sources throughout Waterford Regional Hospital where a patient died of nosocomial Legionnaires' disease in April 2003 were sent to the laboratory for analysis (Coleman & O'Donnell, 2003). Sixteen of these isolates were selected at random for macrorestriction fragment analysis using the *Sfi*I restriction enzyme, as described in Section 4.2.8. All 16 of these isolates were shown to have identical PFGE-generated DNA patterns, as shown in Figure 4.5, indicating that one strain of *L. pneumophila* had spread through the water systems of the entire hospital. Unfortunately, *L. pneumophila* isolates recovered from clinical specimens taken from the patient were not available for analysis by this laboratory so this particular *L. pneumophila* strain could not be definitely attributed to the death of the patient.
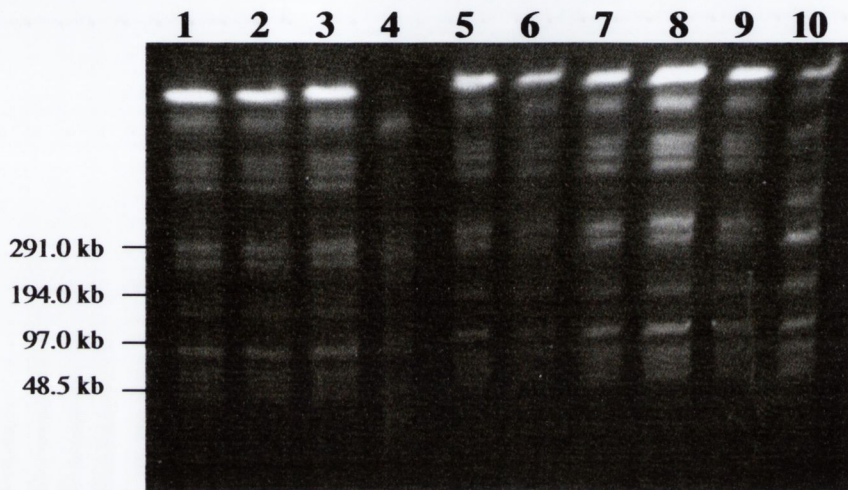
Figure 4.5. Photograph of a section of a 1% agarose gel showing *Sfi*1-digested DNA from *L. pneumophila* isolates following separation of DNA fragments by pulsed field gel electrophoresis (PFGE), as described in Section 4.2.8.1. Lanes 1-3 and 5-10 show patterns from separate *L. pneumophila* isolates recovered from the water outlets at Waterford Regional Hospital. Lane 4, *L. pneumophila* serogroup 2 (ATCC 33154); Lane 10, *L. pneumophila* serogroup 3 (ATCC 33155).

4.4 Discussion

Contamination of DUW output water with relatively high numbers of *Legionella* bacteria has been reported in previous studies from England, the US and Italy with at least one case of Legionnaires' disease associated with exposure to DUW output water (Atlas *et al.*, 1995; Challacombe & Fernandes, 1995; Williams *et al.*, 1996; Pankhurst *et al.*, 1998; Zanetti *et al.*, 2000). While *L. pneumophila*, particularly serogroup 1, is the main causative agent of Legionnaires' disease, other species, such as *L. micdadei*, *L. dumoffii*, and *L. bozemanii*, have also been implicated in infection (Atlas *et al.*, 1995; Luck *et al.*, 1995; Knirsch *et al.*, 2000). In the case of an outbreak of Legionnaires' disease, it is essential to rapidly find the source of infection in order to prevent further cases. However, there are many difficulties associated with the cultivation of *Legionella* from both clinical samples and potential sources of infection such as water samples, in particular the fact that *Legionella* may take up to ten days to grow on agar plates (Walker *et al.*, 2000). Other difficulties associated with the cultivation of *Legionella* include the fastidious nutritional requirements of the bacteria, e.g. a requirement for cysteine, and the fact that some *Legionella* may be sustained within amoebae and are therefore uncultivable (Fields *et al.*, 2002).

4.4.1 Culture of *Legionella* species from DUW output water

Recent literature searches have shown that this was the first study that attempted to characterise the extent of DUW output water contamination with *Legionella* in the Republic of Ireland. Viable *Legionella* bacteria were recovered from only one of 19 (5%) DUW output water samples tested, despite the fact that none of the DUWs tested had been previously subjected to disinfection. This indicates that *Legionella* may not be particularly prevalent in the DUWs in the Republic of Ireland, although a much more extensive study with periodic sampling of the DUW output water of many DCUs located throughout the Republic of Ireland needs to be undertaken in order to confirm this. These results are in agreement with two recent studies undertaken in Great Britain, which found that *Legionella* was present in less than 2% of DUW output water samples tested (Walker *et al.*, 2000; Pankhurst *et al.*, 2003). The *Legionella* isolated from the only culture-positive DUW output water sample was found to be a non-*L. pneumophila Legionella*. It is of note that the culture-positive DUW output water sample was located in the dental clinic of a prison staffed by personnel from the Dublin Dental Hospital that was only used on a once-weekly basis and had not previously been disinfected. Following the recovery of *Legionella* from the DUW output water, this DCU has since been decommissioned as the

DUWs could not be readily disinfected (Prof. D. Coleman, Personal Communication). The stagnation of the water within the DUWs during the six days when this DCU was not in use probably facilitated the build-up of *Legionella* within the water supplies as *Legionella* species multiply in stagnant water where they have more opportunity to form biofilms (Steinert *et al.*, 2002). The fact that most of the other DUWs were used five days a week may explain the low prevalence of *Legionella* recovered from the DUWs of the DCUs investigated in this study compared to previous studies from Italy and the US (Atlas *et al.*, 1995; Zanetti *et al.*, 2000). Another explanation for the lower prevalence of *Legionella* in DUW output water of DUWs in Great Britain and Ireland could be the cooler temperature of municipal waters held in reservoirs and lakes, from which the DUWs of DCUs are fed, compared to warmer climates such as Italy and the US, which would limit the presence of *Legionella* species (Pankhurst *et al.*, 2003).

4.4.2 Culture of *Legionella* from water taken from other healthcare institutions

In order to better calibrate the system for detection of *Legionella* species, 77 water samples from various healthcare institutions in the Mid-Western Health Board region were also tested. *Legionella* isolates were recovered from 18 of 77 (23%) water samples tested, representing water from nine separate healthcare institutions (Table 4.6). All *Legionella* isolates recovered were identified as *L. pneumophila* by the *mip* assay, with three of these isolates belonging to serogroup 1, the serogroup most often associated with Legionnaires' disease. It is of grave concern that the levels of *L. pneumophila* serogroup 1 cultured from two of these water samples were relatively high (90,000 cfu/l and 30,000 cfu/l). These water samples were taken from one particular Mid-Western Health Board hospital (Table 4.6), which undoubtedly has immunocompromised patients attending who could be exposed to these high levels of *Legionella*. One study has investigated *Legionella* contamination of water supplies in Ireland and is available on the PubMed database. This study was published in 1990 and consisted of periodic and random sampling of showers and water tanks in two Dublin hospitals with 5.3% of the samples tested yielding *Legionella* species (Haugh *et al.*, 1990). From 1989 to date, three studies in the PubMed database have reviewed the epidemiology of Legionnaires' disease in Ireland (Hone *et al.*, 1989; Carr *et al.*, 1991; Smith *et al.*, 2002). The two earlier studies reported a low prevalence of pneumonia due to *Legionella* species and indicated that there was little cause for concern (Hone *et al.*, 1989; Carr *et al.*, 1991). In contrast, however, a review of all 17 Legionnaires' disease case notifications in Ireland from 1998-2001 was undertaken by Smith *et al.* (2002) who reported that all 17 cases were attributed to *L. pneumophila*

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serogroup 1 with nine cases associated with travel abroad. The authors of this paper suggested that the seemingly low prevalence of Legionnaires' disease in Ireland compared to Great Britain (Table 4.7) is due to under-diagnosis and under-reporting of this disease in Ireland. This disparity suggests that cases of Legionnaires' disease in Ireland may be going unreported and also that the problem of *Legionella* contamination of water supplies may be of greater significance than has previously been acknowledged. The first proven case of nosocomial Legionnaires' disease in Ireland was reported in 2003 and resulted in the death of the patient (Coleman & O'Donnell, 2003). A systematic investigation of water supplies in healthcare and other institutions needs to be undertaken to gain a greater understanding of the prevalence of *Legionella* species in the Republic of Ireland in order to prevent further cases of Legionnaires' disease (Coleman & O'Donnell, 2003).

4.4.3 *Real-time* PCR assay for detection of *Legionella* in water samples

In order to overcome difficulties associated with culture of *Legionella* species, a *real-time* PCR assay for quantification of *Legionella* in DUW output water samples suitable for use with the TaqMan Sequence Detection System was developed. Recent literature searches have shown that this is the first study to use *real-time* PCR technology with the Applied Biosystems TaqMan system to determine numbers of *Legionella* species in water samples. The 16S rRNA gene was used as the target gene and is quite suitable in this regard as it exists in multiple copies per genome and thus allows for a high sensitivity of the PCR (Wellinghausen *et al.*, 2001). The total time taken for sample preparation, DNA extraction and PCR analysis was 4 h. The genus-specific 16S rRNA gene PCR assay was specific for all 11 *Legionella* species tested and the *L. pneumophila*-specific *mip* assay was specific for all 14 serogroups tested without cross-reaction with other non-*L. pneumophila Legionella* species tested. The minimum detection limit of both assays was 80 fg of *Legionella* DNA per PCR, which is equivalent to 19 *Legionella* cells, as *Legionella* contains an average of 4.3 fg DNA per cell (Bender *et al.*, 1990). Given the assay conditions of this study and the fact that only 2.5 µl of the test DNA was included in each assay (see Sections 4.2.3 and 4.2.4), this corresponds to a minimum detection limit of approximately five *Legionella* organisms per ml of the original 500-ml water samples. Further concentration of DNA samples could have been achieved by centrifuging the DNA samples and resuspension in a smaller volume or by using a commercially available PCR-clean up kit. However, the introduction of more processing steps would have also increased the possibility of errors in the quantification of *Legionella* in the water samples due to contamination of DNA samples or loss or damage of DNA.

Table 4.7. Reported Cases of Legionnaires' Disease in Ireland, England & Wales from 1998 - 2002

Country	Year	Nosocomial			Travel	Comm.	Sex		Total
		Def	Prob	Poss		acq.	M	F	cases
Ireland	1998	0	0	0	1	1	1	1	2
	1999	0	0	0	1	1	1	1	2
	2000	0	0	0	6	3	6	3	9
	2001	0	0	0	2	2	3	1	4
	2002	0	0	0	2	2	1	3	4
	Total	**0**	**0**	**0**	**12**	**9**	**12**	**9**	**21**
England +Wales	1998	1 (-)	-	2 (-)	115 (24)	108 (35)	161	65	226
	1999	4 (-)	-	2 (-)	90 (20)	104 (16)	150	51	200
	2000	8 (3)	2(-)	2 (-)	103 (15)	69 (5)	147	37	184
	2001	3 (-)	-	1[a](-)	101[b] (17)	73[b] (14)	139	39	178
	2002	4 (-)	2(-)	0 (-)	136 (18)[c]	80 (21)	168	54	222
	Total	**20 (3)**	**4(-)**	**7 (-)**	**545 (94)**	**434 (91)**	**765**	**246**	**1010**

Data taken from Coleman & O'Donnell, 2003.

Abbreviations: Comm. acq.- Community acquired
                          Def.- Definite
                          Prob.- Probable
                          Poss.- Possible

Figures in brackets represent the number of cases associated with outbreaks

[a] 2001- one possible nosocomial case also included in travel

[b] 2001- one community case also included in travel abroad

[c] 2002- one UK travel case also included in a community outbreak

The mean average numbers of *Legionella* estimated from the pan-*Legionella* PCR results were 108-fold higher than those obtained by culture. Similar results were reported by Wellinghausen *et al.* (2001) using the LightCycler *real-time* PCR system. These authors reported that, compared to culture results, the mean average numbers of *Legionella* calculated in hospital water supplies were 68 times higher. These results are not surprising because, in contrast to the difficulties associated with culture methods, *real-time* PCR detection methods detect non-cultivable *Legionella* and have also been shown to exceed the sensitivity of culture (Wellinghausen *et al.*, 2001). Furthermore, rates of recovery in culture are usually less than 100% due to fastidious growth requirements, overgrowth by other bacteria, and damage to *Legionella* bacteria by the concentration steps. Damage to *Legionella* in this study would have most likely occurred during the bead-beating step. However, preliminary work for this study indicated that the greatest recovery of *Legionella* species was achieved by filtration and bead-beating compared to filtration and vortexing or centrifugation (results not shown). The high densities of *Legionella* detected by PCR most likely represent non-viable cells, dead *Legionella* cells or *Legionella* DNA that is not infectious to humans. Amplification and detection of DNA from bacteria that are not *Legionella* may also occur, since alignment of the target sequence of the primers using the BLAST programme can never include all environmentally occurring bacteria, as there are undoubtedly many environmental species that are not yet known. Therefore, the high densities of *Legionella* detected by the *real-time* PCR assays should be critically interpreted and do not necessarily represent a health risk for exposed individuals. However, the high numbers of *L. pneumophila* detected using the *mip*-specific primers and probe indicate that *Legionella* DNA is being detected by the assays.

Positive correlation was found between culture results and both pan-*Legionella* and *L. pneumophila*-specific *real-time* PCR results in that, overall, the mean average *real-time* PCR results for the culture-negative samples were lower than those for the culture-positive samples (see Figures 4.3 and 4.4). These results are similar to the results reported in the study done by Wellinghausen *et al.*, (2001). These authors reported that the mean average PCR results of culture-negative samples (175 fg DNA) were lower than the mean average culture-positive samples (231 fg DNA). The combination of the speed with which results are achieved (< 4 h) and the fact that this study showed a correlation between culture results and *real-time* PCR results suggests that these assays might be useful for initial screening of water samples for determining the source of an outbreak or for routine testing of large numbers of samples. Water samples that are found to have a high amount of

Legionella DNA by the PCR method should be investigated further for the presence of cultivable *Legionella* organisms using culture methods.

4.4.4 *Real-time* PCR assay for detection of *L. pneumophila* in water samples

As *L. pneumophila* is the main cause of Legionnaires' disease, a quantitative *L. pneumophila*-specific *real-time* PCR assay, based on the *mip* gene of *L. pneumophila*, was developed, suitable for use with the Applied Biosystems TaqMan Sequence Detection System. This PCR assay was shown to detect all 14 serogroups of *L. pneumophila* but none of the non-*L. pneumophila Legionella* species. This assay was as sensitive as the general *Legionella* assay, with an identical minimum detection limit of 19 *Legionella* cells per PCR assay. All the 16S rRNA gene PCR-positive water samples were also positive in the *mip* gene PCR assay, thus suggesting the presence of *L. pneumophila*; however, numbers of *L. pneumophila* were lower than numbers of *Legionella* detected using the pan-*Legionella* assay. This indicates the presence of other non-*L. pneumophila Legionella* DNA in the DUW output water and healthcare institution water supplies. Another possibility is that the 16S rRNA PCR pan-*Legionella* assay detected non-*Legionella* DNA because, as discussed above, the BLAST search may not have included all environmentally occurring bacteria as some may not yet be described and may represent non-cultivable bacterial species.

4.4.5 Macrorestriction fragment analysis of *Legionella* DNA

Following PFGE of *Sfi*I restriction enzyme-digested DNA, all 16 *Legionella* isolates recovered from water outlets of Waterford Regional Hospital where a fatal case of Legionnaires' disease was contracted in April 2003 were shown to have identical PFGE-generated DNA patterns (Figure 4.5) indicating that one strain of *Legionella* was present throughout the water systems of the entire hospital.

4.4.6 Conclusions

In conclusion, there seems to be a low prevalence of *Legionella* in DUW output water in Ireland compared to previous studies from other countries, but more extensive studies need to be undertaken to confirm these results. However, DUWs may potentially be a source of Legionnaires' disease in the Republic of Ireland if they are not routinely disinfected or if they are left to stagnate as occurred in the *Legionella*-positive DCU in the dental clinic of a Dublin prison tested in this study. This point is particularly important, as there are several dental clinics in Irish hospitals that are used infrequently and *Legionella*

may well proliferate inside the stagnant DUWs during the periods of time that the DCUs are not in use. Further work also needs to be undertaken in order to investigate the extent of *Legionella* contamination of water supplies in other healthcare institutions as this study has indicated that high densities of *L. pneumophila* are present in the water supplies of some of these institutions. This study has also developed *Legionella* genus-specific and *L. pneumophila* species-specific quantitative *real-time* PCR assays that proved to be both sensitive and specific for quantitative determination of *Legionella* in DUW output water and other potable water samples. As there was a correlation between culture results and PCR results, these assays may be particularly useful for initial screening of routine DUW output water samples or large sample amounts during outbreak investigations in a minimum amount of time.

Chapter 5

Serotyping and molecular fingerprinting of
Pseudomonas aeruginosa **isolates**
to trace the origin of dental chair unit baseplate contamination

5.1 Introduction

5.1.1 Corrosion and bacterial contamination of DCU baseplates

Within six months of the opening of the new Dublin Dental Hospital in September 1998 areas of dampness and corrosion were observed on many of the baseplates of the hospital's DCUs at the site of attachment of the suction hoses (Figure 5.1). A previous study from this laboratory showed that the corroded areas were heavily contaminated with *Pseudomonas* species and related genera posing a risk of cross-contamination and cross-infection, particularly for immunocompromised patients (O'Donnell, 2002). These species were then used as marker organisms to investigate the source of the contamination. *Pseudomonas aeruginosa* was the predominant species recovered from 41 selected DCU baseplates (61% prevalence), whereas both *P. putida* (46% prevalence) and *P. aeruginosa* (43% prevalence) were predominant at the attachment ends of 37 selected high-volume suction hoses (O'Donnell, 2002). Selected isolates of *P. aeruginosa* from 13 DCU baseplates, 16 high-volume suction hoses and 12 coarse filter housings (another suction system site) from 19 separate DCUs were selected as marker organisms for further investigation in the present study in order to determine the source of contamination of the baseplates. Isolates were subject to phenotypic and genotypic analysis in order to determine if the strains of *P. aeruginosa* recovered from the DCU base plates were the same as those recovered from various other sites within the DCU suction system

5.1.2 Phenotypic analysis of *P. aeruginosa*

Both phenotypic and genotypic methods can be used to distinguish between *P. aeruginosa* strains for the purpose of epidemiology. Phenotypic methods include pyocin typing, serotyping, and phage typing (Römling *et al.*, 1994; Grundmann *et al.*, 1995). However, pyocin typing has been shown by at least one study to be unstable: pyocin typing patterns of *P. aeruginosa* strains studied over a long period of time changed, and partial and complete loss of pyocin production was also observed for some strains (Römling *et al.*, 1994). In the same study, typing by phage lysis was also shown to be unreliable with all initial *P. aeruginosa* isolates being typeable by phage lysis, while nine of the 11 final isolates of the persisting clones were non-responders to this type of phenotyping (Römling *et al.*, 1994). While serotyping is a more stable method of phenotypic analysis of *P. aeruginosa*, it can only distinguish between 16 different types. Furthermore, there may be cross-reaction between different serotypes, and some strains are known to be non-typeable (Grothues *et al.*, 1988).
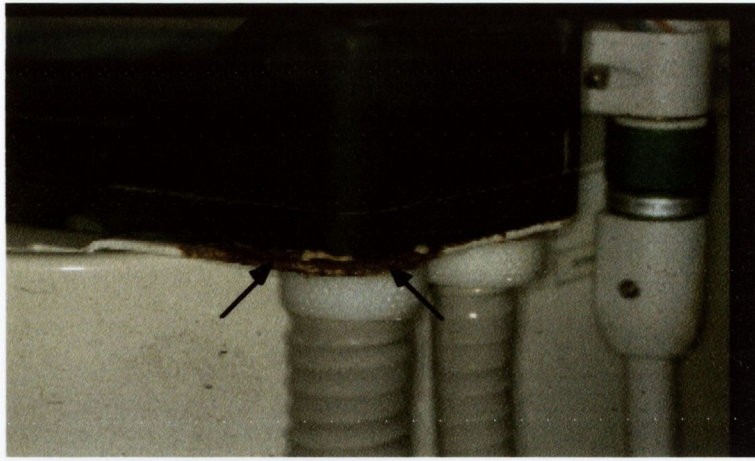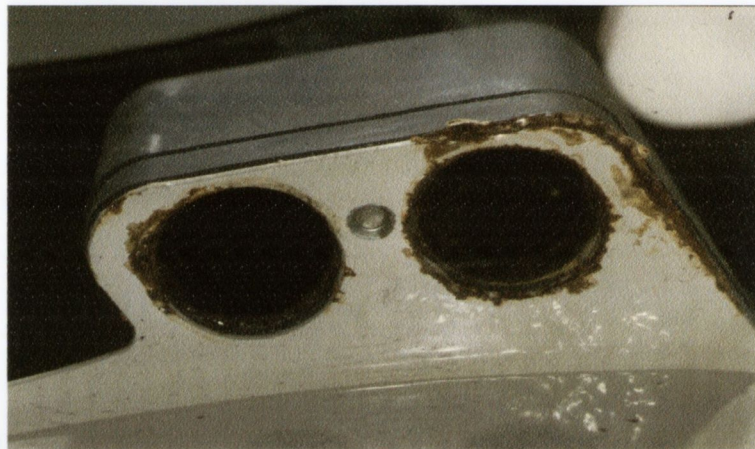
Figure 5.1. Views of different sections of a Planmeca Prostyle Compact DCU showing suction hose attachment sites. (a) Portion of a pedestal section with the ceramic spittoon removed showing the high-volume (left) and low-volume (right) suction hoses attached to the DCU by plastic connectors. Arrows indicate areas of corrosion visible on the side section of the metal baseplate through which the suction hoses are connected. (b) View of DCU baseplate *in situ* photographed from underneath showing the orifices into which the suction hoses connectors are inserted. Evidence of corrosion is visible around the orifices and adjacent areas. (c) Suction connectors attached to a high-volume (left) and low-volume (right) suction hose. The staining evident on both connectors was due to biofilm formation by *Pseudomonas* spp. and related bacterial species.

5.1.3 Genotyping *P. aeruginosa*

Due to the inadequate discriminatory capacity of phenotypic methods for distinguishing between isolates of *P. aeruginosa*, DNA-based techniques that compare isolates by generating type-specific DNA fragment patterns following restriction endonuclease cleavage have become widely used (Grundmann *et al.*, 1995). These genomic fingerprinting methods are now generally regarded as the most accurate methods for the typing of microorganisms for epidemiological purposes (Speijer *et al.*, 1999). These methods include ribotyping, PCR-based fingerprinting methods and pulsed-field gel electrophoresis (PFGE) of restriction endonuclease-digested total genomic DNA (Speijer *et al.*, 1999).

Macrorestriction analysis of DNA by PFGE involves the separation of high molecular weight DNA fragments that have been generated from genomic DNA using restriction enzymes that cleave infrequently. It is the most commonly used method for genotyping *P. aeruginosa* because it has been shown to be the most powerful and discriminatory method for the classification of isolates (Grundmann *et al.*, 1995; Römling & Tümmler, 2000). However, this method has occasionally been associated with DNA degradation, as endogenous nucleases released from *P. aeruginosa* following cell lysis digest the DNA, resulting in small random fragments ranging from 40 to 150 kb, which can prevent the typing of a specific strain (Römling & Tümmler, 2000). In order to combat this degradation, a recent paper suggested modifying the standard protocol for PFGE of *P. aeruginosa* to reduce this DNA degradation by increasing the incubation time of the agarose plug in proteinase K buffer, from 24 to 72 h, and by increasing the concentration of proteinase K five-fold. This study also recommended the addition of 50 µM thiourea to the gel buffer to further hinder *P. aeruginosa* DNA degradation during genotypic analysis (Römling & Tümmler, 2000).

5.1.4 Aims and objectives

This part of the present study aimed to use DNA fingerprinting as an investigative tool to determine if the strains of *P. aeruginosa* isolated from the DCU baseplates were related to those isolated from different sites within the suction system. If the *P. aeruginosa* strains recovered from different sites on the same DCU were shown to be identical, it would indicate that the bacterial contamination found on the baseplates had originated from leakage from within the suction system. A further aim was to determine if a single strain of *P. aeruginosa* was colonising each individual DCU at the various sites sampled, or if many strains colonised each individual DCU. Also, it was hoped to determine

whether one strain predominated throughout the DCUs in the Dublin Dental Hospital or if there were many *P. aeruginosa* strains present.

5.2 Materials and Methods

5.2.1 Serotyping *Pseudomonas aeruginosa* isolates

Pseudomonas aeruginosa isolates were recovered from the DCU baseplates and other locations within the DCU suction system over a 20-month period from March 1999-December 2000. Isolates were identified using the API 20 NE system, as described in Section 2.1.5.1.3, and were then serotyped by slide agglutination using 17 *P. aeruginosa* O-specific antisera supplied by the Public Health Laboratory Service, Laboratory of Hospital Infection, Central Public Health Laboratory, Colindale, London, UK. The antisera were supplied as individual antisera and as four pools of antisera, as shown in Table 5.1. Each isolate was tested for agglutination with antiserum from pool I by placing a drop of this antiserum (approximately 50 µl) onto a clean glass microscope slide (15 mm x 75 mm). A single colony from a fresh PBA plate was picked using a sterile disposable plastic loop (Sigma-Aldrich) and mixed with the pool I antiserum. If cells agglutinated with the antiserum within 1 min, a positive reaction was recorded. A negative reaction was recorded if the cells failed to agglutinate after one minute. In the case of a negative test result with the pool I antisera, additional colonies from the same plate were tested with the antisera from the other pools as above. Each isolate tested agglutinated with antisera from only one of the four pools tested. Once this pool was identified additional colonies from the PBA plate were further tested for agglutination with the individual antisera present in the pool (see Table 5.1), resulting in the identification of the test isolate's serotype. All *P. aeruginosa* isolates were also tested for spontaneous agglutination with sterile saline. Isolates were serotyped on two separate occasions. Serotyping was carried out in conjunction with Mary O'Donnell.

5.2.2 Preparation of *Pseudomonas aeruginosa* chromosomes in agarose plugs

Agarose plugs containing *P. aeruginosa* were prepared as described in Section 2.2.2.1.

5.2.3 Restriction endonuclease digestion of genomic DNA and agarose gel electrophoresis

5.2.3.1 Spe*I endonuclease digestion*

For restriction enzyme digestion, a three-mm sample of each plug was cut using a clean scalpel and placed in a 1.5 ml Eppendorf microfuge tube with 60 µl *Spe*I buffer. The buffer contained 6 µl 10X RE buffer (New England Biolabs), 6 µl dithiothreitol (DTT), 1.2 µl BSA (10 mg/ml) and 1 µl *Spe*I enzyme (10 U/µl) made up to 60 µl buffer with sterile

Table 5.1. *Pseudomonas aeruginosa* O-specific antisera used for serotyping

Serum			
Pool I	**Pool II**	**Pool III**	**Pool IV**
O:1	O:2a	O:6	O:9
O:3	O:5	O:15	O:10
O:4	O:16	O:17	O:11
O:7			O:12
O:8			O:13
			O:14

distilled water. The *P. aeruginosa* DNA within the plug was digested at 37°C for 7 h. While other papers (Grundmann, *et al.*, 1995; Grothues, *et al.*, 1988) recommend overnight (15 h) digestion with the restriction enzyme, it was found in the present study that this led to complete degradation of the *P. aeruginosa* DNA and thus the shorter period of 7 h was adopted. After this period, plugs were removed from the buffer for separation of the DNA fragments contained within them on 1.1% (w/v) agarose gels.

5.2.3.2 Xba*I endonuclease digestion*

In order to confirm the results obtained following *Spe*I digestion, high molecular weight chromosomal DNA from nine isolates was selected for further analysis with the *Xba*I restriction enzyme. A three-mm sample of each plug was cut using a clean scalpel and placed in a 1.5 ml Eppendorf microfuge tube. Digestion of the DNA within the plugs was carried out at 37°C for 15 h in *Xba*I buffer. The restriction enzyme buffer contained 1X RE buffer (New England Biolabs), 1.2 µl BSA (10 mg/ml) and 2 µl *Xba*I enzyme (10 U/µl) made up to 100 µl with sterile distilled water. After this period, plugs were removed from the buffer for separation of the DNA fragments within them on 1% (w/v) agarose gels.

5.2.4 Pulsed-field gel electrophoresis

5.2.4.1 PFGE of Spe*I-digested DNA fragments*

The *Spe*I restriction-enzyme digested DNA fragments in agarose plugs were separated in 1.1% (w/v) agarose gels made with ultra-pure DNA grade agarose (Bio-Rad) in 0.5X TBE in two ramps for 37 h at 14°C. Pulse times were increased linearly in 1 s increments from 5 to 25 s during the first ramp and from 5 to 60 s during the second ramp with a field strength of 6 V/cm, modified from Römling *et al.* (1994). λ phage DNA oligomers (New England BioLabs) were used as size reference markers and were applied to the outermost lanes of each gel. Plugs containing *Spe*I restriction-enzyme digested DNA fragments from the predominant hospital strain *P. aeruginosa* serotype O:10 were applied to the left-hand lane of each gel adjacent to the lane containing the λ ladders and the resulting DNA fragment patterns following PFGE were used for gel-to-gel comparisons.

5.2.4.2 PFGE of Xba*I-digested DNA fragments*

The *Xba*I restriction-enzyme digested DNA fragments in agarose plugs were separated in 1% (w/v) agarose gels made with ultra-pure DNA grade agarose (Bio-Rad) in

0.5X TBE in two ramps for 20 h at 14°C. Pulse times were 5 s for the first 10 h and 10 s for the final 10 h with field strength of 6 V/cm, modified from Hla *et al.* (Hla *et al.*, 1996). λ phage DNA oligomers (New England BioLabs) were used as size reference markers and were applied to the outermost lanes of each gel. Plugs containing *Xba*I restriction-enzyme digested DNA fragments from the predominant hospital strain *P. aeruginosa* serotype O:10 were applied to the left-hand lane of each gel adjacent to the lane containing the λ ladders and the resulting DNA fragment patterns following PFGE were used for gel-to-gel comparisons.

5.2.5 Staining and photography of gels

Following electrophoresis, all gels were stained, photographed and the fingerprint profiles subject to analysis with the DENDRON fingerprint profile analysis software package (Soll, 2000), as described in Section 2.2.2.3.

5.3 Results

5.3.1 Serotyping of *P. aeruginosa* isolates

Selected isolates of *P. aeruginosa* recovered from corroded baseplates, coarse-filter housings and the attachment ends of high-volume suction hoses in separate DCUs were serotyped in order to determine whether antigenically similar isolates were present in different DCUs and at different sites in individual DCUs. Forty-one separate *P. aeruginosa* isolates recovered from 19 DCUs were serotyped (Table 5.1). The majority of the isolates (28/41, 68.3%) belonged to serotype O:10 and the remainder belonged to four other serotypes, including O:6 (7.3%), O:11(7.3%), O:14(9.8%) and O:5/O:16(7.3%) (Table 5.1). Of the 19 DCUs included in the study, 13 yielded *P. aeruginosa* from two or from three of the separate suction system sites sampled. Of these 13 DCUs, ten yielded *P. aeruginosa* isolates belonging to serotype O:10 from all the sites sampled (Table 5.2). Three separate DCUs yielded isolates of O:6, O:11 or O:5/O:16 from the three sites sampled, respectively (Table 5.2).

Of the 13 *P. aeruginosa* isolates from separate DCU baseplates that were serotyped, additional isolates with the same serotype were recovered from other sites in the respective DCUs in 10/13 (76.9%) cases (Table 5.2). Overall, only *P. aeruginosa* isolates of one serotype were recovered from each of the 19 DCUs investigated (Table 5.2). All of these findings provided evidence that the organisms recovered from the DCU baseplates originated from within the respective DCU suction systems.

5.3.2 DNA fingerprinting of *P. aeruginosa* isolates

The 41 *P. aeruginosa* isolates that were serotyped (Table 5.2) were also subject to *Spe*I-generated DNA fingerprinting and computer-assisted analysis of fingerprint profiles with the computer program DENDRON (Soll, 2000). In general, isolates of different serotypes yielded distinctly different fingerprint patterns, while isolates with the same serotype yielded identical or closely related patterns, apart from O:10 and O:14 isolates (Figure 5.2). To obtain an objective determination of the relationship between the isolates investigated, similarity coefficient (S_{AB}) values were computed for every possible pairwise combination of isolates, and these data (Table 5.3) were used to construct a dendrogram showing the relationships between all of the isolates (Figure 5.3). The 28 serotype O:10 isolates could be readily divided into two clearly distinct DNA fingerprint groups, termed fingerprint groups I (4 isolates) and II (24 isolates), respectively, (Table 5.2 and Figure 5.3). The four fingerprint I isolates yielded identical fingerprint patterns

Table 5.2. Serotypes and fingerprint groups of *P. aeruginosa* isolates recovered from three sites in 19 DCUs in the Dublin Dental Hospital

DCU[a]	Baseplates		Attachment-end of high-volume suction hose[b]		Coarse filter housing	
	Serotype	DNA Type	Serotype	DNA Type	Serotype	DNA Type
2.1.1	NS	-	NI	-	O:10	1
2.1.5	NI	-	O:10	I	NI	-
2.1.8	NS	-	O:10	I	O:10	I
2.2.1	O:10	II	O:10	II	NI	-
2.2.2	O:10	II	O:10	II	O:10	II
2.2.3	O:10	II	O:10	II	O:10	II
2.2.4	NS	-	O:10	II	O:10	II
2.2.5	O:10	II	O:10	II	O:10	II
2.2.6	NS	-	O:10	II	NI	-
2.2.8	O:10	II	O:10	II	O:10	II
2.3.5	O:6	III	O:6	III	O:6	III
2.4.5	NS	-	O:10	II	O:10	II
2.4.6	O:10	II	O:10	II	O:10	II
2.5.4	O:5/O:16[c]	IV	O:5/O:16[c]	IV	O:5/O:16[c]	IV
A/E 2	O:11	V	O:11	V	O:11	V
A/E 3	NI	-	O:10	II	O:10	II
A/E 9[d]	O:14	VI	NI	-	NI	-
	O:14	VII				
A/E 10	O:14	VI	NI	-	NI	-
A/E 11	O:14	VI	NI	-	NI	-

[a]The first digit refers to the clinic number, the second digit refers to the bay number and the third digit refers to the chair number.

[b]The high-volume suction hoses are attached to the DCU baseplate using a hose connector.

[c]serotypes O:5 and O:16 share similar trisaccharide backbone repeat units and the respective antisera cross-react.

[d]Two *P. aeruginosa* isolates from DCU AE9 that were distinguishable on the basis of colony morphology were investigated. The two isolates yielded distinctly different *Spe*I generated fingerprint profiles (S_{AB}=0.67) indicating that the isolates belonged to separate strains.

Abbreviations: NI, *P. aeruginosa* not isolated from site; NS, site not sampled.
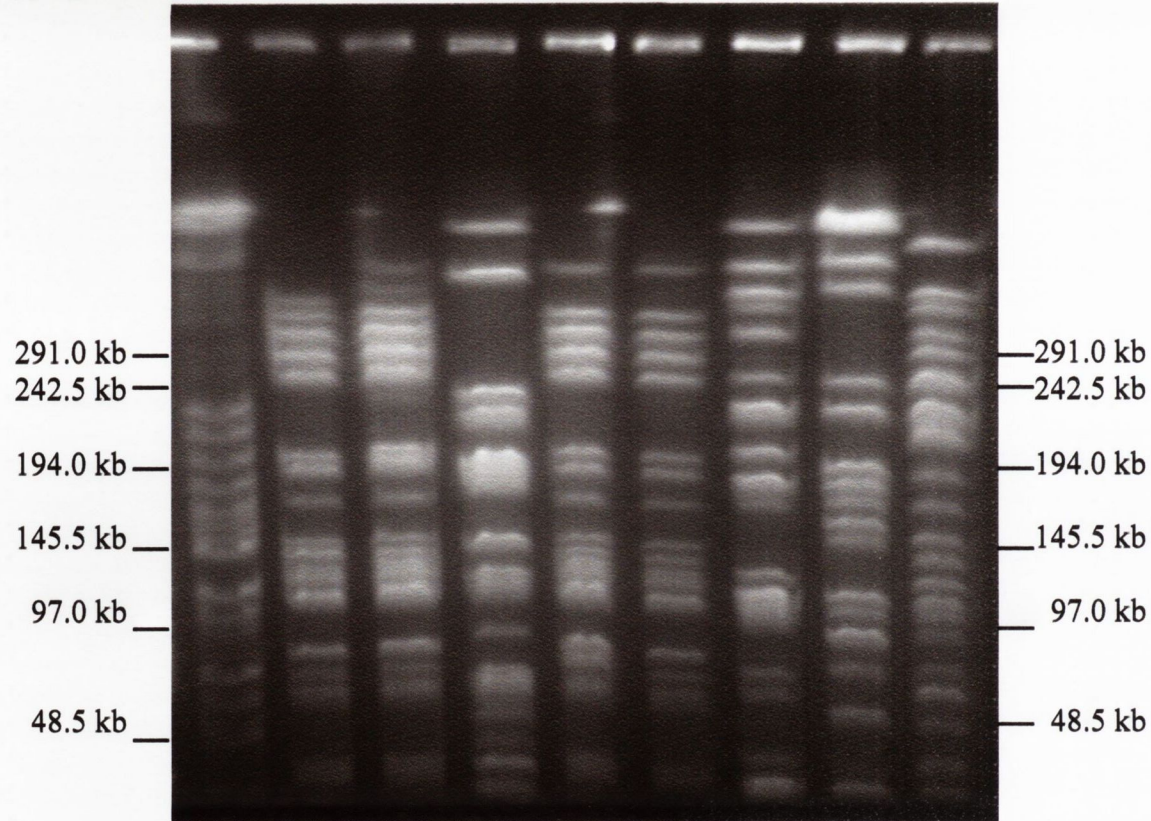
Figure 5.2. Photograph of a section of a 1.1% agarose gel showing DNA fingerprint patterns of nine *Spe*I-digested *P. aeruginosa* isolates recovered from the suction systems of nine separate dental chair units in the Dublin Dental Hospital. DNA fragments were separated by PFGE as described in Section 5.2.4.1. Six distinct genotype patterns are evident on the gel. The isolates included in the gel are as follows: Lane 1, 2.3.5C; Lane 2, 2.4.6H; Lane 3, 2.2.1H; Lane 4, 2.1.5H; Lane 5, A/E 3C; Lane 6, 2.2.6H; Lane 7, A/E 2B; Lane 8, A/E 11B; Lane 9, 2.5.4C. The provenance of isolates is shown in Table 5.2.

Table 5.3.   Matrix showing S_{AB} values for pairwise combinations of *P. aeruginosa* isolates[a] from different fingerprint groups

Isolates[b]	2.1.5H	2.2.1B	2.2.2H	2.2.3B	2.2.3H	2.2.4C	2.2.4H	2.2.5C	2.2.6H	2.4.5C	2.4.5H	2.4.6H	2.3.5C	2.5.4B	AE2B	AE2C	AE9Bi	AE11B
2.1.5H																		
2.2.1B	0.14																	
2.2.2H	0.14	0.88																
2.2.3B	0.21	0.94	0.88															
2.2.3H	0.14	0.91	0.91	0.91														
2.2.4C	0.50	0.91	0.85	0.91	0.88													
2.2.4H	0.14	0.94	0.88	0.94	0.91	0.97												
2.2.5C	0.15	0.91	0.85	0.91	0.88	0.88	0.91											
2.2.6H	0.14	0.94	0.94	0.94	0.97	0.91	0.94	0.91										
2.4.5C	0.14	0.94	0.82	0.88	0.86	0.91	0.88	0.85	0.88									
2.4.5H	0.21	0.94	0.88	0.94	0.91	0.91	0.94	0.91	0.94	0.88								
2.4.6H	0.15	0.91	0.97	0.91	0.88	0.88	0.91	0.88	0.91	0.85	0.91							
2.3.5c	0.31	0.31	0.25	0.31	0.36	0.39	0.38	0.32	0.31	0.31	0.31	0.26						
2.5.4B	0.27	0.61	0.56	0.61	0.65	0.63	0.61	0.57	0.61	0.61	0.61	0.57	0.47					
AE2B	0.07	0.59	0.53	0.53	0.57	0.55	0.53	0.55	0.59	0.59	0.59	0.48	0.38	0.56				
AE2C	0.07	0.61	0.55	0.55	0.59	0.56	0.55	0.56	0.61	0.61	0.61	0.50	0.39	0.51	0.97			
AE9Bi	0.46	0.31	0.25	0.38	0.30	0.32	0.31	0.32	0.31	0.31	0.38	0.26	0.33	0.35	0.44	0.45		
AE11B	0.46	0.31	0.25	0.38	0.30	0.32	0.31	0.32	0.31	0.31	0.38	0.26	0.33	0.35	0.44	0.45	0.93	
AE9Bii	0.48	0.46	0.34	0.46	0.39	0.41	0.40	0.41	0.40	0.40	0.46	0.35	0.18	0.38	0.34	0.35	0.67	0.67
Isolates[b]	2.1.5H	2.2.1B	2.2.2H	2.2.3B	2.2.3H	2.2.4C	2.2.4H	2.2.5C	2.2.6H	2.4.5C	2.4.5H	2.4.6H	2.3.5C	2.5.4B	AE2B	AE2C	AE9Bi	AE11B
	I					II							III	IV	V		VI	

[a]Where several isolates from a particular fingerprint group were indistinguishable (i.e. shared all fingerprint bands in common; S_{AB}=1), a representative was selected for matrix construction.

[b]The provenance of isolates is shown in Table III. H=attachment end high volume suction hose, B=DCU baseplate, C=coarse filter cage housing.
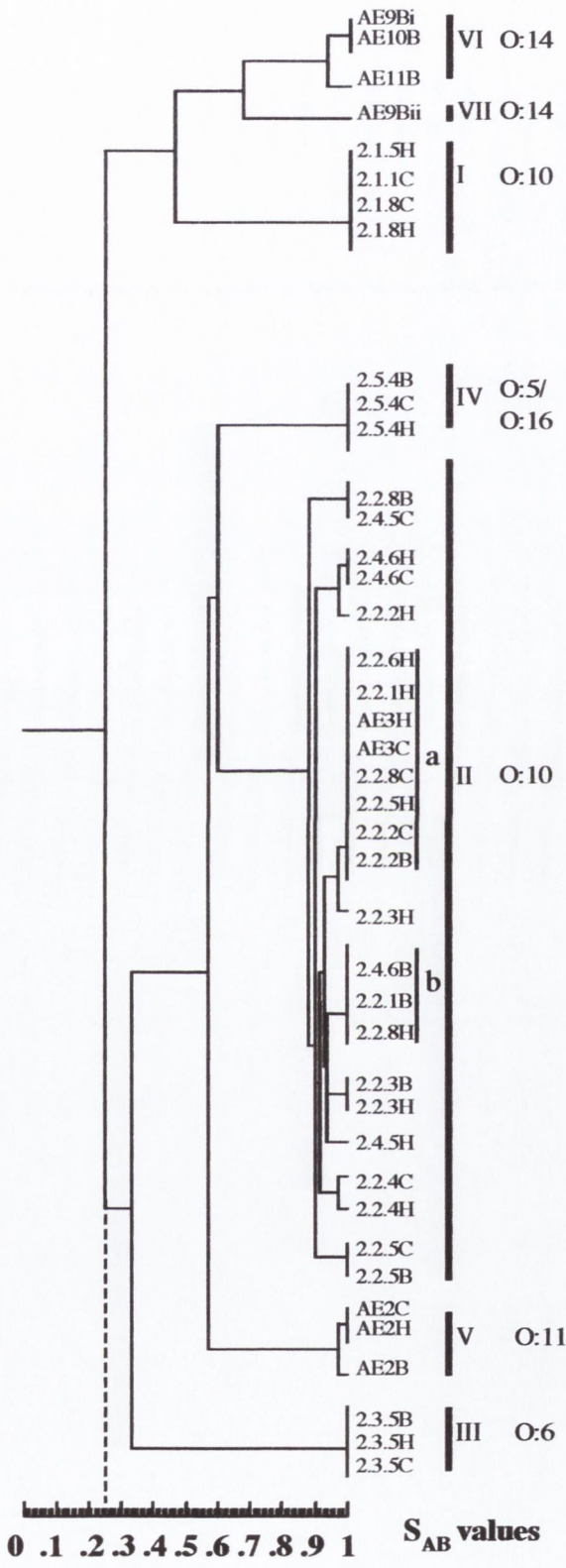
Figure 5.3. Dendrogram generated from the similarity coefficients (S_{AB}s) computed for every pairwise combination of *Spe*I-generated fingerprint profiles of 41 *P. aeruginosa* isolates recovered from three separate sites in 19 DCUs. At an S_{AB} value of 0.24 (short dashed vertical line), the isolates are divided into two main populations. The first main population contains three distinct clades, corresponding to fingerprint groups I (serotype O:10), VI (serotype O:14) and VII (serotype O:14). The second main population contains four distinct clades, corresponding to fingerprint groups II (serotype O:10), III (serotype O:6), IV (serotype O:5/O:16) and V (serotype O:11). The majority of the isolates tested belonged to fingerprint group II (serotype O:10). A number of clusters of indistinguishable isolates (average S_{AB}=1) are evident within the fingerprint group II clade. Two of these containing 8 isolates and 3 isolates are indicated by the notation a and b, respectively. Despite the presence of clusters within the fingerprint group II clade, all of the isolates within this clade were very closely related (average S_{AB} = 0.93). The serotypes and fingerprint groups of the isolates are shown to the right of the dendrogram. The source of the isolates is indicated by the following notation: H=attachment end of high volume suction hose, B=DCU baseplate, C=coarse filter cage housing.

(S_{AB}=1.0) and were recovered from three separate DCUs, whereas the 24 fingerprint group II isolates were recovered from ten separate DCUs (Table 5.2). Of these ten DCUs, seven yielded fingerprint group II isolates from two or from all three sites sampled. Some variability in the DNA fingerprint profile of the fingerprint group II isolates was observed, but all were highly related with an average S_{AB} value of 0.93 (range 0.82-1.0) (Figure 5.3 and Table 5.3). Interestingly, the fingerprint group I and II isolates (all serotype O:10) were very distinct and clearly divided at an S_{AB} value of 0.24 (Figure 5.3). The three serotype O:6 isolates yielded identical fingerprint profiles (S_{AB}=1.0), termed fingerprint group III, as did the three O:5/O:16 isolates, which were termed fingerprint group IV. Two of the three O:11 isolates yielded identical fingerprint profiles (fingerprint group V) and the third differed by a single band (average S_{AB}=0.98) (Tables 5.2 and 5.3). The four O:14 isolates yielded two distinct fingerprint patterns, termed fingerprint groups VI (three isolates; average S_{AB}=0.95) and VII (one isolate), respectively (Tables 5.2 and 5.3), which separated from the other three O:14 isolates at an S_{AB}=0.67 (Figure 5.3 and Table 5.3). These findings provided evidence that individual isolates within each of these fingerprint groups belonged to the same strain in each case.

Multiple isolates (18 single colonies recovered on primary isolation plates) of the same *P. aeruginosa* serotype O:10 strain (with identical or very similar *Spe*I-generated fingerprint profiles) recovered in each case from the high-volume suction hoses of three separate DCUs yielded an average S_{AB} value of 0.97 (S_{AB} range 0.94-1.0).

The relationship between eight selected fingerprint I (6 isolates) and II (2 isolates) isolates was also investigated by *Xba*I-generated DNA profiling following computer-assisted analysis with DENDRON (Soll, 2000) and the results obtained confirmed the findings obtained with *Spe*I-generated DNA profiles (Figure 5.4).

Lane:	1	2	3	4	5	6	7	8	9
Serogroup:	O:10	O:10	O:10	O:10	O:10	O:10	O:10	O:1I	O:10
*Spe*I genotype pattern:	II	II	I	II	I	II	II	V	II
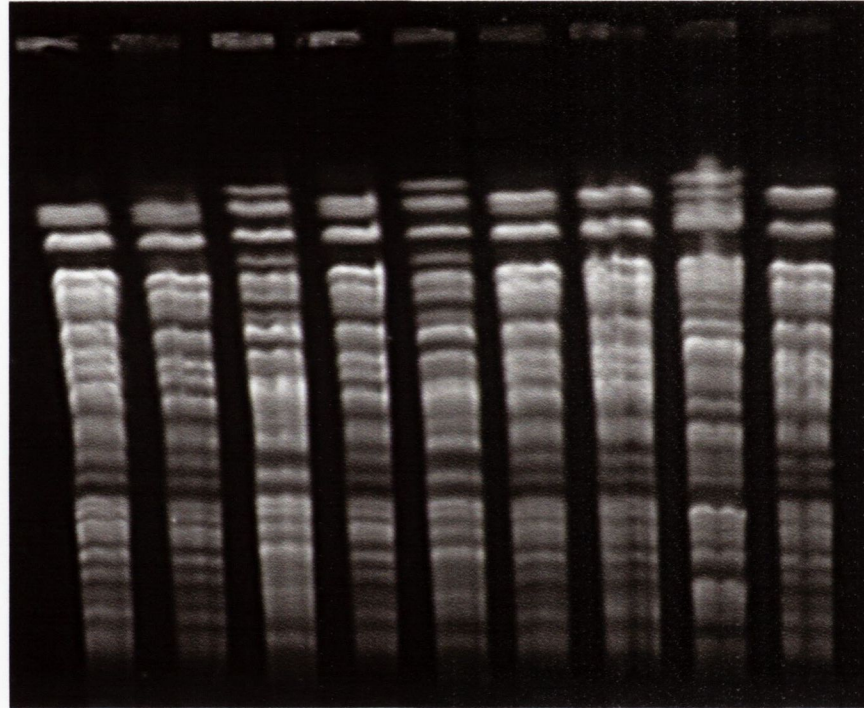
Figure 5.4. Photograph of a section of a 1% agarose gel showing DNA fingerprint patterns of nine *Xba*I-digested *P. aeruginosa* isolates recovered from the suction systems of seven dental chair units in the Dublin Dental Hospital. DNA fragments were separated by PFGE as described in Section 5.2.4.2. Three distinct genotype patterns are evident on the gel, examples of which are in Lanes 1, 3 and 8. Lane 1, 2.2.6H; Lane 2, 2.4.5C; Lane 3, 2.1.5H; Lane 4, 2.2.6H; Lane 5, 2.1.8H; Lane 6, 2.4.6B; Lane 7, 2.4.6H; Lane 8, 2.5.4H; Lane 9, 2.2.5H. The provenance of isolates is shown in Table 5.2 and Figure 5.3.

5.4 Discussion

Within six months of the opening of the new Dublin Dental Hospital in September 1998, areas of corrosion became apparent on many of the baseplates of the DCUs with which the hospital is equipped. The corrosion was localised to the area where the high-volume and low-volume suction system hoses were attached to the main body of the DCUs. Preliminary observations revealed that the areas of corrosion were damp and harboured high concentrations of *Pseudomonas* species and related bacteria.

5.4.1 Distinguishing between *P. aeruginosa* serotypes using DNA fingerprinting

Five different serotypes were found among 41 selected *P. aeruginosa* isolates recovered from DCU baseplates, attachment-end high-volume suction hoses and suction system filter cages from 19 separate DCUs (Table 5.2). Fourteen of the 19 DCUs yielded *P. aeruginosa* from two or three of the sites tested and in all cases isolates of the same serotype were recovered from the different sites in the same DCU. These findings suggested that *P. aeruginosa* isolates recovered from DCU baseplates originated from other areas within the suction system and also indicated that only a small number of *P. aeruginosa* strains had colonised the DCU suction systems throughout the hospital. Because the discriminatory ability of serotyping is limited for strain differentiation (Osmon *et al.*, 2004), and in order to investigate the relatedness of the *P. aeruginosa* isolates in more detail, the population of isolates was analysed by DNA fingerprinting. DNA fingerprinting analysis of *Spe*I-digested high molecular weight genomic DNA of 28 serotype O:10 isolates revealed that the isolates could be separated into two clearly distinct fingerprint groups, termed groups I (four isolates from three separate DCUs) and II, (24 isolates from ten separate DCUs), respectively. Computer-assisted analysis of DNA fingerprint profiles revealed that group I and II isolates were distantly related (Table 5.3 and Figure 5.3). The four group I isolates were indistinguishable by fingerprinting (S_{AB}=1.0) and all belonged to a single strain, whereas some variability in fingerprinting patterns was observed among the 24 group II isolates. Nonetheless, group II isolates were very closely related (average S_{AB}=0.93) and undoubtedly were clonal. These results were confirmed for a subset of eight fingerprint group I and II isolates by *Xba*I-fingerprinting of genomic DNA (Figure 5.4).

5.4.2 Predominance of one *P. aeruginosa* strain throughout Dublin Dental Hospital

Computer-assisted analysis of *Spe*I-generated *P. aeruginosa* isolate profiles showed that the 41 isolates of *P. aeruginosa* examined belonged to seven separate strains, confirming the presence of a small number of *P. aeruginosa* strains in the hospital's DCU suction systems, one of which (serotype O:10, *Spe*I-fingerprint group II) predominated (68.3%). Why this latter *P. aeruginosa* strain should have become predominant in the hospital DCU suction systems is unclear. Perhaps this strain is particularly well adapted for survival within the DCU suction systems. Alternatively, this strain may be particularly resistant to disinfection as the suction system of each DCU was subject to disinfection after each clinical session with the phenolic disinfectant Puli-Jet (Cattani), as described in Section 1.5.5.1. Interestingly, serotype O:10 isolates appear to be relatively rare and this is comparable to a Russian study that reported a prevalence of only 1% among 708 *P. aeruginosa* strains serotyped (Ivanova & Bekina, 1987). However, a recent report described an outbreak of infection caused by serotype O:10 *P. aeruginosa* isolates in a neonatal intensive care unit in France associated with a contaminated milk bank pasteuriser and bottle warmer (Gras-Le Guen *et al.*, 2003). Fourteen cases of infection and 17 cases of gastrointestinal tract colonisation were recorded with four fatalities. In the present study the original source of the fingerprint group II strain, and the other strains also, is unknown. It is possible that they originated from the DCU water supply although an in-depth study of dental unit output water quality from this laboratory did not recover *P. aeruginosa* during a prospective study of multiple DCUs over a period of several months (Tuttlebee *et al.*, 2002). Alternatively, the organisms may have originated from the oral cavities of dental patients (Botzenhardt *et al.*, 1987).

5.4.3 Limitations of current DCU suction system disinfection regimen

Pseudomonas aeruginosa has frequently been recovered from damp or moist environments in hospitals (Kolmos *et al.*, 1993; Bert *et al.*, 1998; Ferroni *et al.*, 1998). These sites often act as a focus for patient contamination and infection. As mentioned above, the hospital DCUs are disinfected twice daily by aspirating a disinfectant solution through the suction hoses, a process that takes approximately one minute. This regular disinfection was evidently ineffective at controlling bacterial contamination in the DCU suction systems. The reasons for this are not immediately clear but may be due to the fact that bacteria present in biofilm, especially *Pseudomonas* species, are more resistant to disinfectants than planktonic organisms (Costerton *et al.*, 1987; Campanac *et al.*, 2002;

Grobe *et al.*, 2002). Secondly the very short contact time the disinfectant has with the inside surfaces of the suction system (i.e. approximately one minute) is likely to be a contributory factor. Because all DCU suction systems operate in an analogous manner to a domestic vacuum cleaner in that material, including disinfectants, is sucked rapidly through the suction hoses to a central receptacle, it is very likely that most DCU suction systems harbour bacterial biofilm due to inadequate disinfectant contact time. In this regard it is interesting to note that ongoing studies in this laboratory with DCUs outside the Dublin Dental Hospital have documented the presence of extensive biofilm in the suction systems of several brands of DCU other than Planmeca units (Prof. D. Coleman, Personal Communication). Empirical studies with a range of disinfectants and disinfection protocols need to be undertaken to assess which is the most effective at eliminating biofilm from DCU suction systems and to determine optimum disinfection conditions.

5.4.4 Cross-contamination potential of DCU suction systems

The presence of *P. aeruginosa* and other related microorganisms within the DCU suction hoses despite regular disinfection is a cause for concern. As discussed in Chapter 1, a small number of studies have demonstrated that under certain conditions liquid from the low-volume suction line can enter a patient's mouth during use (Watson & Whitehouse, 1993; Mann *et al.*, 1996; Barbeau *et al.*, 1998). This indicates that retraction of oral fluids and biofilm-derived microorganisms from contaminated suction hoses could potentially be an important source of cross-contamination and cross-infection. It should be noted that the low-volume suction hoses were not investigated in the present study, however studies from our laboratory revealed that they were just as contaminated as the high-volume hoses (Prof. D. Coleman, Personal Communication). There are virtually no published reports on the extent of bacterial contamination of, or biofilm formation in dental unit suction systems.

5.4.5 Impact of study

The results of this study demonstrated that bacterial contamination of the DCU baseplates was probably due to leakage from the suction hoses. It is very likely that movement of the hoses during use in clinical sessions eventually resulted in loosening of the connectors from the receiving orifices causing leakage of fluid from the suction system (Figure 5.1). Following collaboration between members of the Microbiology Research Laboratory (Prof. David Coleman and Mary O'Donnell) and Planmeca Oy, the manufacturers of the Prostyle Compact DCUs used in the Dublin Dental Hospital,

Planmeca engineers designed a new type of interlocking connector system to secure the suction hoses to the DCUs (Figure 5.5). The new system consists of three components, including a new type of hose connector, a metal bushing receiver for each connector and a securing clip. New suction hoses were supplied, each fitted with the new-design plastic connector firmly attached to each hose. These connectors interfaced with the metal bushings fitted through and extending below the suction hose orifices in the DCU baseplates and were then held in place by the insertion of a plastic clip (Figure 5.5). Each connector interfaces with a groove machined into the receiving bushing and a watertight seal is obtained by insertion of the securing clip. The securing clips also prevent loosening of the suction hoses caused by movement of the hoses during use. This strategy effectively resolved the problem of seepage of liquid from the suction hoses and eliminated further contamination of the DCU baseplates.

5.4.6 Conclusions

The original hose connectors were identified as the primary cause of DCU baseplate contamination. The implementation of an effective practical solution to this problem by retrofitting all of the hospital's DCUs with the new-design hose connectors efficiently resolved the problem of suction-hose-derived DCU baseplate contamination. However, the results of this study highlight the presence of extensive bacterial biofilm in DCU suction systems, which needs to be investigated in more detail in order to understand more fully the potential risk of cross-infection posed by microorganisms within the suction system hosing.
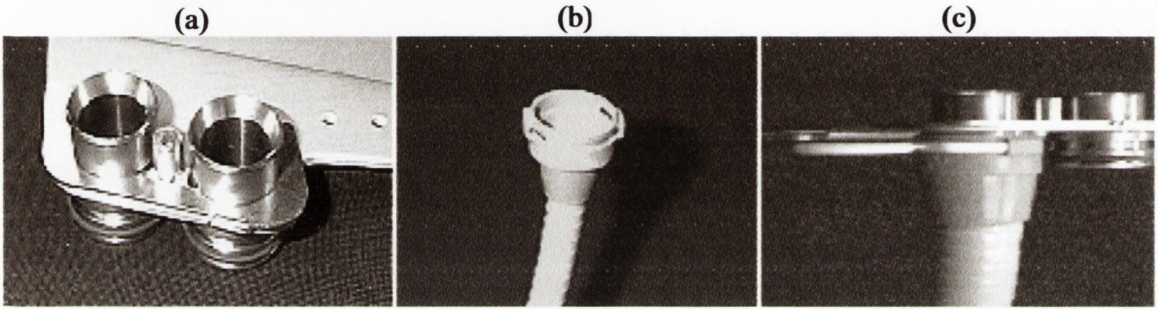
Figure 5.5. Components of the newly-designed interlocking suction hose connector system. (a) DCU baseplate fitted with hose receiver bushings. (b) Suction hose fitted with connector. This interfaces with one of the bushings shown in panel (a). (c) Suction hose with connector interfaced with DCU baseplate bushing with securing clip in place.

Chapter 6

An investigation of microbial diversity of biofilm within dental chair unit suction system hoses

6.1 Introduction

The DCU suction system is designed to remove excess fluids and aerosols that have been generated in the oral cavity during dental work, e.g. DUW output water, saliva and blood. As such, the suction system quickly becomes contaminated with aerial, oral and DUW-derived microorganisms, which can lead to build-up of biofilm as microorganisms aspirated through the suction system adhere to the lumen of the hosing and multiply. Furthermore, the lumen of the hosing itself is frequently damp with a ready supply of nutrients, e.g. from blood, saliva and tissue, which enhances the survival and proliferation of microorganisms within the biofilm of the suction system.

Many previous studies (Abel *et al.*, 1971; Williams *et al.*, 1993; Walker *et al.*, 2000; Putnins *et al.*, 2001; Tuttlebee *et al.*, 2002) have investigated microbial contamination of dental unit waterlines (DUWs), but there have been very few studies that investigated the extent of microbial contamination of DCU suction systems. Some of the studies that have investigated DCU suction system contamination have shown that liquid from low volume suction system hoses can enter a patient's mouth during use (Watson & Whitehouse, 1993; Mann *et al.*, 1996; Barbeau, 1998), indicating that retraction of oral fluids and biofilm-derived microorganisms from contaminated suction lines could be a potential source of cross-infection for patients. Furthermore, maintenance and dental staff are regularly exposed to the potential risk of infection from suction system biofilm during routine maintenance and disinfection of the suction system.

In the Planmeca Prostyle Compact DCUs used in the DDH, the suction system is a fixed and integral part of the DCU, comprising the high volume suction, the saliva ejector and the spittoon (see Figure 6.1). Before treatment of each patient, a new or sterile suction tip is placed on the proximal ends of the suction system hoses to be used during patient treatment and are removed after use to reduce cross-contamination between patients. It is not practical to replace or sterilise the hosing itself between patients as providing new hosing for each patient would be expensive and time-consuming, while sterilisation of hosing after each patient-use would also be very time-consuming. However, water is flushed through the suction hoses following treatment of each patient to elute blood, saliva and other debris generated during dental treatment down through the suction system hoses. In the Dublin Dental Hospital disinfection of the suction system hosing is undertaken twice-daily after morning and afternoon clinical sessions. A phenolic disinfectant, Puli-Jet (Cattani), is used to disinfect the suction system hosing, as recommended by Cattani, the manufacturer of the central suction plant with which the Hospital is equipped. The estimated contact time of the disinfectant with the internal surfaces of the suction hosing is
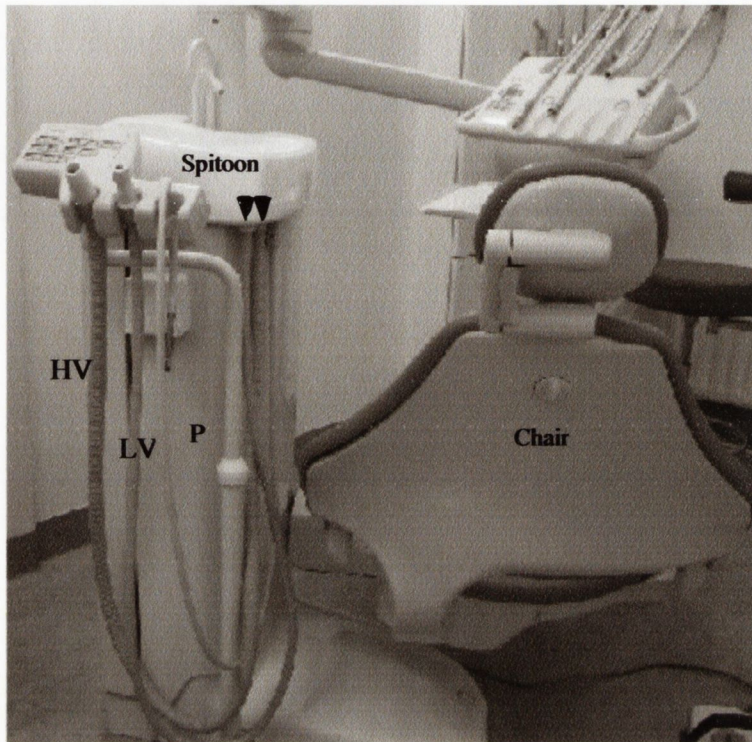
Figure 6.1. View of a Planmeca Prostyle Compact DCU. The pedestal unit (P) is located on the left under the ceramic spittoon. The high-volume (HV) and low-volume (LV) suction hoses are indicated. The arrowheads indicate the attachment sites of the suction hoses under the spittoon.

approximately 90 s because the design of the suction system means that all liquids, including disinfectants, are aspirated rapidly through the hoses. However, even though there is limited contact time of the disinfectant with the biofilm during the disinfection cycle, one would expect that some of the disinfectant would adhere to the lumen of the hoses and have some efficacy against the biofilm.

6.1.1 Aims and objectives

This part of the present study aimed to characterise and compare the microorganisms present in the biofilm lining the proximal and distal ends of hosing of a selected number of DCU suction systems, using non-selective culture and 16S rRNA gene sequence analysis techniques, in order to assess the potential risk of cross-infection because very few studies have investigated contamination of suction system hoses thus far.

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6.2 Materials and Methods

6.2.1 DCUs included in this study

Initially, the high volume suction system hosing of three Planmeca Prostyle Compact DCUs, two of which were located in the Dublin Dental Hospital and one in the dental clinic of a local acute Dublin hospital staffed by personnel from the Dublin Dental Hospital, were investigated. Five-cm sections were removed from both the distal (attached to the DCU) end and proximal (patient) end of the high volume suction system hosing from each of these units by maintenance staff. Biofilm samples recovered from these hose samples were then pooled together to maximise the number of species detected and to gain an overall profile of the bacteria contaminating the lumen of the high volume suction system hoses.

Following this initial investigation, in order to separately compare the microbial diversity at the proximal and distal ends of the high volume suction hosing, a further two DCUs from the Dublin Dental Hospital were selected for inclusion in the study and five-cm sections of hosing were again excised from both the proximal and distal ends of the high volume suction system hosing. However, in this case, the biofilm samples recovered from the proximal ends were pooled together separately from the biofilm samples from the distal ends. No attempt was made to assess the microbial diversity present in each individual DCU suction system hosing as previous studies from this laboratory showed that similar microorganisms were recovered from multiple, separate DCU suction system hoses (O'Donnell, 2002). Each DCU sampled was used routinely during clinic sessions and disinfected twice-daily with Puli-Jet.

6.2.2 Extraction of biofilm from suction system hoses

Each sample of suction hosing was cut in half through the lumen using a sterile scissors. Sterile swabs (Venturi) moistened with sterile distilled water were used to remove biofilm from the inside wall of each half of the suction system hosing by rubbing the whole lumen of the hose thoroughly with the head of the swab until no more biofilm was visible on the lumen surface. Water was used to moisten swabs because suction system hoses are subject to rinsing with water following each patient visit and therefore the microorganisms within the suction system biofilm must be able to survive in water. The head of each swab was broken off aseptically and placed in a sterile glass vial containing 1 ml sterile distilled water. Each vial was vortexed thoroughly to break up the biofilm and to elute microorganisms from the biofilm into the water. The resulting suspensions were divided into two 500-µl aliquots for further analysis.

6.2.3 DNA extraction from suction system biofilm

One 500-µl aliquot was used for DNA extraction and was transferred into a sterile 1.5 ml Eppendorf microfuge tube. This was centrifuged at 7,500 x g for 3 min to pellet the bacterial cells. The supernatant was removed and the pellet was resuspended in 180 µl lysis buffer containing lysozyme (20 mg/ml) (Sigma-Aldrich), as previously described (Singh *et al.*, 2003). The resuspended sample was incubated at 37°C for 30 mins. Nucleic acids were then extracted from the sample using the DNeasy® Tissue Kit (Qiagen) according to the manufacturer's instructions, as described in Section 2.2.1. The concentration of each resulting DNA solution was determined by measuring its absorbance at 260 nm using a Genesys 2 spectrophotometer (ThermoSpectronic) and the concentration was subsequently determined using the formula: 1 optical density unit @ A_{260} = 50 µg DNA. The samples were then stored at -20°C until required.

6.2.4 16S rDNA clone library construction

Biofilm rDNAs were amplified by PCR from 30 ng of DNA from each of the biofilm samples, as described in Section 2.3.2. PCR products were resolved on 0.8% (w/v) agarose gels (Sigma-Aldrich) containing ethidium bromide (0.5 µg/ml). The amplified PCR products were excised from the gels using a clean scalpel and purified using the Wizard® SV Gel and PCR Clean-up system (Promega) as described in Section 2.3.3.

Clone libraries of the purified PCR products were then generated using the pGem® T-Easy Vector System I (Promega), as described in Section 2.3.4. The T-Easy vectors used in this system are prepared by Promega by digestion with *Eco*RV followed by the addition of a 3' terminal thymidine (T) to both ends. Because *Taq* DNA Polymerase adds a single deoxyadenosine to the 3'-ends of PCR products, the 3'-T overhangs at the insertion site of the vector make for easier insertion of amplicons. Transformants harbouring recombinant plasmids were selected using X-Gal-IPTG indicator L agar plates supplemented with ampicillin (100 µg/ml). White colonies (presumed to contain cloned DNA inserts) that grew on the X-Gal-IPTG indicator plates were selected and used to create a clone library for the biofilm samples. Each white colony selected was also inoculated into a sterile test tube containing 2 ml L broth (Oxoid) supplemented with ampicillin (100 µg/ml) and grown at 37°C overnight in a shaking incubator at 200 rpm (Gallenkamp). Following overnight incubation, plasmids were harvested using the Sigma Gen Elute Mini Prep kit as described in Section 2.3.1.

The presence of cloned insert DNA in each plasmid was then confirmed by *Eco*R1 restriction enzyme digestion as described in Section 2.3.6. Those plasmids that contained

an insert between 1 and 1.5 kb in length were sequenced unidirectionally (one DNA strand) by Lark Technologies using the standard pUC/M13R primer (Table 2.2). Some inserts were also sequenced using the pUC/M13F primer in order to confirm results (Table 2.2). Sequences were then compared with published 16S rRNA gene sequences using the BLAST programme, which is run through the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/) for the determination of closest matches. If the highest scoring sequence match (as determined by BLAST) had greater than 95% sequence similarity to the cloned sequence the sequences were considered to be from bacteria of the same species.

6.2.5 Identification of microorganisms cultured from the suction system biofilm

For the culture of DCU suction system biofilm microorganisms, the second 500-μl aliquot of each biofilm suspension sample (see Section 6.2.2 above) was serially diluted using ten-fold dilutions in sterile distilled water to 10^{-4}.

For the biofilm removed from the first three DCUs (biofilm samples from proximal and distal ends pooled together), 100-μl aliquots of the neat suspension and of each dilution were then each spread individually onto NA plates.

For the biofilm removed from the other two DCUs (biofilm samples from proximal and distal ends pooled separately from each other), 100-μl aliquots of the neat suspension and of each dilution were each spread onto NA, PBA and R2A agar. This was done in order to see if a greater diversity of microorganisms could be recovered when using three types of agar rather than just using NA.

Each plate was then incubated at 30°C for up to five days. Colonies of differing morphology, e.g. different colour, size and shape, mucoid or non-mucoid, were purified by subculture for further investigation.

6.2.6 Testing cultured isolates for catalase production

Purified isolates recovered from the suction system hoses were tested for their ability to break down hydrogen peroxide by catalase production as described in Section 2.1.5.1.1.

6.2.7 Identification of cultured isolates by API 20NE

Purified isolates were also tested for oxidase production using the BBL™ DrySlide™ (Becton Dickinson) system, and identified, where possible, by means of the API 20 NE system (bioMérieux), as described in Sections 2.1.5.1.2 and 2.1.5.1.3.

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6.2.8 DNA extraction from cultured isolates

6.2.8.1 DNA extraction from isolates cultured on NA

For DNA extraction, individual test tubes containing 2 ml NB were inoculated with one single colony from those isolates subcultured from NA and were grown overnight at 30°C in a shaking incubator at 200 rpm (Gallenkamp). Following overnight growth, cells were harvested by centrifugation at 7,500 x *g*. The supernatant was removed and DNA was extracted following treatment with lysozyme and Proteinase K, using the DNeasy tissue kit (Qiagen), as described above in Section 2.2.1.

6.2.8.2 DNA extraction from isolates cultured on PBA and R2A

For DNA extraction from those isolates subcultured from PBA and R2A, five colonies were scraped directly from the agar using a sterile inoculating loop into 1 ml sterile distilled water. Cells were then harvested by centrifugation at 7,500 x *g*, the supernatant was removed and DNA was extracted as described above in Section 2.2.1.

6.2.9 Identification of cultured isolates by 16S sequence analysis

The DNA extracted from each cultured isolate was subjected to sequence analysis following 16S rRNA sequence amplification and cloning of the amplicon using the purified pGem® T-Easy Vector System I (Promega), as described in Sections 2.2.1, 2.3 and 2.4. Cloned inserts were sequenced unidirectionally (one strand) using the pUC/M13R primer by Lark Technologies and the sequences were submitted to BLAST for closest match identification. If the highest scoring sequence match (as determined by BLAST) had greater than 95% sequence similarity to the cloned sequence the sequences were considered to be of the same species. Identities of isolates obtained by 16S sequence analysis were then compared to those obtained by use of the API 20 NE system (bioMérieux).

6.3 Results

6.3.1 16S rDNA analysis of total biofilm community DNA from first set of suction system hoses

In order to investigate the microbial diversity of biofilm within the lumen of the suction system hosing, biofilm from DCU suction system hoses was removed and subjected to DNA and culture analysis, as described in Section 6.2. For the first set of suction system hoses investigated (biofilm from the proximal and distal ends of three DCU suction system hoses pooled together), 200 transformants harbouring cloned amplicons were selected for inclusion in the clone library. Plasmids were extracted from these and, following *Eco*R1 restriction enzyme digestion, 123 plasmids that contained cloned inserts (size range, 1-1.5 kb) were sequenced using the pUC/M13R primer. One-hundred and twenty-one sequences (98%) were matched with sequences of bacteria in the GenBank database to at least the genus level. Eleven genera were represented among the cloned inserts with the most predominant sequence identified as belonging to *Stenotrophomonas*, as 41 cloned inserts (33%) corresponded to *S. maltophilia*. Other prevalent sequence identity matches to the species level were *Brevundimonas diminuta* (17%) and *Sphingomonas yanoikuyae* (15%), *Sphingomonas paucimobilis* (5.7%), *P. putida* (4%) and *P. aeruginosa* (2.4%) (see Table 6.1). The other seven genera represented in the biofilm lining the suction system hosing were *Caulobacter* (4%) *Ralstonia* (2.4%), *Delftia* (1.6%), *Propionibacterium* (1.6%), *Sphingobacterium* (0.8%), *Microbacterium* (0.8%) and *Xanthomonas* (0.8%) (Table 6.1). With regard to two cloned inserts that could not be identified to the genus level, one matched a published sequence for bacterial DNA that had been detected in drinking water (GenBank AY328841), while the other corresponded to a sequence for bacterial DNA of an unspecified source (GenBank AF407397).

6.3.2 Identification of cultured isolates from first set of suction system hoses

For the first set of suction system hoses investigated (biofilm from proximal and distal ends of suction system hoses pooled together), 22 morphologically distinct colonies were recognised on NA from the DCU suction system biofilm samples. All 22 (100%) were identified to the species level by 16S sequence analysis. Four genera were represented, including *Stenotrophomonas* (14 isolates), *Pseudomonas* (four isolates), *Microbacterium* (two isolates) and *Delftia* (two isolates) (see Table 6.2). Only 18 of the 22 isolates (81%) were identified to the species level by the API 20 NE system. Ten were identified as *Stenotrophomonas* species, five as *Pseudomonas* species, two as *Delftia* species and one as *Brevundimonas* species. Inconsistent identification occurred for isolates

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Table 6.1. Bacterial diversity of 123 16S DNA sequences amplified from DNA extracted from biofilm lining the inside of DCU suction system hoses

Species/ genus name	No. of clones	Relative abundance
Stenotrophomonas maltophilia	41	33.3%
Brevundimonas diminuta	21	17.1%
Sphingomonas yanoikuyae	19	15.4%
Sphingomonas paucimobilis	7	5.7%
Other *Sphingomonas* sp.	1	0.8%
Pseudomonas putida	5	4.0%
Pseudomonas aeruginosa	3	2.4%
Pseudomonas tolaasii	3	2.4%
Pseudomonas reactans	1	0.8%
Other *Pseudomonas sp.*	4	3.3%
Caulobacter crescentus	2	1.6%
Other *Caulobacter* sp.	3	2.4%
Ralstonia basilensis	1	0.8%
Other *Ralstonia sp.*	2	1.6%
Delftia acidovorans	2	1.6%
Propionibacterium acnes	2	1.6%
Sphingobacterium spiritivorum	1	0.8%
Microbacterium sp.	1	0.8%
Xanthomonas sp.	1	0.8%
[a] Uncultured clone from drinking water	1	0.8%
[b] Uncultured bacterial clone 234 ds	1	0.8%
Methylobacterium sp./ Caulobacter crescentus	1	0.8%

[a] This sequence matched a published sequence for uncultured bacterial DNA that had been detected in drinking water (GenBank)

[b] This sequence matched a published sequence for uncultured bacterial DNA that had been detected from an unknown source (GenBank)

Table 6.2. Identification of 22 isolates cultured from DCU suction system hoses biofilm by the API 20 NE system and 16S sequence analysis

Isolate API 20NE system		16S sequence analysis
1	*Pseudomonas putida*	*Pseudomonas putida*
2	**Inconclusive**	*Microbacterium oxydans*
3	**Inconclusive**	*Microbacterium oxydans*
4	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
5	**Inconclusive**	*Stenotrophomonas maltophilia*
6	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
7	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
8	*Brevundimonas vesicularis*	*Stenotrophomonas maltophilia*
9	*Pseudomonas putida*	*Stenotrophomonas maltophilia*
10	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
11	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
12	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
13	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
14	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
15	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
16	**Inconclusive**	*Stenotrophomonas maltophilia*
17	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
18	*Delftia acidovorans*	*Delftia acidovorans*
19	*Delftia acidovorans*	*Delftia acidovorans*
20	*Pseudomonas fluorescens*	*Pseudomonas tolaasii*
21	*Pseudomonas fluorescens*	*Pseudomonas tolaasii*
22	*Pseudomonas fluorescens*	*Pseudomonas tolaasii*

[a] Isolates in boldface were not identified by the API 20 NE system

8 and 9, which were identified as *S. maltophilia* by 16S sequence analysis but as *Brevundimonas vesicularis* and *P. putida*, respectively, by the API 20 NE system. There was further discrepancy between the two methods with regard to identification to species level for isolates 20-22. These three isolates were identified as *P. fluorescens* by the API 20 NE system, while the 16S rDNA sequence analysis identified them as *P. tolaasii* (see Table 6.2).

6.3.3 Comparison of microbial diversity of biofilm lining proximal and distal ends of second set of suction system hoses by 16S sequence analysis

Forty cloned inserts amplified from the template DNA of biofilm lining the distal ends of two high volume suction system hosing of two separate DCUs contained in the Dublin Dental Hospital were sequenced and the corresponding sequences were submitted to BLAST to search for the closest identity match. Of the 40 cloned inserts, 38 (95%) were matched to the genus level and were representative of nine separate genera (see Table 6.3). The two most predominant sequences obtained were found to correspond to *S. maltophilia* (32.5%) and *Pseudoxanthomonas mexicana* (32.5%) as shown in Table 6.3. Another predominant species was *Variovorax paradoxus*, which was found to represent 12.5% of cloned inserts. The remaining two cloned inserts had 97% sequence identity with a sequence referred to by GenBank as "Bacterium Ellin 362". The remaining genera detected in the biofilm recovered from the distal end of the suction system hosing included *Dialister* (2.5%), *Chryseobacterium* (2.5%), *Pseudomonas* (2.5%), *Acidovorax* (2.5%), *Acinetobacter* (2.5%) and *Bradyrhizobium* (2.5%) (see Table 6.3).

Forty-three cloned inserts amplified from the biofilm DNA recovered from the proximal ends of the two high volume suction system hoses were also sequenced. In contrast to the distal end, a very different range of microorganisms was detected in the biofilm within the proximal end of the suction hoses. Forty-two of the sequences (97.7%) were identified to the genus level. The proximal end of the suction hosing is the end that used in patients' oral cavities and, unsurprisingly, DNA from many bacteria associated with oral flora and/ or oral infections were detected within the biofilm. The most predominant species was found to be *Comamonas testosteroni*, representing 18.6% of sequenced inserts. Other genera represented in the biofilm lining the proximal end of the suction system hosing included *Acinetobacter sp.* (9%), *Dialister sp.* (7%), *Prevotella sp.* (7%) and *Pseudomonas sp.* (9%), including two *P. aeruginosa* (4.8%) (see Table 6.4). Twenty-one different genera were represented in the proximal end of the hosing, although only 43 cloned inserts were sequenced, indicating a greater diversity of microorganisms in

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Table 6.3. Bacterial diversity of the biofilm lining the distal end of the high volume suction system hoses determined by 16S rRNA sequence analysis

Species/ genus name	No. of clones	Relative abundance
Stenotrophomonas maltophilia	13	32.5%
Pseudoxanthomonas mexicana	13	32.5%
Variovorax paradoxus	5	12.5%
Dialister invisus	1	2.5%
Chryseobacterium meningosepticum	1	2.5%
Bacterium Ellin 362	2	5.0%
Pseudomonas fulva/ putida/ parafulva	1	2.5%
Acidovorax sp.	1	2.5%
Variovorax sp.	1	2.5%
Acinetobacter sp.	1	2.5%
Bradyrhizobium sp.	1	2.5%

Table 6.4. Bacterial diversity of the biofilm lining the proximal end of the high volume suction system as determined by 16S rRNA sequence analysis

Name of species/ genus	No. of isolates	Relative abundance
Comamonas testosteroni	8	18.6%
Pseudomonas aeruginosa	2	4.8%
Stenotrophomonas maltophilia	2	4.8%
Sphingomonas yanoikuyae	2	4.8%
Sphingobacterium spiritovorum	2	4.8%
Pseudoxanthomonas mexicana	1	2.3%
Dialister invisus	1	2.3%
Eubacterium brachy	1	2.3%
Delftia acidovorans	1	2.3%
Delftia tsuruhatensis	1	2.3%
Pseudoramibacter alactolyticus	1	2.3%
Chryseobacterium indologenes	1	2.3%
Prevotella tannerae	1	2.3%
Prevotella veroralis	1	2.3%
Janthinobacterium lividum	1	2.3%
Acinetobacter sp.	4	9.0%
Dialister sp.	3	7.0%
Pseudomonas sp.	2	4.8%
Chryseobacterium j11	1	2.3%
Ochrobacter sp.	1	2.3%
Prevotella sp. oral clone	1	2.3%
Veillonella sp. oral clone	1	2.3%
Firmicutes sp. oral clone	1	2.3%
Selenomonas-like species	1	2.3%
[a]*Bacteroides* oral clone	1	2.3%
Uncultured *Duganella*	1	2.3%

[a] This sequence matched an bacterial sequence published on the GenBank database from the class *Bacteroides* of the phylum *Bacteroidetes*

this part of the hosing in comparison to the distal end of the hosing. The one cloned insert that could not be identified to the genus level had identity with a GenBank sequence from the class *Bacteroides* of the phylum *Bacteroidetes*.

These findings showed very distinct differences between the DNA sequences found in the biofilm lining the proximal end of suction system hosing, which is closest to the patient, and the distal end, which is closest to the DCU.

6.3.4 Comparison of microbial diversity of biofilm lining proximal and distal ends of second set of suction system hosing as determined by culture

For the culture of DCU suction system biofilm microorganisms from the second set of suction system hoses (proximal and distal ends analysed separately from each other), the second 500-µl aliquot of each biofilm suspension sample (see Section 6.2.2) was serially diluted ten-fold in sterile distilled water to 10^{-4} and 100-µl aliquots were spread onto three different types of agar, NA, PBA and R2A.

Twenty-three morphologically distinct colony types isolated from the distal end of the DCU suction system hosing were subcultured and identified by 16S sequence analysis and by the API 20 NE system. Ten different genera, as identified by 16S sequence analysis, were represented among these isolates, the most predominant of which were *Stenotrophomonas* (43%), *Chryseobacterium* (13%), *Microbacterium* (8.7%) and *Delftia* (8.7%) (see Table 6.5). The other genera identified by 16S sequence analysis included *Agrobacterium* (1.8%), *Pseudoxanthomonas* (1.8%), *Acinetobacter* (1.8%), *Sphingomonas* (1.8%), *Janthinobacterium* (1.8%) and *Variovorax* (1.8%), as shown in Table 6.5.

For the biofilm lining the proximal end of suction system hosing, 25 morphologically distinct colonies were subcultured onto the same agar from which they had been recovered and identified by the API 20 NE system and 16S sequence analysis. Ten genera (as identified by 16S sequence analysis) were represented among the isolates cultured from the proximal end of the suction system, with the four most predominant genera being *Pseudomonas* (24%), *Sphingomonas* (16%), *Microbacterium* (16%) and *Chryseomonas* (12%), as shown in Table 6.6. The other genera represented were *Stenotrophomonas* (8%), *Ochrobacter* (8%), *Comamonas* (4%), *Ralstonia* (4%), *Agrobacterium* (4%) and *Brevundimonas* (4%).

All 48 isolates (100%) subcultured from the proximal and distal ends of the DCU suction system hosing were identified by 16S sequence analysis, whereas 25/48 isolates (52%) could not be identified by the API 20 NE system. Of the 25 isolates that could not be identified using the API 20 NE system, 13 were identified by 16S sequence analysis as

Table 6.5. Isolates cultured from biofilm of proximal and distal ends of suction system hoses

Hose	Agar	No.	API 20 NE system	16S Sequence analysis
Proximal	NA	1	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
		2	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
		3	*Chryseobacterium* species	*Chryseobacterium meningosepticum*
		4	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
		5	**Inconclusive**	*Stenotrophomonas maltophilia*
	PBA	6	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
		7	*Chryseobacterium meningosepticum*	*Chryseobacterium meningosepticum*
		8	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
		9	**Inconclusive**	*Microbacterium sp.*
		10	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
		11	**Inconclusive**	*M. liquefaciens/ oxydans*
		12	**Inconclusive**	*Agromyces mediolanus*
		13	**No growth on API strip**	*Pseudoxanthomonas mexicana*
		14	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
		15	*Acinetobacter* species	*Acinetobacter* species
	R2A	16	**Inconclusive**	*Stenotrophomonas maltophilia*
		17	*Stenotrophomonas maltophilia*	*Chryseobacterium meningosepticum*
		18	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
		19	**Inconclusive**	*Sphingomonas yanoikuyae*
		20	**Inconclusive**	*Janthinobacterium* sp.
		21	**Inconclusive**	*Delftia acidovorans*
		22	**Inconclusive**	*Variovorax paradoxus*
		23	*Delftia acidovorans*	*Delftia acidovorans*
Distal	NA	24	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
		25	*Ochrobacter anthropi*	*Ochrobacter anthropi*
		26	**Inconclusive**	*Sphingomonas yanoikuyae*
		27	**Inconclusive**	*Pseudomonas aeruginosa*
		28	*Chryseobacterium indologenes*	*Chryseobacterium indologenes*
		29	*Chryseobacterium meningosepticum*	*Chryseobacterium meningosepticum*
		30	**Inconclusive**	*Microbacterium* species
	PBA	31	**Inconclusive**	*Sphingomonas yanoikuyae*
		32	**Inconclusive**	*Sphingomonas yanoikuyae*
		33	**Inconclusive**	*Comamonas testosteroni*
		34	**Inconclusive**	*Microbacterium liquefaciens/oxydans*
		35	**Inconclusive**	*Agrobacterium tumefaciens*
		36	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*
		37	**Inconclusive**	*Pseudomonas putida/ fulva/ parafulva*
		38	**Inconclusive**	*Microbacterium oxydans*
		39	**Inconclusive**	*Microbacterium oxydans*
		40	**Inconclusive**	*Chryseobacterium indologenes*
	R2A	41	**Inconclusive**	*Agrobacterium tumefaciens*
		42	*Ochrobactrum anthropi*	*Ochrobactrum anthropi*
		43	**Inconclusive**	*Brevundimonas vesicularis*
		44	*Sphingomonas paucimobilis*	*Sphingomonas yanoikuyae*
		45	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*
		46	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
		47	**Inconclusive**	*Ralstonia* species
		48	*Pseudomonas putida*	*Pseudomonas putida*

species that are not included in the API 20 NE database (bioMérieux); these were various *Microbacterium* species (six isolates), *Sph. yanoikuyae* (four isolates), *Janthinobacterium* species (one isolate), *Variovorax* species (one isolate) and *Pseudoxanthomonas mexicana* (one isolate).

It was found that R2A and PBA supported the growth of 11 and ten different genera, respectively, while NA only supported the growth of six different genera (see Table 6.5).

6.3.5 Catalase production

The 48 isolates recovered from the proximal and distal ends of the DCU suction system hosing (see Section 6.3.4) were tested for catalase production as described in Section 2.1.5.1.1. It was found that 31/48 (65%) of isolates recovered were catalase-positive, with 19/48 (40%) strongly (immediate rapid effervescence) and 12/48 (25%) weakly (effervescence within one min) catalase-positive, while 17/48 (35%) of isolates recovered were catalase-negative.

6.3.6 Phyla represented by total number of sequenced cloned inserts from both sets of DCU suction system hoses

In total, 206 cloned inserts from amplified 16S rDNA extracted from both sets of DCU suction system hoses examined were sequenced in this study (see Tables 6.1, 6.3 and 6.4). This total figure includes cloned inserts from the DNA of biofilm from both the proximal and distal ends of the suction system hosing. The major group of organisms (87.1%) detected by this method was the *Protebacteria* division (see Figure 6.2). The γ subdivision was the most represented class of this phylum, comprising 47.1% of the total number of cloned inserts sequenced. Genera represented in the biofilm from this class included *Stenotrophomonas* (27.2%), *Pseudomonas* (10.2%), *Pseudoxanthomonas* (6.8%), *Acinetobacter* (2.4%) and *Xanthomonas* (0.5%). The α subdivision comprised 18.7%, including *Sphingomonas* (14.1%), *Brevundimonas* (10.2%), *Caulobacter* (2.5%) and, at 0.5% each, *Ochrobactrum*, *Duganella*, *Bradyrhizobium* and *Methylobacterium*. The β subdivision comprised 11.3% and included *Comamonas* (3.9%), *Delftia* (1.9%), *Variovorax* (3%), *Ralstonia* (1.5%), *Acidovorax* (0.5%) and *Janthinobacterium* (0.5%) (Figure 6.2). The other phyla represented among the cloned inserts from the biofilm rDNAs included *Firmicutes* (4.9%), *Bacteroidetes* (4.5%) and *Actinobacteria* (1.5%) as depicted in Figure 6.2.
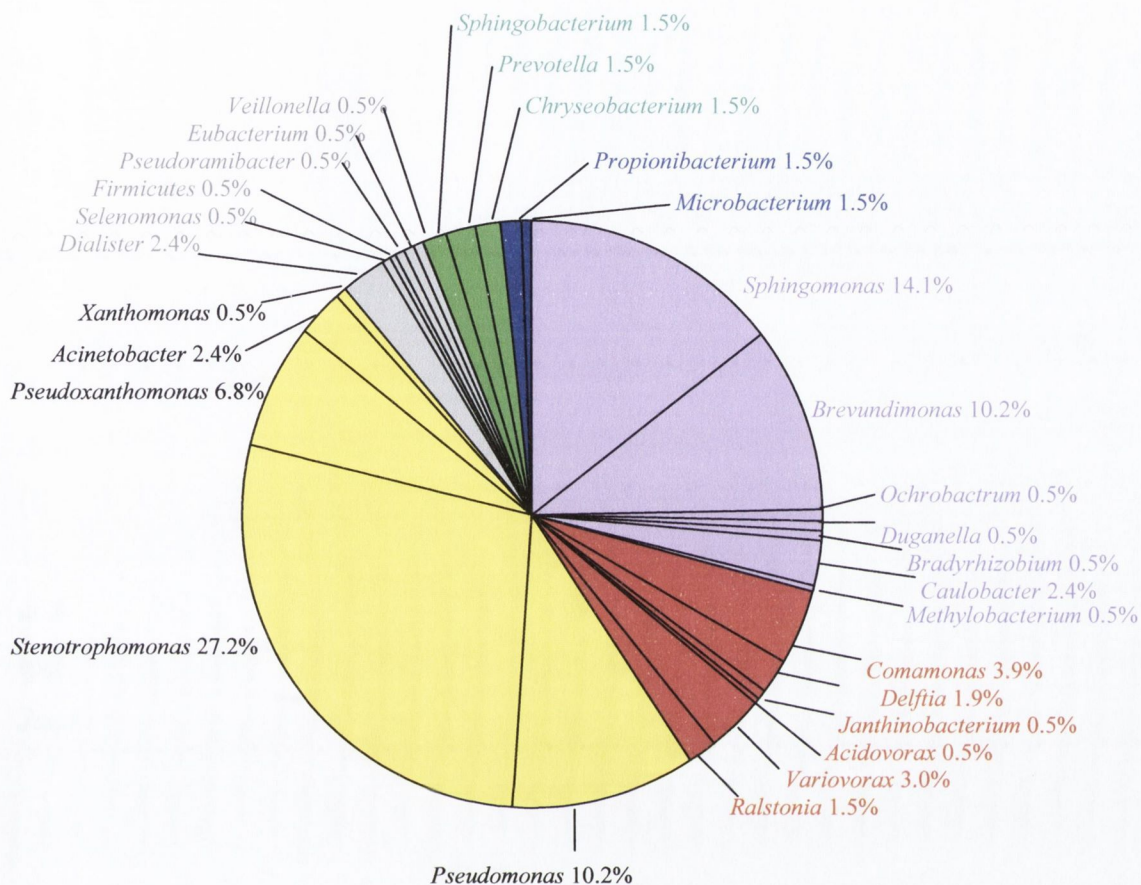
Figure 6.2. Graphical representation showing prevalence of bacterial genera detected in all DCU suction system hoses investigated in this study by PCR analysis of ribosomal 16S rRNA genes. The coloured segments refer to taxonomic phyla to which these genera belong. Purple, red and yellow segments represent genera detected from the α, β and γ subdivisions of *Proteo-bacteria*, respectively. Grey, green and blue segments represent genera detected from *Firmicutes*, *Bacteroidetes* and *Actinobacteria*, respectively.

6.3.7 Phyla represented by total number of cultured isolates

In total, 70 isolates were recovered from the biofilm lining the DCU suction system hosing comprising 22 isolates recovered from the suction system hoses where the proximal and distal ends were pooled together, a further 23 isolates recovered from the distal ends of the suction system hoses of another two DCUs and, finally, 25 further isolates recovered from the proximal ends of these same two DCUs (see Tables 6.2 and 6.5). Similarly to the 16S sequence analysis data, the major phylum detected by culture was the *Protebacteria* division (78.4%) as shown in Figure 6.3. The γ subdivision was the most represented class of this phylum, comprising 52.8% of the total number of isolates identified, including *Pseudomonas* (12.9%), *Stenotrophomonas* (37.1%), *Pseudoxanthomonas* (1.4%) and *Acinetobacter* (1.4%). The second most prevalent group of bacteria was from the phylum *Actinobacteria* and comprised 21.4%, including *Microbacterium* (11.4%), *Agromyces* (1.4%) and *Chryseobacterium* (8.6%). The α-*Proteobacteria* subdivision comprised 14.3% and included *Brevundimonas* (1.4%), *Sphingomonas* (7.1%), *Ochrobactrum* (2.9%) and *Agrobacterium* (2.9%). The β-*Proteobacteria* subdivision comprised 11.3% of the total number of isolates identified and included *Delftia* (5.7%) and, at 1.4% each, *Janthinobacterium*, *Comamonas*, *Ralstonia* and *Variovorax* (Figure 6.3).
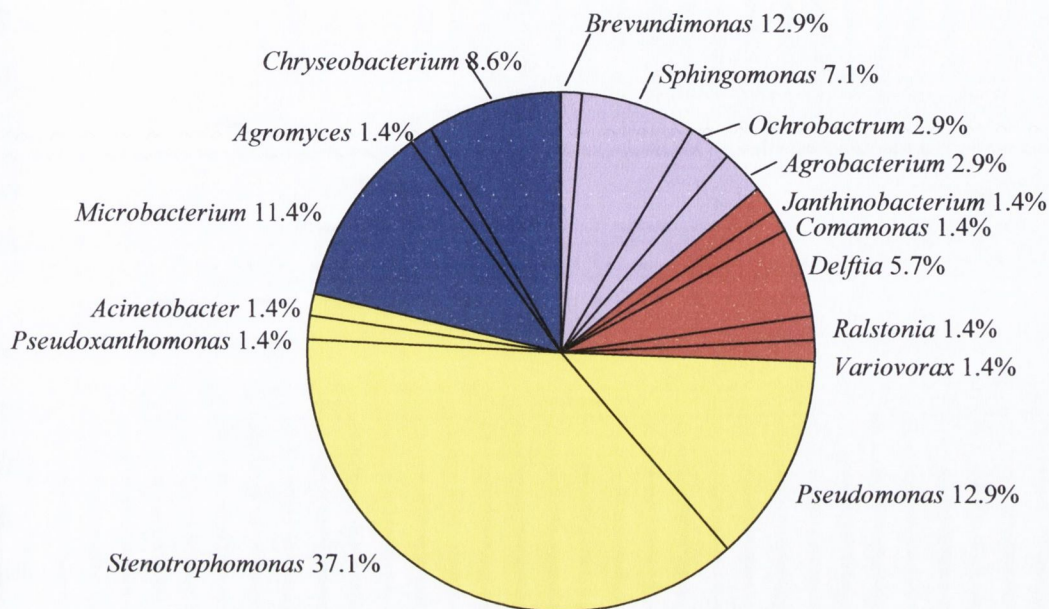
Figure 6.3. Graphical representation showing prevalence of the 70 cultured bacterial genera recovered from all DCU suction system hosing investigated in this study. Coloured segments refer to the taxonomic phyla to which these genera belong. Purple, red and yellow segments represent genera belonging to the α, β and γ subdivisions of *Proteobacteria*, respectively. Blue segments represent genera belonging to the phylum *Actinobacteria*.

6.4 Discussion

6.4.1 Microbial diversity of biofilm lining DCU suction system hosing

This study has revealed a very diverse microbial community in DCU suction system hosing as determined by both culture methods and 16S sequence analysis, but particularly the latter. Culture methods detected 16 separate genera and 20 species, while 16S sequence analysis detected 28 separate genera and 37 species. Both the 16S sequence analysis and culture methods detected three classes, α, β and γ, of the *Proteobacteria* division. However, the 16S sequence analysis method also detected bacteria from three other phyla, *Firmicutes*, *Bacteroidetes* and *Actinobacteria*, while the culture method only detected organisms from one other phylum, *Actinobacteria* (see Figures 6.2 and 6.3).

The major group within the biofilm lining the DCU suction system hosing was the γ-*Proteobacteria* subdivision, comprising 47.1% and 52.8%, respectively, for both 16S sequence analysis and culture-dependent analysis. In fact, the same species, *S. maltophilia*, was the most prevalent organism detected within the biofilm by both methods, with prevalence rates of 27.2% and 37.1% for 16S sequence analysis and culture, respectively. The consistency in this regard between the two methods is of interest as a previous study investigating microbial diversity of biofilm lining dental chair unit waterlines using both culture and 16S sequence analysis also reported that both these methods detected the same subdivision of bacteria (in their case, α-*Proteobacteria*) as the most prevalent group of organisms within the biofilm (Singh *et al.*, 2003). However, Singh *et al.* also reported differences in the prevalence of particular species within the subdivision as detected by both methods.

Stenotrophomonas maltophilia is increasingly recognised as a nosocomial pathogen, particularly for patients who are immunocompromised due to cystic fibrosis or cancer (Denton *et al.*, 1998). This species has been isolated from clinical specimens from cystic fibrosis patients with increasing frequency, probably because of the extensive use of antibiotics for treatment and control of *P. aeruginosa* infections (Denton *et al.*, 1998). It is assumed that patients acquire *S. maltophilia* from a wide variety of natural environments, including soil and water, and on various contaminated objects such as taps (Khardori *et al.*, 1990) and showers (Denton *et al.*, 1998). A recent study reported a case of nosocomially acquired empyema with infected ascites caused by a strain of *S. maltophilia* the source of which was found to be a disinfectant solution (Mukhopadhyay *et al.*, 2003).

Pseudomonas species represented 12.9% of organisms isolated by culture and 10.2% of the species detected by 16S sequence analysis. *Pseudomonas* species, especially *P. aeruginosa*, are well-documented opportunistic pathogens that can survive on a meagre

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supply of nutrients and often exhibit resistance to antibiotics and disinfectants (Michel-Briand *et al.*, 1981; Wilson & Dowling, 1998). *Pseudomonas aeruginosa* usually causes infections in people who are immunocompromised in some way, particularly burns victims and those suffering from the genetic disorder, cystic fibrosis, and it also has significant morbidity and mortality in AIDS patients and neutropenic patients undergoing chemotherapy (Govan & Deretic, 1996; Lyczak *et al.*, 2000; Maschmeyer & Braveny, 2000). Because there has been an increase in the number of medically and immunocompromised individuals attending dental clinics, it is of concern that this microorganism is present in relatively high abundance in the biofilm of suction system hosing.

The presence of bacteria within DCU suction system hosing that have been associated with oral infections, such as *Prevotella veroralis*, *Pseudoramibacter alactolyticus*, *Eubacterium*, *Dialister* and *Selenomonas* species is also of concern (Nakazawa & Hoshino, 1993; Rawlinson *et al.*, 1993; Nakazawa *et al.*, 1999; Rolph *et al.*, 2001; Siqueira & Rocas, 2003). This is of concern especially because these microorganisms were recovered from the proximal (patient) end of the hosing, thus increasing the likelihood of these microorganisms entering patients' oral cavities should backflow occur.

Of course, as this was a preliminary investigation, a limited number of suction system hosing samples from only five Planmeca Prostyle Compact DCUs were investigated in this study. Future studies investigating the microbial diversity should study a greater number of DCUs, including DCUs of different makes and models in order to more fully understand the diversity of microorganisms in the biofilm of DCU suction systems.

6.4.2 Comparison of organisms detected at proximal and distal ends of hosing

The difference in the diversity of microorganisms isolated from the proximal end of the suction system hosing compared to the distal end, as determined by 16S sequence analysis, is of considerable interest. While *S. maltophilia*, an opportunistic pathogen, and *Pseudoxanthomonas mexicana*, a species that has not yet been implicated in disease, according to recent PubMed searches, are the predominant microorganisms (65%) found in the biofilm lining the distal end, these species only account for 8.1% of microorganisms found at the proximal end. Furthermore, over 25% of identified sequences at the proximal end were from anaerobes, including representatives from *Prevotella*, *Dialister*, and *Eubacterium* (Haraldsson & Holbrook, 1999; Nakazawa *et al.*, 1999; Downes *et al.*, 2003).

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In contrast, only one sequence (2.5%) from the distal end of the hosing biofilm was found to be from an anaerobe, *Dialister invisus*; this was also the only sequence from the distal end of the biofilm that corresponded to an oral microbe. However, DNA sequences from oral microbes were quite prevalent in the proximal end of the suction system, comprising 31% of the sequences identified. This is to be expected, given that the suction system is used to aspirate fluids from the patient's mouth and that the proximal end is closer to the patient. Oral microbes identified from the proximal end corresponded to seven genera, including *Dialister*, *Eubacterium*, *Pseudoramibacter*, *Prevotella*, *Veillonella*, *Firmicutes* and *Selenomonas* (Haraldsson & Holbrook, 1999; Nakazawa *et al.*, 1999; Downes *et al.*, 2003). One further sequence was not matched to a specific genus, but was identified as a sequence that matched an uncultured DNA sequence from the class *Bacteroides* of the class *Bacteroidetes*.

It could be argued that many of these sequences detected by 16S sequence analysis do not represent viable bacteria from the biofilm and that the DNA may have been isolated from lysed bacterial cells that pose no risk of illness to dental staff and patients. However, the most predominant genera detected by 16S sequence analysis of the biofilm, *Stenotrophomonas* (27.2%) and *Pseudomonas* (10.2%), were also the most predominant genera cultured from the biofilm (37.1% and 12.9%, respectively). These results indicate that many of the microorganisms in the suction system biofilm are indeed viable. While none of the oral bacteria detected by 16S sequence analysis were cultured from R2A, NA or PBA media, many of these DNA sequences analysed were from anaerobes. Therefore, the anaerobic bacteria from which they were derived could not have been cultured as only aerobic heterotrophic microbes were supported by the culture methods used in this study. These results demonstrate the bias of using traditional culture methods alone in assessing the microbial diversity of various environments. This study has demonstrated that a wide range of microorganisms is present in the biofilm of DCU suction system hoses and their presence there demands further investigation by a more extensive study.

It is of interest to note that no oral or anaerobic species were detected when the rDNAs from both the proximal and distal ends of the suction system hosing were pooled. This is probably because there was a much denser layer of biofilm on the distal end of the suction system hosing, compared to the proximal end (O'Donnell, 2002), which would have meant that DNA from the organisms lining the distal end of the suction system hosing was in much greater abundance compared to DNA from the proximal end. This would consequently have decreased the likelihood of selecting plasmids that contained cloned partial 16S sequences from organisms lining the proximal end of DCU suction system

hosing. As discussed in previous studies, genera present at low numbers within the biofilm are less likely to be detected unless the number of plasmids screened for the presence of the cloned insert is greatly increased (Rolph *et al.*, 2001; Singh *et al.*, 2003).

This study did not assess the gradation of microorganisms present along the length of the suction system hosing, as this was beyond the scope of the study. Future studies assessing the microbial diversity of the biofilm lining the whole length of the suction system hosing may yield an even more diverse range of genera.

6.4.3 Possible selection of catalase-positive microorganisms

Interestingly, the prevalence of *S. maltophilia* within the biofilm lining the DCU suction system hosing is quite in contrast to the results of a previous study undertaken in 2000 by this laboratory. This previous study investigated the microbial diversity of biofilm lining the suction system hosing and other sites within the suction system of Planmeca Prostyle Compact DCUs located within the Dublin Dental Hospital by culture on PBA medium only as preliminary investigations had indicated that the majority of microorganisms present were *Pseudomonas* and related species (O'Donnell, 2002). The results of this previous study showed that the most predominant organisms isolated from the distal end of the DCU suction system hosing were *P. putida* (recovered from 49% of DCUs sampled) and *P. aeruginosa* (recovered from 46% of DCUs sampled) with *S. maltophilia* recovered from only 38% of DCUs sampled. The proximal ends of suction system hosing were also examined and *P. putida* was recovered from 20% of hoses sampled, while *P. fluorescens* and *S. maltophilia* were each recovered from 9% of hoses sampled; however 69% of proximal hoses yielded no growth. However, in this present study, *Pseudomonas* species represented only 10.2% of the cloned inserts sequenced and 12.9% of isolates recovered from the DCU suction system hosing. *Pseudomonas fluorescens* was not detected by either method. The differences between the two studies may be explained by the fact that since the first study was undertaken in 2000, DCU waterlines in the Dublin Dental Hospital have been subject to weekly disinfection with a hydrogen peroxide- and silver ion-based disinfectant, Sanosil, as discussed in Chapter 3. Catalase-positive microorganisms have increased in predominance within the DUWs (Prof. D. Coleman, Personal Communication), most likely selected by prolonged usage of the hydrogen peroxide-based disinfectant. Following disinfection, the eluate from the DUWs passes down the spittoon into the suction system located within the pedestal unit of the DCU. As many of these microorganisms are motile and conditions within the DUWs, spittoon and suction system are often damp, some catalase-positive microorganisms eluted

from the DUWs may have moved from the suction system within the pedestal unit into the suction hoses during the overnight periods when the DCU suction system is not in use and formed part of a new, more microbially diverse biofilm. Alternatively, some of the hydrogen peroxide-based disinfectant may have entered the suction system hoses and selected for catalase-resistant microorganisms already present within the biofilm of the suction hoses. Either of these explanations could account for the change in the microorganisms within the hosing from the previous study in 2000 to the present study in 2004, as the majority (65%) of microorganisms isolated from the DCU suction system in this present study that were tested for catalase production were shown to be catalase-positive.

6.4.4 Limitations of API 20 NE system

This study has also highlighted some limitations associated with the use of the API 20 NE system for identification of Gram-negative non-enterobacterial bacteria. Of 70 isolates tested, only 38 (54%) were identified consistently to the genus level using the API 20 NE system in this study. While most of those isolates that could not be identified simply gave profiles that did not definitively match any of those in the database, two isolates did not grow in the API 20 NE strip. Thirteen of the isolates that could not be identified by the API 20 NE system were identified by 16S sequence analysis as species that are not included in the API 20 NE database (bioMérieux); these were *Microbacterium* species (six isolates), *Sph. yanoikuyae* (four isolates), *Janthinobacterium* species (one isolate), *Variovorax* species (one isolate) and *Pseudoxanthomonas mexicana* (one isolate). In fact, the API 20 NE system can only distinguish between 61 different species in total (bioMérieux), which is obviously a serious limitation in a study aiming to investigate the microbial diversity of an environment. However, 16S rDNA sequence analysis of cultured isolates provides an attractive alternative as the BLAST database is updated regularly.

6.4.5 Limitations of 16S sequence analysis

Of course, no method that aims to investigate the microbial diversity of an environment is without bias. While culture is more obviously selective for the particular organisms that can grow on the media under the specific growth conditions used, methods for determining microbial diversity that involve DNA extraction, such as that used in the present study, may also give biased results. Thus, the choice of DNA extraction procedure is extremely important to ensure all species are lysed and thus available for inclusion in the study (Rolph *et al.*, 2001). In this present study a standard kit, Qiagen DNeasy Tissue Kit,

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that has been developed for extraction of DNA from both Gram-positive and Gram-negative bacteria was used, which involves both lysozyme and Proteinase K degradation. This would have lysed all but the most resistant microorganisms present in the biofilm sample, ensuring that DNA from almost all microorganisms present in the sample were available for inclusion in the study.

Other factors that may lead to biased results in this type of study include the number of genomes and number of genes coding for 16S rRNA, which vary widely among prokaryotes. Thus, microorganisms that have a higher copy number of genes coding for 16S rRNA will be represented more frequently in the overall analysis, while those with a lower copy number will be under-represented. The number of genomes per bacteria may also vary with bacteria possessing more than one genome appearing to be more prevalent in the environment (Farrelly *et al.*, 1995). All these factors contribute to bias with the non-culture dependent 16S sequence analysis method for determining the microbial diversity of an environment and mean that this method can only be semi-quantitative. However, despite these limitations, this method remains a useful tool for the investigation of environments where not all bacteria can be detected by traditional culture methods.

6.4.6 Conclusions

This study has highlighted the failure of the Puli-Jet (Cattani) disinfection regimen used in the Dublin Dental Hospital for disinfection of suction system hoses. Despite twice-daily disinfection, there are still high densities of bacteria in the suction system hoses, posing a real risk of infection to patients as previous studies showed that backflow from suction hoses can result in the aspiration of fluid from suction hoses into the oral cavities of patients during use (Watson & Whitehouse, 1993; Mann *et al.*, 1996; Barbeau *et al.*, 1998). Furthermore, when the suction system is not in use, these motile microorganisms within the suction hoses may move to the external side of the hoses from where there is an even greater likelihood of cross-infection. In order to fully assess the potential risk of infection for dental staff and patients, it is imperative that the diversity of microorganisms within the suction system is investigated in greater detail and that a more effective disinfectant and disinfection regimen for the suction system is found.

Chapter 7

General Discussion

7.1 Bacterial contamination of dental chair units

It is well recognised that a wide range of medical devices such as ventilators, medical suction systems, central venous catheters, urinary catheters, contact lenses, endo-tracheal tubing and prosthetic heart valves can be sources of cross-contamination and cross-infection for patients, particularly those who are immunocompromised (DePaola *et al.*, 2002; Donlan & Costerton, 2002). In fact all instruments "used for human beings for the purpose of diagnosis, prevention, monitoring, treatment or alleviation of disease" are defined as medical devices, according to the EU Medical Devices Directive (Anonymous, 1993). As such, the highly complex modular pieces of equipment, dental chair units (DCUs), should also be classed as medical devices. While DCUs are obviously not permanently indwelling devices like the medical devices mentioned above, there is still much potential for cross-contamination and cross-infection. Bacterial contamination of DCU components used in patient treatment should be regarded as potential sources of infection. Dental procedures carried out during the clinical session in which DCUs are used and routine maintenance of DCUs expose both staff and the many patients treated during the day to potential pathogens originating from the biofilm within DUWs and the DCU suction system and, thus, to the risk of infection.

There has been a great increase in the number of immunocompromised patients attending the dental surgery as the many advances in medicine and technology over the last two decades have ensured their continued survival. Furthermore, in order to maintain good oral hygiene and to reduce infections in immunocompromised individuals, regular dental treatment is now an important part of their treatment. Such individuals include the elderly, organ transplant recipients, cancer patients, cystic fibrosis patients, HIV-positive patients and those on immunosuppressive therapy etc. (Shearer, 1996; Pankhurst *et al.*, 1998; Pontón *et al.*, 2000; Al Shorman *et al.*, 2002). These individuals are much more susceptible to cross-infection and rigorous cross-infection control procedures should be followed when treating such patients. However, the fact that a patient seeking dental treatment is immunocompromised is not always obvious to the dental practitioner or indeed to the individuals themselves. Therefore, all patients should be treated as if they are immunocompromised with the implementation of appropriate cross-infection control measures for all patients seeking dental treatment.

In this study the DUW output water and the suction systems of Planmeca Prostyle Compact DCUs were investigated for bacterial contamination and the consequent potential for cross-infection of patients and dental staff.

7.1.1 Bacterial contamination of DUW output water

Over the past thirty years the problem of high bacterial densities in DUW output water has been increasingly highlighted and discussed in many peer-reviewed journals (Abel *et al.*, 1971; Furuhashi & Miyamae, 1985; Douglas & van Noort, 1993; Pankhurst & Philpott-Howard, 1993; Williams *et al.*, 1993; Williams *et al.*, 1994; Williams *et al.*, 1995; Pankhurst *et al.*, 1998; Walker *et al.*, 2000; Putnins *et al.*, 2001; DePaola *et al.*, 2002; Tuttlebee *et al.*, 2002). However, the full impact of such studies has yet to be realised in the dental community. The high bacterial densities in DUW output water are due to the flow of output water sloughing off bacteria from the biofilm within the DUWs when the waterlines are in use. The build-up of biofilm is due to the design of DCUs in that the DUWs are of such narrow bore that the surface area: volume ratio is very high allowing high numbers of bacteria to adhere to the lumen of the tubing and form biofilms. Furthermore, bacteria also adhere readily to the hydrophobic polymeric plastic material used in DUWs, e.g. polyvinyl chloride, polyurethane (Pankhurst *et al.*, 1998). The USA, Japan and the European Commission have set national standards of 500, 100 and 100 cfu/ml, respectively, for aerobic heterotrophic bacteria in drinking water (Furuhashi & Miyamae, 1985; Anonymous, 1998; Anonymous, 2002a). Clearly the quality of DUW output water should be at least as good as that recommended for human consumption, as DUW output water is going directly into patients' mouths and also into their upper respiratory tracts as aerosols. Currently, there are no standards for the microbiological quality of dental unit water set by the European Commission, however, the ADA set a goal for the year 2000 of no more than 200 cfu of heterotrophic bacteria per ml of dental unit output water (Anonymous, 1996). Unfortunately, however, water samples taken from DUWs of DCUs located in Irish dental clinics in this study had bacterial densities that far exceeded all potable water standards, as well as the ADA's recommendations of no more than 200 cfu/ml. This indicates that while there may be many research papers and discussion in journals, very little action is actually being undertaken by the dental community, including dental practitioners and DCU manufacturers, in response to the problem.

It is clearly unethical to deliver water containing such high bacterial density into the mouths of patients, many of whom may be immunocompromised. As discussed above, it is not always evident to the dental practitioner which patients are compromised, therefore dental practitioners should assume all patients to be immunocompromised and treat them accordingly. There is an onus on both the dental practitioners and DCU manufacturers to develop and implement effective solutions to this situation.

The potential pathogens that have been isolated from the DUW output water both in this study and other studies are of particular concern. Known opportunistic pathogens such as *B. cepacia*, *S. maltophilia* and *P. fluorescens* were isolated from the DUW output water in this study. All three species have been associated with infection in immunocompromised patients (Pankhurst & Philpott-Howard, 1996; Denton *et al.*, 1998; Hsueh *et al.*, 1998). *Burkholderia cepacia* has been particularly associated with infection in cystic fibrosis patients (Pankhurst & Philpott-Howard, 1996), while *S. maltophilia* is a microorganism that is being increasingly recognised as a nosocomial pathogen, particularly for patients who are immunocompromised due to cystic fibrosis or cancer (Denton *et al.*, 1998). Both of these species are being isolated from clinical specimens from cystic fibrosis patients with increasing frequency as a consequence of the extensive use of antibiotics for treatment and control of *P. aeruginosa* infections (Denton *et al.*, 1998). *Pseudomonas fluorescens* has been implicated in an outbreak of nosocomially-acquired bacteraemia among oncology patients (Hsueh *et al.*, 1998).

Other studies have detected even more pathogenic bacteria from DUW output water, such as *P. aeruginosa* and *Legionella* species (Martin, 1987; Atlas *et al.*, 1995), which have been implicated in illness in dental patients and death in the case of one dental practitioner (Atlas *et al.*, 1995). The fact that such bacteria are present in high densities in DUW output water should be of grave concern to both DCU manufacturers and dental practitioners, yet, in the vast majority of cases, very little is being done in dental clinics and private practices to reduce the numbers of bacteria present in DUW output water.

Even in situations where disinfection procedures are implemented to reduce the bacterial densities within DUWs, periodic monitoring of the DUW output water should be undertaken as part of routine quality control procedures in order to identify inadequate disinfection its causes. Studies in the Dublin Dental Hospital, for example, reveal a three-way potential for reduced efficacy of the disinfection regimen (Prof. D. Coleman, Personal Communication). Firstly, there is the potential for human error during the disinfection/ flushing cycle for various reasons, including the busy schedules of dental personnel, which may lead to failure to perform the procedure properly. Secondly, there may be technical problems with the DCU itself and thirdly, regular disinfection with the same disinfectant may select for microorganisms that are resistant to the active ingredient of the biocide.

With regard to human error, in the Dublin Dental Hospital, weekly disinfection of all DUWs is undertaken by different dental nurses throughout the Hospital. It has been noted, however, that there are varying degrees of compliance with the disinfection protocol, leading to reduced efficacy of the disinfection regimen in some cases. For

example, during the disinfection/ flushing cycle, disinfectant is aspirated automatically through all the DUWs apart from the nurse's air/water syringe, which needs to be operated manually. However, some dental nurses were not aspirating the disinfectant through the dental nurse's air/water syringe during the disinfection cycle and the result of such an oversight was that water of unacceptable quality was still being delivered into patients' mouths despite the implementation of a once-weekly disinfection regimen (Prof D. Coleman, Personal Communication). Personnel undertaking the disinfection regimen should be fully informed by the clinical microbiology team as to why such a regimen is being pursued and as to its importance and the potential consequences of failure to implement the disinfection procedure properly. Education of dental personnel in this regard and regular updates with the success/ failure of the disinfection regimen by the clinical microbiology team should reduce the likelihood of human error during the disinfection process.

With regard to technical and engineering considerations, DCU manufacturers must also accept responsibility for ensuring that their product is designed to deliver the highest possible standard of water to dental patients. As such, DCU manufacturers should research all appropriate means of disinfecting their DUWs or, indeed, should consider ways of designing new types of DUWs that do not lend themselves to biofilm formation. The Planmeca Waterline Cleaning System is one example of how collaboration between the clinical microbiology team and DCU manufacturers can ensure the delivery of water that meets the European Commission potable drinking water standards, as well as the ADA's recommendations. However, there also needs to be ongoing co-operation among the DCU manufacturers, microbiologists, DCU maintenance staff and the dental team so that if any problems with disinfection are encountered, they can be investigated and resolved. Such co-operation is ongoing in the Dublin Dental Hospital and has resulted in further modification of the Waterline Cleaning System so that all Planmeca Prostyle Compact DCUs are now sold complete with the Waterline Cleaning System integrated into the pedestal unit of the DCU and almost fully automated, ensuring that all dental practitioners who buy these DCUs have the ability to disinfect their DUWs on a regular basis, even if the DCU is connected to the mains water supply. This integration of the waterline cleaning system into the DCU was necessary because the disinfectant intake valves of some of the DCUs in the Dublin Dental Hospital that had been retro-fitted with the Planmeca Waterline Cleaning System were not working sufficiently and disinfectant was leaking from the DUWs during overnight disinfection. This led to an insufficient volume of disinfectant

acting on the biofilm and, consequently, reduced efficacy against the biofilm (Prof. D. Coleman, Personal Communication).

As this study has shown, regular use of one disinfectant may select for bacteria within the biofilm that are already resistant to the active ingredient of the disinfectant. In the case of the study described in Chapter 3, which involved DCUs in the Dublin Dental Hospital, following two years of regular weekly disinfection of the DUWs with a hydrogen-peroxide based disinfectant, a proliferation of catalase-positive microorganisms cultured from DUW output water was observed five days following the disinfection and rinsing cycle. The most predominant catalase-positive isolates recovered from the DUWs were identified as *Sphingomonas subarctica*, *Bacillus pumilus*, *Micrococcus luteus*/ *Arthrobacter* sp., *Variovorax paradoxus* and *Flectobacillus speluncae*, none of which had been isolated from the DUW output water during the study period described in Chapter 3. This highlights the potential for bacterial resistance within the biofilm community of the DUW tubing to develop following prolonged usage of the same disinfectant over a considerable period of time.

To ensure continued efficacy of the disinfection regimen, the clinical microbiology team, the engineering team and those responsible for disinfection of DUWs should undertake periodic monitoring of the DUW output water and be in regular contact with each other to ensure that the water delivered to patients is of the highest quality.

7.1.2 Bacterial contamination of DCU suction systems

The DCU suction system is also very likely to be a source of cross-contamination and infection for dental personnel, maintenance staff and patients alike. Very little investigation of biofilm within DCU suction system hosing has been undertaken thus far, but the few studies that have been published have reported high densities of bacteria in biofilm within the suction system (Barbeau *et al.*, 1998; O'Donnell, 2002). Some studies have also demonstrated backflow of liquids from the low volume suction system hoses into patients' mouths, indicating that these systems could be a possible source of cross-infection (Watson & Whitehouse, 1993; Mann *et al.*, 1996; Barbeau *et al.*, 1998). Furthermore, cross-infection of dental staff and maintenance personnel from the suction system may also occur during routine maintenance and cleaning of the DCUs. For example, in the Dublin Dental Hospital, the coarse filters located in the suction system are removed daily from inside the pedestal unit of the DCUs by dental nurses, which exposes them to the potential risk of cross-infection from bacteria and other microbes trapped in the filter cage or its housing.

The first part of the present study investigating bacterial contamination of DCU suction systems was undertaken in collaboration with Ms M. O'Donnell and investigated the source of corrosion, dampness and bacterial contamination that was localised to the area where the high-volume and low-volume suction system hoses were attached to the main body of the DCUs. *Pseudomonas aeruginosa*, a well-documented nosocomial pathogen (Govan & Deretic, 1996; Hatchette *et al.*, 2000; Lyczak *et al.*, 2000; Donlan & Costerton, 2002) was used as a marker organism and was recovered by Ms M. O'Donnell from the baseplates and three separate suction system sites of many DCUs located throughout the Dublin Dental Hospital. Following serotyping of *P. aeruginosa* isolates and fingerprinting of the DNA of *P. aeruginosa* isolates using the PFGE technique, it was demonstrated in the present study that the source of the dampness and bacterial contamination was from within the suction system itself, probably due to the movement of the hoses during use in clinical sessions, resulting in loosening of the hose connectors from the receiving orifices causing leakage of fluid from the suction system. The DNA fingerprinting results also indicated that a small number of strains, particularly one of serogroup O:10, had colonised suction systems of DCUs throughout the hospital. It is not known from where the *P. aeruginosa* isolates originated but one possibility is that *P. aeruginosa* colonising patients' oral cavities or in DUW output water was aspirated into the suction system hoses and from there into the central suction plant that creates the vacuum used for the operation of the DCU suction systems in the Dublin Dental Hospital. However, *P. aeruginosa* was not recovered from DUW output water at any time during the study period. A further possibility is that *P. aeruginosa* was already contaminating the central suction plant when it was supplied to the Dublin Dental Hospital. The spread of this small number of *P. aeruginosa* isolates to the DCU suction systems throughout the Hospital most likely occurred from the Hospital's central suction system plant. As the suction system is often damp, motile bacteria such as *P. aeruginosa* could have moved back into the suction system hoses of separate DCUs throughout the Hospital during periods when the suction system was not in use, e.g. over the weekend or during the holiday periods. This illustrates the point that unless hospital-wide cross-contamination controls are fully implemented, potential pathogens may spread throughout the building, as occurred in this case, exposing more people to the potential risk of infection.

The results of the second part of this investigation of bacterial contamination of suction systems demonstrated that the high volume suction system hosing is lined with biofilm that contains many opportunistic aerobic heterotrophic pathogens such as *S. maltophilia* and *P. aeruginosa* and oral anaerobic pathogens such as *Dialister*,

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Eubacterium, *Pseudoramibacter* and *Prevotella*. Further work needs to be undertaken in order to investigate more fully if these anaerobic pathogens within the biofilm are actually viable bacteria or, indeed, really part of the biofilm, as they were not cultured during the course of the project. Furthermore, other pathogens such as viruses may also be present in the biofilm and fungi and yeasts such as *Aspergillus* and *Candida* have also been recovered (Prof. D. Coleman, Personal Communication) but as the purpose of this study was to investigate bacterial contamination of the suction systems they were not investigated here.

The US Center for Disease Control (CDC) have recommended that any DCU surface, including suction system hosing, contaminated with patient material should be disinfected using a chemical germicide with mycobactericidal activity, such as a phenolic, iodophor or chlorine-containing compound (Centers for Disease Control and Prevention, 1993). Accordingly, since the opening of the new Dublin Dental Hospital in 1998, the suction system hosing of the Planmeca Prostyle Compact DCUs included in this study has been subject to disinfection twice-daily with the phenolic-based disinfectant Puli-Jet (Cattani). It is of particular concern then that extensive biofilm was present in the suction system hosing despite this twice-daily disinfection regimen. It should be obvious, therefore, that the implementation of a disinfection regimen of suction systems (or indeed of any DCU surface) without appropriate monitoring of its effectiveness is pointless. Indeed, it may lead to a cavalier attitude by dental practitioners towards the practice of further cross-contamination controls in the mistaken belief that the disinfection procedure is eliminating or significantly reducing the risks of cross-infection. One explanation for the ineffectiveness of the disinfection regimen of the DCU suction systems in the Dublin Dental Hospital is the limited contact time (approximately 90 s) of Puli-Jet with the biofilm. This is due to the fact that in all DCU suction systems, aspiration of the liquid through the suction system hosing is immediate and is undertaken in a manner analogous to a household vacuum cleaner. It is well documented that bacteria within biofilms are much more resistant to disinfection than planktonic organisms (Donlan & Costerton, 2002; Drenkard & Ausubel, 2002) and therefore a more appropriate technique for disinfection would involve flooding the suction system hosing with the disinfectant for a longer period of time, e.g. overnight. Empirical studies should also be undertaken in order to assess which type of disinfectant (e.g. chlorhexidine gluconate, phenol, hydrogen peroxide etc.) would be most effective at reducing bacterial contamination of the biofilm within the suction system hosing. In this regard, collaboration has been established between Planmeca and the clinical microbiology team of the Dublin Dental Hospital and research is underway to ascertain the best method of disinfecting the DCU suction system hoses.

This study has also demonstrated the value of non-culture techniques for the detection of potential pathogens both in water supplies and biofilm. The use of non-culture techniques could be further expanded in the investigation of DCU suction system contamination. For example, primers and probes suitable for use with the TaqMan sequence detection system could be designed that detect the presence and levels of certain viruses such as the Hepatitis B and C viruses, oral Herpes group viruses, including Epstein-Barr Virus (EBV) and cytomegalovirus (CMV) in the suction systems of the DCUs. Hepatitis C virus (HCV) is of particular concern in the dental surgery, as a study from Italy detected the presence of HCV-RNA on dental equipment in the surgery, including inside the suction system, following treatment of HCV-positive dental patients (Piazza *et al.*, 1995). Furthermore, one study showed that dental treatment was the only demonstrable risk factor in 9% of acute Hepatitis C and Hepatitis non-A non-B community-acquired hepatitis, indicating that there may be much more cross-contamination and cross-infection in the dental surgery than is currently acknowledged (Piazza *et al.*, 1995). EBV has recently been detected by *real-time* PCR in the saliva of both patients presenting with periodontitis and other healthy patients, although it is more prevalent and is present in higher levels in periodontitis patients than in healthy patients (Idesawa *et al.*, 2004). As this virus is present in patients' saliva, it will undoubtedly be aspirated into the suction system during dental treatment of the patient from where it could be passed to another patient should backflow from the suction system hosing to the next patient occur (Watson & Whitehouse, 1993; Mann *et al.*, 1996; Barbeau *et al.*, 1998).

7.1.3 *Legionella* contamination of DUW output water and other healthcare institution water supplies

Virtually nothing is known about the contamination of water supplies in the Republic of Ireland with *Legionella* species. The only published study in the PubMed database investigating *Legionella* contamination of water supplies in the Republic of Ireland was published in 1990 (Haugh *et al.*, 1990). That study consisted of both periodic and random sampling of showers and water tanks in two large Dublin hospitals. Only 5.3% of the samples tested yielded *Legionella* species with *L. pneumophila* serogroups 3, 5 and 6 being isolated from these samples (Haugh *et al.*, 1990). Similarly, it seems that little or no work has been undertaken to investigate the extent of bacterial contamination of DUW output water with *Legionella* species in the Republic of Ireland as, in this regard, there are no published studies available in the PubMed database. In fact, from 1989 to date, only three further studies in the PubMed database have even investigated

Legionnaires' disease in Ireland (Hone *et al.*, 1989; Carr *et al.*, 1991; Smith *et al.*, 2002). The two earlier studies reported a low prevalence of pneumonia due to *Legionella* species and indicated that there was little cause for concern (Hone *et al.*, 1989; Carr *et al.*, 1991). In contrast, however, a review of all 17 Legionnaires' disease case notifications in Ireland from 1998-2001 was undertaken by Smith *et al.* (2002) in order to assess the level of concern Irish health authorities should have in relation to this disease. They reported that all 17 cases were attributed to *L. pneumophila* serogroup 1 with nine cases associated with travel abroad. While the prevalence of Legionnaires' disease in this country appears to be low, the authors of this paper believe that this is due to under-diagnosis and under-reporting of this disease in Ireland, as the number of reported cases in Ireland is conspicuously low compared to other European countries. This indicates that cases of Legionnaires' disease may be going unreported and also that the problem of *Legionella* contamination of water supplies may be of greater significance than has previously been acknowledged. Since the publication of that study in 2002, a report has been published of another patient dying following contraction of Legionnaires' disease while an in-patient at an Irish hospital in 2003, which is the first proven case of nosocomial Legionnaires' disease in Ireland (Coleman & O'Donnell, 2003).

This present study found that cultivable *Legionella* species were present in 23% of water samples received from various healthcare institutions in the Midwestern Health Board region, with up to 90,000 cfu/l recovered from some water samples. All isolates tested were *L. pneumophila* with isolates from three separate water samples belonging to serogroup 1, the most frequent cause of Legionnaires' disease. This is a much higher rate of *Legionella* prevalence than the 5.3% prevalence reported by Haugh *et al.* in 1990, indicating that the prevalence of *Legionella* in water supplies may be increasing in the Republic of Ireland, which may lead to increased cases of Legionnaires' disease unless action is taken.

Legionella species were cultured from only one DUW output water sample in this study out of 19 samples tested, a prevalence of 5%. This seems to indicate that *Legionella* contamination of DUW output water is not as prevalent in Ireland compared to some previous reports from other countries (Atlas *et al.*, 1995; Challacombe & Fernandes, 1995; Williams *et al.*, 1996; Pankhurst *et al.*, 1998; Zanetti *et al.*, 2000). Two recent papers from Great Britain have indicated that levels of *Legionella* in DUW output water are less than 2% and the results of this present study concur with these low prevalence rates (Walker *et al.*, 2000; Pankhurst *et al.*, 2003). Of course, if effective disinfection of DUWs is being

undertaken regularly, then high levels of *Legionella* in the DUW output water can be avoided altogether, along with high levels of other bacteria.

This present study has also shown that molecular methods such as *real-time* PCR may well be of benefit in detecting *Legionella* in water systems as the mean average *Legionella* genome equivalents were greater in the culture-positive samples compared to the culture-negative samples. It may be of benefit to use *real-time* PCR assays in the preliminary analysis of water samples suspected to contain high levels of *Legionella* in the event of an outbreak as the culture of *Legionella* species can take up to ten days (Walker *et al.*, 2000). However, cultivation of *Legionella* from water samples should still be carried out as *real-time* PCR may detect dead or non-viable *Legionella* cells, which are not detrimental to humans.

Judging from the paucity of studies published investigating the prevalence of *Legionella* contamination of general water supplies and DUW output water in the Republic of Ireland there seems to be a tendency to avoid investigating the prevalence of this microorganism in the community. But is ignorance bliss? Perhaps if studies investigating the prevalence of *Legionella* in water supplies had been undertaken in Irish hospitals followed by appropriate remedial action, the recent death of a patient from nosocomial Legionnaires' disease acquired during a hospital visit in 2003 (Coleman & O'Donnell, 2003) would never have occurred. In 2002, the National Disease Surveillance Centre (NDSC) published guidelines for the management of Legionnaires' disease in Ireland and the presence of *Legionella* in water supplies (Anonymous, 2002b) and it is hoped that these will be implemented widely throughout healthcare institutions in the coming years. The implementation of these guidelines together with routine *Legionella* environmental testing in Irish healthcare institutions should help to reduce the incidence of Legionnaires' disease in Ireland and result in earlier and more accurate diagnosis. However, there will most likely be an initial increase in the number of reported cases of Legionnaires' disease due to better detection as it is widely suspected that Legionnaires' disease is underreported in Ireland (Smith *et al.*, 2002; Coleman & O'Donnell, 2003).

7.2 Conclusions

There appears to be a belief within the dental community at large that unless large numbers of patients contract life-threatening disease following attendance at the dental surgery there is no need to investigate potential sources of cross-infection associated with various components of the DCU. However, it is simply unacceptable to dispense DUW output water into the mouths of patients that is contaminated with up to a million aerobic

heterotrophic bacteria per ml (Williams *et al.*, 1994; Williams *et al.*, 1995; Pankhurst *et al.*, 1998; Putnins *et al.*, 2001; DePaola *et al.*, 2002). Part of the problem is that there are currently no national or European commission standards regarding microbial contamination of DUW output water and, therefore, there is no motivation for dental practitioners to improve the quality of DUW output water. However, should effective disinfection of DUWs be implemented with appropriate monitoring to check for failures, then the levels of potential pathogens such as *Pseudomonas* and *Legionella* species in the DUW output water would also be dramatically reduced, along with the potential for cross-infection of patients and dental staff. It is also unacceptable to expose patients to human-derived and biofilm-derived pathogens in DCU suction systems. Further research must be undertaken in order to reduce the risk of cross-infection from this component of the DCU too. The extent of *Legionella* contamination of water supplies of healthcare institutions in Ireland also needs to be investigated in further detail and appropriate action taken in order to avoid further preventable cases of Legionnaires' disease.

This study investigated levels of microbial contamination, including *Legionella* species, in DUW output water of DCUs in Dublin and other parts of the Republic of Ireland and showed that the novel Planmeca Waterline Cleaning System, the first DCU disinfection system custom-made for the disinfection of DUWs, used in conjunction with the disinfectant Sanosil, was an effective means of reducing this microbial contamination to potable drinking water standards. This study also showed that periodic monitoring of the effectiveness of any disinfection regimen should be undertaken, in order to identify inadequate disinfection and the causes for failure. Bacterial contamination of DCU baseplates in the Dublin Dental Hospital was investigated in collaboration with Ms M. O'Donnell and was shown to be as a result of leakage of material from the suction system hosing by serotyping and genotyping isolates of *P. aeruginosa* recovered from the DCU baseplates and various sites within the suction system. As a consequence of this study, a solution for this problem was consequently found following collaboration between members of the clinical microbiology team and the DCU manufacturer, Planmeca. Finally, this study also investigated the bacterial diversity of biofilm within the high volume suction system hoses of Planmeca Prostyle Compact DCUs using 16S rDNA sequence analysis and culture methods in order to assess the potential risk of cross-infection because very few studies have investigated this phenomenon thus far. This study revealed the presence of many oral and other opportunistic bacterial pathogens in the suction system biofilm and highlighted the possibility that other microorganisms such as viruses may also be present, posing a risk of cross-infection to dental patients and staff.

In these ways this study has helped to reduce the potential for cross-contamination and cross-infection in DCUs and has also highlighted the need for further investigation of DCU contamination.

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