UNIVERSITY OF Southampton

Corrigendum

Hutchins, Chloe Frances (2018) **Investigating engineering and human behavioural factors influencing the colonisation of hospital taps with *Pseudomonas aeruginosa***. *University of Southampton, Doctoral Thesis*, 260pp.
https://eprints.soton.ac.uk/429744/

In the above thesis by Hutchins, the following text on page 110 (section 5.3.2.1.1 Culture-based analysis) should be corrected from:

"Nitrile SVs had a median *P. aeruginosa* recovered with 8.3x105 CFU/diaphragm, with recovery ranging from 4.2x103 to 4.8x106 CFU/diaphragm. No significant difference (p=0.33) was observed in *P. aeruginosa* recovered from diaphragms (Figure 5.6)."

to:

"Nitrile SVs had a median *P. aeruginosa* recovered of 5.3x105 CFU/diaphragm, with recovery ranging from 4.2x103 to 6.7x105 CFU/diaphragm. No significant difference (p=0.55) was observed in *P. aeruginosa* recovered from diaphragms (Figure 5.6)."

An error bar on the accompanying graph (Figure 5.6) has also changed. The corrected graph is shown below:

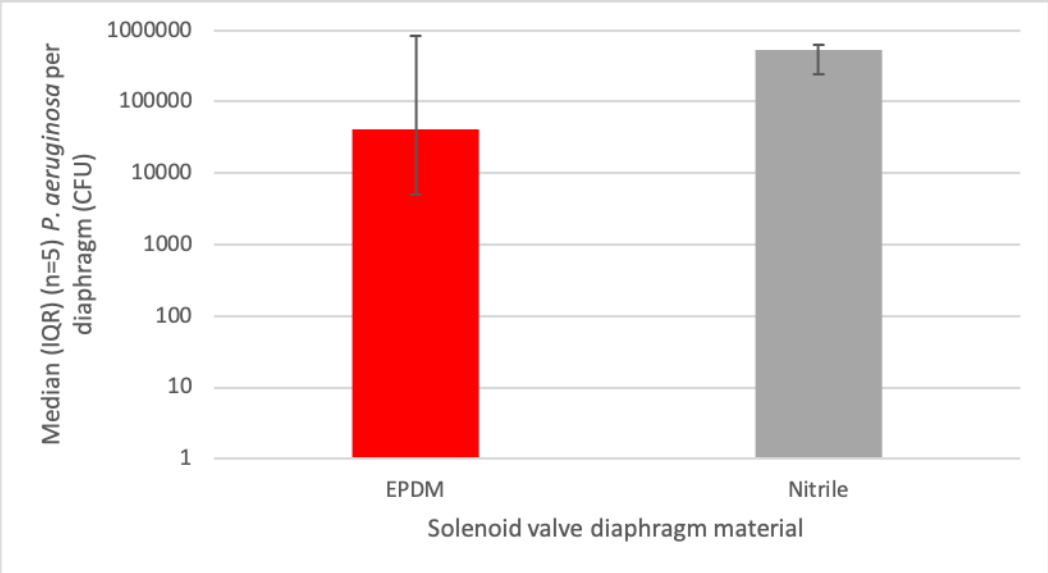

Figure 5.6P. aeruginosa recovered from solenoid valve diaphragms after 12 weeks in the EWDS(n=5). Solenoid valve diaphragms(EPDM(red) and nitrile(black)) were extracted and *P. aeruginosa* recovered.

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UNIVERSITY OF SOUTHAMPTON

FACULTY OF BIOLOGICAL SCIENCES

Institute for Life Sciences

Investigating engineering and human behavioural factors influencing the colonisation of hospital taps with *Pseudomonas aeruginosa*

by

Chloe Frances Hutchins

Thesis for the degree of Doctor of Philosophy

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UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF BIOLOGICAL SCIENCES

Microbiology

Thesis for the degree of Doctor of Philosophy

Investigating engineering and human behavioural factors influencing the colonisation of hospital taps with *Pseudomonas aeruginosa*

Chloe Frances Hutchins

Pseudomonas aeruginosa is an important nosocomial pathogen and its contamination of hospital water poses a threat to immunocompromised patients and is frequently associated with colonised taps. Engineering and human behavioural factors are reported to play a role in tap contamination. Conventional plumbing components and novel alternatives were investigated to determine the role that materials and designs play on *P. aeruginosa* survival and persistence *in vitro (*bioreactor model) and *in situ* (experimental water distribution system (EWDS)). A questionnaire was developed to explore the knowledge, attitudes and beliefs/opinions of hospital staff surrounding water hygiene and their role in maintaining it. A cloth contamination model was developed to investigate behaviour-derived contamination of taps.

Antimicrobial effects were only evident on copper-based materials *in vitro* ($3.1x10^1$-$1.7x10^2$ CFU/material coupon; significantly reduced planktonic survival compared to non-antimicrobial controls (>6-$\log_{(10)}$ reduction (p<0.05)); no antimicrobial effects were observed on silver-impregnated material ($1.5x10^5$ CFU/material coupon; planktonic reduction of <1.3-$\log_{(10)}$ (p>0.05)). Within the EWDS, no significant differences in water- or biofilm-contamination levels were observed between solenoid valves (SVs) using nitrile rubber diaphragms compared to conventional EPDM. Silicone SVs also supported *P. aeruginosa* biofilm and some SVs arrived pre-contaminated with *P. aeruginosa*. Evidence was provided for taps to be flushed pre-use. The cloth-contamination model demonstrated that whilst all outlet fittings (OFs) tested could become contaminated with *P. aeruginosa*, alternative OFs were more effective at reducing contamination over a series of flushes. No significant difference in *P. aeruginosa* survival over 24-hours was observed between conventional OFs (4.4-$\log_{(10)}$ reduction) and alternatives (3.5-5.2-$\log_{(10)}$ reductions). Only 59% of staff surveyed believed that tap water could act as a vector for infection. Staff responses demonstrated varying knowledge of water hygiene, but a willingness to change practices. A standardised, staff-wide water hygiene training programme should be developed. Best practice should, in-turn, allow for effective engineering solutions to reduce *P. aeruginosa* contamination unhindered.

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Academic Thesis: Declaration Of Authorship

I, CHLOE FRANCES HUTCHINS

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Investigating engineering and human behavioural factors influencing the colonisation of hospital taps with *Pseudomonas aeruginosa.*

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as:
8. HUTCHINS, C. F., MOORE, G., THOMPSON, K. A., WEBB, J. & WALKER, J. T. 2017. Contamination of hospital tap water: the survival and persistence of *Pseudomonas aeruginosa* on conventional and 'antimicrobial' outlet fittings. *Journal of Hospital Infection,* 97.

Signed:

Date: 26/02/2018

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The list of names of those who have provided moral support at various stages throughout this PhD is too extensive for this page, however I am grateful to each and every one of them.

Definitions and Abbreviations

=	Equal to
%	Per cent
<	Less than
>	Greater than
±	Plus or minus
≤	Less than or equal to
≥	Greater than or equal to
°C	Degrees Celcius
µg	Microgram
µL	Microlitre
µm	Micrometre
Ag	Silver
$AgNO_3$	Silver nitrate
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
BP	Binder point
$CaCO_3$	Calclium carbonate
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
c-di-GMP	Cyclic di-guanosine monophosphate
cm	centimetre
cm^2	centimetre squared

CN	Cetrimide
CO_2	Carbone dioxide
COSHH	Control of Substances Hazardous to Health
Cu	Copper
$CuCl_2$	Copper chloride
D/E	Dey-Engley
DEFRA	Department for Environment, Food and Rural Affairs
dH_2O	Deionised water
DIPC	Director of infection prevention and control
DNA	Deoxyribonucleic acid
DoH	Department of Health
DPD	Diethyl-p-phenylene diamine
DWI	Drinking Water Inspectorate
e.g.	Exempli gratia
eDNA	Extracellular DNA
EPDM	Ethylene propylene diene monomer
EPS	Extracellular polymeric substances
EWDS	Experimental water distribution system
F1	Flush 1
F2	Flush 2
F3	Flush 3
g	Gram
H_2O	Water

HCAI	Healthcare associated infection
HCU	Heater-cooler unit
HMDS	Hexamethyldisilazane
HNO_2	Nitrous acid
HPA	Health Protection Agency
HPC	Heterotrophic plate count
HRA	Health Research Authority
HSE	Health and Safety Executive
i.e.	Id est
ICP-MS	Inductively coupled plasma mass spectrometry
IQR	Inter-quartile range
IPA	Isopropanol
IRAS	Integrated Research Application System
IRB	Institutional Review Board
KED	Kinetic energy discrimination
L	Litre
LMIC	Lower-middle income countries
$\log_{(10)}$	Logarithm to base 10
MALDI-ToF	Matrix assisted laser desorption/ionization time of flight
MBC	Minimum bactericidal concentration
mg	Milligram
MIC	Minimum inhibitory concentration
mL	Millilitre

mm	millimetre
MoO_3	Molybdenum trioxide
MRSA	Meticillin-resistant *Staphylococcus aureus*
MSSA	Meticillin-susceptible *Staphylococcus aureus*
n	Sample size
n/a	Not applicable
NHS	National Health Service
nm	Nanometre
OD	Optical density
OF	Outlet fitting
OPP	Opportunistic plumbing pathogen
PCR	Polymerise chain reaction
PEX	Cross-linked polyethylene
PHE	Public Health England
PI	Principle investigator
POU	Point-of-use filter
ppb	Parts per billion
ppm	Parts per million
ppt	Parts per trillion
R&D	Research and development
ROS	Reactive oxygen species
rpm	Revolutions per minute
S1	Sample 1
S2	Sample 2

S3	Sample 3
SD	Standard deviation
SEM	Scanning electron microscopy
SNP	Single nucleotide polymorphism
SSI	Surgical site infection
SV	Solenoid valve
T_0	Time nought
TMV	Thermostatic mixing valve
TSA	Tryptone Soya agar
TVC	Total viable counts
UK	United Kingdom
UNICEF	United Nations International Children's Emergency Fund
UTI	Urinary tract infection
UV	Ultraviolet
v/v	Volume/volume
VAP	Ventilator-associated pneumonia
VBNC	Viable but non-culturable
VNTR	Variable number tandem repeat
vs.	Versus
w/v	Weight/volume
WGS	Whole genome sequencing
WHO	World Health Organization
WRAS	Water Regulations Advisory Scheme

WSG	Water safety group
X^2	Chi-squared

Chapter 1 Literature review

1.1 Healthcare associated infections

1.1.1 Overview

Healthcare associated infections (HCAIs) are infections that occur as a result of healthcare provided in either the hospital or community environment (e.g. care homes). Such infections can be as a direct result of a procedure and/or aftercare, or from exposure to the healthcare environment. The most common HCAIs are surgical site infections (SSIs) and urinary tract infections (UTIs), with surveillance data demonstrating variable prevalence depending upon the year, ward and location (Cotter *et al.*, 2012, Lahsaeizadeh *et al.*, 2008, Smyth *et al.*, 2008, Emori and Gaynes, 1993), however gastrointestinal disease and respiratory tract infections have also been reported as major HCAIs (Smyth *et al.*, 2008, Liu *et al.*, 2016). It is important to acknowledge that figures and statistics on HCAI prevalence are constantly changing (updated), which can lead to some contradiction in the literature. SSIs have been reported to account for ~15% of HCAIs, with 1,632 SSIs reported in England between 2015 and 2016 (Smyth and Emmerson, 2000, Public Health England, 2016). Data collected in England between 2013 and 2016 indicates that the most common causative organism for SSIs is *Staphylococcus aureus*, responsible for 32% of cases, followed by Enterobacteriacaea, causing 26% (Public Health England, 2016). *Pseudomonas* was the third most common genus, responsible for 5% of SSIs.

UTIs have been reported to account for 19% of HCAIs and are frequently catheter-associated (43-56% of cases) (Cotter *et al.*, 2012, Loveday *et al.*, 2014). UTIs are most commonly caused by *Escherichia coli* (Foxman, 2014), which has been reported to cause >80% of UTIs (Stamm and Hooton, 1993), however other common organisms include *Klebsiella* spp. and *Pseudomonas aeruginosa,* the latter particularly associated with catheter associated UTI (Ronald, 2002).

HCAIs can also arise as a result of exposure to the healthcare environment as opposed to medical intervention, leading to infections such as nosocomial gastroenteritis (Lopman *et al.*, 2004) or waterborne opportunistic infections from exposure to water in healthcare facilities (Stout *et al.*, 2007). Common causative agents of nosocomial gastroenteritis include *Clotridium difficile,* norovirus and rotavirus at variable prevalence across epidemiological studies (Lopman *et al.*, 2004, Polage *et al.*, 2012, Garey *et al.*, 2006). HCAIs caused by exposure to the hospital environment can occur not just in patients but in staff and visitors also (Public Health England, 2015, HO *et al.*, 2003).

In the UK, mandatory surveillance (conducted by Public Health England (PHE)) for HCAIs is conducted primarily using four types of infection: Meticillin-resistant *Staphylococcus aureus* (MRSA) bacteraemia, Meticillin-susceptible *Staphylococcus aureus* (MSSA) bacteraemia and *E. coli* bacteraemia, as well as *C. difficile* infections (Public Health England, 2017a, Public Health England, 2017g, Public Health England, 2017h, Public Health England, 2017b). In 2011, the prevalence of HCAIs in acute wards in the UK was 6.4%, of which, 32.4% were caused by Enterobacteriaceae, 12.6% by *C. difficile*, 9.1% by MSSA, and 2.4% by MRSA (Health Protection Agency, 2012). Since then, cases of MRSA bacteraemia and *C. difficile* have decreased by 26.3% and 28.8% respectively (Public Health England, 2017g, Public Health England, 2017a). In contrast, *E. coli* and MSSA bacteraemias have increased over the last six years by 26.2% (to 40,580 cases) and 31.0% (to 11,486 cases) respectively (Public Health England, 2017h, Public Health England, 2017b). Currently, case fatality rates of the mandatory reported infections range between 15.1% (*C. difficile*) and 29.4% (MRSA) (Public Health England, 2017j) and it has been estimated that HCAIs cost the NHS approximately £900 million per year (Plowman *et al.*, 2001).

Several other HCAI data sets, based on voluntary reporting, are collated by PHE. These include surgical site infections (voluntary for surgical procedures other than hip and knee replacements, reduction of long bone fractures and neck and femur repairs, which are mandatory) (Public Health England, 2016) and other bacteraemias caused by organisms such as (non- *E. coli*) Enterobacteriaceae *(*e.g. *Klebsiella* spp.) (Public Health England, 2017d).

1.1.2 Waterborne HCAIs and biofilm

1.1.2.1 UK water quality

In the UK, water suppliers must legally provide water free from microorganisms at concentrations which would "constitute a potential danger to human health" with the focus being on gastrointestinal disease (Drinking Water Inspectorate, 2016). In England and Wales, the drinking water is provided by public water supplies, which are thoroughly regulated by the Drinking Water Inspectorate (DWI), and private companies, who are answerable to local authorities. Despite the relatively high quality of UK water, there have still been outbreaks of waterborne diseases attributed to drinking water supplies: between 1992 and 2003 there were 24 and 25 outbreaks from public- and private water supplies respectively (Smith *et al.*, 2006). In 2002 alone, there were seven individual outbreaks implicating drinking water as the source (causative organisms not stated) (World Health Organization, 2009b). Between 1993 and 2013, the DWI led 49 prosecutions and gave 27 cautions to public water suppliers. The majority of cautions (23/27) and prosecutions (40/49) were associated with organoleptic issues, i.e. discolouration, taste or odour

problems. However, seven of the 49 prosecutions and 2/27 cautions were for issues relating to cross contamination of the supply (with sewage), inadequate disinfection or consumer disease (cryptosporidiosis) (Drinking Water Inspectorate, 2014, Drinking Water Inspectorate, 2017b, Drinking Water Inspectorate, 2017a).

1.1.2.2 Important waterborne opportunistic pathogens

Water is an important vector for infection, which has the potential to directly lead to infection by contact, ingestion or inhalation (Braeye *et al.*, 2015, Petrini, 2006, Bauer *et al.*, 2008), or indirectly lead to infection by introducing organisms to medical devices, for example, endotracheal tubes for ventilators, which if exposed to water, can lead to device contamination and/or colonisation and patient infection when the device is implemented (Exner *et al.*, 2005). "Opportunistic premise plumbing pathogens," ((OPPs) e.g. *Pseudomonas* spp., *Legionella* spp. and non-tuberculous *mycobacteria* (Falkinham *et al.*, 2015b)) can impact public health through contamination and colonisation of domestic-based medical devices such as contact lenses (Kilvington *et al.*, 2004) and lead to pneumonias such as Legionnaires disease through aerosol production during showering (Collins *et al.*, 2017) and the use of items such as humidifiers (Hahné *et al.*, 2002) and spa pools (Coetzee *et al.*, 2012, Phin *et al.*, 2014). Whilst there is a legal requirement for premise owners to control for certain OPPs, particularly *Legionella* spp. (Health and Safety Executive, 2017a), there is no such requirement to reduce the concentration of other OPPs. *Legionella* spp. can cause Pontiac fever, non-pneumonic legionellosis and, more commonly, pneumonia. *Legionella* pneumonia, also known as Legionnaires' disease, is a notifiable disease; there is a statutory obligation for practitioners/healthcare laboratories to inform local authorities of cases, who in turn inform PHE (Public Health England, 2017i). In the UK the primary cause of legionellosis (any disease caused by *Legionella* spp.) is *L. pneumophila*. Legionnaires' disease occurs when *Legionella* are inhaled as aerosols or contaminated water is aspirated. There were 345 cases of Legionnaire's disease in residents of England and Wales in 2016, of which 42% were associated with travel abroad and 1.7% were nosocomial (Public Health England, 2017f).

In a healthcare setting, the OPPs, many of which are not traditionally considered as waterborne pathogens (i.e. by causing disease via ingestion) (Falkinham *et al.*, 2015a), pose a serious risk to immunocompromised patients (World Health Organization, 2011) and as such hospitals must take extra precautions. This is especially true of the most susceptible patients, such as those in haematology-oncology units, where patients undergo immunosuppressive treatments and/or have reduced white blood cell counts to combat otherwise non-pathogenic organisms (Urrea *et al.*, 2004).

A major waterborne pathogen, responsible for at least 6% of all HCAIs, is *P. aeruginosa* (Health Protection Agency, 2012) which commonly infects cystic fibrosis (CF) sufferers (Bjarnsholt *et al.*, 2009, Henry *et al.*, 1992) and burns victims (Turner *et al.*, 2014, Markley *et al.*, 1957).

As an opportunistic pathogen, *P. aeruginosa* also poses a threat to other immunocompromised patients including neonates (Cipolla *et al.*, 2011), chemotherapy patients (Ohmagari *et al.*, 2005) and acquired immune deficiency syndrome (AIDS) sufferers (Dropulic *et al.*, 1995). In haematology- oncology patients, *P. aeruginosa* pneumonia and septicaemia has been reported to have high morbidity and mortality rates, with blood stream infections leading to a 67% mortality rate in some incidences (Ciofi degli Atti *et al.*, 2014) and environmental sources (contaminated hospital hand wash stations) implicated (Gillespie *et al.*, 2000, Engelhart *et al.*, 2002). Recently, there has been an increase in reports of *P. aeruginosa* bacteraemia cases, with an 11.1% rise to 3,553 *P. aeruginosa* seen between 2012 and 2016 (Public Health England, 2017e), however it is unclear whether this is reflective of increased reporting or increased incidence. *P. aeruginosa* reporting in the UK has been for bacteraemia cases only and, until September 2017, on a voluntary basis.

1.1.2.3 Biofilm formation

Waterborne opportunistic pathogens are not only a threat in their planktonic state (free floating), as delivered by the water supply, but also when constituting a biofilm. Biofilm is a term given to a mixed or exclusive population of sessile cells enveloped as a community in a matrix of extracellular polymeric substances (EPS), often attached to a surface (Yang *et al.*, 2011). Bacterial existence within the environment and *in vivo* commonly occurs in the form of biofilm and can be a major obstacle for treating infection. Biofilm also causes problems in preventing/minimising contamination in healthcare and industrial settings (such as the food processing industry) (Kumar and Anand, 1998, Lindsay and von Holy, 2006).

There a five key stages to biofilm formation. The initial stage is reversible attachment to a surface through proximity and electrostatic interactions (dependent both upon the number and type of bonds formed (covalent, hydrogen etc.) (Berne *et al.*, 2015)) (Figure 1.1 (1)). Following initial attachment, irreversible attachment takes place, whereby adhesins (structures on the bacterial cell surface), either specific (usually for targeting host cells (Gaastra and de Graaf, 1982)) or non-specifc (more typical for abiotic surfaces) (Berne *et al.*, 2015), anchor cells to a surface (Figure 1.1 (2)). Once adhered to a surface, microcolonies form (i.e. colonies consisting of ~50 cells (Zhao *et al.*, 2013)), which produce EPS (maturation stage-1) (Figure 1.1 (3)). Major constituents of EPS include exopolysaccharides, extracellular DNA (eDNA) and extracellular proteins (Flemming and Wingender, 2010). The protection provided to cells within a biofilm from exogenous stresses is

mostly afforded by the EPS. The biofilm structure continues to develop until it reaches maturation stage-2 (Figure 1.1. (4)). Biofilms can be architecturally complex and the appearance and structure of biofilm varies depending on the type of exopolysaccharide that is present and dominant in the EPS (Stoodley *et al.*, 2002b). Finally, triggered by endogenous (cell-cell signalling) or exogenous (environmental triggers such as nutrient availability) stimuli, previously sessile biofilm cells convert to planktonic status and disperse from the biofilm (Figure 1.1 (5)) (Petrova and Sauer, 2016).

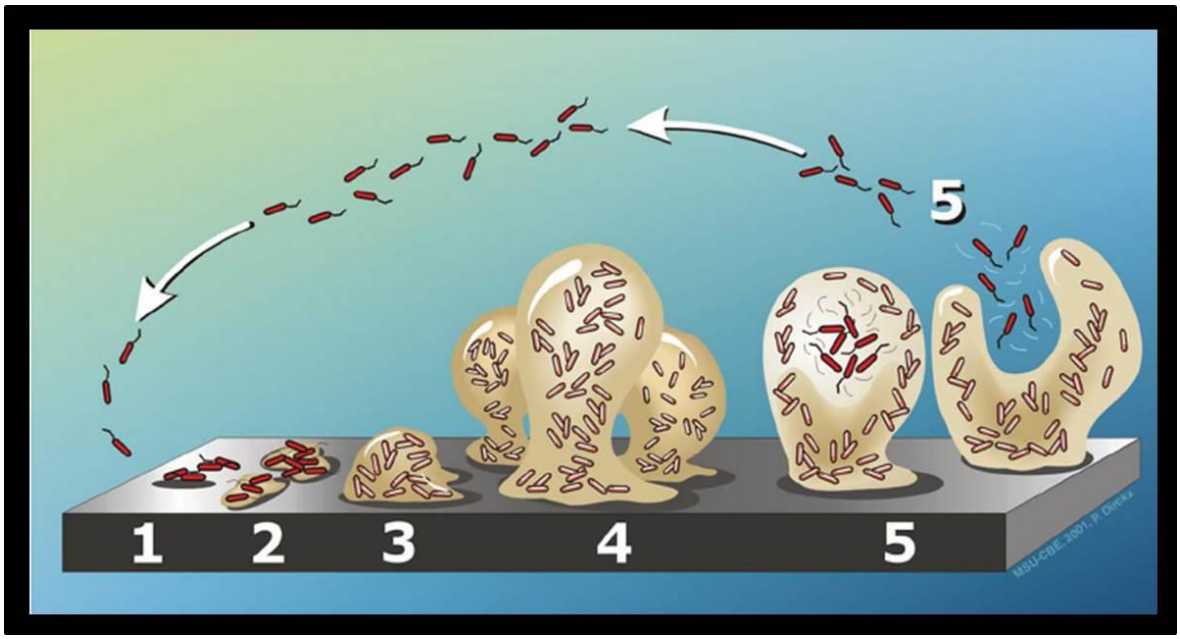


Figure 1.1 Biofilm life cycle stages. 1) initial attachment to a surface; 2) irreversible attachment; 3) microcolony formation and the production of EPS; 4) development of a complex biofilm structure; 5) dispersal of cells from the biofilm. *(Figure reprinted (Stoodley et al., 2002b))*

The final stage, dispersal, is a key process in the spread of bacteria, and can occur in several ways, including the detachment of planktonic cells, sloughing of bacterial aggregates or movement of entire biofilm including EPS (Hall-Stoodley *et al.*, 2004). Dispersal of cells from a biofilm can be problematic in many environments, leading to infection *in vivo*, contamination of food during production or water within distribution systems (Kaplan, 2010, Stoodley *et al.*, 2002a). Within a hospital drinking water distribution system, the newly planktonic cells are able to contaminate water used for patient care. Depending on the circumstances (immune status of those exposed, bacterial species and strain virulence), such dispersal has to potential to lead to infection. Triggers for dispersal range from induction by shear force, lysis triggered by exogenous sources such as bacteriophage (Hall-Stoodley *et al.*, 2004) and controlled subpopulation lysis in response to environmental stimuli (McDougald *et al.*, 2012). One environmental trigger is temperature, with bacteria shown to disperse from biofilm to reach a temperature more favourable for growth along

a gradient (from 32°C to 36°C) (Kaplan and Fine, 2002). Increased nutrient availability is another environmental stimulus and the extent of dispersal can depend upon carbon source, with glucose shown to lead to lower dispersal of *P. aeruginosa* biofilm (54% of the total biomass dispersed) than succinate (~80%) (Sauer *et al.*, 2004). Dispersal can also occur as a consequence of starvation, due to the resultant low levels of cyclic di-guanosine monophosphate (c-di-GMP; an intracellular second messenger that decreases cell motility and associated with biofilm formation (Ma *et al.*, 2011)) (Schleheck *et al.*, 2009). Both temperature and nutrient availability fluctuate within drinking water distribution systems, influencing not only biofilm dispersal but also the microbiota colonising the plumbing and regrowth of biofilms after sloughing events caused by shear stress (flushing) (Douterelo *et al.*, 2016).

1.1.2.4 *P. aeruginosa* biofilm

P. aeruginosa is a widely studied model organism for biofilm (O'Toole *et al.*, 2000, Heydorn *et al.*, 2000) and there are three major exopolysaccharides produced by *P. aeruginosa*: alginate, Psl and Pel (Franklin *et al.*, 2011). Overproduction of alginate leads to a mucoid phenotype which has been associated with the viscosity of EPS (Tielen *et al.*, 2005), linked to antibiotic tolerance (Alkawash *et al.*, 2006) and poor patient prognosis (Mayer-Hamblett *et al.*, 2014). It is thought that the acquired *P. aeruginosa* infection tends to be non-mucoid, converting to a mucoid phenotype *in vivo* (Govan and Deretic, 1996), a notion supported by the relatively effective clearance of early infections through use of antibiotics (Taccetti *et al.*, 2012, Frederiksen *et al.*, 1997). Conversion to a mucoid phenotype has been associated with mutations in *mucA*, a gene within the operon regulating alginate production (Mathee *et al.*, 1999). *mucA* mutations can be induced by stresses found *in vivo* such as reactive oxygen species (ROS), which are produced by neutrophils and macrophages and lead to cell membrane, protein and DNA damage (Ezraty *et al.*, 2017). Increased alginate production resulting from *mucA* mutations can provide protection against ROS by reducing penetration and scavenging oxygen radicals (Bylund *et al.*, 2006, Cochran *et al.*, 2000).

Psl has been hypothesised to play a role in encouraging planktonic cells and cells attached to surfaces to join existing biofilm. Motile *P. aeruginosa* secrete a Psl 'trail' on surfaces, which is followed by exploring bacteria, leading to localisation of new cells at a central aggregation and/or encouraging microcolony formation along the way (Ma *et al.*, 2009, Zhao *et al.*, 2013). Rapid microcolony formation is a trait that has been associated with successful competitors in multispecies biofilms (Rao *et al.*, 2005).

The role of Pel is the least well understood of the three major polysaccharides, with contradictory reports of its role in attachment (Colvin *et al.*, 2012, Cooley *et al.*, 2013). More recently, it has

been reported that Pel has an important structural role in mature biofilms, binding eDNA to the base of the biofilm (Jennings *et al.*, 2015). eDNA is released by lysed cells, a controlled process to maintain the structure of the matrix (Whitchurch *et al.*, 2002, Flemming and Wingender, 2010). The dominance of eDNA within the EPS varies depending upon species and strains, but is a major constituent of *P. aeruginosa* biofilms (Flemming and Wingender, 2010, Yang *et al.*, 2007).

1.1.2.5 Guidance on *P. aeruginosa* control in the healthcare environment

Originally isolated from wound dressings, *P. aeruginosa* was first described in 1882 and has been associated with the healthcare environment ever since (Gessard, 1882, Florman and Schifrin, 1950, Kinsey *et al.*, 2017, Moore and Forman, 1966). Despite this, *P. aeruginosa* control within water distribution systems did not become a priority for the NHS until 2013 (Department of Health, 2013b). In contrast, a major waterborne pathogen to cause non-gastrointestinal disease that has received a lot of attention over the last few decades is *Legionella* spp.. In the UK the control of *Legionella* spp. is a duty under the Health and Safety at Work Act (1974) and covered by Control of Substances Hazardous to Health (COSHH) Regulations (2002) legislation (Health and Safety Executive, 2017b, Health and Safety Executive, 2017a). As such, there are several guidance documents, codes of practice and British Standards based around controlling *Legionella* spp. in buildings, particularly hospitals (British Standards Institution, 2006, Department of Health, 2013a).

The 2006 Department of Health (DoH) guidance for water hygiene in healthcare premises (HTM 04-01) focused on the control measures for *Legionella* spp.. However, in proceeding years it became apparent that this guidance did not translate to other waterborne opportunistic premise pathogens after a series of high profile incidents of *P. aeruginosa* infection, so an addendum was produced to cover the control of *P. aeruginosa* in augmented care (Department of Health, 2013b).

The *P. aeruginosa* incidents that triggered the DoH addendum occurred in Northern Ireland, during the winter of 2011/2012 (Troop, 2012). One of the affected wards was the Belfast maternity hospital neonatal unit. This *P. aeruginosa* incident saw 25 babies infected, of whom four died (Walker *et al.*, 2014), with at least two cause of deaths reported as *P. aeruginosa* infection (Troop, 2012). Water sampling of the taps on the unit revealed that five out of six sampled taps were positive for the same strain of *P. aeruginosa* as that isolated from the patients. Results from destructive microbiological analysis of the taps identified the thermostatic mixing valves and solenoid valves as the components most heavily contaminated with waterborne organisms. The outlet fittings (devices that straighten and regulate the flow of water from the tap at point of use) were associated with the highest *P. aeruginosa* counts (Walker *et al.*, 2014). At

the same time (December 2011), another hospital in Northern Ireland declared an incident of *P. aeruginosa.* Strain typing revealed that isolates recovered from tap components were the same as those from water samples and patient samples, but different to those associated with the Belfast incident. A positive outcome resulting from these incidents was the publication of the addendum to the HTM 04-01 regarding the control of *P. aeruginosa* in augmented care (Department of Health, 2013b). However, despite the additional guidance and as previously stated, reported incidence of *P. aeruginosa* bacteraemia across England, Wales and Northern Ireland has increased by 11.1% since 2012 (Public Health England, 2017e).

1.1.2.6 Other OPPs of growing concern

Besides *P. aeruginosa* and *Legionella* spp., there are several other waterborne pathogens of growing concern, one of which is *Stenotrophomonas maltophilia.* Cases of *S. maltophilia* bacteraemia are reported on a voluntary basis and in 2016 there were at least 478 cases of *S. maltophilia* (482 *Stenotrophomonas* spp.) bacteraemia in England, Wales and Northern Ireland, a 4% increase since 2012 (Public Health England, 2017e). Although lower in incidence than *P. aeruginosa* bacteraemia, *S. maltophilia* infections should not be underestimated. Studies have demonstrated average mortality rates of between 37% and 40% (Tseng *et al.*, 2009, Falagas *et al.*, 2009), with one hospital reporting a mortality rate of 69% over a 2-year period (Jang *et al.*, 1992).

S. maltophilia has been known to co-infect with *P. aeruginosa*, especially in CF patients (Kollef *et al.*, 1995). *P. aeruginosa* and *S. maltophilia* are not only found as co-contaminants of human lungs; they have also been shown to co-exist in multi-species biofilm under laminar and turbulent flow models *in vitro* (Stoodley *et al.*, 1999), which could be representative of varying flows within hospital water distribution systems.

Another emerging Gram-negative opportunist is *Acinetobacter* spp., for which data is collected on a voluntary basis. There were 965 reported bacteraemias of *Acinetobacter* spp. in 2016, a 30% increase since 2012 (Public Health England, 2017c). The primary causative agents of reported bacteraemias between 2012-2016 have consistently been *A. lwoffii (*34-38% of cases) and *A. baumannii (*19-22% of cases). As such, *A. lwoffii* has been dubbed as an 'emerging pathogen' in immunocompromised patients including neonates (Mittal *et al.*, 2015). Like *P. aeruginosa, Acinetobacter'*s ability to survive on dry surfaces for extended periods under controlled conditions has been demonstrated (Wendt *et al.*, 1997, Kramer *et al.*, 2006), with some *Acinetobacter* strains maintaining counts of ~10^7 CFU over several months. Isolates from water sources have confirmed *Acinetobacter*'s abundant nature in the environment, with counts of up to 10^3 CFU/ mL in pre-treated surface water and 10^2 CFU/mL in treated potable water (Narciso-da-Rocha *et al.*, 2013). *Acinetobacter*'s ability to colonise, survive and persist on a variety of surfaces, from

medical devices to pillow feathers, highlights the versatile opportunistic pathogen's risk to augmented care environments (Weernink *et al.*, 1995, García-Garmendia *et al.*, 2001).

Water has been recognised as a source of pathogenic nontuberculous mycobacteria for many years (Goslee and Wolinsky, 1976, Falkinham 3rd, 1996). Nontuberculous mycobacteria are slow growing organisms which can be widespread throughout hospital water distribution systems and can be commonly found in water samples taken from clinical taps (Shin *et al.*, 2007). Nontuberculous mycobacteria have a high tolerance to disinfection procedures, including chlorination, after which competition for nutrients from chlorine-sensitive organisms is reduced (Falkinham, 2016). Nontuberculous mycobacteria infection most commonly manifests as pneumonia (Donohue *et al.*, 2015). Wallace Jr *et al.* (2002) report that, certain patients, particularly those with nodular bronchiectasis (widened and mucus-filled bronchioles, such as those found in CF patients or patients who have previously had serious pulmonary illnesses such as tuberculosis) can have recurrent nontuberculous mycobacteria pneumonia despite successful treatment; relapses are unusual and repeated pneumonia is usually caused by new infections. In 2004, a new species of nontuberculous mycobacteria was described (Tortoli *et al.*, 2004), *M. chimaera*, and it was retrospectively discovered that *M. chimaera* had been misidentified as other nontuberculous mycobacteria such as *M. intracellularae* (Schweickert *et al.*, 2008). The clinical significance of *M. chimaera* has been and is still debated; recent investigations have found that patients who have undergone cardiac surgery have had a delayed onset of *M. chimaera* surgical site infection, with an incubation period ranging from three months to six years (Chand *et al.*, 2016) with a mortality rate of 50% (van Ingen *et al.*, 2017).

1.1.3 Biofilm and device-related waterborne HCAIs

1.1.3.1 Heater-cooler units

Machines and devices requiring water for proper function can lead to waterborne HCAIs. Design flaws or maintenance issues can lead to patient exposure to potential pathogens if such devices have been filled using non-sterile water, especially if there has been biofilm formation within the device. If there is a failure in the machinery, such as inappropriate water backflow or generation and subsequent release of aerosols, patients (and staff) can be exposed to the waterborne organisms (Hedge *et al.*, 2017, Rao *et al.*, 2009). Investigations into the increase in *M. chimaera* post-cardiac surgery infection were carried out on a global scale, including UK, Switzerland, the Netherlands, Germany, Australia and the US, and it was concluded that the source of infections was the heater-cooler units (HCUs) used during cardiopulmonary bypass. HCUs are used for extracorporal circulation (and oxygenation) and maintaining temperature of the blood and body.

The devices rely upon water reservoirs and a series of plastic piping, some of which led to stagnation and biofilm that in certain units was visible by eye (Walker *et al.*, 2017, van Ingen *et al.*, 2017). The mode of transmission for *M. chimaera* from the water within HCU devices to the surgical sites was through aerosolisation. Flaws in design, such as gaps and holes, in combination with a cooling fan, were able to create and release aerosols into the operating theatres (Chand *et al.*, 2016). Recent studies used whole genome sequencing to link strains from both patients and HCU water samples across Europe to a common source, which has been postulated as the production site of the units (van Ingen *et al.*, 2017, Hedge *et al.*, 2017).

1.1.3.2 Ventilator-associated pneumonia

A similar risk is posed by reusable medical devices, particularly those that are washed with water between usages; one such example is bronchoscopes. Bronchoscopes are devices used to visualise the airways via fibre optic cables (Rath *et al.*, 1973). Although bronchoscopes undergo disinfection procedures including the use of various chemicals including succinic dialdehyde and isopropanol, there have been reports of post-sterilisation rinsing with tap water, and some cleaning procedures involve water-based washing techniques (washer-disinfectors) (Gubler *et al.*, 1992, DiazGranados *et al.*, 2009, Brooks *et al.*, 2004). Bronchoscope contamination has been associated with contaminated tap water used within washer-disinfectors (Schelenz and French, 2000), leading to bronchoscopes acting as a fomites for *P. aeruginosa*, *L. pneumophila , S. maltophilia* and *Acinetobacter* spp. (Schelenz and French, 2000, Schuetz *et al.*, 2009, Brooks *et al.*, 2004, Behnia *et al.*, 2010). The use of bronchoscopes requires the patient to be artificially ventilated, and as such any infections that result from artificial ventilation of the patient are termed 'ventilator associated pneumonias' (VAPs) (Safdar *et al.*, 2005, Chadha *et al.*, 2015). VAPs could come about in a number of ways, including washing the patient, contaminated nebulised medication, and as discussed, contamination of reusable equipment (Safdar *et al.*, 2005). Endotracheal tubing for ventilation has also been linked to infection (Feldman *et al.*, 1999, Smith and Howland, 1959). A study by Gil-Perotin *et al.* (1999) found biofilm on 95% (n=75) of endotracheal tubes from patients, with 28% and 23% colonised with *A. baumannii* and *P. aeruginosa.* Not all endotracheal tube colonisation is due to exogenous sources; the patient's microbiota can use the surface of the tube to colonise and form biofilm, later leading to infection (Inglis *et al.*, 1989). However, manually washing endotracheal tubes by scrubbing in tap water has been reported as standard practice, with some methods including a final rinse with tap water after chemical disinfection (presumably to remove residual chemicals) (Leonhard *et al.*, 2016). The implication of staff scrubbing biofilm and patient fluids from tubes using tap water is that this practice occurs at, or in proximity to, a tap and is therefore likely to introduce organisms present on the medical device to, at the very least, the sink basin and drain. Although this kind of

procedure should not be done at hand wash stations, staff compliance in use of sluices and misuse of hand wash stations have both been reported and linked to infection, and can result in colonisation of the tap itself (Balm *et al.*, 2013, Reuter *et al.*, 2002). Balm *et al.* (2013) demonstrated that hand wash stations that had been exposed to misuse had a significant correlation with colonisation of the tap outlet fittings. Where colonisation of taps and tap assemblies leads to contaminated hospital tap water, the water poses a risk to patients, including a risk of VAP, which is especially apparent in cases of nosocomial ventilator-associated legionellosis; *Legionella* is not part of the human microbiome (Divan Khosroshahi *et al.*, 2015, Muder *et al.*, 1989).

1.1.3.3 Catheter-associated urinary tract infection

Urinary catheters are devices that allow draining of patient urine from the bladder via the urethra via tubing and are commonly used in instances of prolonged surgical procedures, obstruction of the bladder or urinary retention and/or where patients are immobilised (Meddings *et al.*, 2013). When catheters are in place, standard healthcare procedures continue, including personal care, which can expose the catheter to tap water and its microbial contents directly (for example, whilst bathing) or indirectly (via staff hands) (Ferroni *et al.*, 1998). A study of nosocomial infections in the United States, incorporating 181,993 patients between 1992 and 1997, found that 95% of UTIs were associated with urinary catheters, with the three major causative organisms as *Candida albicans*, *E. coli* and *P. aeruginosa* (Richards *et al.*, 1999). A report of catheter associated *P. aeruginosa* UTIs identified the source of *P. aeruginosa* as the tap water, noting the contamination of sink traps and outlet fittings (Ferroni *et al.*, 1998). Sterilisation of the catheter prior to insertion has been found to be ineffective at reducing rates of UTIs, however the disinfection of the periuthreal area prior to catheter insertion is common practice, aimed to minimise risk of contamination by microorganisms endogenous to the patient (Schiøtz, 1996, Webster *et al.*, 2001). A study by Webster *et al.* (2001) demonstrated that cleaning with antiseptic (chlorhexidine wipes) was no more effective at minimising UTIs than cleaning with soap and tap water in short term catheterisation (<24 hours) (n=436). Webster's study did not isolate *P. aeruginosa* from patients using tap water (n=1 *P. aeruginosa* isolate from patients using chlorhexidine) however a limitation of this study was that the organisms isolated were only from patients who developed bacteraemia (n=38). The authors could not find justification for using sterile water, deciding that, as sterile water did not have antimicrobial effects, tap water was just as suitable, and also concluded that use of the antiseptic should be abandoned, a policy which was put into place at the hospital involved in Webster's study. Both of Webster's conclusions fail to recognise the risk of contamination of the catheter by organisms exogenous to the patient.

Within the healthcare environment, there is a plethora of niches for *P. aeruginosa* and other waterborne opportunistic pathogens, with problem areas including urinary catheters (Nickel *et al.*, 1985a), intravascular catheters and attachments (Apisarnthanarak *et al.*, 2012), ventilator equipment (Planquette *et al.*, 2013, El Solh *et al.*, 2008, Kollef *et al.*, 2014), cleaning equipment such as spray bottles and cloths (Sifuentes *et al.*, 2013, Department of Health, 2012), spa pools (Hollyoak *et al.*, 1995, Moore *et al.*, 2015a) and even soap dispensers (Lanini *et al.*, 2011). However, some of the highest risk environments acting as reservoirs of *P. aeruginosa* include patient showers (Kerr and Snelling, 2009), clinical taps and hand-wash basins (Department of Health, 2013b, Blanc *et al.*, 2004).

1.1.4 Hand wash stations as a reservoir for infection

Where tap water has been implicated in infection, subsequent investigations often conclude that the source of contamination is biofilm colonising the tap and/or tap components as opposed to the water supply itself being highly contaminated (Blanc *et al.*, 2004). Although genotypic comparison of environmental and clinical isolates can help to determine whether the strain causing the infection is endogenous or exogenous (to the patient) in origin, the route of transmission is not always clear. Blanc *et al.* (2004) carried out genomic analysis of 132 clinical *P. aeruginosa* isolates; 42% of patient isolates could be linked to those recovered from biofilm swabs taken during the dismantling of hospital taps. However, as no water samples were positive for *P. aeruginosa,* patient acquisition was not be explained. Weber *et al.* (1999) readily recovered *S. maltophilia* from biofilm colonising tap outlet fittings, yet, the organism could only be detected in water samples through concentration (filtration) of large volumes (10 L). Nonetheless, within four days of the outlet fittings being replaced, one was again positive for *S. maltophilia,* suggesting that the low level of contamination within the water was sufficient to result in bacterial attachment. However, laboratory studies have shown minimal attachment of *P. aeruginosa* to outlet fitting despite continual exposure to highly contaminated water (Moore *et al.*, 2015b). It seems likely that in Weber's study, the outlet fitting became colonised via a source exogenous to the water system (retrograde contamination). A study by Reuter *et al.* (2002) demonstrated transmission of organisms from the ward environment and/or patient to the tap as well as from the tap to the patient. Reuter suggested that in both directions of transmission, staff hands and particular staff behaviours could act as vehicles for transmission. One example provided was staff disposing of contaminated fluids down the hand wash stations, from which splashing could lead to contamination of the entire sink and outlet fitting. Reuter supported the hypothesis that staff influenced retrograde contamination by investigating patient and tap water isolates from transferred patients (from one hospital ward to another). Strain types from immobilised patients

(i.e. unable to make contact with the taps) were isolated from the taps of their new ward post-transfer. Staff hands have also been associated with the transmission of *P. aeruginosa* (Widmer *et al.*, 1993, Döring *et al.*, 1996, Zawacki *et al.*, 2015).

It has also been proposed that cloth contamination could play a role in retrograde contamination. Engelhart *et al.* (2002) investigated a *P. aeruginosa* outbreak in a haematology-oncology unit and recovered multiple strains from patients and the environment. Genetically identical isolates were recovered from patients, taps and cleaning equipment, primarily cleaning cloths but also cleaning solutions, mops and drains. Original direction of transmission was not determined (i.e. whether the cloths were contaminated by tap water/biofilm and further spread contamination, or whether a cloth was involved in a cross-contamination event on the ward and introduced patient-sourced *P. aeruginosa* to the taps). Regardless, Engelhart's study highlighted the risk of cleaning cloths propagating *P. aeruginosa*.

The risk of retrograde contamination was recognised by the DoH and raised in the addendum, stating that, "where the origin is due to retrograde contamination by patients and/or staff, other local management control measures *[unspecified]* and monitoring will be necessary" (Department of Health, 2016a). Retrograde contamination has also been inferred through recovery of antimicrobial resistant strains of *P. aeruginosa* from tap water*;* these strains have been exposed to a broader range of antibiotics in the clinical environment than isolates found in non-clinical environments or in community-acquired *P. aeruginosa* (Paterson, 2006). The lack of distinction between environmental and clinical strains in healthcare settings can leave a chicken and egg scenario of what came first: a contaminated water supply or a retrograde contamination event? Often retrograde contamination can only be hypothesised (Vonberg *et al.*, 2005b, Berthelot *et al.*, 2001). Similar epidemiological problems have occurred in determining whether healthcare workers' hands that are contaminated with the same strain as an infected patient are the cause of patient infection or contaminated as a result of patient infection (Widmer *et al.*, 1993). However, from the evidence available, it seems likely that retrograde contamination of taps is associated with human behaviour (e.g. the misuse of a hand wash station or unknowingly using contaminated cleaning equipment (Balm *et al.*, 2013, Inglis *et al.*, 2010, Hutchins *et al.*, 2017, Engelhart *et al.*, 2002)). Several studies have investigated compliance in hospital staff with hand hygiene protocols (O'Donoghue *et al.*, 2016, Duggan *et al.*, 2008), however their attitudes towards water hygiene, perception of risk and behaviour around taps has not been fully investigated.

It has been concluded that the cause of waterborne HCAIs is multifactorial, with non-sterile potable water, susceptible individuals, and a lapse in infection control practices listed as contributory factors (Williams *et al.*, 2013a). However, materials used within water systems and

plumbing components have been shown to facilitate microbial attachment and their role in HCAI should not be underestimated. Other than outlet fittings, there are several other tap components that have been shown to harbour biofilm, including the tap body (spout) (Pottage *et al.*, 2012), Thermostatic mixing valves (Quick *et al.*, 2014), which are responsible for mixing water to a pre-determined temperature (to minimise the risk of scalding) and solenoid valves (Kilb *et al.*, 2003), which are required for the operation of automatic (sensor) taps.

Installation of automatic taps within unchlorinated drinking water distribution systems in Germany resulted in the number of coliform bacteria present in the water increasing to above the legal limit (i.e. to ≥1 CFU/100 mL) (Kilb *et al.*, 2003). The diaphragms associated with the solenoid valves were composed of either ethylene propylene diene monomer (EPDM) or nitrile rubber, both of which were heavily contaminated, but significantly higher number of bacteria were recovered from the EPDM (n=14, median counts of 1×10^8 CFU/cm^2) than nitrile solenoid valves (n=6, median counts of 2.5×10^7 CFU/cm^2). Other studies investigating the colonisation of EPDM and nitrile rubbers have found no significant differences in terms of bacterial attachment and/or biofilm formation (Suárez *et al.*, 1992). However, rubbers have been found to facilitate higher rates of colonisation than other materials used in plumbing such as copper (often used for piping), cross linked polyethylene (PEX; often used as a flexible plastic hose such as those used for detachable shower heads (Brûlet *et al.*, 2008)) and stainless steel (an alternative pipe material to copper) (Waines *et al.*, 2011).

As discussed, HCAIs can cause serious complications in patients, including death, despite often being preventable. A study investigating cases of HCAIs in the United States concluded that evidence-based interventions (e.g. hand hygiene) could have led to a reduction of up to 75% in catheter-associated infections and 55% in VAP cases (Umscheid *et al.*, 2015). Since the incidents in Northern Ireland, there has been a drive by the clinical tap industry to improve designs and incorporate antimicrobial materials into plumbing products to try to reduce HCAIs by minimising bacterial colonisation and/or survival. However, evidence of the efficacy of these products is lacking. Occasionally incorporation of substances with proven antimicrobial properties, such as metallic ions (e.g. copper (Arijit Kumar *et al.*, 2014) and silver (Chernousova and Epple, 2013)), is relied upon to justify marketing new designs as an antimicrobial product. There is no requirement for these products to be tested using standard methodology to prove claims of antimicrobial efficacy. In contrast, there are standards to ensure that materials and designs do not promote the growth of organisms; if a fitting will carry or receive water that has been sourced from the public mains supply, it is statutory for the product to comply with The Water Supply Regulations (Anonymous, 2010). Products and materials in contact with a drinking water distribution system

must undergo British Standards testing to determine whether they could have an impact on water quality.

1.2 Assessment of suitability of materials and products used in drinking water distribution systems

1.2.1 Water Regulations Advisory Scheme and testing of materials

The British Standards Institute has a series of tests, the BS 6920 (Table 1.1), that are used to determine whether non-metallic materials used in plumbing components/fittings will affect the flavour or appearance of water, whether materials leach toxic metals or substances harmful to human health and whether the materials promote microbial growth (British Standards Institution, 2014d, British Standards Institution, 2014f, British Standards Institution, 2014e, British Standards Institution, 2014a). A commonly sought after and renowned certification of suitable materials and products is that awarded by the Water Regulations Advisory Scheme (WRAS), which is advised by the Environment Agency (Department for Environment Food and Rural Affairs, 2002). WRAS certification is recognised across the UK water supply industry and is confirmation of whether a product or material has been tested against and 'passed' the British Standards Institute BS 6920. WRAS approval lasts for a maximum of five years and approval is non-transferable to similar or rebranded products. WRAS does not carry out the testing itself but does offer an independently organised BS 6920 test report analysis, allowing inspectors and customers to easily identify whether a product or material complies with The Water Supply Regulations. If so, the product or material requires no further investigation.

Table 1.1 British Standards Institute BS 6920 tests for determining suitability of non-metallic materials and products for use in contact with water intended for human consumption with regard to their effect on the quality of the water.

Test no.	Test description	Principle/method	Reference (s)
2.2	Odour and flavours imparted to water by hoses and pipes.	Hoses/pipes are filled with test water for 24 and 72 hours. If no discernible odour is detected, the water is tasted to assess flavour. Tests are conducted by panellists.	BS 6920- 2.2.2: 2000+A1:2014 (British Standards Institution, 2014b)

Test no.	Test description	Principle/method	Reference (s)
			BS 6920- 2.2.3: 2000+A2:2014 (British Standards Institution, 2014c)
2.3	Appearance of water.	Product is immersed in water for 24 and 72 hours. Water colour and turbidity are measured.	BS 6920- 2.3: 2000+A1:2014 (British Standards Institution, 2014d)
2.4	Growth of aquatic microorganisms.	Product is immersed in water and inoculated with tap water containing 'naturally occurring aquatic organisms' (>10 CFU/100 mL presumptive coliforms; >1 CFU/100 mL presumptive pseudomonads). Water is changed every 3-4 days and growth determined by measurement of dissolved oxygen depletion prior to water change. Tests are carried out over seven weeks. Depleted oxygen levels of >1.69 mg/L indicate an unsuitable level of microbial growth.	BS 6920- 2.4: 2000+ A1:2014 (British Standards Institution, 2014a)
2.5	The extraction of substances that may be of concern to public health.	Product is immersed in water. After 24 hours a mammalian cell line is exposed to the water and incubated. Cell morphology is examined microscopically.	BS 6920- 2.5: 2000+ A2:2014 (British Standards Institution, 2014e)

Test no.	Test description	Principle/method	Reference (s)
2.6	The extraction of metals.	Product is immersed in water for 24 and 72 hours. Detection methods vary depending upon the metal being tested for.	BS 6920- 2.6: 2000+A2:2014 (British Standards Institution, 2014f) BS 6068-2 (British Standards Institution)
3	High temperature tests.	Product is immersed in water at temperatures of 30-85°C and/or 100°C. Odour, flavour and appearance of the water is assessed (as above) as is the extraction of metals and other substances that may be of public health concern.	BS 6920- 3: 2000 (British Standards Insititution, 2000)
4	Identification of leachable organic substances.	Product is immersed in water for 24, 48 and 72 hours. Gas chromatography mass spectrometry is used to detect (unspecified) organic compounds.	BS 6920- 4: 2001 (British Standards Institution, 2001)

To gain WRAS certification, a product for use in contact with potable tap water must comply with the test requirements stipulated by BS 6920. However, WRAS certification itself is not a legal requirement and manufacturers can choose not to apply. Some, for example, consider WRAS accreditation an unnecessary expense, particularly if the product has not changed during the five years that WRAS certification is valid. Despite this, it is often assumed that products without WRAS certification have failed to achieve WRAS approval. In contrast, if an application results in a product failing to achieve WRAS approval, it will have also failed the BS 6920 and is therefore unsuitable for use.

Nevertheless, WRAS certification is not fool-proof. Counterfeited products are not necessarily composed of the same materials as the originals, but are manufactured to appear the same, so could be mistaken for a WRAS approved product. If a counterfeit material does not perform to the standard of the original, its use within a product or component could result in the manufacturer

of the final product suffering financial and/or reputational damage. Counterfeiting of products intended for maintenance of public health can have serious implications. In the pharmaceutical industry, substandard medications are sold as legitimate products (Almuzaini *et al.*, 2013), leading to ineffective treatment of disease. It has been reported that fake anti-malarials result in approximately 450,000 preventable deaths per year (Karunamoorthi, 2014). The counterfeiting of products with WRAS approval is a cause for concern for 'original' manufacturers as, should a product be associated with contamination or implicated in an infection, proof of origin is difficult.

Within water distribution systems, water quality can be influenced by the leaching of inorganic and organic materials from piping. Lehtola *et al.* (2004) demonstrated that polyethylene pipes release phosphorus (low levels of which can act as a limiting factor for biofilm formation) and assimilable organic carbon (the fraction of dissolved carbon that is most readily taken up and used by bacteria (Ma *et al.*, 2012)). In comparison to the source water, there were higher levels of both phosphorous and assimilable organic carbon in water exposed to polyethylene piping. Microbially available phosphorous levels increased during the first 20 days, whilst the level of assimilable organic carbon increased on the first day and remained constant. Similarly, copper ions have been shown to leach from copper piping. Lehtola monitored the presence of copper in water over time and measured a copper concentration four times that of the source water during the first 76 days, which increased to 10-times that of the source water for the remainder of the 308-day experiment. The antimicrobial properties of copper are well documented (Arijit Kumar *et al.*, 2014, Grass *et al.*, 2011) and it is tempting to think of the leaching of copper as beneficial to microbial control. However, whilst biofilm is initially slower to form on copper piping, longer term (200 days) colonisation of copper is no different to that of polyethylene pipes (Lehtola *et al.*, 2004). It has long been recognised that corrosion of copper piping facilitates biofilm formation, thus hospitals cannot rely upon the natural antimicrobial properties of their historical (or newly replaced) pipework to act as a microbial control strategy (Arens *et al.*, 1995, Walker *et al.*, 1991).

One of the chemical properties of water that varies across the UK is hardness, or calcium carbonate ($CaCO_3$) concentration. The effect of water hardness on bacterial abundance in water systems is variable between and across species, with negative correlations reported for *Mycobacteria* and variable effects on *Legionella (Kusnetsov et al., 2003, Borella et al., 2005).* However, some studies have reported that disinfection strategies (e.g. ultraviolet (UV) irradiation, chlorine) are more effective when used to treat hard water than artificially softened water (Rand *et al.*, 2013). Nonetheless, results are inconsistent, with variations arising from the type of material colonised and whether UV decontamination was carried out before or after chlorine treatment (Rand *et al.*, 2013).

One of the notable peculiarities of the 2011/2012 Northern Ireland *P. aeruginosa* incidents was the visible biofilm and debris built up in the outlet fittings, which were reported to have been in place for only four months, however after two years of testing in the EWDS at PHE, no obvious soiling other than lime scale was visibly present (Moore *et al.*, 2015b). After replacing the taps with UV taps, Belfast neonatal unit still had water samples returning positive for *P. aeruginosa,* and these outlets were consequently dismantled after only a few months of installation and sent to PHE for analysis. The report produced noted the visible soiling and rust around various components (Walker, 2012). These findings raise the question of whether the biochemical composition of Belfast water could have played a role in facilitating *P. aeruginosa* contamination and biofilm formation (in addition to retrograde contamination factors). The BS 6920 does not take into consideration chemical composition of regional waters and the impact this might have on the materials tested, and relies upon tap water local to the testing laboratory.

The test for growth of aquatic organisms in the BS 6920 (Table 1.1) relies upon depleted oxygen measurements to determine whether there has been an inappropriate amount of microbial growth and is also carried out without any shear flow conditions that would be found in a plumbing system. Within a biofilm, there is an oxygen gradient whereby cells furthest from the surface survive in hypoxic or anoxic conditions (De Beer *et al.*, 1994b, Peters *et al.*, 1987). Reduction of metabolic activity due to oxygen concentrations can lead to advantages such as increased antibiotic tolerance (Walters *et al.*, 2003). In addition, alternative metabolites such as nitrates can be used for respiration instead of oxygen (Dietrich *et al.*, 2013).

Thus, within a biofilm, some organisms will not be able to access oxygen and/or will have entered a low metabolic state. Oxygen depletion, therefore, should not be considered directly proportional to the number of organisms present. A reason for using depleted oxygen as an indicator is that this method removes the chance of missing microorganisms as a result of not using appropriate agar for plate counts (British Standards Institution, 2014a). The use of 16S qPCR would provide a quantitative evaluation of the microbial DNA present (Salter *et al.*, 2014). Alternatively, the water could be inoculated with a known level of marker organisms, thus appropriate media could be selected in advance to detect growth.

There are several materials that have passed the BS 6920 and achieved WRAS accreditation that have also been shown to facilitate biofilm formation at rates substantially higher than other materials, such as EPDM and PEX (Waines *et al.*, 2011, Moritz *et al.*, 2010). The notion held within healthcare and manufacturing environments that ‘WRAS approval = safe’ should be challenged and microbial testing methods that rely upon counts would provide a more realistic indication of

microbial levels facilitated by the material/product that may be underestimated by reduced oxygen consumption in biofilm cells.

1.3 Methods of studying biofilm development

There are numerous methods by which bacterial biofilm can be investigated *in vitro*, allowing the investigation of biofilm development under static and flow conditions, as well as in the presence of thermal-, chemical- and/or shear stress. There are also multiple quantitative and qualitative approaches to analyse biofilms developed *in vitro* or *in situ*.

1.3.1 Investigating biofilm under static conditions

Investigating biofilm under static conditions with a single batch of media allows for isolation of single factors that may influence biofilm formation. Microtitre plate assays therefore allow controlled investigations into biofilm formation across multiple strains, growth conditions or the influence of antimicrobial presence. This method, first described in 1977 (Fletcher, 1977), is commonly used with modifications across the literature; the basic principle is adding inoculum suspended in a medium that will support growth of the organism into wells of a flat-bottomed microtitre plate, allowing the plate to incubate and biofilm to form around the surfaces of the wells (Merritt *et al.*, 2005). Planktonic or loosely attached cells are washed away and the remaining cells (representative of biofilm) are stained, typically with crystal violet, then the strain is resolubilised and quantified by measuring optical density (Figure 1.2).

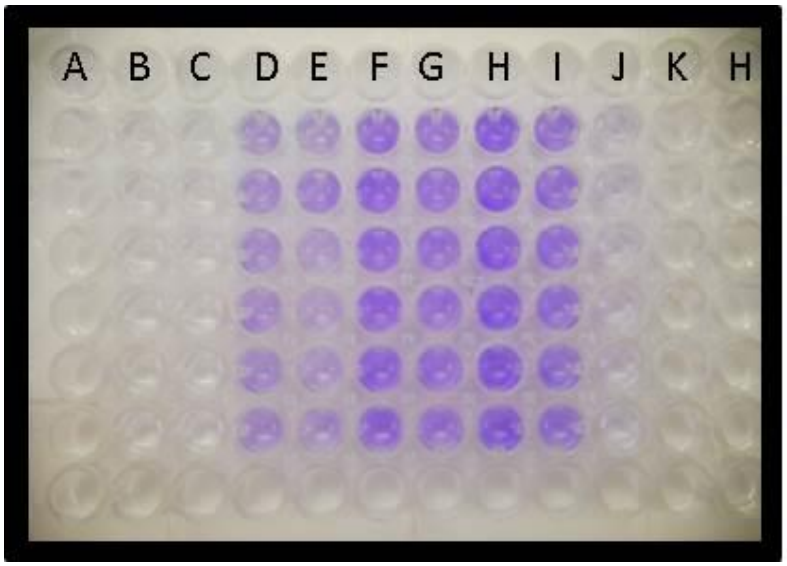


Figure 1.2 Photograph of a static biofilm assay using a flat-bottomed microtitre plate, stained with crystal violet. Lid removed from a 96-well plate, showing resolubilised crystal violet used to stain biofilm (darker staining implies greater biofilm levels, which is

confirmed by absorbency readings). Columns D-J containing different isolates of *P. aeruginosa* and columns B-C are negative controls (media without inoculum). Columns A, K and H, as well as the top and bottom rows of the plate are empty.

A popular modification to this assay is the inclusion of a microtitre lid onto which with pegs are fixed. Biofilm forms on the peg surfaces, removing the requirement for efficient washing steps (to exclude/remove non-sessile cells) for quantification. The pegged lids can be relocated to a new microtitre plate which is used for biofilm recovery stages (Harrison *et al.*, 2010). Quantification of biofilm from the pegs requires removal of the cells by agitation, a method which does not guarantee an accurate representation of the cells present in the biofilm due to inefficiencies in recovery (Azeredo *et al.*, 2017). Whilst not an accurate representation of biofilm formation conditions in real-life (due to protection from fluctuations in environmental conditions) microtitre assays are considered a reliable, effective and relatively cheap/easy method for biofilm investigation (Waters *et al.*, 2014).

For qualitative analysis of static biofilm, microscopy techniques can used. An easy way to visualise biofilm (without requiring a microscope stage capable of holding a microtitre plate) is through the addition of a surface on which biofilm can develop, e.g. glass slides. Inoculated media (enough to cover the slides) is incubated and the slides removed, gently rinsed and stained with dyes such as crystal violet (Prouty *et al.*, 2002). The stained biofilm on these glass slides can be visualised by microscopy. Again, this will not lead to biofilm representative of those found *in situ*, especially where there are fluid dynamics, such as biofilm found in pipework or aquatic environments.

1.3.2 Investigating biofilm under flow conditions

Biofilm development under conditions that exhibit shear force, representative of those that may be found in the environment, particularly the aquatic environment, can be carried out in either batch or continuous flow phases. The major difference between the two conditions is the introduction of fresh media. Under batch conditions no period of stagnation occurs, and shear forces can be maintained through rotors and stir plates as opposed to the current caused by media flow (Wand *et al.*, 2012). The media is not replaced throughout the experiment, however, additives can be made, such as the introduction of antimicrobials after biofilm formation has occurred (i.e. several days after the beginning of the experiment) to determine the effect on controlled, pre-established biofilm under non-static conditions (Touzel *et al.*, 2015). In contrast, continuous flow systems (chemostats) supply fresh media consistently over the course of the assay (Figure 1.3). Chemostats can be used to study planktonic cultures including evolutionary adaptations over time (Ziv *et al.*, 2013), but can also be designed to investigate biofilm formation,

such as flow cell systems. For biofilm investigations, the continuous flow of media is typically initiated after a period of stagnation to allow for bacterial attachment/initial biofilm development (Sternberg and Tolker-Nielsen, 2005, Labbate *et al.*, 2004). Continuous flow culture methods have been used to represent biofilms formed under stagnant conditions, such as storage tanks, which are later exposed to shear forces, perhaps from the turbulence caused by emptying and refilling the storage tank (Rogers *et al.*, 1994, Zurigat *et al.*, 1988).

Some technologies, such as flow chambers coupled with digital time lapse microscopy, allow for imaging of the biofilm over the course of its development without extraction or disruption of the chamber (Tolker-Nielsen and Sternberg, 2011). There is a risk of bubble formation along the system, which can lead to disruption of biofilm with severe impact on its architecture; bubble formation can be mediated by the inclusion of a bubble trap, however the potential for leakage may require gluing of the apparatus (Crusz *et al.*, 2012).

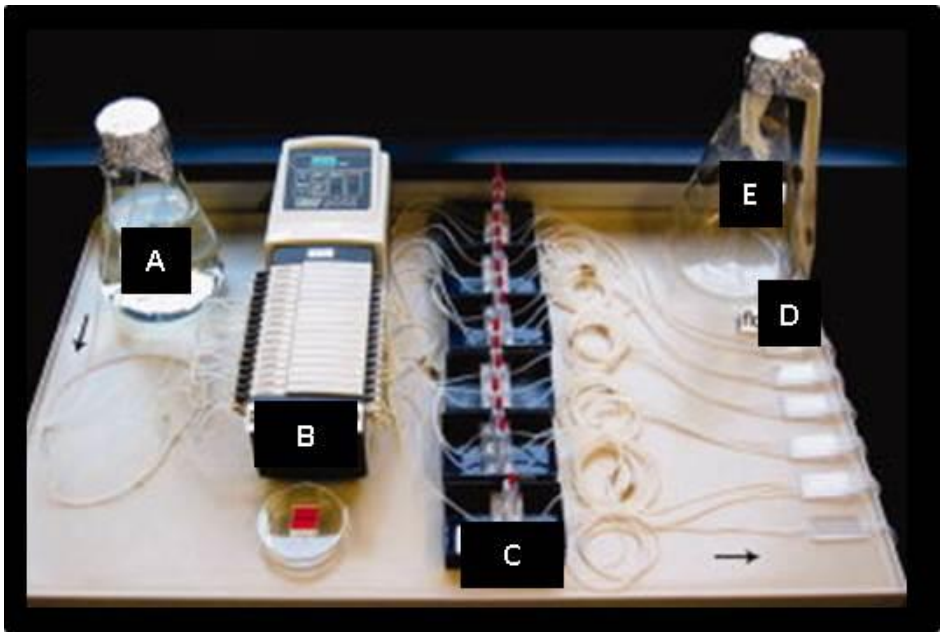


Figure 1.3 Flow cell apparatus. Photograph of the experimental set up of a flow cell chemostat. A: Influent media; B: peristaltic pump to ensure media flow; C: bubble traps; D: flow cell chamber (i.e. point of biofilm formation/cultivation); E: effluent. Figure adapted from (Pamp *et al.*, 2009).

Cover slips used within the flow cell chambers can be analysed using different microscopic techniques. Qualitative analysis, such as biofilm architecture and distribution, can be investigated through confocal and scanning electron microscopy (Wood *et al.*, 2000, Lawrence *et al.*, 2003). Fluorescence microscopy allows for semi quantitative determination of biofilm viability through use of live/dead staining kits such as those incorporating SYTO9 and propidium iodide (SYTO9 staining all cells and propidium iodide staining only cells with permeable membranes (i.e. dead

cells)) (Nagant *et al.*, 2013). The flat and level nature of glass coverslips allows for clear 2D micrographs which allows for percentage coverage (of biofilm on the surface material) calculation.

An advantage of using batch-flow culture is that the biofilm is developed in conditions that produce shear force, as opposed to a stagnation period prior to introduction of shear forces, as with continuous culture methods (Rogers *et al.*, 1994). Additionally, systems that allow for the insertion of material coupons (as opposed to glass cover slips) offer a wider opportunity for investigation of material surface role on biofilm formation.

Quantification of biofilm can be carried out via culturing or molecular methods. The recovery of microorganisms from biofilm via culture is a commonly used method that does not depend upon high-performing equipment, and is relatively inexpensive to conduct (Williams *et al.*, 2013b). Some of the limitations of culture include the risk of not recovering all organisms if using selective media, and on non-selective media, slow growing organisms may be outcompeted (Verhagen *et al.*, 2011, World Health Organization, 2003). This is less problematic if investigating a monospecies biofilm, however culture will not detect cells that have entered a dormant or viable but nonculturable (VBNC) state, which have been reported within biofilms (Azeredo *et al.*, 2017, Pasquaroli *et al.*, 2013). Options such as polymerase chain reactions (PCR) will detect DNA from all cells present, dead or alive, and 16S analysis for multispecies biofilms will provide qualitative data on organisms present (Lyautey *et al.*, 2005).

1.4 Biofilm and water decontamination strategies

1.4.1 Biocides and biofilm

P. aeruginosa possesses various mechanisms of antimicrobial tolerance and antibiotic resistance, including efflux pumps (transport proteins involved in the removal of noxious substances from within the cell) and a low outer membrane permeability (i.e. membrane pores available for hydrophilic-antibiotic entry to the cell). The outer membrane of *P. aeruginosa* is up to 100-fold less permeable than other Gram-negative organisms such as *E. coli* (Angus *et al.*, 1982, Livermore, 2002). As such, very few patients are able to eradicate pulmonary infection despite aggressive antibiotic therapy (Frederiksen *et al.*, 1997) and hospitals struggle to completely clear their plumbing of contamination (Garvey *et al.*, 2016c). In addition to the inherent antimicrobial resistance of *P*. *aeruginosa*, biofilm formation also offers protection from antimicrobials, causing chronic infection and contamination. Cells within a biofilm are more tolerant to drugs that are otherwise bacteriostatic or bactericidal to the cells in planktonic state (prior to biofilm formation

and as dispersed cells) (Nickel *et al.*, 1985b). Tolerance can be inferred by limited metabolic activity (Walters *et al.*, 2003), physiological adaptions as a result of stress responses independent of antimicrobial presence, such as in response to starvation (Nguyen *et al.*, 2011) and limited penetration into the biofilm due to the EPS (Tseng *et al.*, 2013).

P. aeruginosa biofilms also show tolerance to non-antibiotic antimicrobials such as those used for disinfection and treatment of hospital water systems and surfaces. Chlorine causes damage to the cell membrane, and it has been proposed that intracellular disruption could also be responsible for its bactericidal effects (Virto *et al.*, 2005). However, the tolerance of both active and dormant *P. aeruginosa* cells to chlorine has been demonstrated (Kim *et al.*, 2009) and when treated with chlorine, only ≤20% of available chlorine penetrated a mixed *P. aeruginosa* and *Klebsiella pneumoniae* the biofilm (De Beer *et al.*, 1994a).

It is likely that cells at the bottom of a biofilm, that have reduced access to oxygen and nutrients, will be prone to entering a state of dormancy, which has been shown to alter antimicrobial tolerance (Kim *et al.*, 2009). Additionally, the natural occurrence of persister cells, that are not necessarily nutritionally challenged and do not have any genetic advantages over the rest of their antimicrobial sensitive community, also contributes to overall biofilm antimicrobial tolerance (Lewis, 2005). Lewis (2005) suggests that the persisters in the biofilm community could also re-establish the biofilm population once the levels of antimicrobial are reduced (Lewis, 2010).

1.4.2 Water system disinfection measures

1.4.2.1 Chlorine and chlorine dioxide

Commonly used chemicals for decontamination of water systems include chlorine and chlorine dioxide (Collivignarelli *et al.*, 2017). Chlorine dioxide, like chlorine, is able to induce intracellular disruption including protein denaturation (Ogata, 2007), but also causes stronger oxidative stress, damaging the cell membrane more effectively than chlorine (Cho *et al.*, 2010). Chlorination is a common control for *Legionella* and can be effective when successfully administered (i.e. concentrations reach and are sustained at target levels) (Srinivasan *et al.*, 2003) and/or when combined with secondary measures such as thermal disinfection procedures (i.e. increasing the temperature of the water) (Zhang *et al.*, 2007). Srinivasan *et al.* (2003) installed a chlorine dioxide water treatment system to control *Legionella,* and found over a 17-month period that the proportion of taps tested (n=28) eluting contaminated water reduced from 41% to 4%, noting that the only tap with contaminated water at the end of the testing period was located furthest away from the treatment source. Concentrations of chlorine dioxide were lower the further from the source, which could in part explain the microbial results. However, distal points in the plumbing

system are not the only areas that can struggle to maintain appropriate biocide concentrations. Chlorination concentrations in hospital water tanks can also drop and lead to microbial proliferation (Walker *et al.*, 1995, Kool *et al.*, 1999). Additionally, there are reports of recolonisation hospital water systems within a few months of 'successful' heat and chlorination treatment (Liu *et al.*, 1995), highlighting the need for frequent monitoring of biocide levels both at the source and distal points.

Other than the increased tolerance to chlorination displayed by cells surviving in a biofilm state, not all tolerance is due to lack of penetration or low metabolic state. It has long been reported that microorganisms isolated from chlorinated water display resistance to higher concentrations of chlorine than their non-exposed equivalents (Ridgway and Olson, 1982). As such, there is the potential to simply select for organisms with higher tolerance to chlorination rather than decontaminating the water system. Additional control measures are required to target organisms that survive source water decontamination regimen.

1.4.2.2 Ultraviolet radiation treatment

UV radiation has long been recognised as an effective method for killing bacteria, protozoa (Hijnen *et al.*, 2006b), fungi (Rotem *et al.*, 1985, Levetin *et al.*, 2001), and even inactivating viruses (Chang *et al.*, 1985, Harris *et al.*, 1987, Gerba *et al.*, 2002). The wavelength of UV light ranges from 100-400 nm and is characterised as UVA (320-390 nm), B (280-320 nm) and C (200-280 nm) (Wellmann, 1983). The absorption of UV light by DNA bases and their subsequent excitation leads to mutagenic effects and oxidative damage (Cadet *et al.*, 2005), the extent of which depends on the UV wavelength (UV A, B or C). UV C (at 254nm) induces DNA damage at a rate 1000-fold higher than UVB (Yakobson *et al.*, 1989, Hijnen *et al.*, 2006b). As a water treatment, UV radiation has been attractive option due primarily to its lack of by-products, unlike those associated with chlorination, e.g. trihalomethanes, the presence of which in drinking water has been associated with cancers (Hijnen *et al.*, 2006a, Zoeteman *et al.*, 1982). UV radiation is effective against protozoan contaminants such as *Giardia* and *Cryptosporidium (Craik et al., 2001, Craik et al., 2000)* and as such is used to disinfect public water supplies to reduce the chances of outbreaks (Widerström *et al.*, 2014). UV radiation can also be installed at individual water outlets, sometimes dubbed "UV taps". Gerba (2015) investigated the presence of *P. aeruginosa* in a range of taps (in clinical and non-clinical areas) across four hospitals (Gerba, 2015). Of the water samples collected (n=112), 22% of non-UV taps (18/82) were positive for *P. aeruginosa.* No taps with a UV device in place (n=30) delivered water contaminated with *P. aeruginosa*. UV taps can achieve a 7-$\log_{(10)}$ reduction against *P. aeruginosa* in tap water (Parks, 2012).

However, UV taps installed on the Belfast neonatal unit after the 2011/2012 incidents continued to dispense *P. aeruginosa* positive water samples (Walker, 2012). Deconstruction and microbiological analysis of these taps revealed bacterial growth at connection points of the spout and, although no *P. aeruginosa* was recovered, there was a clear niche for the organism to colonise. The inability of UV to reach all niches, nooks and crannies in which biofilm can develop was also noted by Liu *et al.* (1995) who concluded that UV light installed on water outlets at point-of-use is not sufficient to prevent *Legionella* colonisation and that UV should be complementary to a chlorination regimen rather than a replacement. Additionally, the presence of limescale and/or other deposits on the UV lamp reduces UV transmission to the water and, therefore, the efficiency of disinfection (Duke *et al.*, 1996, Liu *et al.*, 1995).

There is also evidence that different strains of *P. aeruginosa* may differ in terms of their sensitivity to UV radiation and that some may be resistant to low levels of UV radiation (Abshire and Dunton, 1981). A study by Elasri and Miller (1999) showed that biofilm formation by *P. aeruginosa* offered cells protection against UV damage. It was observed that alginate-heavy biofilms only allowed between 13-33% of the UV radiation to penetrate the biofilm, effectively shielding cells encased in the matrix. This work has two important implications for hospitals: the first is that if there were any fault in the UV radiation system at point-of-use, such as a reduction in or UV lamp failure, this would offer an opportunity for dispersed *P. aeruginosa* to form biofilm at the outlet in any weak points, such as the connection points previously highlighted as a microbial niche. If the UV lamp were further back in the plumbing (i.e. not at point-of-use), the UV radiation would be too far back in the system to make a difference to distal contamination (such as retrograde contamination of the outlet fittings/spout). A second implication is that UV radiation may be less effective within a clinical environment if the predominant strains of *P. aeruginosa* produce alginate and readily form biofilm; such strains would not be as susceptible to sterilisation by UV light.

Therefore, it should be acknowledged that although UV treatment of aquatic micro-organisms capable of causing opportunistic infection has been shown to be effective, as with all disinfection measures, it is not infallible. Autoclaving and cleaning components of UV taps (Parks, 2012) should be strictly adhered to, as well as complementation by additional control measures such as chlorination (Liu *et al.*, 1995).

1.4.2.3 Copper-silver ionisation

Copper-silver ionisation is a method that allows release of copper and silver into the water from electrodes (Stout and Yu, 2003b). Silver ions have a bactericidal effect on *P. aeruginosa* and a wide variety of other microorganisms (Davies and Etris, 1997). However, the concentrations

reported to be effective (minimum inhibitory/bactericidal concentrations) vary throughout the literature (0.1 ppm (Chernousova and Epple, 2013)- 50 ppm (Sintubin *et al.*, 2011)) and as such is likely to be strain dependent (Salmon and Watts, 2000). Silver ions can bind to the thiol groups of enzymes and proteins, denaturing them (Feng *et al.*, 2001), leading to disruption of intracellular processes and cell death. Silver ions also bind to DNA, causing it to condense (Feng *et al.*, 2001), thus inhibiting DNA replication, which concludes in cell death (Park *et al.*, 2009). Another mechanism by which silver ions can damage DNA (as well as many other intracellular components) is bonding to the thiol groups of enzymes within the respiratory pathway, creating reactive oxygen species (ROS) (Park *et al.*, 2009).

The antimicrobial properties of copper are well documented (Grass *et al.*, 2011, Yu-sen *et al.*, 1998, Zevenhuizen *et al.*, 1979), as is the rising resistance against copper ions (Santo *et al.*, 2008, Mergeay *et al.*, 1978, Sarma *et al.*, 2010). Copper ions disrupt cells through the same mechanisms as silver ions, including thiol group bonding (Kusnetsov *et al.*, 2001), ROS production and inhibition of DNA replication (Arijit Kumar *et al.*, 2014).

Treating hospital water systems with silver and copper ions can reduce microbial colonisation within the system (Stout *et al.*, 1998, Rohr *et al.*, 1999, Liu *et al.*, 1994), but due to microbial resistance, silver and copper ion treatment should not be the sole measure of antimicrobial control in hospital water distribution systems. Resistance mechanisms for silver and copper are similar, and have been linked to upregulation of ion binding proteins and efflux pumps, removing potentially toxic ions from the cell and reducing access to the cytoplasm (Wu *et al.*, 2007, Cha and Cooksey, 1991, Franke *et al.*, 2003). Several hospitals, within the first year of installing copper-silver ion treatment systems, have reported to have reduced *Legionella spp.* contamination. However, after several years of treatment, outlets can become positive again, due, it is postulated, to the development of tolerance or resistance. Release of suboptimal concentrations of silver/copper ions has also been suggested (Rohr *et al.*, 1999, Stout and Yu, 2003a).

1.4.2.4 Point of use filters

When water supply disinfection measures have failed, hospitals can use point-of-use filters to provide filter-sterilised water (Health and Safety Executive, 2000). Point-of-use filters are detachable heads that can be mounted to the end of a spout, filtering through pores of 0.2 µm. These have been shown to reduce heterotrophic plate count bacteria by more than 99% (Sheffer *et al.*, 2005). Filters are often 'single use', generally with a lifespan of 30-60 days, and should be used as a temporary measure in hospitals whilst remediation of the underlying contamination is carried out or until the cessation of an outbreak. Permanent use of point-of-use filters could conceal underlying problems within the water system and/or infection control procedures.

"Single use" filters should not be replaced if removed for cleaning or flushing of the tap due to risks of retrograde contmaination (Department of Health, 2013b); contamination of point-of-use filters with *P. aeruginosa* has been reported, with a strain identical to a clinical isolate from a patient located in the tap's proximity (Garvey *et al.*, 2016b). Filters can cause practical problems in a healthcare setting by reducing the activity space (area between the water outlet and basin), as well as reducing water flow rate (Walker and Moore, 2014). Another important reason for filters to be seen only as a temporary measure is one of cost. Filters are expensive, at roughly £50 per filter, and are an additional (as opposed to replacement) cost to the routine disinfection procedures (Garvey *et al.*, 2016b). Furthermore, longevity can depend on the quality of water; hospitals in hard water areas have reported that their filters 'clog up', so require more frequent replacement, leading to increased costs. Nonetheless, the effectiveness of 0.2 µm filters has led to their recommendation in the DoH guidance (2013) for controlling *P. aeruginosa* or other pathogenic colonisation of taps (Department of Health, 2013b).

1.5 Hospital staff and their role in water safety

Responsibility of water hygiene management, as recommended by DoH guidance, primarily falls upon Water Safety Groups (WSGs) (Department of Health, 2016a). It is recommended that the WSG create a water safety plan, which should be an holistic approach to maintaining water hygiene on the premises, something that can be achieved by inclusion of multi-disciplinary representatives within the WSG. Various staff groups have interactions with water outlets and thus have influence on water hygiene. As such, the experience required for an WSG include personnel with a high level of infection control knowledge, those who have an understanding of water application around the most susceptible patients, and those who maintain the plumbing system and outlets. The listed traits can be found in medical staff, particularly the director of infection prevention and control (DIPC), nursing staff (and augmented care nursing staff), estates staff who are responsible for plumbing and domestic (housekeeping) staff who maintain the condition of the taps through cleaning and disinfection. Additional staff who would benefit a WSG as recommended by the newly updated DoH guidance (2016) include microbiologists and independent advisors (Department of Health, 2016a). Some of the challenges in forming a WSG include deciding who to chair the group; this is undoubtedly a large responsibility and guidance recommends it falls to persons with, 'management responsibility, knowledge, competence and experience,' as opposed to hiring an individual whose sole duty would be coordinating the WSG.

Decision making on appropriate water safety plan strategies is a challenge, especially with conflicting evidence and variable efficacy of decontamination strategies. The marketing of 'bio-safe' plumbing products to the healthcare sector without sufficient evidence to substantiate

claims can lead to WSGs having to gamble limited resources on products that may either not work or not be appropriate for their situation. Additionally, some recommended water hygiene measures, such as regular flushing of taps (Department of Health, 2013b), rely upon staff compliance and/or behaviour change. A major factor influencing behaviour is motivation and capability, which includes training and education provided to induce a behaviour to be carried out (Michie *et al.*, 2011). Whilst staff surveys have been conducted to explore issues surrounding hand-hygiene compliance (Erasmus *et al.*, 2009), these tend to focus on clinical staff. Maintenance of water hygiene does encompass hand hygiene, but is a more diverse and multifactorial issue and can be impacted by a number of staff types besides clinical staff. The DoH has clearly highlighted the importance of multidisciplinary awareness and cooperation for the implementation of a successful water safety plan. The knowledge, attitudes and beliefs of staff regarding issues surrounding water hygiene are yet to be investigated, thus motivation and capability (required for behaviour change/compliance) of staff to follow water safety infection control procedures is unknown.

The use of questionnaires to investigate knowledge attitudes and beliefs or opinions of staff is an approach taken in preparation for the planning or implementation of intervention for behaviour change, for both those carrying out the behaviours and those in charge of decision making (Behera, 2009, World Health Organization, 2008). This style of questionnaire can also be used to evaluate behaviour change strategies (World Health Organization, 2008) as well as aid in predicting obstacles that may arise in implementing strategies and whether particular approaches would be sufficient alone (Grol and Wensing, 2004, Kong *et al.*, 2009). Knowledge, attitudes and beliefs surveys are often used to inform training programmes and have been used to determine whether training is desired or would be well received (Happell *et al.*, 2002, Kong *et al.*, 2009). Whilst questionnaires require more cognitive effort on behalf of participants, they allow participants to remain anonymous and therefore reveal information that they might otherwise change (i.e. answers can be influenced by social bias in a face-to-face or focus group style method of surveying) (Bowling, 2005).

1.6 Hypothesis

The contamination of hospital tap components with *P. aeruginosa* is a multifactorial problem, encompassing human (behavioural) and engineering (design) elements, both of which can either enhance, or pay detriment to, the other's impact on water hygiene.

1.7 Aim and objectives

Understanding factors affecting the occurrence and persistence of nosocomial pathogens in water systems is essential to allow the development of interventions to reduce the human and financial costs of HCAIs. The aim of this study was to investigate engineering and human behavioural factors influencing *P. aeruginosa* survival, biofilm formation and persistence within hospital tap assemblies, and the consequences on water quality.

The main objectives were to:

- Create and distribute a questionnaire to determine multidisciplinary hospital staff attitudes and opinions regarding issues surrounding water hygiene and maintenance of hospital taps.
- Investigate *P. aeruginosa* attachment and biofilm formation on conventional and alternative/antimicrobial materials used within healthcare plumbing *in vitro* using a CDC biofilm reactor.
- Install conventional, antimicrobial/alternative solenoid valves to an experimental water distribution system (EWDS) to investigate formation and persistence of biofilm.
- Apply remedial interventions using the EWDS to investigate the effect of these measures on contaminated tap components and water contamination.
- Use the EWDS to create a cloth-contamination model to investigate the potential for retrograde contamination of hospital taps.

Chapter 2 Materials and methods

2.1 Questionnaire development

2.1.1 Inclusion and exclusion criteria

A questionnaire investigating staff knowledge, attitudes and beliefs/opinions was developed, with the intention to distribute to four key staff groups: domestic, estates, medical (doctors) and healthcare (healthcare assistants, midwives, nurses). It has been recommended that representative from these staff groups are included in hospital water safety groups and be responsible for maintaining water hygiene within hospitals (Department of Health, 2016b). The inclusion criteria was staff working within an NHS England hospital (and therefore of working age and with capacity to consent), with an exclusion criteria of non-staff (and therefore children), and no other discriminatory factors.

2.1.2 Questionnaire design

A questionnaire incorporating seven main questions (34 sub-questions in total) was designed by the researcher. All questions were close-ended (respondents must select an option from predetermined answers (Williams, 2003)) other than requesting participants to state their job title and leaving room for feedback at the end of the questionnaire. The rest of the questionnaire was multiple-choice, with responses in the form of a five to seven-point Likert scale (determining the extent to which the respondents agreed with a statement or believed something to be likely) to produce ordinal data (Williams, 2003, Allen and Seaman, 2007).

Questions were designed to determine:

- Staff appreciation for water as a vector for infection.
- staff perceptions of retrograde and systemic contamination of taps;
- staff opinions on the misuse of hand wash stations and behaviour change;
- staff awareness and perception of risks surrounding tap maintenance and hand hygiene and;
- staff risk assessment of physical injury vs. infection risk with regards to water safety.

Questions were designed to minimise risk of initiating a blame or victimisation culture in participants, i.e. no questions asked staff to report whether behaviours had been witnessed, rather whether they thought certain behaviours could have an effect or were likely to change.

2.1.3 Anonymity, sensitive information and consent

Consent was obtained by participants ticking a box which indicated that they had read and understood the participant information sheet and consented to volunteer for the study. Participants were instructed not to write their name on the questionnaires. Anonymity was limited to job role and no personal or sensitive information was requested.

2.1.4 Pilot study response rate

The questionnaire was piloted (primarily to obtain feedback regarding question structure and relevance) at one London hospital. 100 questionnaires were distributed and left to circulate for six weeks. 45 questionnaires were returned (an overall response rate of 45%) and grouped according to the respondent's job role (21 (47%) domestic staff, 4 (9%) estates staff, 14 (31%) healthcare staff, 1 (2%) medical staff and 5 (11%) other (e.g. administrative staff)). Lessons learned included the removal of background information prior to questions to reduce the reading/time demand, the standardisation of Likert scales to five-points (from seven-points, removing the option of "somewhat agree/disagree") and the inclusion of a "not applicable" option within one of the seven questions.

The pilot study also highlighted the importance of being able to explain the study to leaders/staff representatives who were responsible for the distribution of questionnaires to each staff group. Feedback from the estates staff representative (received when the researcher collected the completed questionnaires), was that the relevance of their inclusion as a staff group was not understood and as a result participation was not encouraged.

2.1.5 Multi-centre study

Lessons learned from the pilot were incorporated into the design and distribution of the final questionnaire. Any question perceived by participants to be confusing or difficult to understand was amended and those deemed irrelevant were removed. The final questionnaire is reproduced in Appendix A.1 (pages 163-169).

Hospitals were not selected due to a known history of *P. aeruginosa* problems. At the time of questionnaire distribution, *P. aeruginosa* bacteraemia incidence was reported on a voluntary

basis only (Chapter 1.2.2) so the burden of *P. aeruginosa* in the hospitals may have been underreported. It is possible that the hospitals which were willing to participate were motivated to do so due to an interest in *P. aeruginosa (*i.e. potential for bias in participating hospitals). However, the most recent reports for bacteraemia cases in the three hospitals which participated had varying levels (reported between April 2017 and March 2018) (Public Health England, 2018), two with hospital onset cases falling at or below the median for the UK, with one at a level below the 25th percentile, whilst the third hospital was above the median but within the 75th percentile.

2.1.6 Approvals

The University of Southampton's Faculty of Medicine Ethics Committee granted approval for the questionnaire (approval number 18724). Further approval was granted by the Health Research Authority (HRA) (Integrated Research Application System (IRAS) project ID 206594). Individual hospital research governance officers were contacted prior to visiting each hospital to arrange for local agreement and, where deemed necessary by individual Trusts, a letter of access.

2.1.7 Questionnaire dissemination and collection

No hospital or participant was credited with any accruals (e.g. remuneration) and no expenses were incurred by the hospitals.

Questionnaires were printed as hard copies and distributed so as not to bias the participant responses to those with access to and/or competence in computer and internet usage.

The opportunity to participate in the study was initially advertised by email to infection control representatives at NHS England Trusts. A contact (i.e. a member of staff with a leading role in infection control; either the director of infection prevention and control, an infection control nurse, or clinical microbiologist) from each hospital was held as principle investigator (PI) at their associated Trust. These contacts were visited by the researcher who explained the research questions and overarching PhD project. Further to this, the PIs permitted communication with heads/representatives of the individual staffing group departments, to whom the study was explained and who were left with questionnaires for distribution to willing volunteers.

One hundred questionnaires were distributed within each hospital. Where possible, questionnaires were divided into groups of 25 for dissemination among the four target staff groups. Where this was not possible (e.g. when no responsible staff representative for medical and nursing staff identified by the PI), questionnaires were divided across wards and the ward managers encouraged both medical and nursing staff to participate. PIs and staff representatives

were responsible for word-of-mouth advertisement of the study. PIs collected the questionnaires from the responsible staff members and deposited them into the central box for collection by the researcher. Questionnaires were left in circulation for a maximum of six weeks before collection; PIs were contacted after two weeks to determine the preliminary uptake of the questionnaire and to encourage them to actively support voluntary participation (e.g. by checking whether their staff representatives had engaged in recruiting volunteers). Two Trusts requested collection of the questionnaires at this stage and any additional questionnaires were collected at the six-week time point.

2.1.8 Data analysis

Results from all hospitals (excluding the pilot study) were entered into an Excel spreadsheet (Microsoft Office Professional Plus 2016) and combined. Responses were both grouped as 'all respondents' and separated into four staffing groups (domestic, estates, healthcare and medical staff). Response percentages were calculated using Excel. Analysis was limited to descriptive and modal analysis. Responses were collapsed down from five categories into three, with "very likely" or "strongly agree" responses merged with "likely/agree" responses (Allen and Seaman, 2007). The number of responses (%) were calculated per staff group and cumulatively. The responses to question six ("In your opinion, are staff likely to carry out the following practices?"; page 170), which offered an option of "already standard practice", were divided in three subgroups: total number of respondents who selected standard practice (A), number of respondents who selected standard practice as their only response (B) and number of respondents who selected 'standard practice' alongside another response (C).This prevented those participants who provided more than one response from artificially increasing the response rate.

Themes arising from the open-ended feedback (i.e. when participants were allowed to respond freely (Williams, 2003)) were used to group comments. Responses were not been edited to correct spelling or grammar.

2.2 *P. aeruginosa* biofilm formation on conventional and 'antimicrobial' plumbing materials

2.2.1 *P. aeruginosa* strain selection

2.2.1.1 Test organisms

For this and further investigations beyond this chapter, three strains of *P. aeruginosa* were selected for potential use, (Table 2.1, isolates A, B and C). Isolates A and B had been previously recovered from the experimental water distribution system (EWDS) at Public Health England ((PHE); Porton Down, Salisbury) so were known to survive within the water supplied to the laboratory. Isolate C was considered due to its history of having colonised the taps implicated in the Northern Ireland incidents (Walker *et al.*, 2014). However, to determine whether isolates A, B and C were exceptionally high biofilm formers (and therefore possibly not representative of environmental strains across the UK), the biofilm-forming ability of each strain was assessed alongside nine other isolates of *P. aeruginosa (*Table 2.1), six of which (D-I) were isolated from the hospital environment (all from UK hospitals) and one from a domestic shower (J) for previous Public Health England investigations. Additionally, two *P. aeruginosa* strains were selected as they had been previously characterised as having high and low biofilm forming ability (isolates K and L respectively (Perumal *et al.*, 2014); serially collected Cystic Fibrosis isolates (UK patients)). These were used to determine whether the potential test strains (A-C) could be considered representative of those isolated from the clinical environment or exceptionally high- or low-biofilm formers. All isolates were stored on cryopreservative beads (Technical Service Consultants Ltd., Bury, UK) at -20°C.

Table 2.1 *P. aeruginosa* isolates used for biofilm formation comparison

Isolate/Strain	PHE reference	Source
A	Tap 20	PHE EWDS- water sample
B	FS12	PHE EWDS- outlet fitting
C	Belfast	Hospital environmental
D	PaCC	Hospital environmental
E	PaCC 127	Hospital environmental
F	Pa6	Hospital environmental

Isolate/Strain	PHE reference	Source
G	RFH79	Hospital environmental
H	RFH82	Hospital environmental
I	RFH87	Hospital environmental
J	Shower 26	Domestic environmental
K	GH12	Clinical- cystic fibrosis
L	GH97	Clinical- cystic fibrosis

2.2.1.2 Static biofilm assay

Static biofilm assays were performed using a modified version of the microtitre plate assay (Merritt *et al.*, 2005). To prepare test suspensions, each cryopreserved *P. aeruginosa* isolate (Table 2.1) was first resuscitated on tryptone soya agar (TSA) (Oxoid, Basingstoke, UK) and then subcultured onto agar selective for *P. aeruginosa* (cetrimide (CN) agar (Oxoid)). Liquid cultures were prepared by transferring a single colony to 100 mL nutrient broth (Oxoid) and incubating at 37°C with shaking (120 rpm) for ~16 hours. 10 mL of each *P. aeruginosa* culture was centrifuged at 4400 rpm for 20 minutes (Eppendorf 5702 centrifuge) and resuspended in 10 mL of 1/10 strength nutrient broth. Aliquots (200 µL) of the resulting suspensions were used to inoculate wells (n=6) of a flat-bottomed 96-well microtitre plate (polystyrene, non-treated; Fisher Scientific, Basingstoke, UK). Uninoculated 1/10-strength nutrient broth was added to control wells (n=12). Plates were incubated at ambient temperature, which was recorded every 10 minutes using a TinyTag TV4500 (Gemini Data Loggers UK Ltd., Chichester, UK) and ranged from 20°C to 22.4°C. *P. aeruginosa* was allowed to incubate for three days to reach early maturation (Southey-Pillig *et al.*, 2005), after which planktonic cells were removed by vigorously shaking the inoculum from the plates and washing the wells once with sterile water. The retained biofilm was then heat fixed by incubating the plates at 50°C for one hour. To allow visualisation of the biofilm, the wells were then stained with 200 µL crystal violet (0.2% (w/v); Fisher Scientific) for 30 minutes. The crystal violet was then removed by vigorously shaking out the contents of the wells and washing the plates with sterile water three times, or until the rinsings were colourless. The microtitre plate was then blotted dry using tissue paper and left to air dry for 15 minutes. The crystal violet was solubilised for quantification by adding 200 µL acetic acid (30% (v/v); Fisher Scientific) to each well and incubating at room temperature for a further 15 minutes. Absorbance at 590nm was measured using a FLUOstar Omega microplate reader (BMG LABTECH Ltd., Buckinghamshire, UK). Readings were taken in triplicate and each experiment was repeated three times.

2.2.2 Material selection

Circular material coupons (1.27 ± 0.013 cm in diameter with a thickness of ~3.0 mm) were purchased from BioSurface Technologies Corporation (Bozeman, USA) or provided by manufacturers. No financial contribution to the production of the material coupons was made to tap manufacturers, who were also made aware that no product endorsement could be made. Three main material groups were selected for investigations: plastics (hostaform ± silver ions; rialene ± molybdenum trioxide; basell), antimicrobial metals (100% copper; brass (>76% copper)) used in conventional and 'antimicrobial' outlet fittings and rubbers (EPDM; nitrile; silicone) used in solenoid valves (Table 2.2).

Table 2.2 The category, claims and usages of materials investigated

Category	Material	Claim	Usage	Photograph
Plastic ('unenhanced')	Polycarbonate	No antimicrobial/anti-biofilm claims	Control coupon	
Plastic ('unenhanced')	Hostaform	"Anti-biofilm": (or anti-fouling/anti-stick) plastic, reduces scale and impurity attachment and thus bacterial attachment.	Outlet fittings	
Plastic ('unenhanced')	Rialene	No antimicrobial/anti-biofilm claims	Outlet fittings	
Plastic ('unenhanced')	Basell	No antimicrobial/anti-biofilm claims	Outlet fittings	
Rubber	Ethylene propylene diene monomer (EPDM)	Conventional rubber.	Solenoid valve diaphragms	
Rubber	Silicone	An alternative to EPDM.	Solenoid valve diaphragms	

Category	Material	Claim	Usage	Photograph
Rubber	Nitrile	An alternative to EPDM.	Solenoid valve diaphragms	
Metal	Brass	Antimicrobial: through direct surface contact and through contact with copper ions released (leached) into the water.	'Antimicrobial' outlet fitting	
Metal	Copper	Antimicrobial: both surface contact and leached copper into the water.	'Antimicrobial' outlet fitting	
Plastic	Silver ion-impregnated hostaform	Antimicrobial: silver leached into the water.	'Antimicrobial' outlet fitting	
Plastic	Molybdenum trioxide coated rialene	Antimicrobial: surface contact	Concept antimicrobial outlet fitting	

2.2.3 Investigating whether *P. aeruginosa* recovery from coupons is influenced by material

An overnight culture of *P. aeruginosa* was diluted to ~6x10^5 CFU/mL, of which 20 µL was used to inoculate coupons (~10^4 CFU/coupon). Each coupon was placed into 3 mL Dey-Engley (D/E) neutralising broth (Sigma-Aldrich, Poole, UK; Lab M Ltd., Bury, UK) within 15 seconds of inoculation and vortexed for 10 minutes. The resulting suspension was serially diluted using sterile water before being plated onto CN agar and incubated at 37°C for up to 48 hours. Each experiment comprised three replicate coupons and was repeated three times. *P. aeruginosa* recovery (i.e. sampling efficiency) was compared across materials.

2.2.4 CDC bioreactor and experimental conditions

The CDC Biofilm Reactor (Biosurface Technologies) is a bioreactor system, used to provide a controlled environment for microbial growth, and assess biofilm development on materials under

user-defined conditions. The main components of the bioreactor system are a 1 L side-arm glass beaker and magnetic rotor, which are seated on a temperature-controlled stir plate, and in which the inoculated medium of choice is contained (Figure 2.1). Vent filters are used to maintain a sterile air-exchange between the bioreactor system and the laboratory environment.

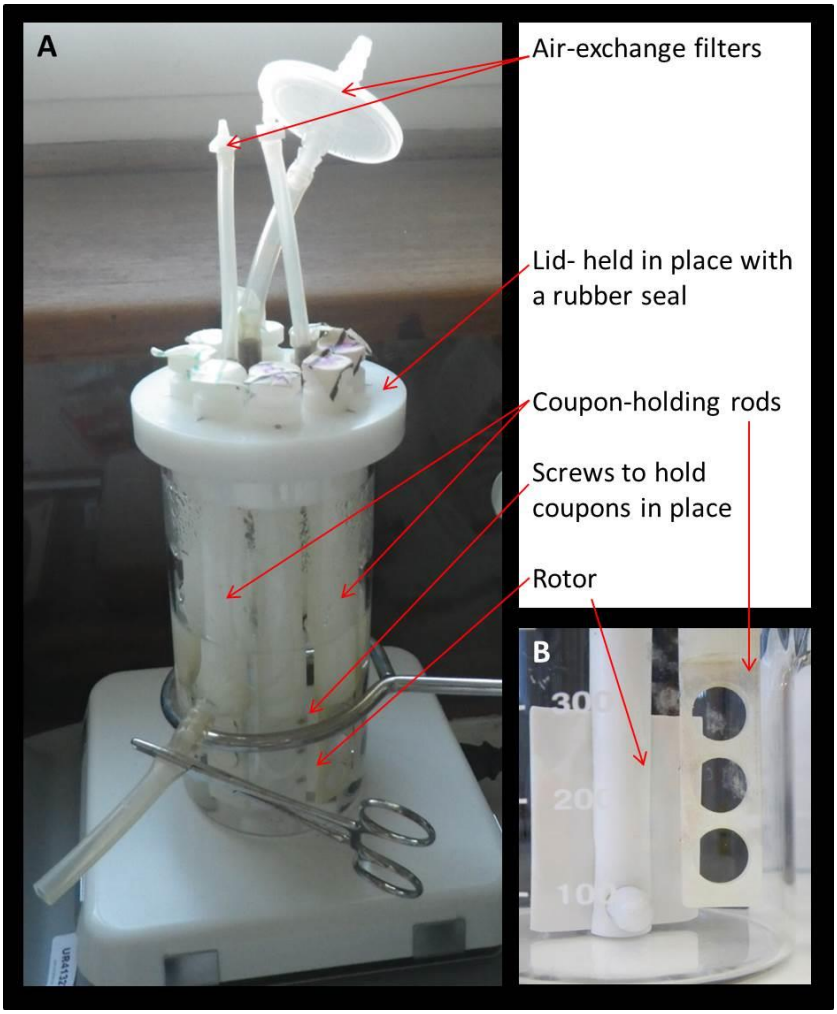


Figure 2.1 Photograph of the CDC Biofilm Reactor (bioreactor) as used in this study. A: Bioreactor experimental set up on a stir plate with 500 mL filtered tap water as media; B: positioning of the stirring rotor and rods in an empty bioreactor.

Material coupons (Table 2.2) were inserted into the rods of the CDC bioreactor and screwed in place. Rods were suspended from the lid of the bioreactor and immersed in 500 mL filtered tap water (AQIN 0.2 µm filters, Pall Life Sciences, Portsmouth, UK). Filter sterilising tap water does not influence water chemistry (Colbourne *et al.*, 1988) and, therefore, sodium thiosulphate [20 mg/L] (Scientific Laboratory Supplies, Nottingham, UK) was added to the tap water to neutralise any chlorine present. Both the filtered tap water and the sodium thiosulphate solution were cultured onto TSA to confirm sterile starting conditions. The bioreactor temperature controlled stir-plate was heated to ~41°C (±1°C) (the recommended temperature of water delivered from healthcare hand wash station taps (Department of Health, 2017)) and rotor set to 130 rpm. An overnight

culture of *P. aeruginosa* was resuspended in the filtered tap water sodium thiosulphate solution (mean concentration $2.4x10^9$ CFU/mL; n=23). An aliquot (1 mL) of the resulting suspension was added to the bioreactor (mean concentration 4.8×10^6 CFU/500 mL; n=23).

The bioreactor was run for 72 hours under batch conditions. Comparisons of material coupons which did not leach antimicrobial agents were tested within the same bioreactor run. This minimised experimental variability due, for example, to different planktonic cultures and any non-chlorine elemental fluctuations from the filtered tap water source.

2.2.4.1 Cleaning and disinfection of the CDC bioreactor

All coupons and bioreactor apparatus underwent physical and chemical cleaning between usages. Coupons were manually wiped with force using paper towels before being immersed in 3 mL 70% IPA and vortexed for 10 minutes. A secondary wipe with paper towels was followed by autoclaving at 126°C for 15 minutes. This cleaning procedure was validated through culture and direct microscopy of the coupons. A toothbrush was used to remove bacterial contamination from the bioreactor apparatus, which was also wiped (with force) using paper towels. The bioreactor was then rinsed in deionised water, chemically disinfected using 70% IPA and then autoclaved.

2.2.5 Investigating *P. aeruginosa* biofilm on material coupons

After the 72 hours had elapsed, the rods containing coupons were removed from the bioreactor and excess media removed through careful blotting so as not to bring blotting tissue in contact with the coupons. Each rod was immersed in sterile water for five seconds, reblotted, washed a second time in sterile water and blotted again. Each coupon was unscrewed and allowed to drop from the rod into individual 30 mL universal tubes containing 3 mL of recovery media (either thiosulphate Ringer's solution (Oxoid) or, to neutralise potential antimicrobial agents, D/E neutralising broth). Inter-assay controls included the incorporation of polycarbonate coupons in every bioreactor run as well as consistent use of the bioreactor apparatus (i.e. not changing the rods, rotor or glass beaker materials). Coupons were then agitated by vortex mixing at 2000 rpm for 10 minutes (Wand *et al.*, 2012). The resulting suspension was serially diluted (ten-fold) and 100 µL aliquots plated onto CN agar and incubated at 37°C for up to 48 hours. The bioreactor medium was also serially diluted and plated onto CN agar to assess the planktonic concentration of *P. aeruginosa* and to determine if there had been any loss in viability over time.

2.2.6 Detection of leached antimicrobial agents

Materials which manufacturers claim have antimicrobial properties were incorporated within the bioreactor as previously described. The bioreactor medium (filtered tap water) was not inoculated with *P. aeruginosa.* Samples of the filtered water were taken before and after each experimental run (i.e. before and up to 72 hours after exposure to any leached antimicrobial agents). Water exposed to non-antimicrobial plastics served as control samples.

2.2.6.1 Investigating the presence of silver nanoparticles

The presence of silver nanoparticles in water exposed to silver ion-impregnated hostaform was analysed using NanoSight (Malvern Instruments Ltd., Amesbury, UK). NanoSight allows for nanoparticle tracking using a light-scattering method and assesses particle size and concentration using a conventional microscope (with an adapted stage) and a charge-coupled device camera (Filipe *et al.*, 2010, Gardiner *et al.*, 2013). 1 mL of deionised water was injected into the NanoSight sample chamber, followed by 1 mL of sample. After analysis, an additional 1 mL of sample was injected such that each water sample was tested in duplicate. Nanoparticles were visualised using a 488nm laser and samples were analysed using NanoSight Nanoparticle Tracking Analysis software. Video footage was captured using an Hamamatsu C11440 ORCA-Flash camera 2.3 (Hamamatsu, Japan). A suspension of colloidal silver [10 ppm] (Livoa Vital ™, West Sussex, UK) was used as a positive control.

2.2.6.2 Investigating the presence of 'antimicrobial' ions

The presence of silver, copper and molybdenum ions was assessed using inductively coupled plasma mass spectrometry (ICP-MS). ICP-MS allows detection of ions by aerosolising the sample and introducing it to an argon plasma beam, which ionises the elements and compounds within the sample (Liu *et al.*, 2014). Ions are further separated according to their mass/charge ratio detected by mass spectrometry, which reports both signal intensity (concentration of ions) and the identity of the ions detected (Ammann, 2007). Calibration standards were prepared from a multi-element solution [0-10 µg/mL] (Spex Certiprep, Stanmore, UK). Iridium solution [0.01 µg/mL] (Spex Certiprep) was used as an internal standard to all samples and nitrous acid (HNO_2; 2% (v/v)) was added for acid digestion. Samples were measured using iCAP Q ICP-MS (Thermo Fisher Scientific, Hemel Hampstead, UK) run in kinetic energy discrimination (KED) mode. The isotopes that were monitored were Ag-107 (silver), Cu-63 (copper) and Mo-98. (molybdenum). 5 mL of each prepared water sample was tested in duplicate, with five reads per replicate (i.e. ten reads per sample). Mean values (± standard deviation) of the reads were calculated to provide an average concentration of ions present in the samples (reported as parts per billion (ppb)).

2.2.7 Investigating the minimum inhibitory and bactericidal concentrations of silver and copper against *P. aeruginosa*

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of silver ions and copper ions against *P. aeruginosa* was investigated using silver nitrate ($AgNO_3$) (Randall *et al.*, 2013, Gaisford *et al.*, 2009) (Spex Certiprep) and copper chloride ($CuCl_2$) (Spex Certiprep) respectively.

The silver nitrate was diluted with sterile deionised water to achieve test concentrations of 0.1, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005 and 0.0025 ppm. Likewise, the copper chloride was diluted with sterile deionised water to achieve test concentrations of 0.8, 0.6, 0.4, 0.2, 0.1, 0.05 and 0.025 ppm. Aliquots (160 µL) of each ionic suspension were added to 3 wells of a sterile microtitre plate and inoculated with 20µL of a diluted overnight culture of *P. aeruginosa (*~1 x 10^6 CFU/mL). To each well, 20 µL 1/20-strength nutrient broth (Oxoid) was added (a total well volume of 200 µL). Negative controls for each ionic suspension (i.e. no inoculum) and positive controls (sterile deionised water (dH_2O) in place of ionic solution) were also included (in triplicate) to each 96 well plate. Plates were incubated at 37°C for 24-hours. To investigate the MICs, absorbance readings at 600nm were taken using a FLUOstar Omega microplate reader (BMG Labtech). To investigate the MBCs, the contents of each well was plated onto Tryptone soya agar (TSA; Oxoid) and incubated at 37°C for up to 48 hours. The MIC was defined as the minimum concentration at which OD_{600} was <0.05 (i.e. not higher than the absorbance from negative controls). The MBC was defined as the minimum concentration at which *P. aeruginosa* could not be recovered.

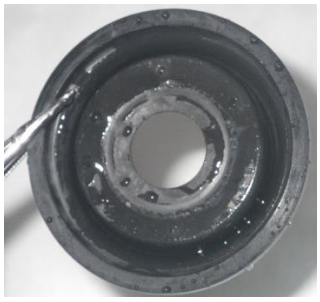
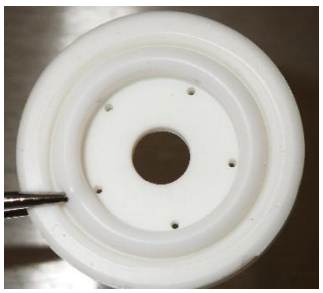
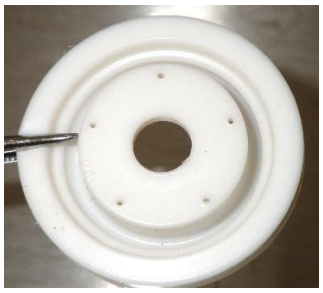
2.2.8 Data analysis

Data analysis was performed using GraphPad Prism (version 7.00 for Windows). For data sets with ≥3 groups, parameteric data were compared using one-way ANOVA, followed by a Tukey's multiple comparison test. Non-parametric data were compared using Kruskal-Wallis one-way ANOVA in combination with Dunn's multiple comparisons tests. Data sets for comparison of two groups was conducted using t-tests (parametric data) or Mann-Whitney tests (nonparametric data) unless otherwise stated. Significance was set at $p<0.05$.

2.3 Investigating *P. aeruginosa* biofilm formation and persistence on conventional and alternative solenoid valves

Four different SV designs were investigated. All SVs were provided by one manufacturer (confidential) and differed primarily in terms of diaphragm material. These included conventional EPDM and alternative nitrile rubber, silicone rubber and 'antimicrobial' silver ion-impregnated silicone (Table 2.3).

Table 2.3 Solenoid valve materials and designs

Diaphragm material	Body material	Diaphragm image
Ethylene propylene diene monomer (EPDM)	Brass	
Nitrile rubber	Brass	
Silicone rubber	Plastic	
Silver ion-impregnated silicone rubber	Plastic	

2.3.1 Model experimental water distribution system

The EWDS used throughout this project was based at Public Health England, Porton Down, Salisbury, and had been constructed to model the tap outlets removed from the neonatal unit involved in the 2011/2012 incidents of *P. aeruginosa*. The EWDS (Figure 2.2) consisted of 27 tap assemblies, each fitted with an automatic tap (Aquarius deck mounted short neck tap with copper tails; Dart Valley Systems, Paignton, UK) and accompanying SV (Phoenix Contact Ltd, Telford, UK). Taps were rewired so as not to require the stimulation of the infrared sensor to open the SVs and switch on the taps. Instead, the SVs were activated via a control panel, which, when operated, was programmed to switch taps on for 30 seconds individually and consecutively. Manual gate valves (Screwfix Direct Ltd, Yeovil, UK), installed below the SVs (Figure 2.2) were used to manually control the flow of water to the tap, overriding the SV when necessary.

Mains supplied water was stored in two indirect copper combination cylinders (RM Cylinders, Castleford, UK) that held a total capacity of 155 L. Water in one of the tanks was heated to 60°C. Water in the second tank was stored at ambient temperature ("cold tank"). Tanks were installed in the laboratory at the same level as the EWDS and, as the EWDS was not gravity fed, the system was kept under negative pressure by an on-demand centrifugal shower pump operating at 3 bar (Salamander Pumps, Sunderland, UK). A thermostatic mixing valve (TMV) (Pegler Prestex P402 15 mm TMV3; Pegler Yorkshire Group Ltd, Doncaster, UK) associated with each tap assembly ensured the water delivered from each outlet was ~43°C.

Binder-style testing points (referred hereafter as Binder points; Macro Construction, Portsmouth, UK) (Figure 2.2 A) were installed between the tap spout and the SV. Binder points act as a point of access to water distribution systems and are commonly used as probe insertion points to test water temperature and pressure. Within the EWDS model, Binder points were used to artificially inoculate each tap assembly with *P. aeruginosa*.

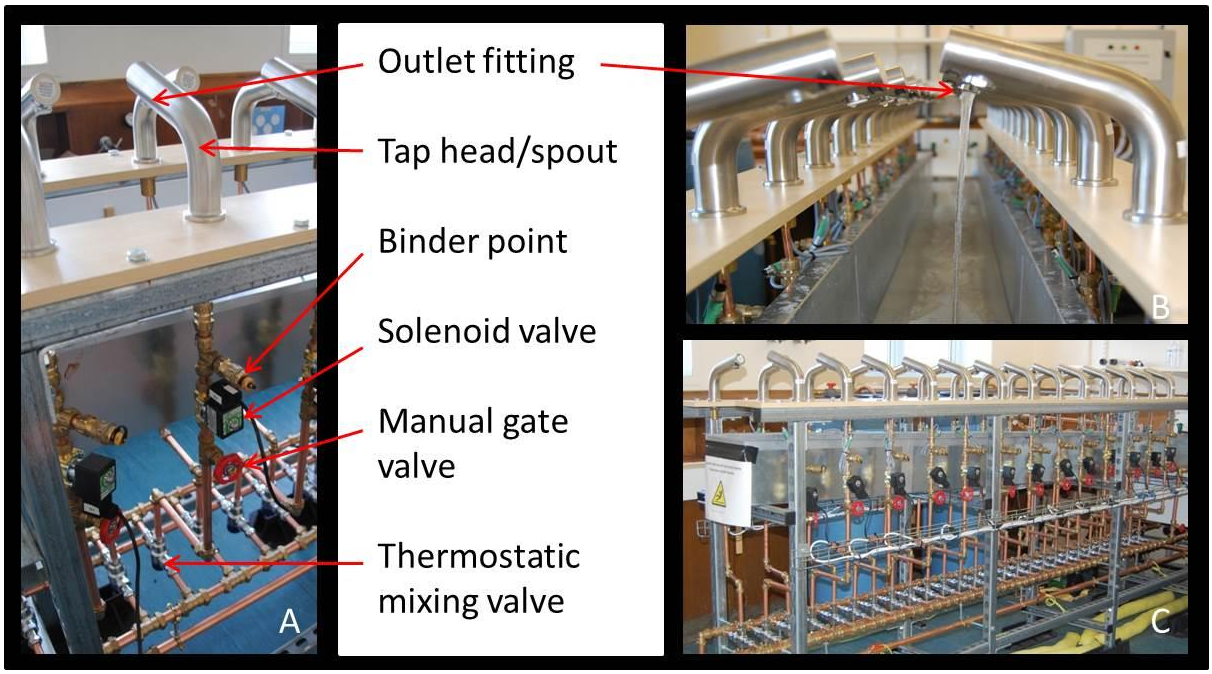


Figure 2.2 The experimental water distribution system (EWDS) at Public Health England. A) The tap assembly main components include an outlet fitting, tap head/spout, Binder point, solenoid valve, manual gate valve and thermostatic mixing valve; B) the EWDS running; C) the 27 taps of the EWDS and water storage tanks (visible behind the tap assemblies).

2.3.2 Inoculation of the experimental water distribution system

New SVs were installed within the EWDS below the Binder point associated with each tap assembly (Table 2.4). To reduce the risk of contamination from previous studies, outlet fittings and their housings were removed, sterilised using 70% v/v isopropanol (IPA) (VWR, Leicestershire, UK) and autoclaved at 126°C for 15 minutes before being reinstalled in the taps. The first sample of water collected post-installation was collected in sample bottles dosed with [20 mg/L] sodium thiosulphate (Scientific Laboratory Supplies, Nottingham, UK) and sampled via membrane filtration onto CN agar. Plates were incubated at 37°C for up to 48 hours. To provide comparison between *in vivo* and *in situ* results, isolate A, used for the assessment of materials within a bioreactor model (Chapter 3) was selected for use. This isolate had demonstrated the ability to thrive in the water conditions of the EWDS. Overnight liquid cultures of *P. aeruginosa* were prepared as previously described (Chapter 3.3.1). 10 mL aliquots of the resulting suspensions (mean counts of 3.9×10^9 CFU/mL; n=2) were drawn into a syringe and injected into each Binder point. The EWDS was allowed to stagnate for five days, after which time each tap assembly was flushed for 30 seconds, twice-daily, five days per week for 12 weeks. Modifications to the methodology will be discussed where appropriate. Whilst WHO guidance recommends

handwashing take place over a 40-60 second period (World Health Organization, 2009a), observational studies have found that staff often fall short, averaging at closer to 10 seconds (Clark *et al.*, 2016, Lam *et al.*, 2004). NHS guidelines state that handwashing should take approximately 15-30 seconds (National Health Service, 2007). It has been demonstrated that significantly more bacteria are removed in a 30-second handwash than a 15-second handwash (Jensen *et al.*, 2017), thus a flushing time of 30-seconds in line with NHS guidelines and current literature was selected for the EWDS.

Table 2.4 Solenoid valves present or installed onto the EWDS: nitrile and EPDM

SV type	Status	Inoculation status	Tap number
EPDM	New	Inoculated	2
EPDM	New	Inoculated	4
EPDM	New	Inoculated	6
EPDM	New	Inoculated	21
EPDM	New	Inoculated	22
Nitrile	New	Inoculated	1
Nitrile	New	Inoculated	7
Nitrile	New	Inoculated	15
Nitrile	New	Inoculated	19
Nitrile	New	Inoculated	26
EPDM	New	Control	18
Nitrile	New	Control	10
EPDM	Old	Control	5
EPDM	Old	Control	11
EPDM	Old	Control	14
EPDM	Old	Control	27

2.3.3 Monitoring water contamination over time

The first sample of water (500 mL) delivered from each outlet following overnight stagnations was collected in sample bottles dosed with [20 mg/L] sodium thiosulphate. Water samples were serially diluted (10-fold) and 100 µL of each dilution plated onto CN agar. Thereafter water samples were taken 18 times over the 12-week period. On each sampling occasion *P. aeruginosa* was cultured by plating 100µL of serially diluted sample onto CN agar or filtering volumes of the sample (1-, 10- or 100 mL) through a 0.2 µm membrane (Pall Life Science, Portsmouth, UK), which was then transferred onto CN agar and incubated at 37˚C for up to 48 hours. Water samples were also taken from the hot and cold storage tanks using sterile Dippas (Scientific Laboratory Supplies) and tested for *P. aeruginosa* as described.

2.3.4 Genomic analysis of *P. aeruginosa*

Phenotypically distinct isolates from water and biofilm cultures were subcultured, confirmed as *P. aeruginosa* by matrix assisted laser desorption/ionization time of flight (MALDI-ToF) mass spectrometry analysis and transferred to 3 mL nutrient agar slopes (Oxoid) to be sent for variable number tandem repeat (VNTR) analysis (Genomic Services and Development Unit, Public Health England, Colindale, UK). These isolates were cryopreserved on beads (Technical Service Consultants Ltd., Lancashire, UK) and stored at -20°C. VNTR analysis is a PCR-based method that detects the number of tandem repeats within the genome at predetermined marker loci for an organism (Vu-Thien *et al.*, 2007). The number of repeats at each locus provides a value, giving strains a distinct VNTR profile (Turton *et al.*, 2010). To achieve higher resolution and investigate strains, with identical or similar VNTR profiles with a change in the ninth locus (reported to provide discrimination between otherwise genetically similar strains (Turton *et al.*, 2010)), were sent for whole genome sequencing (WGS) to compare isolates on the basis of single nucleotide polymorphisms (i.e. mutations of a single DNA base) (den Bakker *et al.*, 2011). DNA extractions for WGS were performed using QIAmp DNA Mini Kit (Qiagen, Manchester, UK) as per manufacturer's instructions. Genomic DNA was stored at -20°C.

2.3.5 Biofilm investigations

SVs were removed from the EWDS at the end of the flushing period and dismantled using aseptic technique. The diaphragms were extracted and rinsed with 60 mL of sterile water (P A M Medical Supplies, Willenhall, UK) to remove any residual water and loosely attached cells. Diaphragms were held in place using sterile, disposable forceps and cut in half using 70% IPA-treated scissors. Each half analysed via microscopy or culture.

2.3.5.1 Investigating biofilm by microscopy

The presence of biofilm on SV diaphragms was detected by fluorescence- and scanning electron microscopy (SEM). The section of each diaphragm retained for microscopic analysis was cut using IPA-treated scissors into two triangular sections (approximately 10 mm x 10 mm x 5 mm) allowing analysis of either side of the diaphragm. Samples investigated by fluorescence microscopy were stained with 200-300 µL Syto9 (a volume sufficient to ensure the test surface was fully immersed) and Propidium Iodide (*Bac*light®; Thermo Fisher Scientific, Basingstoke, UK) and imaged using a 10x, 20x, 50x and 100x dry lens or 50x water lens on a Nikon microscope (Eclipse Ni-U, Nikon UK Ltd., Surrey, UK). Samples investigated by SEM were fixed in 1 mL formalin (VWR) [4% v/v] for up to 24-hours, followed by a 1-2 hour fixation in osmium tetroxide [1% w/v] (Sigma-Aldrich, Irvine, UK) and a graded ethanol dehydration over 75 minutes. Samples were then transferred into hexamethyldisilazane (HMDS) (Sigma-Aldrich), air dried and gold-coated using an ultra-fine grain sputter coater (AtomTech Z705) and examined using a Philips FEI XL30 FEG SEM.

2.3.5.2 Investigating biofilm by culture

The section of diaphragm retained for culture was placed in 10 mL sterile thiosulphate Ringers solution (Oxoid) containing five glass beads (3 mm diameter; Sigma-Aldrich) and vortexed at 2000 rpm for 10 minutes. Alternatively, to neutralise potential antimicrobial agents, Dey-Engley neutralising broth (Sigma-Aldrich, Poole, UK; Lab M Ltd., Bury, UK) was used as the recovery media in place of thiosulphate Ringers solution.

The resulting suspension was serially diluted and plated onto TSA and CN agar and incubated at 37˚C for up to 48 hours.

Isolates from TSA agar were identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) (Bruker Microflex; Bruker, Massachusetts, United States), which produces mass spectra characteristic of different bacterial species by monitoring the mass/charge ratio of ions released in gas form following the exposure of the sample to a laser (Carbonnelle *et al.*, 2011). The spectra are run against a database library and identification confidence scores are provided; the higher the score, the more reliable the identification.

2.3.6 Investigating the release of active antimicrobial agents from 'antimicrobial' solenoid valves

Water samples were collected from tap assemblies incorporating silver-ion impregnated silicone, silicone, and EPDM SVs (n=4). Water from both the hot and cold tanks associated with the EWDS was collected to assess the background level of silver ions in the water. Pre- and post-flush water

samples (collected at the beginning and end of a 30-second flush) were tested for silver ions using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (iCAP Q ICP-MS; Thermo Fisher Scientific) as described in Chapter 3.3.6. Pre-flush water samples were tested for silver nanoparticles using a NanoSight microscopy (Malvern Instruments Ltd., Amesbury, UK), as described in Chapter 3.3.6.

2.3.7 Remedial strategy investigation

Taps incorporating EPDM, silicone rubber and silver-ion impregnated silicone SVs, which had continually dispensed water contaminated with *P. aeruginosa (*i.e. were likely colonised with *P. aeruginosa* biofilm) were selected for investigation into the effect of two remedial measures employed by hospitals: flushing and chlorination.

2.3.7.1 Flushing

Each tap was flushed for 30 seconds and the first (S1), second (S2) and third (S3) sample of water from each flush was collected in 500 mL sample bottles dosed with [20mg/L] sodium thiosulphate. This was repeated five times over a twenty-minute period (i.e. each tap was flushed once every five minutes) and repeated on four separate occasions. Water samples were tested for *P. aeruginosa* via membrane filtration onto CN agar, with serial dilutions carried out where necessary.

2.3.7.2 Chlorination

Chlorine granules (ClearWater™; distributed by Wilton Bradley Ltd, Newton Abbot, UK) were dissolved in deionised water and added to the cold water storage tank at a final concentration of 50 ppm, confirmed using a Palintest photometer in combination with DPD 1 and DPD 3 comparator reagents (Palintest Ltd., Gateshead, UK) to detect total chlorine, used as per manufacturer's instructions. Palintest analyses the presence of chemicals in water samples via a colour change induced by comparator reagents (specific to whichever chemicals are being tested for). The absorbency readings are compared to a blank (water sample not treated with comparator reagents) and, reported as a concentration of the chemical being tested for. Taps were flushed for several minutes until chlorine was detected in the water dispensed from the taps at levels above the starting concentration. The taps were left to stagnate for two hours before SVs (n=5) were removed from the EWDS and analysed via microscopy and culture as described. The remaining SVs were kept *in situ* (n=3) and subjected to a series of flushes over the course of two weeks. The chlorine levels were monitored over this two-week period and water samples were

collected and plated onto CN, TSA and R2A, after which SVs were removed and analysed as previously described.

2.3.8 Data analysis

2.3.8.1 Statistical analysis

Statistical analysis was performed using GraphPad Prism for Windows (version 7.00) and Microsoft Excel (Microsoft Office Professional Plus 2016). Median values were used for microbial water counts due to the detection limit of >1 CFU/100 mL. Comparison of two groups was conducted using Mann-Whitney tests, and comparison of three groups or more was conducted using Kruskal-Wallis one-way ANOVA for unpaired data or a Friedman one-way ANOVA for paired data. To calculate microbial reductions from key values and to allow for statistical analysis of data below the detection limit of the assay, microbial count data was increased by a value of 1 before being $\log_{(10)}$ transformed (Feng *et al.*, 2014). Transformed data was compared using one-way ANOVA or where appropriate a repeated-measures one-way ANOVA. Significance was set to $p<0.05$.

2.3.8.2 Genomic analysis

Genomic DNA from isolates of interest and from two reference strains (a PA14 strain used to inoculate the EWDS in a previous study (Moore *et al.*, 2015b) and the PA14 variant used to inoculate the EWDS in this study (strain "A")) were dispatched to PHE Collindale (London, UK) for Next Generation Sequencing on Illumina sequencers. WGS data was returned in FastQ format and analysed using PHE Galaxy (http://bioinformatics-galaxy.phe.org.uk) (Afgan *et al.*, 2016) and Integrative Genomics Viewer (IGV) (version 2.3 for Windows) (Thorvaldsdóttir *et al.*, 2013). Reference genomes were assembled via PHE Galaxy using an in-house workflow (Wand *et al.*, 2017), which involved the use of SPAdes 3.1.1 to assemble contigs (Wand *et al.*, 2017, Bankevich *et al.*, 2012) and SSPACE to order contigs into scaffolds (Boetzer *et al.*, 2010). The output FASTA file was then used as a reference strain for FastQ files of other isolates to be mapped against. An in-house mapping workflow produced BAM and GATK2 files for SNPs (Wand *et al.*, 2017). IGV was used to visualise SNPs from BAM files; SNPs were disregarded where allele frequency was <80%, where >3 SNPs occurred within 1000 bp of each other (Wand *et al.*, 2017), or where SNPs occurred in close proximity (<1000 bp) to the end of a contig (Briskine and Shimizu, 2017, Read *et al.*, 2002)

2.4 Investigating the attachment and persistence of *P. aeruginosa* on conventional and alternative outlet fittings

2.4.1 *P. aeruginosa* biofilm formation on conventional outlet fittings

Cryopreserved *P. aeruginosa (*'isolate A'; Chapter 3.4.1) was resuscitated on TSA Oxoid and incubated at 37°C, then further subcultured onto agar selective for *P. aeruginosa* CN agar and stored at 4°C.

New conventional outlet fittings (OFs) (Table 2.5A) (manufacturer confidential) were placed into 30 mL universal tubes (VWR, Leicestershire, UK), containing 10 mL nutrient broth (Oxoid). The broth was inoculated with a single colony of *P. aeruginosa* suspended by vortexing at 2003 rpm for one minute. Broths were incubated for 5 days at 37°C without shaking. After incubation, each OF was removed from the broth using sterile forceps and shaken vigorously to remove retained media before being rinsed with 20 mL sterile water to remove loosely attached cells and residual media.

2.4.1.1 Visualisation of biofilm

OFs (n=3) were dismantled, cut into segments and stained with *Bac*light® (Syto9 and propidium iodide) (Thermo Fisher Scientific, Hemel Hampstead, UK) as per Chapter 4.3.5.1. Segments were exposed to stain for 20-30 minutes protected from light, and then rinsed using syringe-filtered deionised water (Ministart 0.2 µm filter; Sartorious, Epsom, UK). OF sections were visualised using an epifluorescent microscope (Eclipse Ni-U) (Nikon UK Ltd., Surrey, UK). Image analysis was carried out using Leica Application Suite software and further assessed using ImageJ imaging software.

2.4.1.2 Effect of contaminated OFs on water hygiene

Artificially contaminated OFs (n=15) were inserted into taps within the environmental water distribution system (EWDS; Chapter 4.3.1) known to be negative for *P. aeruginosa* and left *in situ* for up to 10 days (n=3 per time point). OFs in the EWDS were exposed to one 30-second flush immediately prior to removal (after one hour, 24 hours, 72 hours, five days and 10 days). Water delivered through the OFs was collected in sample bottles containing 20 mg/L sodium thiosulphate (Scientific Laboratory Supplies, Nottingham, UK) and sampled as previously described (Chapter 4.3.3). Plates were incubated at 37°C for up to 48 hours. Water contamination levels were recorded as above (≥10 CFU/100 mL) or below (1-9 CFU/100 mL) the alert level for

augmented care, or below the detection limit of the assay (<1 CFU/100 mL). This experiment was repeated on three separate occasions.

2.4.1.3 Recovery of *P. aeruginosa* from outlet fittings

Immediately after the water samples were taken, OFs (n=3 per time point) were removed from the EWDS, rinsed with 20 mL sterile water to remove any loosely attached cells and/or residual water, and transferred to 30 mL universal tubes containing 5 mL sterile thiosulphate Ringers solution (Oxoid) and 5 sterile glass beads. OFs were vortex mixed for two minutes and the resulting suspension serially diluted (ten-fold). Aliquots (100 µL) of each dilution were plated onto CN agar and incubated at 37°C for up to 48 hours. Control OFs (i.e. artificially contaminated OFs that were not inserted into the EWDS (T_0)) were sampled in the same way. This experiment was repeated on three separate occasions.

2.4.2 Cloth contamination of outlet fittings

2.4.2.1 Contamination of outlet fittings and *P. aeruginosa* recovery

Test suspensions were prepared by inoculating 100 mL of nutrient broth with a single colony of *P. aeruginosa* and incubating at 37°C with shaking (120 rpm) for ~16 hours (i.e. overnight).

Microfibre cleaning cloths (80% polyester and 20% poly-amide; Arco, Hull, UK) were cut into 10 cm^2 swatches, weighed and inoculated with 10 mL of the bacterial test suspension (~10^8 CFU/mL). Each swatch was wrung and reweighed, before being used to wipe the accessible surfaces of between one and nine OFs for 10 seconds. Cloth-contaminated OFs (all types; Table 2.5) were inserted into taps on the EWDS (known to be negative for *P. aeruginosa*) and left in situ for 15 minutes, one-, four-, eight-, 12-, or 24 hours.

At each time point, OFs (n=3) were removed from the taps and transferred without rinsing to 5 mL recovery medium (i.e. thiosulphate Ringer's solution or Dey-Engley (D/E) neutralising broth (Sigma-Aldrich, Poole, UK); see 5.3.3) containing five glass beads. *P. aeruginosa* recovered as previously described (5.3.1.3). Control OFs (i.e. cloth contaminated OFs that were not inserted into the EWDS (T_0)) were sampled the same way.

Table 2.5 Conventional and alternative outlet fittings investigated

Outlet fitting	Manufacturer	Description	Main materials	Claim (s)	Image
A	Manufacturer confidential	Conventional outlet fitting, multi-layered plastic rosette. Plastics included rialene, hostaform and basell	Rialene, hostaform, basell	Straightens and regulates flow of water	
B	Manufacturer confidential	Single-bore with a copper (100%) interior lining	Copper	Antimicrobial Straightens and regulates flow of water	[Photograph removed for confidentiality]
C	Manufacturer confidential	Single-bore made from hostaform	Hostaform	Anti-biofilm Straightens and regulates flow of water Non-splash* * *Since reporting the results of this study to the manufacturer, this claim has been retracted*.	[Photograph removed for confidentiality]
D	Manufacturer confidential	Conventional-style multi-layered silver-ion impregnated plastic rosette surrounded by a sheath that	Silver-ion impregnated hostaform, brass (>76% copper)	Antimicrobial Straightens and regulates flow of water	[Photograph removed for confidentiality]

Outlet fitting	Manufacturer	Description	Main materials	Claim (s)	Image
		projects beyond the internal device			

2.4.2.2 Neutralisation of antimicrobial ions

Materials associated with OF-B and –D were previously shown to release copper-ions (Chapter 3.4.3.2). Copper-leaching during *P. aeruginosa* recovery from OFs was investigated by contaminating OF-B and -D (n=5) with *P. aeruginosa* as per 5.3.2 and recovering in D/E neutralising broth or thiosulphate Ringer's solution as described in 5.3.2. Control OFs (i.e. OF-A which contains no antimicrobial materials) were tested in the same way (n=3). *P. aeruginosa* recovered as previously described (5.3.1.3).

2.4.2.3 The effect of flushing on cloth-contaminated outlet fittings

Outlet fittings (contaminated as described in 5.3.2) were inserted into the EWDS. Each tap was flushed (30 seconds, 4.5 L/min) either once over a 24 hour period (OF-A only), or five times over a 20 minute period (all OF types) to simulate an infrequently or frequently used tap respectively. Chlorine levels were approximately 0.03-0.22 mg/L. During each flush, water samples (500 mL) were collected in sample bottles containing 20 mg/L sodium thiosulphate. The presence of *P. aeruginosa* was determined by filtering 100 mL of each water sample through a 0.2 µm membrane. Membranes were transferred to CN agar and incubated at 37°C for up to 48 hours. Experiments were repeated on four separate occasions.

2.4.3 'Antimicrobial outlet fitting' release of active antimicrobial agents

Two of the three OFs (OF-B and-D) claimed antimicrobial properties and were tested for leaching of antimicrobial agents. Leaching of copper ions from materials incorporated in OF-B and –D was confirmed in Chapter 3.4.3.2. OF-D was inserted into taps on the EWDS and the first sample of water collected. OF-B was inserted into a separate secondary EWDS, which incorporated a corresponding tap by the same manufacturer, allowing a secure, water-tight fit for the OFs. The secondary EWDS was run under the same temperature and flow rates as the primary EWDS and was fed by the same water supply. Water samples were tested for copper ion presence using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (iCAP Q ICP-MS; Thermo Fisher

Scientific) as described in Chapter 3.3.6. Water samples from taps containing OF-D were also tested for silver ions by ICP-MS and silver nanoparticles using a NanoSight microscopy (Malvern Instruments Ltd., Amesbury, UK) as described in Chapter 3.3.6.

2.4.4 Data analysis

Statistical analysis was performed using GraphPad Prism (version 7.00 for Windows). Parameteric data were compared using unpaired *t*-tests or one-way analysis of variance (ANOVA) followed by Šidák's multiple comparisons test when comparing select groups or Tukey's multiple comparison test to compare all groups. Non-parametric data were compared using Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparisons test or a Mann-Whitney test to compare two groups. Statistical significance set at $p<0.05$. X^2 –tests were performed to test for independence between contamination levels of flushed water and the number of flushes. The probabilities of the X^2 results were calculated using the GraphPad Prism tool http://graphpad.com/quickcalcs/pValue1/.

Water contamination levels were recorded as above (≥10 CFU per 100 mL) or below (1-9 CFU per 100 mL) the hospital alert limit for augmented care, or below the detection limit of the assay (<1 CFU per 100 mL).

Chapter 3 Water hygiene and the use and maintenance of clinical taps: the knowledge, attitudes and beliefs/opinions of healthcare workers.

3.1 Introduction

Since the publication of the DoH guidance on *P. aeruginosa* control for augmented care (Department of Health, 2013b), UK hospitals are more aware of the presence and associated risks of *P. aeruginosa* in their water systems. The responsibility for producing and executing water safety plans (i.e. control measures required to maintain water safety) lies with hospital WSGs. WSGs should be a multidisciplinary team, with DoH guidance recommending the incorporation of domestic, estates, healthcare and medical staff (Chapter 1.5). Involving representatives from different staff groups allows for a range of expertise and perspectives when deciding on control strategies. Staff roles can dictate interactions with water outlets, which range from using water for patient care (i.e. healthcare staff) to cleaning and maintenance/repairs (i.e. domestic and estates staff).

It can be argued that just as WSGs are responsible for maintaining the hygiene of the water system leading up to the outlet, ward staff are responsible for maintaining the water hygiene between the outlet and the patient. This cannot be achieved if staff are not protecting the tap from retrograde contamination (contaminants being introduced to the tap (and thus tap water) from sources exogenous to the water system; Chapter 1.1.4). The potential for this to occur was highlighted by Garvey *et al.* (2016b), who demonstrated the costs of installing point-of-use (POU) filters to the end of taps (designed to provide filter-sterilised tap water) and the potential for these filters to become contaminated with *P. aeruginosa* introduced from the ward environment (retrograde contamination).

One potential method of retrograde contamination has been addressed on a large scale throughout UK hospitals, in line with DoH guidance (Department of Health, 2013a). Locating the basin drain directly beneath the tap's flow of water led to splashing and dissemination of the microbial content of the drain (Kotsanas *et al.*, 2013). It is now common place for hospitals to use basins with the drain located away from the flow of water.

However, whilst some routes of retrograde contamination can be engineered-out, many others stem from human behaviour and the interaction between user and taps. For example, direct contact with outlet fittings by patients, staff or visitors (e.g. during the learning process with

automatic taps, if there is a delay between stimulating the sensor and water being eluted) (Vonberg *et al.*, 2008); a cross contamination event during cleaning (Hutchins *et al.*, 2017); or inappropriate disposal of patient fluids down hand-wash basins (Balm *et al.*, 2013), leading to splashback from the contaminated basin to the tap (Hota *et al.*, 2009). Misuse of hand-wash stations by staff has been correlated to contaminated outlet fittings and linked to incidents of waterborne infection (Inglis *et al.*, 2010, Balm *et al.*, 2013). Despite this, few pre-emptive measures have been suggested and/or implemented.

Retrograde contamination of clinical taps has been shown to pose a significant threat to the maintenance of tap and water hygiene in the healthcare environment (Reuter *et al.*, 2002, Rogues *et al.*, 2007). The implications of contaminated water being used on or around the most vulnerable patients has been discussed (Chapter 1.1.2). With WSGs taking extensive measures to control or reduce the microbial burden of their water systems, as well as efforts by the tap industry to engineer-out microbial niches, it is important that these efforts are not made redundant by actions that facilitate retrograde contamination.

The behaviour and actions of all staff, regardless of role, should reduce the potential for retrograde contamination and there is strong evidence that behavioural change and compliance is supported by awareness (Pittet, 2004, O'Donoghue *et al.*, 2016, Schönberger *et al.*, 2006). If staff are not aware of the role water plays as a vector for infection and the role taps play in harbouring opportunistic pathogens, then it should be no surprise that there is poor compliance to recommendations made by their WSGs to minimise these risks. Behaviour has also been reported to be 'sourced' from capability (i.e. whether someone is physically or psychologically able to do something, encompassing training and education), opportunity (i.e. whether there is the social and physical possibility to carry out or prompt behaviour) and motivation (i.e. that which leads to the incentive to act), referred to by Michie *et al.* (2011) as the COM-B system (Figure 3.1).

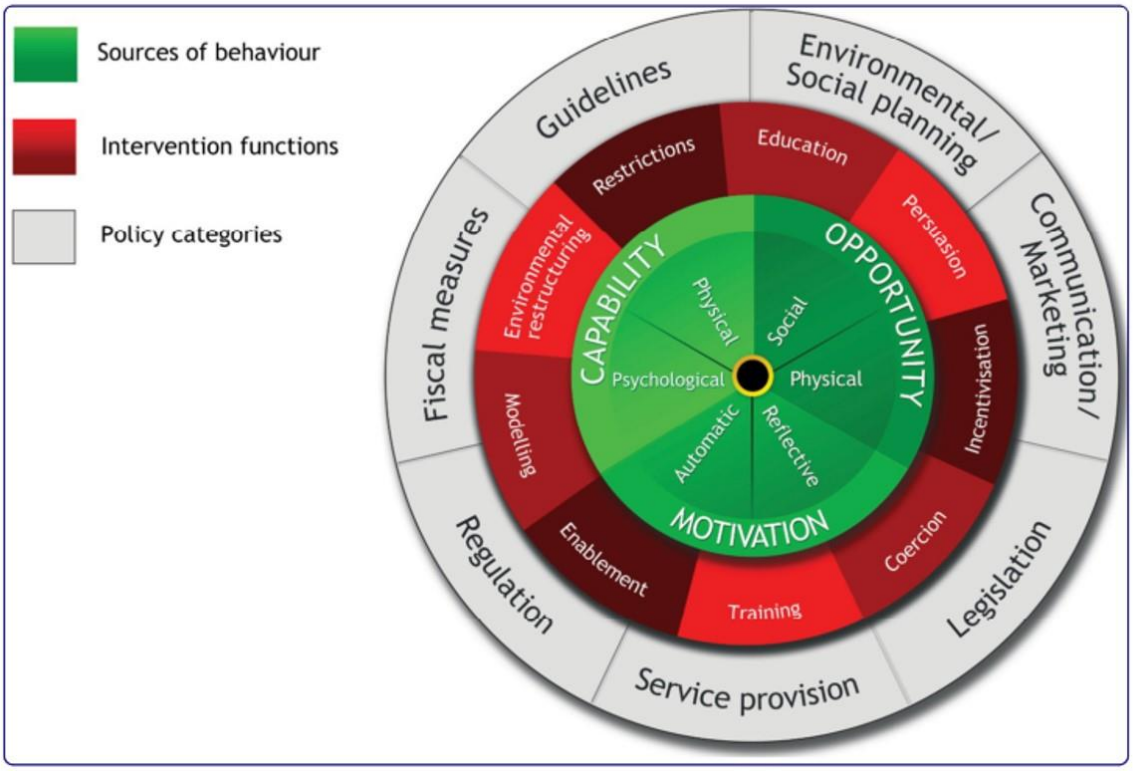


Figure 3.1 The behaviour wheel (COM-B system). Behaviour can be derived from: capability (i.e. whether a person has the physical or psychological capability that results from training and skill sets); opportunity (i.e. factors exogenous to the person such as having the physical possibility to do something (for example, being allotted time and provided with the means) and; motivation (i.e. the psychological processes that drive action). All three are able to influence each other, culminating in a particular behaviour. The behaviour wheel incorporates potential interventions and ways in which these interventions can be introduced (i.e. policies) **(***Figure reprinted (Michie et al., 2011))*

It is important to investigate what staff perceive to be risks to provide insight into motivation for or against carrying out particular behaviours. Balm *et al.* (2013) reported that staff misusing hand wash basins do so due to time restrictions and convenience. It is possible that staff consider measures put in place by the WSGs and/or infection control teams to be not as safe as alternative actions/existing practices. For example, staff may judge the transportation of patient fluids to the sluice room a greater risk than (mis)using a hand wash basin in closer proximity to the patient to dispose of the fluid (Walker and Moore, 2014). Whatever the reason for misuse, it is likely that staff are either unaware, or do not comprehend, that such actions on could adversely affect water hygiene (and thus patient health).

It is possible that staff are so task-focused that they do not recognise their responsibilities when indirectly overlapping with the roles of other staff types; the priority of plumbing staff is to

maintain a functional water system, the priority for healthcare workers is the treatment of the patient. If the importance and reasons behind indirect multidisciplinary cooperation are not explained to them, staff may not appreciate how their actions can impact the responsibility of colleagues.

The complexity of overlap in job roles and responsibilities for infection control is immense; Figure 3.2 outlines just a few of the factors that can impact the maintenance of water quality used for patient treatment. For example, it may not instinctively occur to the plumbing staff that the tools they use on outlets/hand-wash stations need to be disinfected between jobs and wards to prevent cross-contamination. If, for example, during routine maintenance of a plumbing component (e.g. a thermostatic mixing valve; TMV), the use of contaminated equipment (e.g. tools/rags/buckets) resulted in contamination of the TMV and/or other plumbing components and, subsequently, contamination of the tap water, the plumbing staff would have impacted the healthcare team's efforts to treat the patients. Similarly, the misuse of hand-wash stations (e.g. by healthcare staff inappropriately disposing of waste material) could cause blockages within the plumbing system impacting the ability of the plumbing staff to maintain an efficient water distribution system. In both cases, such actions would impact the infection control team and their efforts to minimise and prevent transmission of infection.

The misuse of taps/hand-wash stations has been widely reported (Balm *et al.*, 2013, Inglis *et al.*, 2010, Florentin *et al.*, 2016), however such accounts are usually provided by managerial personnel who perhaps spend less time on the wards than the staff they manage. This provides bias in the evidence base, particularly when evidence is anecdotal (e.g. feedback to Public Health England or discussed at conferences/meetings) or is the opinion of the authors rather than resulting from an observational study. Suchomel *et al.* (2013), who provided evidence that flushing taps pre-use to reduce microbial accumulation at the tap, postulated that it would be "impossible to implement this measure," suggesting staff would- or could not find time to comply with the suggested strategy.

It is important to investigate whether general staff have an appreciation for water hygiene; their behaviours and actions that may influence water hygiene and if; they would be willing and/or have the opportunity to change such behaviours. Engaging with general staff may highlight areas where they believe there is a lack of opportunity to comply with WSG control measures, provide insight into why noncompliance occurs and/or inform whether there is a requirement for standardised water hygiene training across all hospital staff, an initiative that is already being trialled and implemented in some Trusts that have dealt with *P. aeruginosa* problems

(Anonymous, 2017). Such training may provide staff with the necessary capability and motivation to lead to behavioural change (i.e. compliance with control measures) (Michie *et al.*, 2011).

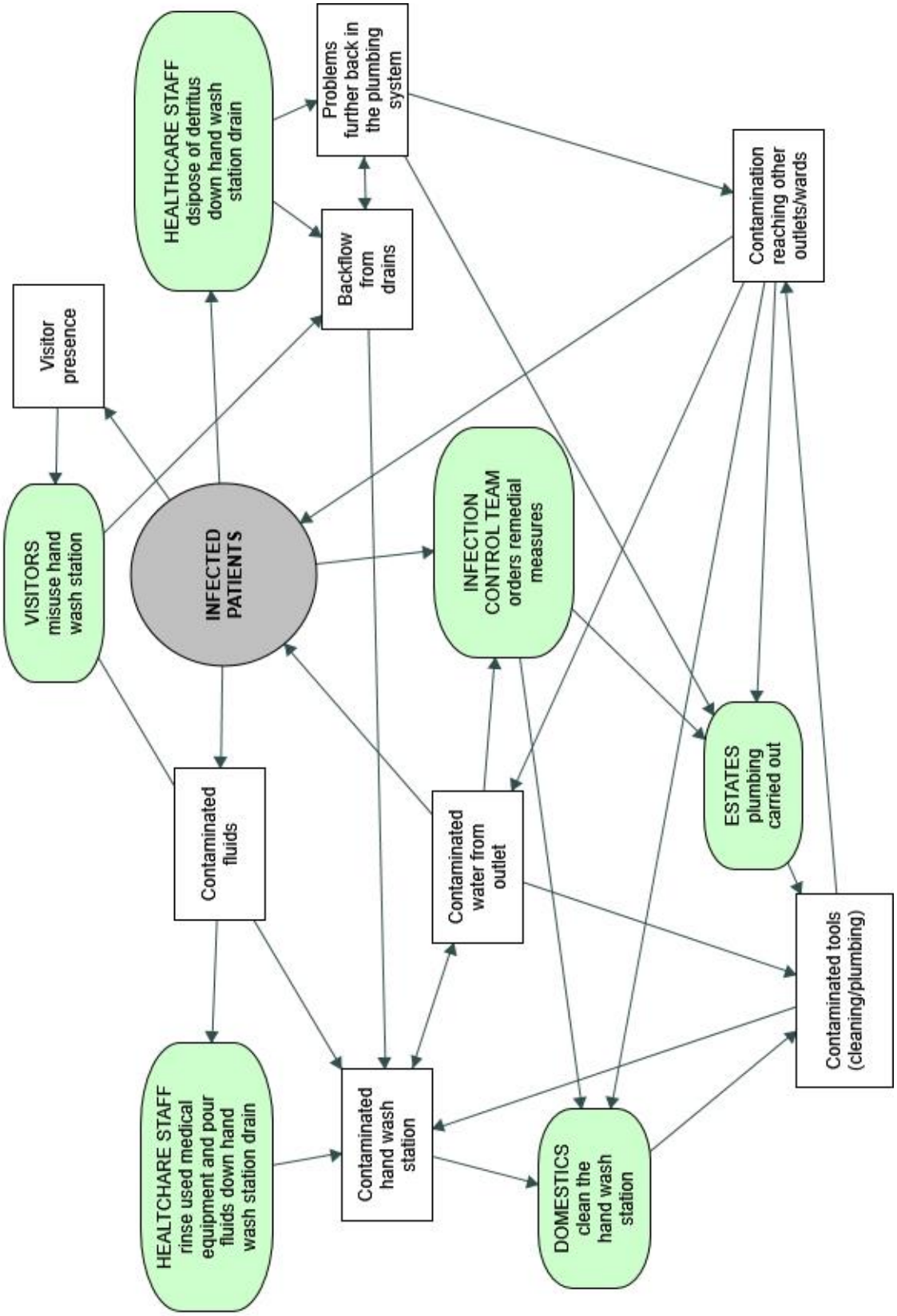


Figure 3.2 Interaction and overlap of behaviours by staff, visitors and patients and the potential impact on infection control.

3.2 Aims

To investigate NHS hospital staff groups' opinions, attitudes and knowledge on issues surrounding water hygiene to determine whether there is a knowledge gap and/or determine areas that require training. The objectives were to:

- Design and pilot a questionnaire to NHS staff investigating knowledge and opinions on issues surrounding water- and tap hygiene.
- Modify the questionnaire appropriately for distribution to NHS England hospitals.
- Analyse questionnaire data to assess:
  - staff appreciation for water as a vector for infection;
  - staff perceptions of retrograde and systemic contamination of taps;
  - staff opinions on the misuse of hand wash stations and behaviour change;
  - staff awareness and perception of risks surrounding tap maintenance and hand hygiene and;
  - staff risk assessment of physical injury vs. infection risk with regards to water safety.
- Determine whether there is a need for staff-wide water hygiene training and/or specific behaviour change.

3.3 Results

3.3.1 Approvals, willingness and responsibility

Of the hospitals initially approached to consider whether they would be willing to partake in the study, four of the five Trusts listed on the IRAS form participated (including the pilot hospital). A common issue was the availability of the principle investigator (PI) designated to each hospital and/or breakdown in communication between them and the researcher (i.e. a lack of reply to queries or failure of both parties to maintain regular communication (weeks-months between responses)). It was reported by one hospital that the poor communication/availability was due to other pressing priorities, such as Care Quality Commission inspections and general workload.

Another challenge was engaging and communicating with the research and development (R&D) offices at the hospitals, with different R&D groups interpreting the study to require different approvals and complications associated with staff turnover (i.e. R&D staff leaving resulting in departments becoming short-staffed) leading to delays in processing approval requests. One particular Trust requested impracticable changes to the questions within the survey despite approval being granted by the HRA; the changes requested would have removed any identification of staff type, meaning that individual staff group responses could not be assessed. The difficulty in appeasing individual institutions in multi-site studies, especially where particular sites have requested changes despite appropriate approvals granted elsewhere, has been reported (Menikoff, 2010). Such changes introduce variability and bias at the detriment of the study, as such it was decided that questionnaires would not be distributed within this Trust.

A somewhat paradoxical problem was that many Trusts would not consider permitting the study within their hospitals unless HRA approval had been granted. A requirement of HRA approval (within the IRAS form) is a list of Trusts and their contacts who have agreed to participate in the study, including written evidence of their willingness of participation.

An additional issue highlighted complications in using a system established primarily for clinical studies for non-clinical, non-patient research. The HRA provides an online advisory "decision tool" (http://www.hra-decisiontools.org.uk/research/) to aid researchers in deciding whether their study requires HRA approval. The questions are provided alongside a glossary to define key terms. The HRA system is so strongly focused on studies involving patient participation that the definitions provided to answer their decision tool are exclusive to clinical studies, for example, "*are your findings going to be generalisable?"* where it states, "*generalisable in this context means the extent to which the findings of a clinical study can be reliably extrapolated from the subjects who participated in the study to a broader patient population and a broader range of*

clinical settings." By the definition provided by the HRA decision tool, this staff survey would not be considered generalisable; a non-clinical definition of generalisable, i.e. the findings of the survey could be applied to settings other than those from which they were taken, indicates approval would be needed. Upon request for clarification by HRA enquiries team, it was confirmed via email that approval was not required; this was later contradicted (due to the generalisable nature of the study) and approval was applied for and granted.

In the United States, research carried out at institutions (hospitals) require an Institutional Review Board (IRB) approval from any IRB involved in a multi-site study, but Menikoff reports an unwillingness of some institutes to accept IRB approval from one another, leading to increased and repetitive workloads for the researchers and individual sites (Menikoff, 2010). Additionally, if an individual site IRB raises concerns with the study, these are not shared with other institutions included in the study; cross-site communication and/or awareness of where IRB approval has been awarded could lead to a breach in the confidentiality agreed to obtain approval. Menikoff's report demonstrates that paradoxical problems are not unique to the UK's approvals system.

3.3.2 Response rates

A requirement of a voluntary anonymous survey is that the researcher should not distribute the questionnaires individually. Participation was dependent upon word-of-mouth and staff head-of -department encouragement. Adoption of this responsibility was highly variable both inter- and intra-study sites. This could be partially explained by communications between the PI and heads of departments/contacts for staffing groups; in the case of one participating hospital, distribution of the questionnaire had to be postponed as a result of breakdown in communications between the PI and staff group contacts (lack of responses due to busy schedules), and in another hospital, this was provided as a reason for no- and low participation in two particular staffing groups.

In total, four hospitals participated in the staff survey: one used to pilot the questionnaire and three used for the final questionnaire.

The overall mean response rate was 43% (128/300 surveys), with the lowest and highest participation at 32% and 53% respectively. The highest staff group participation was from healthcare staff followed by domestic staff with overall responses of 38% (48/128) and 34% (43/128) respectively. The lowest participation was by the medical staff, responsible for only 9% of responses, followed by estates staff at 16%. Within the questionnaires, question response rates ranged from 94-100%. The modal response rate for questions was 97% (124/128 of participants answering). No question was answered by fewer than 120 participants (i.e. the response rate to all questions was ≥94%).Only two questions received 100% response rates (participants were

asked whether they agreed that, “hospital tap water can spread infection,” and, “splashing water from the tap onto medical equipment is not an infection risk”). Seventeen (13%) of participants provided free-text comment and feedback.

3.3.3 Themes arising from open-ended feedback

Four main themes arose from the open-ended feedback option at the end of the questionnaire (Table 3.1): 1) confusion about what was being asked; 2) compliance; 3) training and; 4) the survey providing a platform to express opinions on the topic. Four of the respondents stated they were confused by some of the questions or felt there was too much ambiguity to answer. Two respondents commented on compliance, associating non-compliance with time-pressure (Table 3.1). The most common theme was on training, with ten respondents commenting on training received or training needs/lack of awareness. The second most common theme was the opportunity to use the questionnaire as a platform to express their opinions on the topic, with six participants doing so. Three respondents’ comments fall under both training and opinions, and have been grouped together (Table 3.1). Individual comments will be referred to further throughout the discussion.

Table 3.1 Open-ended feedback/commentary

Theme	**Comment number**	**Comment**
Compliance	1	*“Standard procedure doesn't always mean procedure is being followed and likely that most people don't follow procedure i.e. running taps before handing out water, this is a standard procedure but most people won't follow that procedure as it's too time consuming”*
	2	*“The inability of staff in hospitals to follow all the clinical rules associate with their job is that, they work under pressure. Not enough staff and the few has to rush to satisfy all the patients”*
Training	3	*“We have had training following a pseudomonas outbreak a couple of years ago and we were all informed about cleaning the taps first basin after and to change our cloths between sinks and to change our water frequently. We also have coloured coded buckets and cloths”*
	4	*“As a clinician I am unsure about many of these issues- I probably should know more”*

Theme	Comment number	Comment
	5	*"There should be a general training on tap/water usage to everyone in clinical areas and non clinical areas"*
	6	*"We were advised to gel our hands after each hand washing to avoid contaminated tap water being transferred to patients (pseudomonas)- all staff did initially but not anymore. We run taps that "we do not get used often" once a week to reduce risk of pseudomonas- do not know why"*
	7	*"This survey is an eye opener"*
Platform to express opinions	8	*"It is very good to have my views on this issues. This will help the association to get opinions from the public. Thank you"*
	9	*"Remove most TMVS in most areas. We do not need TMVs in all areas"*
	10	*"Given the number of water outlets across the NHS the risk of Pseudomonas transmission is very very low and continual testify in disproportionate to the risk"*
Training and platform to express opinions	11	*"Ensuring good standards of hygiene within your water ventilation systems is essential. Regular monitoring and testing, along with cleaning will not only ensure that you meet all health and safety requirements it will also show your staff that you take health and safety and their well being seriously. Therefore all of the staff in this hospital should get an advance training about water hygiene"*
	12	*"Hospitals to have special water taps that reduce bacteria contamination. All hospitals to have a standard principles of disposing waste water apart from body fluids or faeces. Infection control to be mandatory for all staff apart from the E-learning session"*
	13	*"Regular checking of our tap water to avoid spread of infection! We need thermometer to measure the temperature of water for patient use. Training staff as hospital tap water can spread infection"*

3.3.4 Staff appreciation for water as a vector for infection

Staff were asked whether they agreed that tap water can spread infection (question 4; page 168), and overall 59% (76/128) of staff agreed (Table 7.1). However, the 17% (21/128) disagreeing and 24% (31/128) of staff responding that they were uncertain highlights a gap in the knowledge of hospital staff.

When asked if disinfection of the hospital water supply would lead to 'safer' water than that delivered from domestic taps, almost half (48%; 9/19) of the estates staff agreed. In contrast 44% (21/48) of healthcare staff disagreed, a difference of opinion that may be due to how the different staff groups interact with hospital water.

When asked about routes of transmission, 66/127 (52%) of respondents correctly disagreed that tap water only presents an infection risk if spray, or water droplets, are inhaled. However, whilst the majority of medical (92%; 11/12) and healthcare staff (60%; 29/48) disagreed that aerosolisation was the only route of tap-associated waterborne infection, many of the domestic staff (46%; 20/43) were uncertain. Estates staff were divided, with 42% (8/19) agreeing and 47% (9/19) disagreeing that aerosolisation was the only route of tap-associated waterborne infection.

The majority of staff (59%; ranging from 50% (healthcare staff) to 83% (medical staff)) disagreed that the splashing of water from a tap onto medical equipment is not an infection risk. A large majority (75%; 15/20) of estates staff believed that medical device contact with tap water could lead to contamination/ an infection risk.

When asked whether staff had received water hygiene training, 53% (64/122) responded that they had, with large majorities from the estates (94%; 17/18) and domestic staff (73%; 30/41). Seventy-seven percent of healthcare staff either had not (30/47) or were uncertain (6/47) whether they had received training.

3.3.5 Staff perceptions of systemic and retrograde contamination of taps

Fifty-seven percent (71/124) of respondents believed that taps are more likely to become contaminated by bacteria from the ward than from the water supply (i.e. retrograde contamination) (question 5; page 169 (Table 7.2)). This belief was most common among estates (79%; 15/19) and medical staff (83%; 10/12). None of the medical staff respondents believed the water supply to be a greater cause of contamination.

3.3.6 Staff opinions regarding the misuse of hand-wash stations and behaviour change

Regardless of staff group, the majority of staff (89%; 113/127) agreed that poor maintenance, misuse or inappropriate cleaning of taps can lead to an increased risk of infection from tap water (question 4; page 168 (Table 7.3)). Overall, only 3% (4/127) of staff disagreed

Staff were provided with an example of misuse: disposal of patient fluids down the hand-wash basins instead of sluices (question 3; page 166). Overall, 66% (84/127) of staff believed that this would have a negative impact on infection control, with the highest proportion in agreement from the medical staff (75%; 9/12). Staff were also asked whether they thought clinical staff (i.e. healthcare and medical staff) would be willing to comply with only using sluices for disposal of patient waste (question 6; page 170). Overall, 69% (85/124) thought that clinical staff would be likely to follow this procedure (Table 7.4). Thirty-three percent (4/12) of medical staff and 15% (7/48) of healthcare staff stated it was already standard practice. The majority of healthcare staff believed clinical staff would be willing to dispose of patient fluids in the sluice room (75%; 36/48).

When asked about the behaviour of patients, the majority (60%; 76/127) of staff believed patients misusing hand-wash stations could lead to an increased infection risk. The domestic staff, who would be responsible for maintaining the hand-wash station during and after patient stay, had the lowest proportion of respondents believing (52%; 22/42) and highest proportion of respondents disagreeing (38%; 16/42) that patient misuse could lead to an increased risk of infection.

Forty-eight percent (61/127) of staff believed blocking hand-wash basins could increase risk of infection. Thirty-seven percent of healthcare staff did not believe this would increase the risk of infection (37%; 18/48). In contrast 26% of domestic staff did not agree this would increase the risk of infection whilst 52% (22/42) agreed.

Staff were also asked whether they believed the risk of infection is increased if plumbing tools are not disinfected between jobs/wards (question 3; page 166). The majority of the estates staff (75%; 15/20) agreed with this statement (Table 7.3), yet only 37% of these respondents believed their own staff group would disinfect tools between tasks (Table 7.5). Only one of the 19 estates staff (5%) reported that the disinfection of tools was already standard practice.

3.3.7 Staff awareness and perception of risks surrounding tap maintenance and hand hygiene.

Staff were asked how likely infrequently-used taps were to increase the risk of infection (question 3; page 167), and overall 50% (63/126) of staff responded it was likely (Table 7.6). Forty-four percent (54/122) of staff were aware of infrequently used taps located on their ward (s) (question

5, page 169), 31% (38/122) believed their taps to be in use often and 25% (30/122) were uncertain (Table 7.6); medical and estates staff reported the highest level of uncertainty.

A large majority of staff (81%; 100/124) agreed that some taps accumulate "dirt and grime" more than others (i.e. prone to biofilm such as the components investigated in the Northern Irish *P. aeruginosa* incidents (Walker *et al.*, 2014)) (question 5; page 169). However, fewer staff (65%; 82/126) thought it likely that taps which are harder to clean/are easily contaminated pose an infection risk (question 3; page 167) (Table 7.6).

Fifty-six percent (71/126) of staff agreed that non-compliance in hand hygiene procedures would be detrimental to infection control. Very few staff were uncertain (6%; 7/126) however the largest proportion of staff selecting this option were the medics (17%; 2/12).

3.3.8 Staff risk assessment of physical injury (immediate consequences) vs. infection risk (delayed consequences)

When staff were asked whether they believed the risk of scalding from hot water (a potential immediate consequence of not having a TMV in place) was greater than the risk of bacterial infection (a potential delayed consequence of having a TMV in place), more staff disagreed than agreed, but there was a large amount of uncertainty (42%; 52/124 (Table 7.8)) (question 7; page 171). The estates group had the largest majority of opinion (63%; 12/19), disagreeing that the risk of scalding outweighed that of infection. However, when asked whether they thought clinical staff would be willing to use a thermometer for water for patient use rather than relying upon a TMV (question 6; page 170), 47% (9/19) of estates staff stated it was unlikely (Table 7.9), and healthcare staff had the highest proportion of responding the same (54%; 26/48). Nine percent (11/124), including 15% (7/48) of healthcare staff, stated use of a thermometer in place of temperature controlled taps was "not applicable".

When asked whether they believed the physical risks associated with splashing were greater than the risk of patients coming into contact with contaminated water (i.e. by having outlet fittings in place to regulate the flow), the greatest proportion of staff (39%; 48/124) were uncertain (question 7; page 171).

3.4 Discussion

3.4.1 Staff appreciation for water as a vector for infection

Water is an important vector for infection (World Health Organization, 2014), and many nosocomial outbreaks have been associated with contaminated tap water (Ferroni *et al.*, 1998, Bert *et al.*, 1998, Schvoerer *et al.*, 1999) and its dissemination by staff (Reuter *et al.*, 2002). It is, therefore, important that staff should be aware of the importance of tap water and its potential impact in terms of infection control. Ideally, all staff ought to have known that tap water can and does contribute to the spread of infection, highlighting a gap (or uncertainty) in the knowledge of hospital staff.

The recent (and heightened) attention to waterborne pathogens, particularly *P. aeruginosa,* and subsequent infection control measures (e.g. disinfection of hospital water, taps and enhanced water testing) could give an impression that hospital tap water is 'safe' and/or not likely to spread infection. Estates staff, responsible for installing and/or maintaining water disinfection strategies (e.g. chlorine dosing), may believe their actions to be effective, leading to hospitals having 'safer' tap water than that in a typical domestic setting. It is also possible that healthcare staff, who deal with the consequences of contaminated water in the patients (i.e. HCAIs), may not believe hospital water disinfection strategies are effective, making it no 'safer' than domestic tap water.

Despite their limited healthcare training, a large majority of estates staff believed that medical device contact with tap water could lead to contamination/ an infection risk, but only half of healthcare staff believed this. Medical device contamination in association with tap water is a common problem for catheters and pulmonary devices such as endotracheal tubes for ventilators (Ferroni *et al.*, 1998, Vallés *et al.*, 2004). The primary group involved in the insertion of medical devices such as urinary catheters, as well as maintenance and routine patient care (such as washing) of patients with medical devices in place, is healthcare staff. Catheter-associated urinary tract infections, such as *P. aeruginosa*, have been associated with contact with contaminated tap water used for daily washing procedures (Ferroni *et al.*, 1998). Vallés *et al.* (2004) linked *P. aeruginosa* colonisation of intensive care patients' stomachs to presence of gastric tubes exposed to contaminated tap water, either directly (using water to flush through medication) or indirectly (healthcare worker's hands). The same study reported ventilator-associated pneumonias (VAPs) caused by *P. aeruginosa* were sourced from tap water in 50% (4/8) cases. Although precise routes of transmission for VAPs were not defined, it is conceivable that indirect transmission of contaminated water via healthcare workers' hands could have led to bacterial contamination of ventilator tubing in these cases also.

It may therefore be a cause for concern that half of healthcare staff agreed or were uncertain that such devices coming into contact with (non-sterile) tap water would not pose a risk of infection. The other staff group involved in medical device insertion, such as endotracheal tubes, is medical staff. However, in contrast to healthcare staff, a large majority of medics were in agreement that contact with tap water posed an infection risk.

These results demonstrate that staff generally had an appreciation for water as a vector of infection and various waterborne pathogen transmission routes. Staff who reported being trained in water hygiene (i.e. domestic and estates staff) demonstrated the poorest knowledge of waterborne transmission routes outside of aerosolisation. Estates staff are likely to be familiar with building regulations and codes of practice associated with the control of *Legionella* in water systems (Health and Safety Executive, 2017b, Health and Safety Executive, 2017a), for which risk assessments are focused on Legionnaire's disease (contracted by inhaling or aspirating contaminated water as droplets or aerosols (Fields *et al.*, 2002)). The microbiological training delivered to estates for *Legionella* awareness may have a role to play in them having the largest staff proportion agreeing aerosolisation was the only transmission route of waterborne pathogens. However, overall staff awareness of transmission routes for waterborne pathogens could be improved and existing water hygiene training programmes should focus on this as a major learning objective.

Staff may not be aware they are behaving contrary to best practice if training (or an education from which they could extrapolate and apply relevant information) has not been provided. The reported lack of training (or confidence that water hygiene training had been provided) implies that the capability via education is not universal. This could lead to problems when hospitals expect good water hygiene practices but specific behaviours are undefined or rely upon 'common sense'.

Training was a main theme of open-ended feedback. Fifty percent (4/8) of training-related responses (Table 3.1; comments 5, 11-13) stated that training ought to be in place. Two respondents indicated a need for all staff (clinical and non-clinical) to receive water hygiene training (comment 5) An additional two respondents admitted to a personal lack of awareness, (comment 7 and 4). These comments demonstrate a belief that more training is required (i.e. that staff have not been provided with the capability to reduce waterborne infection). Comments also imply that this should be resolved by hospitals.

One respondent commented staff had received training in response to a *Pseudomonas* outbreak (Table 3.1; comment 3). This comment implies the scope of the training provided was task/role specific as opposed to a broad water hygiene training session, further evidenced by domestic staff

generally demonstrating poor water hygiene knowledge in the questions discussed in Table 7.1. Another respondent stated that they were made to carry out a practice (flushing taps) without knowing why (Table 3.1; comment 6). This indicates poor communication between WSGs (who were likely to have been responsible for this control measure) and the staff relied upon to carry out the task. Comment 6 demonstrates not only a lack of communication/education (i.e. capability) for staff, but also a lack of awareness of the WSG's motivation (which could be adapted for their own motivation, e.g. patient safety). Capability and motivation have been reported as key factors for behaviour/behaviour change (Michie *et al.*, 2011).

3.4.2 Staff perceptions of systemic and retrograde contamination of taps

Contamination is likely to be introduced to hospital taps by one of two directions: from the water system leading up to the outlet (systemic contamination) or from the ward environment back to the tap (retrograde contamination) (Department of Health, 2016a, Department of Health, 2013b). Systemic contamination could be the result of biofilm within the plumbing system including storage tanks, pipework and tap components (Best *et al.*, 1983, Walker *et al.*, 2014, Kilb *et al.*, 2003), whilst retrograde contamination can be caused by misuse of hand-wash stations (Balm *et al.*, 2013, Inglis *et al.*, 2010).

The DoH has stated that where water decontamination protocols are in place and effective against *Legionella* (systemic contamination), the origin of *P. aeruginosa* tap contamination could be due to staff or patients (Department of Health, 2016a). It is therefore important that staff have an appreciation for this reality as there is a potential impact on patient health.

These results imply that staff are generally aware of retrograde contamination (i.e. have capability), and any strategies put in place to reduce retrograde contamination should perhaps focus on motivation and opportunity.

3.4.3 Staff opinions regarding the misuse of hand-wash stations and behaviour change

Retrograde contamination, acknowledged by staff as a risk to tap hygiene (Chapter 3.3.5), has been associated with misuse of hand-wash stations (Chapter 1.1.4).

The disparity both within and between staff groups regarding what is considered standard infection control practice with regards to disposal of patient fluids highlights a training/re-education requirement. For this particular example (i.e. use of hand-wash basins as sluices) DoH guidance states, "do not dispose of body fluids at the clinical wash-hand basin. Use the slophopper or sluice in the dirty utility area to dispose of body fluids," (Department of Health,

2016c). Despite a low proportion of healthcare staff believing disposal of patient fluids in sluices to be standard practice, the majority believed behaviour change was likely. As capability appeared not to be a major issue (i.e. the majority of staff are aware of the risks associated with the behaviour) hospitals should focus on opportunity (i.e. facilitating the correct disposal of patient fluids) and maintaining motivation using visual prompts such as posters used in behaviour change campaigns such as the 'cleanyourhands' campaign, encouraging compliance with hand hygiene (Fuller *et al.*, 2015).

Patient misuse of hand-wash stations has been reported, including hand contact with point of use filters and their removal to increase water flow (Florentin *et al.*, 2016). Vonberg *et al.* (2008) highlights the risk that such misuse poses to tap hygiene through retrograde contamination and recommends detailed instructions are provided to patients to prevent this from happening.

Domestic and healthcare staff, who are in most frequent contact with patients, should appreciate the risk of patient misuse of taps so that they can prompt patient compliance with hygienic use the taps.

Additionally, anecdotal evidence (presented by healthcare professionals and estates staff at conferences, meetings and/or teleconferences) suggests patients hang cloths and clothing over taps and also use the sinks to dispose of foods and patient fluids. Anecdotal evidence, although unpublished, should not to be ignored; a common piece of evidence is the discovery of foods in hand-wash station drains (such as grapes), which introduce nutrients to drain traps, already known to harbour bacteria (Breathnach *et al.*, 2012). Unpublished investigations from Public Health England have provided evidence of clinical and miscellaneous waste present in traps (tap U-bends) removed from hospitals (Figure 2.3).

Modern hand-wash stations include a simple drainage hole at the back of the basin and do not usually come supplied with a plug. These holes can be 'plugged' using materials such as paper towels, allowing the basin to fill with water, which, anecdotally (observations passed on unofficially at conferences and/or meetings), has been carried out by both patients and healthcare staff.

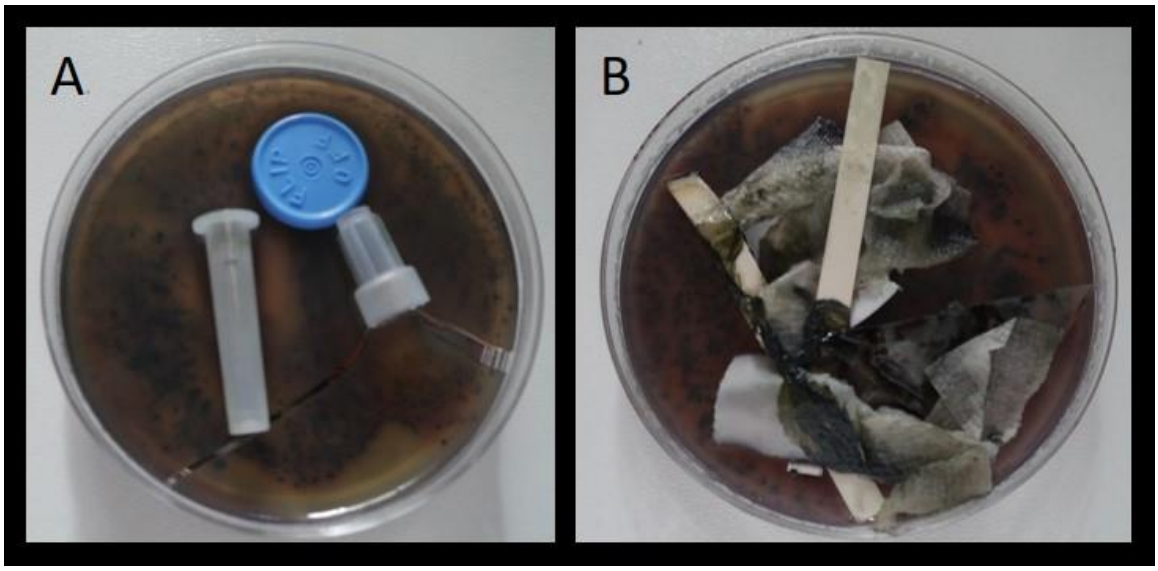


Figure 3.3 Detritus removed from hospital drain traps. Used drain traps were sent to Public Health England from hospitals and detritus removed and placed onto chromID™ CARBA agar (selective for Carbapenemase-Producing *Enterobacteriacaea*). Evidence of tap misuse and inappropriate disposal of medical equipment (lids and caps from syringes etc.) (A) and wipes (B) amongst other detritus. Images taken and provided by Ginny Moore (Public Health England; Ginny.Moore@phe.gov.uk).

Healthcare staff, who may be most likely to witness this behaviour by patients, had the highest proportion of respondents stating that this would not cause an infection risk. Domestic staff, who clean the sinks, had the highest proportion of respondents stating that blocking the drain would increase the risk of infection. Blockages would not only lead to the water collected being contaminated with whatever may be present in the sink, but also have the potential to lead to contaminants from the plug hole area being introduced back into the sink; under normal circumstances the running water would continue to run down the pipework and not stagnate as it waits to drain (i.e. stagnation may provide a means for bacteria to spread from the drain back to the sink). Furthermore, materials such as paper towels or tissue have the potential to become detached from the 'plug' and enter the drainage system. Inappropriate disposal of items such as patient wipes and paper towels have been reported to cause blockages in other types of hospital plumbing, such down as toilets, leading to backflow in other plumbing areas such as the shower drains (Breathnach *et al.*, 2012). The public health implications of not maintaining an efficient drainage system was also demonstrated in the 2003 severe acute respiratory syndrome (SARS) outbreak in Hong Kong, which led to 321 infections and 42 deaths (Gormley *et al.*, 2012). Blockage of hand-wash basins should therefore be discouraged during water hygiene training and highlighted as misuse.

The general degree of uncertainty as to whether estates staff would be willing to disinfect tools between wards among domestic, healthcare and medical staff was possibly due to a lack of evidence, as this mode of transmission has only been suggested in literature and anecdotally but not proven (Walker and Moore, 2014).

3.4.4 Staff awareness and perception of risks surrounding tap maintenance and hand hygiene.

Taps which are under-/infrequently-used can act as a deadleg in a plumbing system (Department of Health, 2002, Curran, 2012), causing water stagnation, which allows for bacterial growth and biofilm development (Suchomel *et al.*, 2013). This means that water dispensed from taps which have undergone stagnant periods is likely to have a higher microbial concentration than water dispensed from a more frequently/recently used tap (Lipphaus *et al.*, 2014).

The groups reporting highest levels of uncertainty as to whether there were infrequently used taps were medical and estates staff; this may reflect the slightly nomadic nature of their roles.

It has been shown that the concentration of *P. aeruginosa* in water dispensed from a hospital tap reduces over the course of a single flush (Charron *et al.*, 2015) and regular flushing of taps is recommended by the DoH, particularly in augmented care units (Department of Health, 2013b). Not all hospitals rely upon a designated flusher, and instead require other hospital staff to ensure all taps are flushed. As previously discussed, one respondent stated that "…*We run taps that "we do not get used often" once a week to reduce risk of pseudomonas- do not know why," (*Table 3.1; comment 6). Increased awareness has been shown to increase compliance, and requesting actions without reason has been associated with non-compliance (O'Donoghue *et al.*, 2016, Erasmus *et al.*, 2009). Anecdotally, staff compliance with flushing taps can be variable, and it is possible that a lack of motivation could be linked to a lack of appreciation for the consequences of non-compliance on patient health. The results highlight the reality of taps remaining stagnant on wards, however the lack of awareness of the potential consequences highlight a training need in staff.

It is possible that 'dirt and grime' was interpreted as non-microbiological accumulation, such as limescale. One respondent demonstrated an awareness of the role of engineering in maintaining water hygiene and a belief that hospitals ought to have antimicrobial/anti-biofilm taps (Table 3.1; comment 12), a goal the clinical tap industry are currently striving towards but as of yet have not achieved/proven.

Staff were further asked whether non-compliance with NHS handwashing procedures would increase infection risks (question 3; page 166); these procedures state that soiled hands should be washed with soap and water and non-soiled hands should be washed with either soap and water or alcohol gel, but not together due to the risks of contact dermatitis (Kampf and Löffler, 2003). The use of alcohol gel instead of handwashing (where appropriate, i.e. no soiling to the hands (Teare *et al.*, 2001)) has been encouraged due to the reduced risk of recontamination of hands by tap water and the reduced time demand compared to that required for soap and water usage (Petignat *et al.*, 2006, Trampuz and Widmer, 2004). The largest proportion of staff uncertain at the impact of hand washing on infection control were the medical staff, whose uncertainty over hand hygiene effectiveness has been previously reported (Erasmus *et al.*, 2009). The launch of the NHS 'cleanyourhands' campaign coincided with increased procurement of alcohol gel and decreases in MRSA bacteraemia and *Clostridium difficile* infection (Stone *et al.*, 2012). The campaign was generally regarded to be a success based on these reasons, and there is evidence that features of the campaign (including raising awareness via posters and introducing alcohol gel at patient bedsides) were sustained (Fuller *et al.*, 2015). However, the proportion of staff who, when asked as part of this questionnaire, did not agree that non-compliance with handwashing procedures would lead to an increased risk of infection, raises questions as to the effective delivery.

Additionally, comments in the open-ended feedback report lack of compliance due to the time-pressure of the job (Table 3.1; comments 1 and 2), i.e. opportunity, a key 'source' of behaviour in the COM-B model (Michie *et al.*, 2011). WSGs/infection control teams need to be made aware of procedures that seem, to general staff, impossible to follow due to lack of opportunity; noncompliance could seriously impede infection control efforts. Raising awareness of these issues by opening communication channels between general staff and WSGs could allow for appropriate solutions to lack of opportunity, such as changing recommended practices, to be decided without compromising water safety.

Taps with a higher risk of contaminated water, from the presence of soiled tap components or biofilm from periods of stagnation, require caution if being used for handwashing, and it has been suggested that in such cases handwashing with potentially contaminated water should be followed by an alcohol gel disinfection (Reuter *et al.*, 2002, Yapicioglu *et al.*, 2012). Post-hand wash alcohol gel application has been associated with the control of a waterborne outbreak of *Chryseobacterium meningosepticum* (Hoque *et al.*, 2001). However it is likely that a secondary control measure, namely the use of sterile water for personal care, had a large role to play. As discussed (Chapter 1) hospitals often apply multiple remedial measures and are unable to distinguish the efficacy of them individually. Staff were divided in their responses as to whether

they would be willing to disinfect hands with alcohol gel after using tap water (Table 7.7). This could have been due to the one-or-the-other approach adopted by hospitals to reduce contact dermatitis as well as the increased time demand for hand-hygiene, reducing opportunity to comply. Thus, it would not be advisable to recommend that hospitals rely upon staff use of alcohol gel after handwashing as a protective measure against poor water quality; any issues with contaminated water should be remediated without disrupting the standard hand-hygiene practices.

3.4.5 Staff risk assessment of physical injury (immediate consequences) vs. infection risk (delayed consequences)

The incorporation of particular tap components to minimise physical injury or increase convenience can sometimes have unintended and undesirable results, for example by providing a bacterial niche. Two such examples include Thermostatic mixing valves (TMVs) to limit the upper temperature of the water and reduce the risk of scalding (Phillips *et al.*, 2011, Quick *et al.*, 2014) and outlet fittings, designed to straighten and regulate the flow of water, reducing splashing (Reuter *et al.*, 2002, Hutchins *et al.*, 2017). In some healthcare settings where TMVs are not in place, water temperature is recorded with a thermometer before allowing patient contact; if this method were widely adapted, it could remove the requirement for a TMV for whole body immersion. In areas such as hand-wash stations, where use of a thermometer would be impractical and time consuming, a TMV is more appropriate.

Staff were asked to prioritise the risks associated with water usage, assessing whether physical injury to themselves or others (avoided by having particular plumbing components in place) was a greater risk than infection from contaminated water (associated with the plumbing components installed to prevent physical injury).

The estates staff, who are responsible for the installation and maintenance of TMVs, were the only group with a majority of opinion disagreed that the risk of scalding was greater than the risk of infection from contaminated water.

Overall, the highest proportion of respondents stated it was unlikely that clinical staff would be willing to use a thermometer for water for patient use rather than relying upon a TMV, with the healthcare staff having the highest proportion of stating this. These results are perhaps reflective of the level of uncertainty in assessing risk (i.e. respondents were doubtful about staff motivation to use a thermometer if risk associated with TMV presence is not appreciated). In contrast, a comment from the open-ended feedback highlighted that some staff would be willing to have TMVs removed and that they felt they were unnecessary in certain places (Table 3.1; comment 9).

Temperature control of water at point of use (either by TMV or use of a thermometer) is important to prevent scalding during patient care, particularly if circulating water levels are raised as a microbial control (i.e. thermal disinfection) (Mirowski *et al.*, 1996). It is possible that the question was misinterpreted (i.e. the concept of a non-TMV tap was not appreciated). Evidence presented by Mirowski *et al.* (1996) of nosocomial scalding highlights the importance of healthcare staff awareness of water temperatures and willingness to use a thermometer where there is no other temperature control.

Outlet fittings have been associated with bacterial contamination and have been recognised as a reservoir of *P. aeruginosa* and other waterborne opportunistic pathogens for many years (Wilson *et al.*, 1961, Mäkinen *et al.*, 2013), and were strongly associated with the *P. aeruginosa* incidents in Northern Ireland (Walker *et al.*, 2014). An advantage of outlet fittings is the regulation of water flow, minimising splashing. Staff were asked whether they believed the physical risks associated with splashing, i.e. slips and falls (a potential immediate consequence of not having an outlet fitting in place), were a greater risk than the risk of patients coming into contact with contaminated water (as a result of contaminated outlet fittings, which could lead to infection as a delayed consequence). Uncertainty demonstrated by staff perhaps reflective of the lack of evidence/studies directly assessing the risks of both.

One respondent's personal risk assessment demonstrated a belief that the efforts made against *P. aeruginosa* contamination of hospitals were disproportionate to the risk they pose (Table 3.1; comment 10). Comment 10 demonstrates a lack of motivation based on personal belief regarding waterborne *P. aeruginosa* and the risk to patients; one that is contrary with the concern of the DoH for *P. aeruginosa* bacteraemia. It is important to remember that controls for *P. aeruginosa (*as well as *Legionella* spp.) are not organism specific, and other waterborne opportunistic pathogens can be influenced by remedial measures in place (Huang *et al.*, 2008), or conversely, conditions allowing *P. aeruginosa* to thrive are likely to also facilitate the survival and growth of other organisms (Mäkinen *et al.*, 2013, Balm *et al.*, 2013).

3.5 Conclusions

The results presented in this chapter have demonstrated that despite some staff having knowledge, attitudes and beliefs in line with those that could aid infection control, there is a lot of discrepancy both across and within staff groups. While the results may not be generalisable to a larger population (with low response rates particularly from medical staff), the responses given provide a snapshot into the knowledge, attitudes and opinions/beliefs of staff surveyed at the time (Kelley *et al.*, 2003), and highlighted important issues in staff who were engaged enough to

participate that should be addressed on a large scale. Motivation to answer the voluntary questionnaire may also have introduced bias in the participants, i.e. those who took part in the survey may have had an interest due to previous/current experience on the topics, however due to the anonymous nature of the survey this was not followed up. It is possible that including an initial question asking about the participants' experience with *P. aeruginosa* or water-associated outbreaks could reduce response rates by making some feel as though the survey was not applicable to them, an issue raised during the pilot study.

Staff reported of lack of capability (i.e. training/education), opportunity (i.e. time and staff availability) and motivation (i.e. reflective motivation such as personal risk assessments or clarity behind behaviour requests), all of which play a central role in forming behaviour change according to the COM-B model (Figure 3.1) (Michie *et al.*, 2011). As mentioned, a limitation of an anonymous survey is that follow-up interviews with participants is not possible, so interpretation of questions (e.g. what was understood by "patient fluids" or "human waste") cannot be investigated and further investigations into particular opinions cannot be carried out on the same staff who provided them. However, whilst face-to-face interviews are likely to give a higher survey response and comprehensive answers, questionnaires can lead to respondents being more open with sensitive or difficult topics than when interacting with the researcher (e.g. in a telephone or face-to-face interview situation), and lead to less bias from 'social desirability' (i.e. answering as they think they ought to instead of honestly) (Bowling, 2005).

Standardised water hygiene training should be mandatory for all staff to eradicate differences in previous training/education. This is imperative due to the potential for one staff group to influence the infection control efforts of another. An important finding from this chapter was that staff groups reporting high attendance to water hygiene training did not demonstrate the best water-hygiene knowledge, possibly due to a task-oriented approach in their training.

A water hygiene training programme should emphasise:

- that hospital tap water is not sterile
- the concept of opportunistic pathogens
- how waterborne microorganisms can reach patients (i.e. the different routes of transmission)
- how behaviour and actions can impact water hygiene and patient safety
- potential routes of retrograde tap contamination
- misuse of hand-wash stations by staff and patients
- the importance of tap cleanliness and maintenance
- the interaction and overlap of different staff groups

- the role of the water safety group

The need for training is supported by some of the open-ended feedback, which demonstrated that awareness on this topic was low and training was both desired and required. Respondents also demonstrated an appreciation for having a platform to express their opinions, perhaps highlighting a lack of interaction between hospital WSGs and the general staff. Improved communication between the WSG and general staff could help WSGs to understand the reasons behind poor water hygiene practices/noncompliance, particularly when caused by lack of opportunity that WSGs may not be aware of.

This chapter also provides evidence of staff personal risk assessment/interpretation, and their belief in staff willingness to comply with infection control strategies, in some cases with a link between the two. Staff generally demonstrated a willingness or belief that other staff groups would take up certain practices except where the practice may be impractical (i.e. lack of opportunity) due to time or staffing constraints.

Staff recognised that tap design also had a role to play in infection control, with open-ended feedback revealing some staff awareness of taps and tap components specifically designed to reduce the contamination of water. However, there is limited evidence regarding the efficacy of novel and existing tap materials and designs marketed as 'antimicrobial' or 'biosafe' and therefore a requirement for further investigation.

The ability of conventional and 'antimicrobial' materials and tap components to support, minimise or prevent the attachment of *P. aeruginosa* and formation of biofilm is the subject of Chapters 4, 5, and 6.

Chapter 4 *P. aeruginosa* biofilm formation on conventional and 'antimicrobial' plumbing materials

4.1 Introduction

4.1.1 Material surfaces and their role in bacterial colonisation

4.1.1.1 Bacterial survival on hospital surfaces

Within a hospital environment, nosocomial pathogens can be spread through contact with contaminated surfaces, such as taps, door handles/push plates, telephones and patient charts, as well as surfaces in the immediate patient surroundings including bedrails, bedside tables and call buttons (Barker *et al.*, 2004, Oie *et al.*, 2002, Getchell-White *et al.*, 1989, Sethi *et al.*, 2010). The survival and persistence of pathogens on inanimate surfaces varies depending on the organism, surface materials and ambient conditions including temperature and relative humidity. Nonetheless, survival on environmental surfaces can be extensive and it has been reported that enterococci can survive for at least 58 days on a countertop (Bonilla *et al.*, 1996) whilst *C. difficile* inoculated onto a floor can persist for five months (Kim *et al.*, 1981). Neely and Maley (2000) highlighted the role material surfaces can play in a study investigating the survival of Gram-positive bacteria on hospital fabrics, with the majority of bacterial isolates tested demonstrating greater survival times on polyester/polyethylene used for privacy drapes and splash aprons than cotton-based materials and cotton-polyester blends used for scrubs, towels and clothing highlighting the role surface material can play in bacterial survival. Neely's results may have been influenced by sampling efficiency, with porous materials such as those containing cotton having lower bacterial recovery than non-porous plastic-based products. Buttner *et al.* (2007) demonstrated that the comparative ease of removing bacteria from a non-porous surface meant the number of bacteria recovered from glass was significantly higher than that recovered from wood.

Waterborne opportunistic pathogens have also been demonstrated to persist on hospital surfaces. *P. aeruginosa* tends to be isolated from inanimate surfaces that are more exposed to high moisture levels, such as cleaning cloths, mops and hand wash station components such as sinks and the plastic casing of tap filters (Engelhart *et al.*, 2002, Orsi *et al.*, 1994, Garvey *et al.*, 2016b). *P. aeruginosa* survival under dry conditions is relatively poor, with low levels of recovery

after two-seven days of desiccation and low correlation between desiccation survival and transmissibility (Skaliy and Eagon, 1972, Panagea *et al.*, 2005).

An advantageous strategy for microbial survival in the environment (as well as *in vivo*) is existence within a biofilm (a community of sessile cells surrounded by protective extracellular matrix (Sauer, 2003) (Chapter 1.1.2)). The tolerance that biofilm imparts against shear and chemical stresses makes cleaning with disinfectants and manual removal of biofilm challenging (Perumal *et al.*, 2014, Simoes *et al.*, 2009, Leonhard *et al.*, 2016).

4.1.1.2 Antimicrobial hospital surfaces

In the UK, a suggested standard is for hospital cleaning to result in fewer than 2.5 CFU/cm^2 (5 CFU/cm^2 internationally) (Griffith *et al.*, 2000, Dancer, 2004). A correlation between hygiene failures (>2.5 CFU/cm^2 on hospital surfaces) and HCAIs has been reported (White *et al.*, 2008).

Surfaces made from materials with antimicrobial properties have been reported to significantly reduce microbial burdens, with copper-based surfaces having levels of <2.5 CFU/cm^2 in 63% of surfaces tested (n=97) compared to only 10% (n=53) of non-copper (wood or plastic) control surfaces (Rai *et al.*, 2012). Incorporation of copper elements into furnishings can also lead to significantly lower levels of bacteria on non-copper elements; an effect dubbed the 'antimicrobial halo' (Rai *et al.*, 2012). However, the reduction observed in hospitals (~2-log$_{(10)}$; (Rai *et al.*, 2012, Schmidt *et al.*, 2012)) are not as great as those reported in the lab, where copper and copper alloys consistently led to up to 6-log$_{(10)}$ reductions compared to stainless steel material surfaces (Warnes *et al.*, 2010).

Other established antimicrobial surfaces include those that incorporate silver. However, unlike copper, the effect of which is not affected by temperature or humidity (Michels *et al.*, 2009), silver exhibits reduced bactericidal effects at lower humidity.

Michels *et al.* (2009) exposed *S. aureus* to silver surfaces under >90% and ~20% relative humidity (RH) and after 24 hours reported log$_{(10)}$ reductions of >5.5 (>90% RH) and <0.2 (~20% RH) regardless of test temperature (20°C or 35°C). Comparatively, copper and copper based alloys gave a difference of <1 log$_{(10)}$ between humidities under the same experimental conditions. As such, silver impregnated materials may not be effective in the hospital environment in dry conditions. This was demonstrated by Wood *et al.* (2007) who trialled silver impregnated stethoscope diaphragm covers in an attempt to reduce HCAIs. All stethoscopes using antimicrobial covers (n=37) were contaminated (some at levels of >400 CFU/diaphragm) and the mean contamination level was significantly higher than control stethoscopes. No antimicrobial

properties were observed and it was speculated that this was due to increased surface area and roughness that resulted from embossing.

In contrast, incorporation of silver in materials that are intended for use in the presence of moisture, has been reported as having good efficacy. Roe *et al.* (2008) demonstrated significant reductions in planktonic survival (n=6) and biofilm (n=12) formation on silver-treated urinary catheters submerged in growth media compared to control catheters, with up to 2-$\log_{(10)}$ reductions in a variety of species including *P. aeruginosa, S. aureus* and *E. coli in vitro*. *In* vivo, Goldschmidt *et al.* (1995) demonstrated that despite similar proportions of colonised catheters for both silver-treated (43%; n=120) and controls (45%; n=113), a significantly lower proportion of patients with silver-treated catheters went on to develop associated infections.

Copper and silver are bactericidal, inducing reactive oxygen species production, leading to DNA damage as well as disruption of intracellular proteins (Grass *et al.*, 2011, Feng *et al.*, 2001). Another metal with antimicrobial properties is molybdenum, which as an oxide (molybdenum trioxide (MoO_3) or molybdic acid in the presence of water) has been shown to have bactericidal effects greater than some antibiotics *in vitro* (Krishnamoorthy *et al.*, 2013). MoO_3 antimicrobial activity is likely to be due to disruption the cell wall (Krishnamoorthy *et al.*, 2013). The antimicrobial effects of MoO_3 have been demonstrated under wet conditions, however the efficacy of MoO_3 treated material was not determined due to the use of non- and semi-quantitative methods (Zollfrank *et al.*, 2012). Zollfrank suggests that as well as high humidity conditions, direct contact of bacteria to the MoO_3 material would be an important factor for any bactericidal effects. Despite manufacturers suggesting incorporation of MoO_3 into materials for tap components, MoO_3 efficacy in conditions representative of a water system is yet to be investigated.

4.1.2 Novel materials and designs marketed to combat *P. aeruginosa* contamination of hospital taps

Through destructive sampling of tap assemblies removed from the neonatal units involved in the *P. aeruginosa* incidents in Northern Ireland, Walker *et al.* (2014) found the outlet fittings to be the most heavily contaminated with *P. aeruginosa (*median counts of 1.8×10^5 CFU/outlet fitting (n=25)). In a subsequent laboratory-based investigation, Moore *et al.* (2015b) demonstrated that solenoid valves, specifically the internal ethylene propylene diene monomer (EPDM) rubber diaphragm, can also became heavily colonised with *P. aeruginosa (*median counts: 1.7×10^5 CFU/cm^{2} (n=10)). In response to these findings and, in an attempt to reduce the risk of microbial

contamination and promote water hygiene, some manufacturers have modified the design of hospital taps and/or the materials used in the manufacture of tap components.

Recent tap designs include detachable and autoclavable tap spouts, spouts with copper exterior surfaces and taps with built-in thermal flushing cycles. Manufacturers are looking to incorporate copper and brass, silver ion- and molybdenum trioxide (MoO_3)-treated plastics in novel outlet fitting designs. 'Anti-biofilm' plastics, which manufacturers claim reduce limescale and bacterial attachment, are also being incorporated. Alternative solenoid valves incorporate diaphragms manufactured using nitrile and silicone-based rubbers.

4.1.2.1 Determining the suitability of novel materials

All materials in contact with drinking water distribution systems must pass the BS 6920 series of tests (Chapter 1.2), including a test to assess for growth of aquatic microorganisms. However, this test assesses growth in the presence of the materials, not growth on the material and thus, despite passing the BS 6920, some materials (e.g. EPDM) have still been associated with microbial colonisation and biofilm formation. It is important to test alternative materials and compare their microbial performance to the surfaces they are intended to replace. Use of the materials simply to market products as an 'antimicrobial', 'anti-biofilm' or 'bio-safe' product is irresponsible, particularly when marketed towards healthcare.

To compare materials like-for-like, the removal of product-dependent variables such as surface area and proximity to water flow can be achieved through the use of material coupons and assessed under controlled laboratory conditions. *In vitro* investigations into the effect of materials on biofilm formation (i.e. when materials are investigated in coupon form and not whole products) may not be exclusive to the tap components of interest. Some of the materials associated with water system contamination, such as the EPDM rubber in solenoid valves, can be found in other areas of plumbing components, e.g. in joins (washers) and piping within plumbing systems (Moritz, 2011, Waines *et al.*, 2011) (i.e. results from such investigations may not be exclusive to the tap components of interest).

P. aeruginosa biofilm formation at the early maturation stage (maturation stage-1) of biofilm development has been reported to occur after a three day incubation period (Sauer *et al.*, 2002). Petrova and Sauer (2009) demonstrated that late stage biofilm formation (maturation stage-2; after six days) can be heavily influenced by strain-specific capabilities. Strains that form poor stage-1 mature biofilms generally continue to produce low levels of biofilm by the six-day time point (i.e. when stage-2 maturation should have been reached). In contrast particular *P. aeruginosa* hypervirulent mutants have been reported to have produced a greater biomass at

maturation stage-1 than at maturation stage-2 (Petrova and Sauer, 2009). Therefore, as long as a strain is capable of forming strong stage-1 biofilms, early maturation is sufficient to provide an indication as to the ability of a material to support biofilm, regardless of strain-specific limitations.

4.2 Aim and objectives

The aim of this chapter was to use an established biofilm model (Chapter 1.3.2) to investigate whether plumbing materials being used as alternatives to conventional materials for solenoid valve diaphragms and outlet fittings, as well as other parts of a tap assembly, have an impact on short-term *P. aeruginosa* biofilm formation. The objectives were:

- To conduct static biofilm assays using the microtitre plate assay to select a *P. aeruginosa* strain for biofilm investigations.
- To validate study methodology and ensure sampling efficiency is not affected by surface material.
- To determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of silver and copper ions (incorporated into 'antimicrobial' materials) against *P. aeruginosa*.
- To use the CDC Bioreactor to investigate short-term biofilm formation on conventional and 'antimicrobial' plumbing materials.

4.3 Results

4.3.1 Strain selection

A one-way ANOVA confirmed significant differences between the biofilm forming abilities of the different isolates. Of the three strains being considered for use (A, B and C), isolate A had the significantly highest biofilm forming ability ($p<0.05$; Figure 4.1), which was statistically comparable ($p>0.05$) to isolate K (selected for its high biofilm forming ability (Perumal *et al.*, 2014)). However, neither isolate formed significantly more biofilm than the other environmental isolates (E-J; Figure 4.1) suggesting isolate A could be considered representative of the isolates tested. The level of biofilm associated with isolate B was comparable to isolate L ($p>0.05$) and thus, was considered a 'low biofilm' forming isolate (Perumal *et al.*, 2014). Isolate C was the weakest biofilm former and produced significantly less biofilm than all other isolates ($p<0.05$). On the basis of these results, and its ability of survival within the Porton regional water, isolate A was selected for further investigations in this chapter.

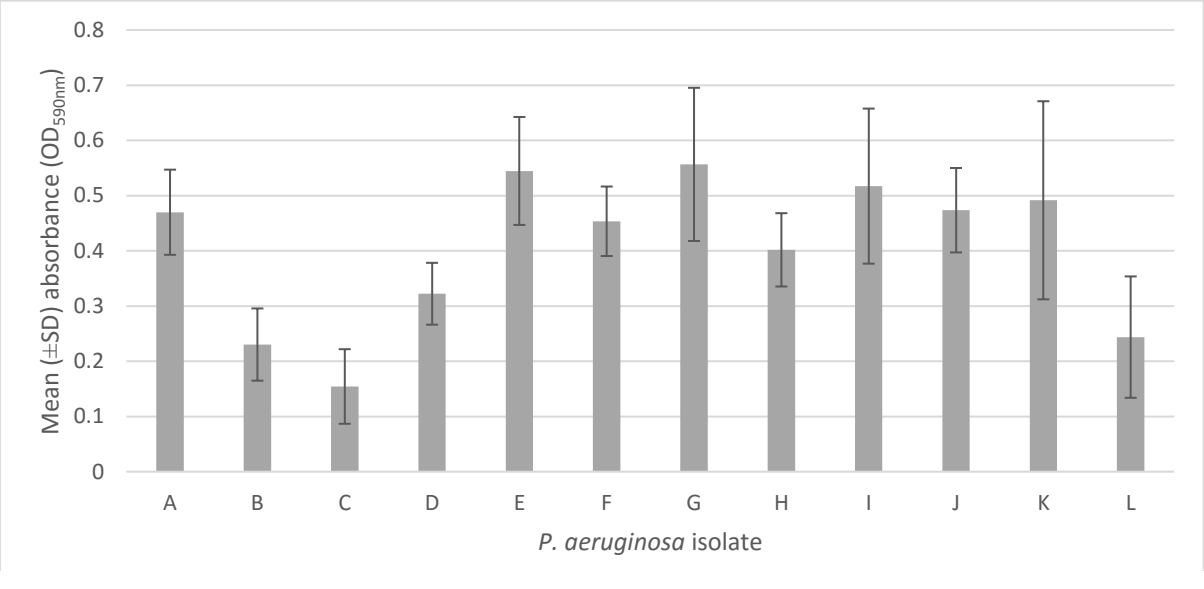


Figure 4.1 Three day static biofilm growth by different *P. aeruginosa* isolates listed in Table 2.1.

Static biofilm assays measured by absorbance from crystal violet staining (mean (±SD) (n=3))

4.3.2 Investigating whether *P. aeruginosa* recovery from coupons is influenced by material

The median number of *P. aeruginosa* recovered from the three rubber materials immediately after inoculation ranged from $1.9x10^4$ (EPDM; n=18) to $2.6x10^4$ CFU/coupon (nitrile rubber; n=18). No differences were significant ($p>0.05$) (Figure 4.2). Similarly, plastic materials had no significant effect upon bacterial recovery, including MoO_3 impregnated rialene ($p=0.58$), silver ion-impregnated hostaform and 'anti-biofilm' hostaform ($p=0.077$) (Figures 4.2 and 4.3). Therefore, the number of *P. aeruginosa* recovered from bioreactor coupons could be considered reflective of the number of cells attached to the surface rather than differences in sampling efficiency.

Recovery was significantly higher from polycarbonate than from both antimicrobial metals ($p=0.0001$), but there was no significant difference in recovery between brass and copper coupons ($p=0.30$) (Figure 4.3). The differences in mean recovery between polycarbonate and copper, and polycarbonate and brass, were $1.2x10^4$ and $1.4x10^4$ CFU/coupon respectively (n=9).

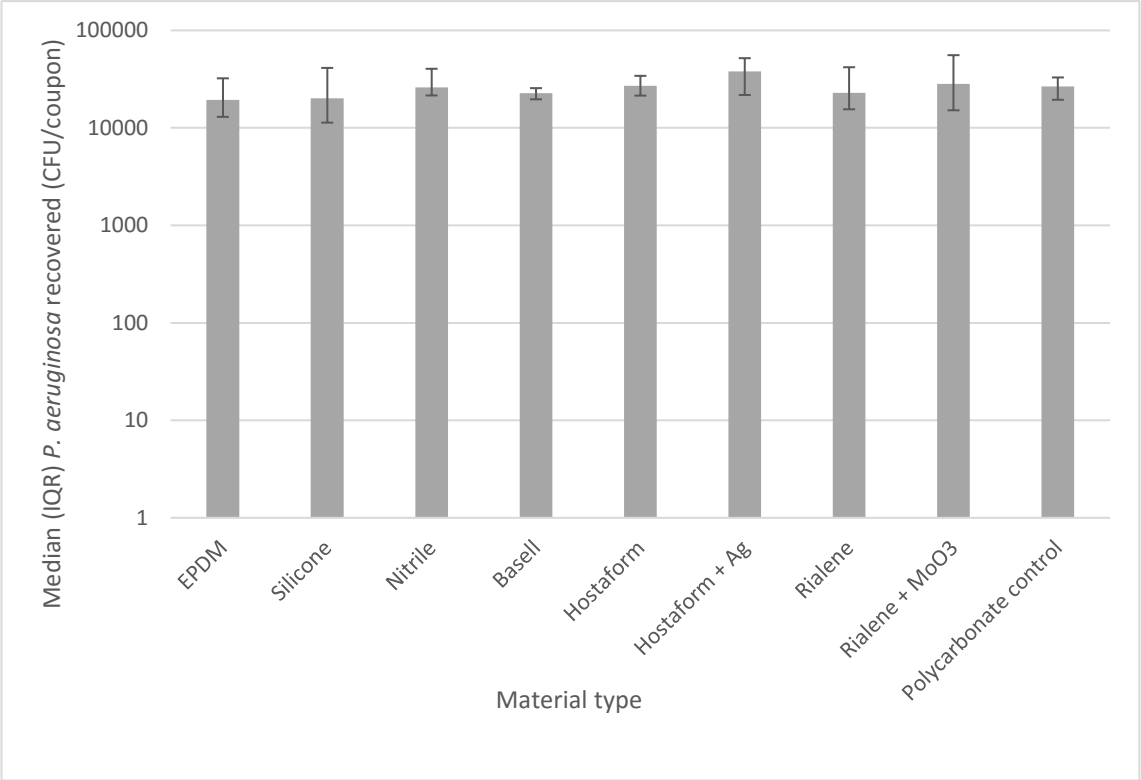


Figure 4.2 **Immediate *P. aeruginosa* recovery from material surfaces.** Coupons were cultured immediately after inoculation. Materials containing non-normally distributed data. Analysis was carried out using Kruskal-Wallis followed by Dunn's multiple comparison. Median (±IQR) (n=18).

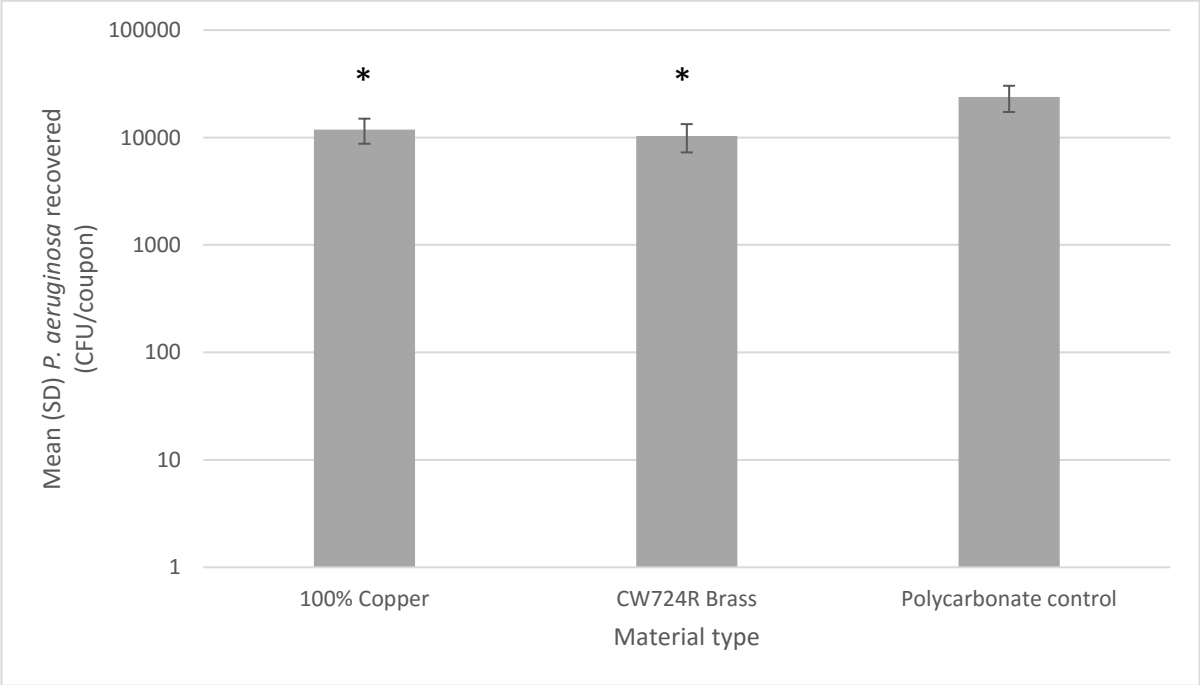


Figure 4.3 **Immediate *P. aeruginosa* recovery from material surfaces.** Coupons were cultured immediately after inoculation. Grouped materials containing normally distributed data only. Analysis was carried out using a one-way ANOVA followed by Tukey's

multiple comparisons tests. Mean (±SD) (n=9). Asterisks (*) indicate significance in comparison to the polycarbonate controls (p<0.05)

4.3.3 Effect and detection of leached antimicrobial agents

4.3.3.1 Investigating the presence of silver nanoparticles

When analysed using NanoSight, the minimum number of particles per frame for a sample to be deemed positive is 20, with an ideal range of 20-60 (Gardiner *et al.*, 2013). The positive control used during this study (10 ppm) had a reading of 52.5 particles per frame (Figure 4.4B). The analysis of water samples exposed to silver ion-impregnated hostaform coupons resulted in 0 particles per frame (Figure 4.4A) implying that no silver nanoparticles had been leached from the material surface.

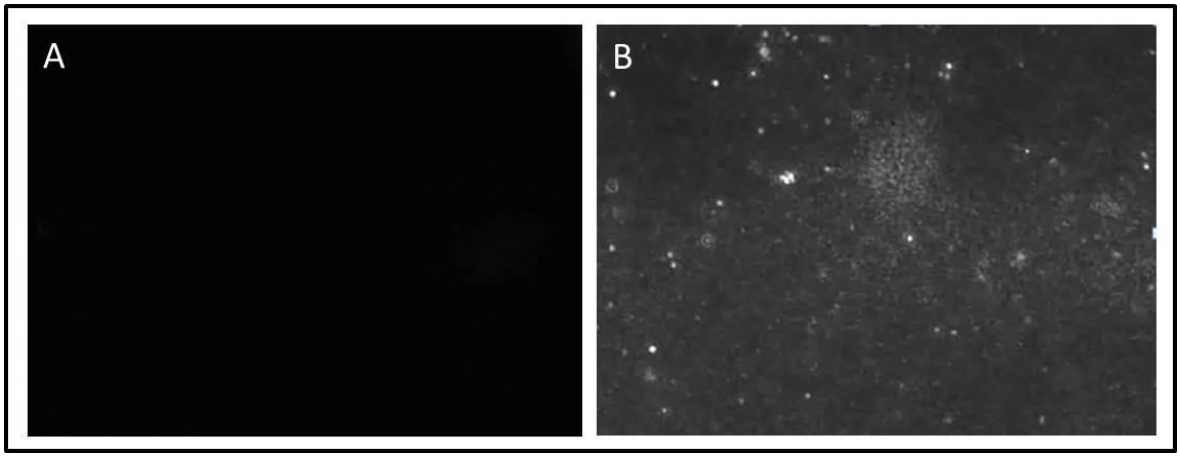


Figure 4.4 Images captured from the NanoSight microscope. A: water exposed to silver ion-impregnated hostaform from the bioreactor (negative for nanoparticles); B: positive control [10 ppm] colloidal silver (white specs are nanoparticles). These results demonstrate that no silver nanoparticles were leached from the silver-ion impregnated hostaform material surface.

4.3.3.2 Detecting the presence of 'antimicrobial' ions

Results of the ICP-MS analysis showed that the level of silver ions present in the bioreactor medium (i.e. filtered tap water) varied considerably. Background silver ion concentration ranged from below the detection limit of the assay (<0.1 parts per trillion) to 0.02 ppb. Water exposed to silver ion-impregnated hostaform for three days contained silver ions at a concentration that ranged from ≤1 ppt to 0.03 ppb. Mean silver ion concentration did not significantly differ from

that of the background water samples (p>0.99) or from water samples exposed to non-impregnated hostaform (p=0.54), implying non-leaching of silver ions.

In contrast, the concentration of molybdenum ions in filtered tap water significantly increased in the presence of MoO_3-impregnated rialene (p<0.0001). The background level of molybdenum ranged from 0.09 ppb and 0.14 ppb. This increased to 4.3 ppb following the three day incubation period, implying molybdenum had leached from the plastic surface. The concentration of molybdenum in water exposed to non-MoO_3 also significantly increased (p<0.0001). However, the concentration detected (≤1.2 ppb) was significantly lower than in the presence of MoO_3.

The background concentration of copper ions in the filtered tap water ranged from 84.0 to 360.2 ppb. This significantly increased during exposure to the copper and brass coupons (p<0.0001). However, results were very variable and equated to a mean increase of 114 ppb (range: 12.1-254.6 ppb) and 338.8 ppb (range: 229.6-448.0 ppb) in the presence of copper and brass respectively.

4.3.3.3 Investigating the minimum inhibitory and bactericidal concentration of silver and copper on *P. aeruginosa*

The MIC and MBC of copper chloride against *P. aeruginosa* was 0.025 and 0.05 ppm (25 ppb and 50 ppb) respectively. The MIC and MBC of silver nitrate was 0.02 and 0.04 ppm (20 and 40 ppb) respectively.

4.3.4 Investigating the effect of material coupons on planktonic *P. aeruginosa*

In the presence of polycarbonate coupons alone, the mean (± standard deviation) reduction of planktonic *P. aeruginosa* in the bioreactor over the three day experimental period was 1.8 (± 0.98)-log $_{(10)}$ (Figure 4.5). In comparison, exposure to both the brass and copper coupons reduced the number of planktonic *P. aeruginosa* by >6 log $_{(10)}$ (p=0.0001). No other material had a statistically significant effect.

Figure 4.5 Mean (±SD) log $_{(10)}$ reduction in *P. aeruginosa* planktonic suspension after three days in the bioreactor (n=3). Asterisks (*) indicate significance in comparison to the polycarbonate controls (p<0.05).

4.3.5 Recovery of *P. aeruginosa* from biofilm on material coupons

Significantly higher numbers of *P. aeruginosa* were recovered from nitrile (6.2x10^5 CFU/coupon; n=17) (p=0.005) and silicone rubber (p=0.002) (5.4x10^5 CFU/coupon; n=17) than from EPDM rubber (2.9x10^5 CFU/coupon; n=17) (Table 4.1). Recovery was significantly higher from all three rubber materials than from the polycarbonate control (1.4x10^5 CFU/coupon; n=17; p<0.05). No other differences were significant.

The median numbers of *P. aeruginosa* recovered from the unenhanced (i.e. non-'antimicrobial') plastics ranged from 2.6x10^4 CFU/coupon (basell) to 3.5x10^4 CFU/coupon (rialene) (Table 4.1; n=15). The type of plastic had no significant effect upon biofilm formation (p>0.05).

The median number of *P. aeruginosa* recovered from hostaform and the silver ion-impregnated hostaform was 1.2x10^5 and 1.3x10^5 CFU/coupon respectively (n=18). Neither significantly differed from the polycarbonate control (2.1x10^5 CFU/coupon) implying the addition of silver ions had no antimicrobial effect. The addition of MoO_3 to rialene was, when compared to the polycarbonate control coupons, equally ineffective (p>0.05; Table 4.2).

In contrast, significantly fewer *P. aeruginosa* were recovered from 100% copper surfaces than from the polycarbonate control coupons. Recovery ranged from below the detection limit of the assay (≤30 CFU/coupon) to $5x10^3$ CFU/coupon. There was no significant difference between brass ($1.7x10^2$ CFU/coupon; n=18) and polycarbonate surfaces ($3.5x10^2$ CFU/coupon; n=18) implying that a reduction in copper content reduces the antimicrobial effect. However, when immersed in the same bioreactor as either the copper or brass coupons, the number of *P. aeruginosa* recovered from the polycarbonate controls was significantly lower than other experimental runs (Table 4.1).

Table 4.1 *P. aeruginosa* biofilm recovery after three days in the bioreactor. Materials containing non-normally distributed data, grouped as per comparison. Analysis carried out using Kruskal-Wallis followed by Dunn's multiple comparison tests, or Mann-Whitney tests (copper-based metal).

Material category	**Material**	**Median (IQR) *P. aeruginosa* recovered (CFU/coupon)**
Rubbers (n=17)	EPDM	2.9×10^5 (2.5×10^5, 4.4×10^5)
	Silicone	5.4×10^5 (3.9×10^5, 9.0×10^5)
	Nitrile	6.2×10^5 (3.7×10^5, 7.2×10^5)
	Polycarbonate (control)	1.4×10^5 (2.4×10^4, 2.0×10^5)
Plastics ('unenhanced'; n=15)	Hostaform	2.8×10^4 (2.6×10^4, 4.0×10^4)
	Basell	2.6×10^4 (1.6×10^4, 3.5×10^4)
	Rialene	3.5×10^4 (2.6×10^4, 1.1×10^5)
	Polycarbonate (control)	3.6×10^4 (2.2×10^4, 2.0×10^5)
Hostaforms (n=18)	Hostaform	1.2×10^5 (7.0×10^4, 3.7×10^5)
	Silver ion-impregnated hostaform	1.3×10^5 (9.9×10^4, 3.6×10^5)

Material category	**Material**	**Median (IQR) *P. aeruginosa* recovered (CFU/coupon)**
	Polycarbonate (control)	2.1×10^5 (1.2×10^5, 2.6×10^5)
Antimicrobial metals: copper (n=18)	100% copper	3.1×10^1 (1.0×10^0, 2.2×10^2)
	Polycarbonate	6.3×10^2 (3.1×10^1, 5.4×10^3)
Antimicrobial metals: brass (n=18)	Brass CW 724R	1.7×10^2 (3.1×10^1, 4.9×10^2)
	Polycarbonate (control)	3.5×10^2 (2.4×10^1, 6.6×10^2)

Table 4.2 *P. aeruginosa* biofilm recovery after three days in the bioreactor. Materials containing normally distributed data, grouped as per comparison. Analysis carried out using an unpaired t-test.

Material category	**Material**	**Mean (±SD) *P. aeruginosa* recovered (CFU/coupon)**
MoO_3 impregnated rialene (n=18)	MoO_3 impregnated rialene	6.4×10^5 (5.9×10^5)
	Polycarbonate (control)	5.0×10^5 (3.8×10^5)

4.4 Discussion

This chapter investigated the attachment of *P. aeruginosa* to different materials during the early maturation stage (maturation stage-1) of biofilm formation, which has been reported in *P. aeruginosa* to occur after a three day incubation period (Sauer *et al.*, 2002). The strain selected for this investigation was shown to have good early biofilm forming ability comparable to a variety of clinical and environmental isolates. It also had a history of surviving within the water supplied to the laboratory and forming strong late-stage biofilm within the EWDS used for previous investigations (Moore *et al.*, 2015b). In contrast, strains isolated from water with lower water hardness levels had historically not survived well in the EWDS (data unpublished). Water samples from Belfast hospital and Porton EWDS were tested for total hardness revealing concentrations of 150 mg $CaCO_3$/L ('slightly hard') and 248 $CaCO_3$/L ('hard') respectively (Drinking Water

Inspectorate, 2009). Preliminary static biofilm assays (Chapter 2.2.1.2) using either Belfast or Porton water (filtered) as media demonstrated that the Belfast strain (isolate C; Table 2.1) formed higher biofilm levels within its own water than Porton water. Thus, it was expected that when added to the bioreactor medium (filtered Porton tap water) the chosen strain (isolate A) would survive and readily attach to the test coupons. Results would be influenced by the surface material rather than the organism itself and/or the organism being in the presence of different water (related to hardness or other chemical constituents) to that from which it was isolated.

Nonetheless, an increased recovery from material coupons may reflect a greater sampling efficiency rather than increased biofilm. Whilst this was not the case for any of the plastic or rubber materials, when sampled immediately after inoculation, significantly fewer *P. aeruginosa* were recovered from copper and brass than from polycarbonate coupons. However, it is likely that this reduced sampling efficiency was due to the bactericidal effect of copper rather than increased bacterial adhesion to the surface. This was further supported by the results of ICP-MS and microbiological culture which demonstrated the presence of copper ions in the bioreactor medium following exposure to copper coupons and a reduced planktonic *P. aeruginosa* counts (Figure 4.5).

Manufacturers producing tap components for the healthcare market are looking to replace or modify materials which have been associated with tap contamination. Some components include materials which, despite having WRAS approval, have had their incorporation in healthcare plumbing advised against, for example EPDM rubber (Department of Health, 2013a, Moore *et al.*, 2015b). The ability of EPDM to facilitate biofilm formation has been demonstrated during field studies (investigating the natural development of biofilm *in situ*) (Kilb *et al.*, 2003) and under controlled conditions within artificially inoculated bioreactors and model water distribution systems (Noble *et al.*, 2016, Moore *et al.*, 2015b, Waines *et al.*, 2011). Controlled studies have focused on biofilms that have established over periods of several weeks to months and have either compared EPDM to non-rubber plumbing materials such as copper and polyethylene (Waines *et al.*, 2011, Moritz *et al.*, 2010), or not made a comparison and merely observed the biofilm development on EPDM alone (Moore *et al.*, 2015b). There is limited evidence in the literature of comparative biofilm studies on multiple rubber materials. Suarez *et al.* (1992) investigated the adherence of bacteria including *Pseudomonas* spp. to EPDM and nitrile rubbers, finding no difference in adherence after one hour of incubation, but did not investigate persistence or further biofilm development, thus providing only a very early indication of potential material colonisation. A study by Ronner and Wong (1993) investigating short-term early stage biofilm (two-day biofilms of *Salmonella* spp. and *Listeria* spp.) demonstrated that nitrile rubber would support biofilm development. However, bacteriostatic effects were observed, with

greater bacteriostatic effects exerted by nitrile rubber than EPDM. A limitation of Ronner's study is that EPDM was only used to demonstrate bacteriostasis and no comparison was made between the two rubbers to determine whether the difference in bacteriostatic effect influenced biofilm formation. A study by Somers and Wong (2004) reported silicone rubber as more 'biofilm resistant' than nitrile rubber. However, statistical significance was not stated and, like Ronner's study, the focus was on food safety and, accordingly, the media used during biofilm development was not representative of water systems.

The current study investigated the attachment of *P. aeruginosa* and the formation of biofilm on three rubbers used in plumbing; EPDM and two considered as alternatives (nitrile and silicone), and was conducted using filtered tap water. Filtration maintains the chemical composition of the water (Colbourne *et al.*, 1988) whilst minimising potential fluctuations in the microbiota of the laboratory water supply, particularly of species reported to readily attach to surfaces such as *Janthinobacterium*, which could lead to competition for colonisation of the material coupons (Douterelo *et al.*, 2014). Other influences the microbiota of the tap water could have stem from cell signalling mechanisms such as quorum sensing, which have been demonstrated to play a role in multispecies biofilm competition (An *et al.*, 2006).

Recovery of *P. aeruginosa* was significantly higher from all three rubber materials than from the polycarbonate controls implying that rubber provides a surface that is advantageous for *P. aeruginosa* biofilm formation. With the proposed alternatives to EPDM (silicone and nitrile) leading to significantly higher levels of *P. aeruginosa* biofilm than EPDM (Table 4.1), the replacement of EPDM rubber could lead to unintended consequences. Comparisons of the three rubbers throughout the literature are normally focused on their physical properties, such as durability under pressures and temperature ranges likely to affect the material *in situ* rather than their microbial performance (Warren, 2008). The significantly higher levels of biofilm on silicone rubber compared to EPDM observed during this study are in contrast to the findings of previous studies which have reported either no significant difference (Noble *et al.*, 2016) or reduced levels of biofouling on silicone rubber (Wagner and von Hoessle, 2003), however these studies took place over several months. These results highlight the importance of a longer-term, controlled study under more realistic conditions than those found *in vitro* to investigate the microbial performance of silicone, nitrile and EPDM rubbers.

Outlet fittings can also become colonised with *P. aeruginosa* (Walker *et al.*, 2014). However, no particular surface material has been implicated and conventional outlet fittings have been condemned as a whole, with hospitals choosing to install new outlet fitting designs (Department of Health, 2013d, Kappstein *et al.*, 2000). It was not clear during the investigations into the

Northern Ireland incidents whether the design (and materials) of the outlet fittings were responsible for the high levels of biofilm or whether operational circumstance (e.g. being installed in automatic taps with restricted flow rates) was the major contributing factor (Walker *et al.*, 2014, Halabi *et al.*, 2001).

In this study, in comparison to the polycarbonate control, no non-antimicrobial plastics had a significant effect upon biofilm formation. This included hostaform, which, some manufacturers claim, has anti-biofilm properties. These claims focus on hostaform having anti-scale properties which prevent limescale and other detritus and bacteria from attaching. The "smooth surface" of hostaform reportedly "reduces bacterial development", yet, the results of this study clearly demonstrate that bacterial attachment to hostaform is not only possible, but attachment and biofilm formation occurs at the same extent as the other plastics used in conventional outlet fittings (i.e. basell and rialene; Table 4.1). Thus, when considering the material in isolation, there is no evidence to support the manufacturer's claims. However, the secondary element to the "anti-biofilm" claim is the simple design of the outlet fitting, highlighting a limitation of the bioreactor, the use of material coupons and the need for a more realistic, applied microbiology investigation.

Manufacturers have modified two of the plastics tested (hostaform and rialene) by impregnating them with silver ions and molybdenum trioxide respectively. The addition of silver ions to hostaform had no effect upon biofilm formation. There was no evidence of surface contact killing or significant losses in planktonic viability, implying little or no leaching of the active agent. This was confirmed via ICP-MS and Nanosight analysis; any silver ions or nanoparticles present in the tap water were at levels far below the MIC/MBC for *P. aeruginosa*. Whilst hard water has been associated with reduced killing effects of silver ions and nanoparticles in comparison to soft water (Anderson *et al.*, 2014), materials that rely upon silver for antimicrobial activity need to be effective to some degree as a large proportion of the UK is supplied with water at harness levels above 100 mg $CaCO_3$/L (Drinking Water Inspectorate, 2009), and silver activity in dry conditions is poor (Michels *et al.*, 2009).

'Antimicrobial' rialene was found to leach molybdenum ions; the World Health Organization's recommended upper concentration for molybdenum in drinking water is 70 ppb (Smedley *et al.*, 2014), and the level detected from water exposed to MoO_3 impregnated rialene was only ~20-fold lower. Across the literature, the few studies investigating the antimicrobial properties of MoO_3 lack consistency in methods, do not always state the concentrations used, or fail to calculate a minimum inhibitory or bactericidal concentration (Lorenz *et al.*, 2011, Qureshi *et al.*, 2016, Zollfrank *et al.*, 2012). The effect of exposing *P. aeruginosa* to MoO_3 within a bioreactor system was, until now, unknown and as such, rialene coupons with and without MoO_3 were tested

separately. In comparison to the polycarbonate controls, the addition of MoO_3 had no effect upon bacterial attachment to rialene. Despite molybdenum ions leaching from the surface, the planktonic suspension was also not affected, implying that MoO_3, at the concentration present within the rialene, is not antimicrobial.

The antimicrobial properties of copper and copper-based alloys are widely documented and have been relied upon throughout history (Borkow and Gabbay, 2009, Warnes *et al.*, 2010). Copper is bactericidal, encouraging the production of reactive oxygen species, protein denaturation, cell membrane damage and DNA degradation (Grass *et al.*, 2011). The antimicrobial effects of copper result from contact-killing (Grass *et al.*, 2011) and/or the release (leaching) of free copper ions from the surface (Stout and Yu, 2003b). The ability of copper ions to leach from copper-based metals such as those found in water systems (Boulay and Edwards, 2001) meant that, in this study, copper and brass coupons had to be tested separately. After three days incubation in the bioreactor, the reduced recovery of *P. aeruginosa* from copper coupons was likely to have been influenced by a combination of reducted planktonic cells, due to release of copper ions into the media, and a reduction in biofilm formation due to contact-killing meaning copper ions can exert an effect on both planktonic and surface attached bacteria (Warnes *et al.*, 2010)

Copper is also known for inducing a viable but nonculturable (VBNC) state bacteria (Jiang, 2014), a state that is common amongst aquatic microorganisms (Oliver, 2000) and can lead to false negative or falsely low counts of metabolically active but non-dividing bacteria (Ramamurthy *et al.*, 2014). The public health implications of VBNC organisms is debated as epidemiological evidence is limited, but amongst pathogenic bacteria such as *E. coli* and *Salmonella typhi,* reconversion or “resuscitation” into culturable and pathogenic state is considered a risk (Oliver *et al.*, 2005, Zeng *et al.*, 2012). A study by Alleron *et al.* (2013) demonstrated that *L. pneumophila* in a VBNC state continually produces proteins associated with virulence, although not at a level that enables virulence to be resumed, as demonstrated by a lack of invasion when cells were resuscitation by co-culturing with amoebae.

Should the incorporation of copper and brass within tap components lead to antimicrobial effects similar to those seen in the bioreactor model, it would be interesting to investigate whether this is a true killing effect or whether there has been some conversion to VBNC state (Bédard *et al.*, 2014).

Grass *et al.* (2011) raises the importance of assessing the antimicrobial effects of copper surfaces under dry conditions, claiming it is more representative of hospital surfaces. However, in the context of plumbing components, copper incorporated within outlet fittings would need to be effective in both wet and dry environments (representative of the tap usage status and/or

exposure of the material to the water flow). Regardless, studies assessing the activity of copper surfaces under laboratory conditions have reported higher antimicrobial efficacy (6-$\log_{(10)}$ reductions) than that demonstrated in real life settings (<2-$\log_{(10)}$ reductions) (Schmidt *et al.*, 2012, Warnes *et al.*, 2010, Rai *et al.*, 2012). Similarly, studies comparing copper and non-copper surfaces have produced variable results (Schmidt *et al.*, 2012). Incorporation of antimicrobial materials does not guarantee an antimicrobial surface, and *in vitro* results do not always translate *in situ* (Muraca *et al.*, 1987, Murga *et al.*, 2001). Previous studies have modified the CDC bioreactor to investigate products such as catheters (Ren *et al.*, 2016). However, the size and shape of many plumbing components makes the adaptation of the bioreactor model to investigate products such as antimicrobial outlet fittings less practicable. Use of laboratory-based whole system models would allow for assessment and comparison of products under more realistic scenarios whilst controlling for variables found *in situ,* such as human behaviour (Muraca *et al.*, 1987, Moore *et al.*, 2015b).

The leaching of copper ions into the bioreactor medium reduced the number of *P. aeruginosa* recovered from the polycarbonate control coupons. It is noteworthy that the background levels of copper ions in the laboratory water were above the MIC/MBC for *P. aeruginosa.* The historical survival of the strain (isolate A; hereafter referred to as strain "A") within the EWDS would imply a tolerance to copper which was not observed *in vitro*.

The bioreactor was run under batch conditions to allow for short term early biofilm formation to be investigated; although continuous culture phase allows for controlled 'representation' of real-life flow and longer term biofilm experiments, it still does not take into account component design or natural fluctuations of a water distribution system.

4.5 Conclusions

Despite being approved for use in healthcare plumbing, the materials tested demonstrated the ability to support bacterial biofilm. Under the bioreactor experimental conditions, there was neither evidence to support the replacement of EPDM with alternative rubbers, nor to replace plastics with their 'antimicrobial' equivalents. No plastic, previously associated with tap or outlet fitting contamination, significantly outperformed another. Of the materials tested, only brass and copper coupons demonstrated antimicrobial effects, with 100% copper coupons leading to the lowest recovery of *P. aeruginosa*.

The incorporation of additives reported to have antimicrobial effects does not guarantee antimicrobial effects in real life. Where design (e.g. shape and/or finish) is also highlighted as an antimicrobial feature of a product, the transferability of *in vitro* material analysis is limited, and the role that design has to play on the microbial performance of products needs to be investigated.

To confirm or rebut any significant findings of material performance *in vitro*, it is also important that a product is tested under conditions more realistic of those it will be expected to perform under in real life, including exposure to different temperatures, flow rates, control regimen and human interaction. *In situ* investigations of whole products that incorporate the materials investigated in this chapter will be the subject of chapters 5 and 6.

Chapter 5 Investigating *P. aeruginosa* biofilm formation and persistence on conventional and alternative solenoid valves

5.1 Introduction

In a drive to reduce cross-contamination via hand-contact with taps, automatic, sensor-operated (non-touch) taps were introduced to hospitals (Chaberny and Gastmeier, 2004, Mäkinen *et al.*, 2013). However the additional components required for the function of automatic taps have called into question the suitability of their use within the healthcare setting (Chaberny and Gastmeier, 2004, Merrer *et al.*, 2005).

Automatic sensor-operated taps, instead of requiring levers/handles to be switched on or off, are stimulated via an infrared sensor which in turn controls a solenoid valve (SV) in the plumbing leading to the spout. SVs can differ in terms of configuration (e.g. normally-open (used to maintain low pressure in piped systems; water/gas can normally pass through the valve) to normally-closed (used for water outlets such as those found in hospitals; water cannot normally pass through the valve)) and electrical stimulation (e.g. latching-current (a pulsed electrical stimulus is required for operation) or maintained-current (a constant electrical stimulus is required for operation)). However the basic concepts and components are the same. In a normally-closed system, to prevent water flowing through the pipes, a spring and plunger normally press against a rubber diaphragm to keep the valve closed (Figure 5.1B). The stimulation of the infrared sensor (e.g. by waving a hand) causes a current to energise an electromagnet (Figure 5.2). When energised, the electromagnet acts upon a spring and plunger, which allow the diaphragm to form the correct shape to allow water to pass through the valve. Both sides of the rubber diaphragm are in contact with water, due to a small hole in the diaphragm that allows equalisation of pressure (Figure 5.1C, indicated with red arrows) so that when the electromagnet de-energises, the diaphragm can be pushed back into shape to block water flow.

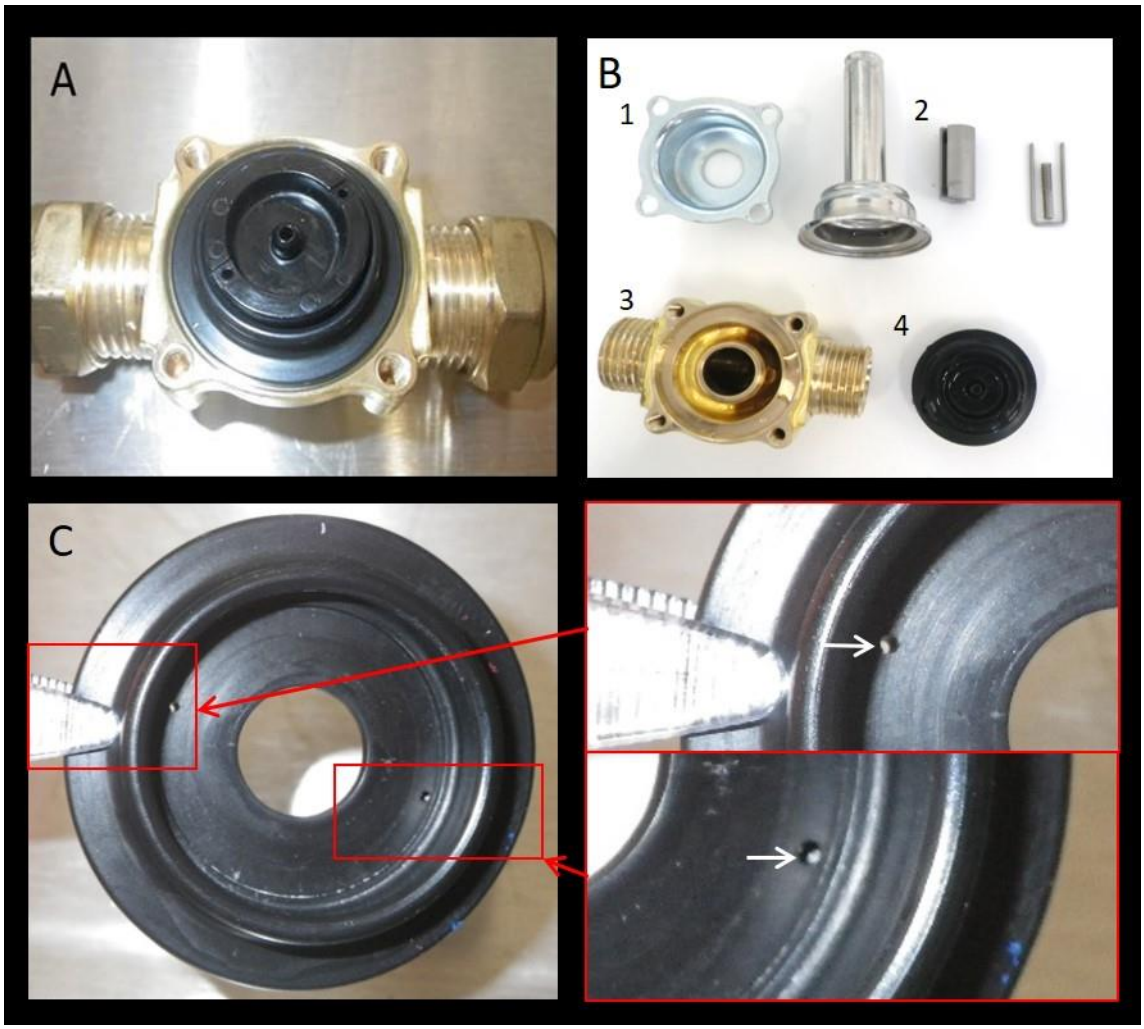


Figure 5.1 Solenoid valve (SV). A) Diaphragm sat within the SV body; B) 1: casing and armature; 2: magnetic plunger; 3: brass body 4: diaphragm; C) diaphragm with holes to allow pressure equalisation (enlarged and highlighted with white arrows).

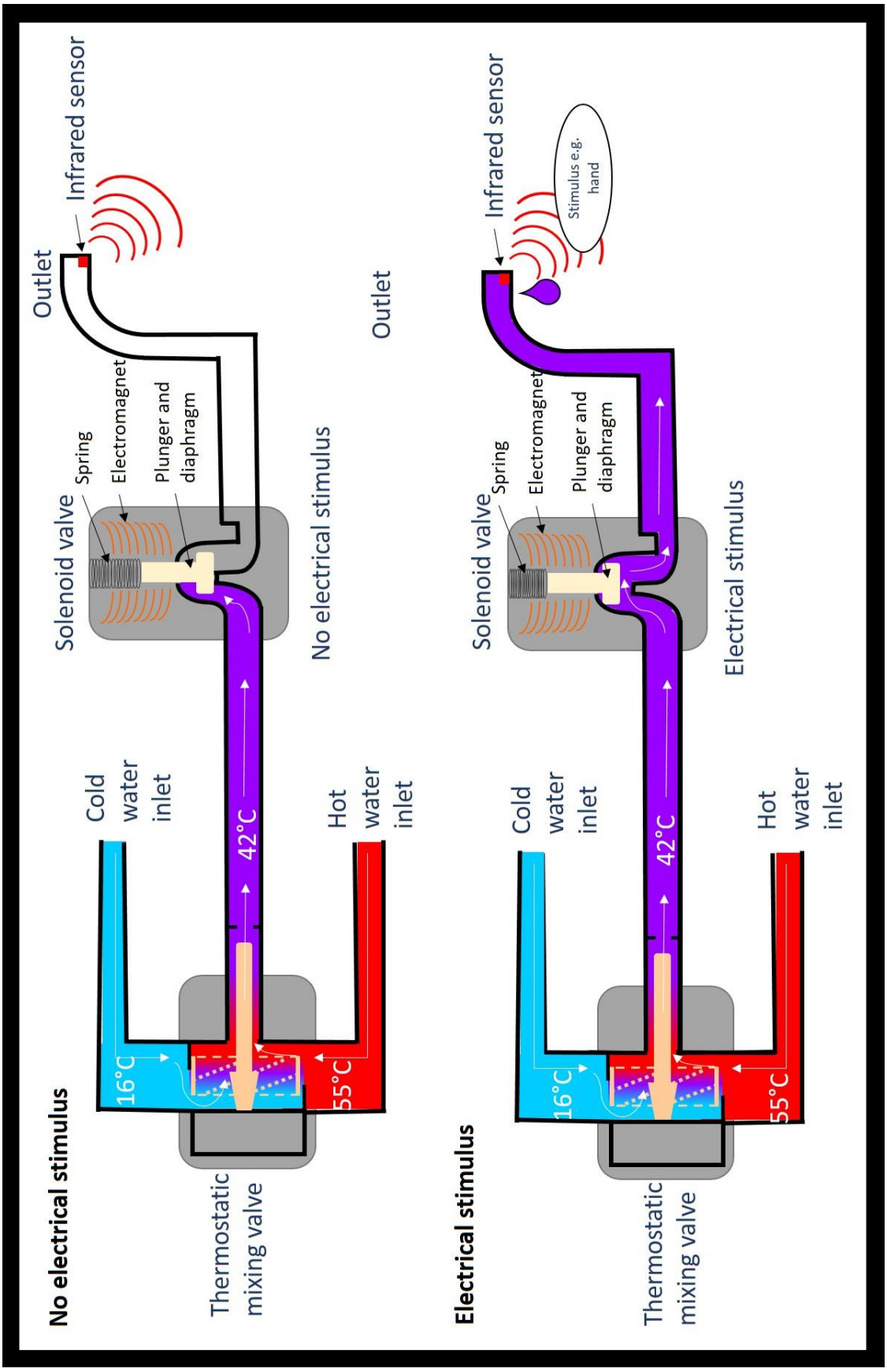


Figure 5.2 The function of a solenoid valve. White arrows represent the flow of water. Water is represented as blue (cold), red (hot) or purple (mixed/warm). With no stimulus to the infrared sensor the electromagnet does not act on the spring, which forces the

plunger and diaphragm to close the valve (i.e. the tap switches off). When the infrared sensor is stimulated (e.g. by waving a hand in front of the sensor) the electromagnet is energised and causes the solenoid valve spring to condense. In turn, the plunger and diaphragm to allow water to pass through the valve to the outlet (i.e. the tap switches on). The Thermostatic mixing valve ensures the water is mixed to an appropriate temperature before water reaches the solenoid valve.

Automatic taps have a predetermined operating time and are often temperature controlled through a thermostatic mixing valve (TMV). TMVs allow either cold water or mixed hot and cold water to pass through the piping, but water above the pre-set temperature cannot pass through (Figure .3). A major reason for installing TMVs within healthcare and domestic settings is to reduce the risk of scalding; incidences of which cost the NHS an average of £25,226 per patient from community cases alone (Phillips *et al.*, 2011). Controlling the temperature and running time of taps offers financial and environmental benefits (Connor *et al.*, 2010) as tap running time is considered an efficient method of conserving water, thus saving on costs (Halabi *et al.*, 2001). The NHS aims to reduce its carbon footprint, one method of which is through better water management (Mayor, 2008). Water usage by the NHS amounts to approximately 40 billion litres per annum, costing ~£47.5 million per year with a carbon footprint of ~0.27 g/L CO_2 (DoH, 2013, Clear *et al.*, 2012). Perhaps unsurprisingly (due to hygiene requirements), it has been reported that NHS hospital staff use ten times the volume of water than staff in a different setting (such as an office) (Clear *et al.*, 2012). The DoH has suggested that these figures could be cut by ~20% through simple remedial measures, including the installation of automatic taps which could reduce water consumption by up to 50% whilst simultaneously aiding infection control by reducing hand-contact surfaces (DoH, 2013). However, such recommendations do not take into consideration costs incurred by waterborne nosocomial infection that could result from the use of automatic taps, which have been reported to act as a reservoir for biofilm (Kilb *et al.*, 2003, Moore *et al.*, 2015b).

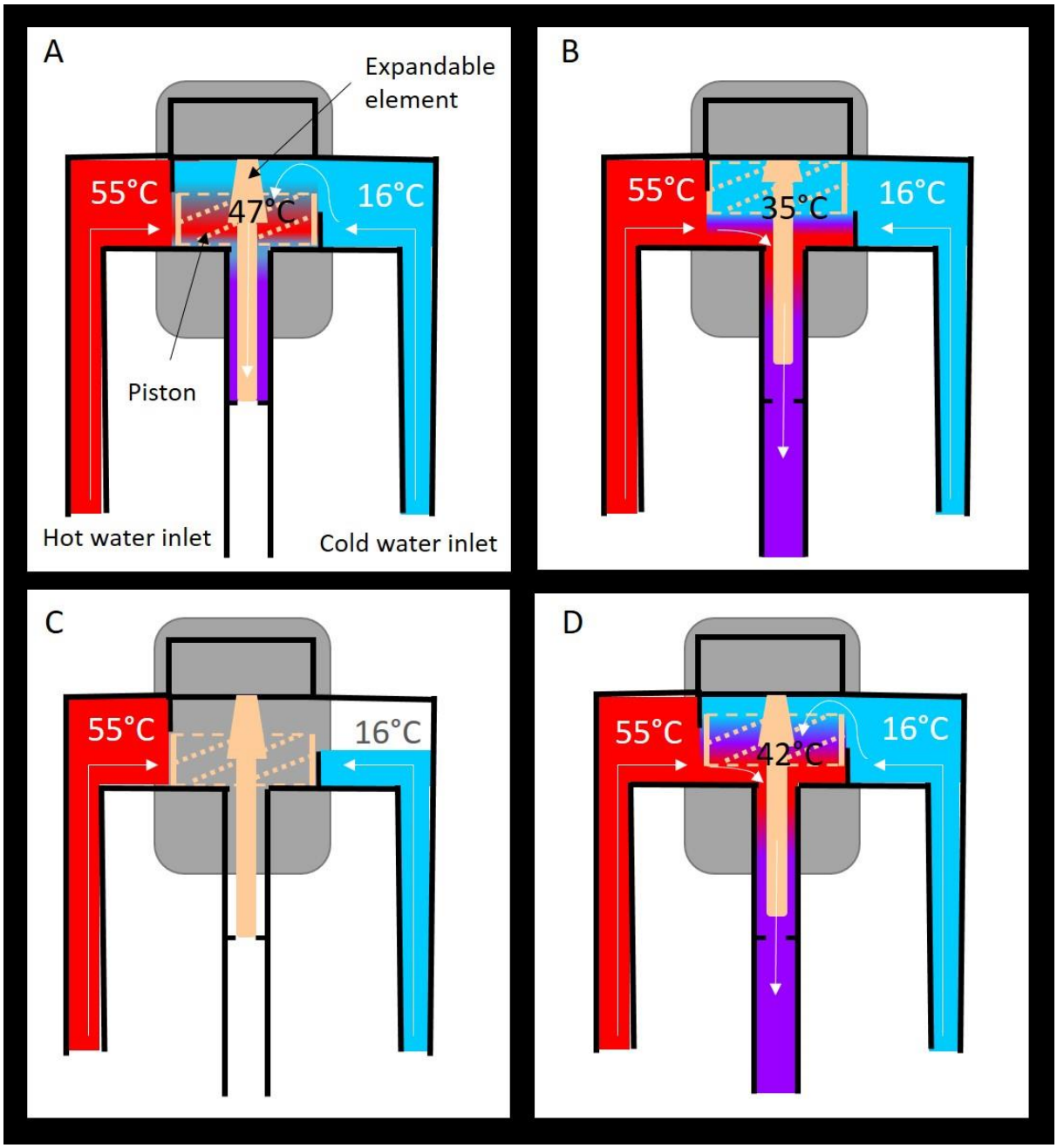


Figure 5.3 The function of a Thermostatic mixing valve under different conditions. White arrows represent the flow of water. Water is represented as blue (cold), red (hot) or purple (mixed/warm). A) Hot water pressure too high: the expandable element (temperature sensitive) has forced the piston to block further hot water from entering the mixing chamber but cold water is able to enter and reduce the temperature. No water is able to exit the TMV and proceed to the outlet; B) Cold water pressure too high: the expandable element has reduced in size, causing the piston to block further cold water entering the mixing chamber but allowing hot water to raise the temperature. Water is still able to flow to the outlet as there is no risk of scalding.; C) Cold water pressure too low: the expandable element has responded only to hot water, blocking any further hot water from entering the

mixing chamber, preventing the risk of scalding; D) Appropriate hot and cold water pressures: the expandable element has kept the piston at a position allowing for both hot and cold water to leave the TMV and reach the outlet at a pre-determined/suitable temperature.

Taps implicated in the *P. aeruginosa* infections of neonates in both Turkey (2010) (Yapicioglu *et al.*, 2012) and Northern Ireland (2011-2012) (Walker *et al.*, 2014) were automated taps. A study by Hargreaves *et al.* (2001) investigated contamination of water from both manual and automatic taps. Hargreaves collected water samples after a thorough decontamination of the tap head including the outlet fittings. The proportion of automatic taps (22%; 13/59) that exceeded Hargreaves' HPC microbial threshold (>500 CFU/mL) was significantly higher than that of manual taps (11%; 12/110). One model/brand of automatic tap had significantly higher levels (32%; 11/34) of this contamination than a second model (8%; 2/25), however differences in design were not specified. Sydnor *et al.* (2012) demonstrated higher occurrence of *Legionella* spp. in automatic taps (95%; 19/20) than manual taps (45%; 9/20), a result which ultimately led to the hospital removing automatic taps from all clinical areas.

In contrast, Mäkinen *et al.* (2013) reported that manual taps support more biofilm than automatic taps. However, this conclusion was based upon biofilm recovered from outlet fittings (e.g. flow straighteners) which are replaceable, interchangeable and not normally exclusive to either automatic or manual taps. Statistically similar heterotrophic plate counts (HPCs) were recovered from water delivered from both tap designs, and automatic taps were associated with the highest *Legionella* spp counts. However, these water results were disregarded to conclude (on the basis of the outlet fitting results) that automatic taps promote hospital hygiene. No investigation into biofilm elsewhere in the tap components was conducted.

A study by Moore *et al.* (2015b) involved artificially inoculating an EWDS with *P. aeruginosa*. Contamination of the water was monitored over a two-year period and *P. aeruginosa* levels remained above the alert limit for augmented care (≥10 CFU/100 mL) (Department of Health, 2013d). Further investigations identified the solenoid valve, specifically the ethylene propylene diene monomer (EPDM) rubber diaphragm, as the source of the contamination (Figure 5.4).

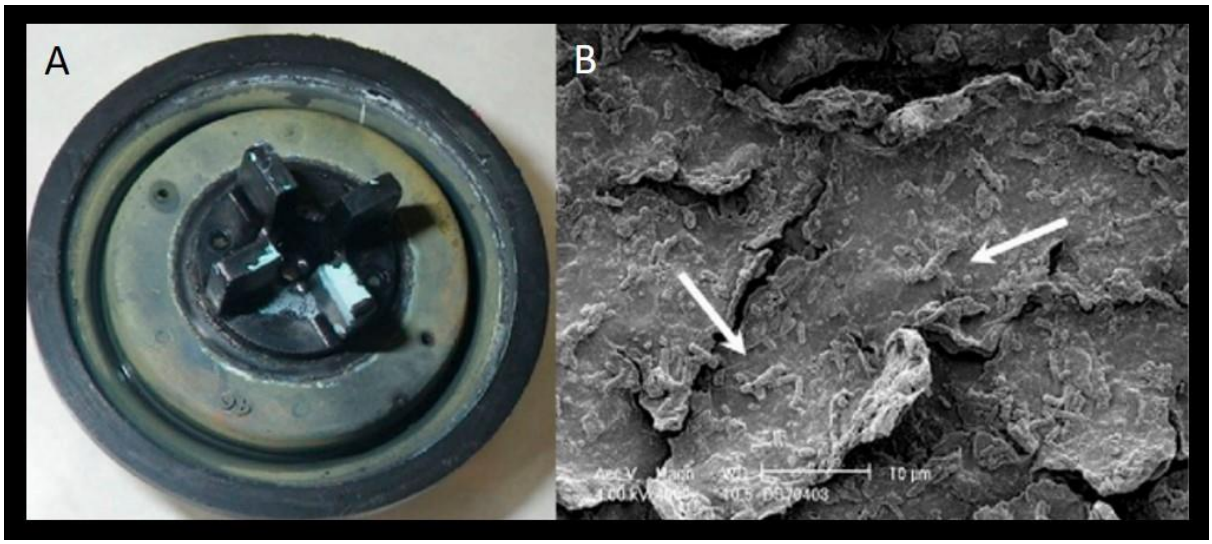


Figure 5.4 Biofilm on a solenoid valve diaphragm. A) Diaphragm and plunger with visible biofilm (green deposits); B) scanning electron microscopy of the diaphragm, showing multi-layered biofilm with visible cells (indicated with white arrows). (*Figure modified from (Moore et al., 2015b);* http://www.tandfonline.com*)*

The prolonged and continuous contact that the SV diaphragm has with water leads to opportunities for colonisation by waterborne microorganisms, including potential pathogens. When the tap is not used, water within the SV stagnates, further enhancing opportunities for biofilm formation. Typically, rubber diaphragms are made from EPDM due to its robust nature against weathering and heat (Chen *et al.*, 2015). Despite being approved for use in the healthcare environment by the Water Regulations Advisory Scheme (WRAS), EPDM has been shown to facilitate biofilm formation by opportunistic pathogens (Moore *et al.*, 2015b, Kilb *et al.*, 2003), and its incorporation into healthcare plumbing is advised against (Moore *et al.*, 2015b).

There is also concern that due to biofilm formation on EPDM components, common hospital water disinfection procedures, which may be effective for manual taps, are insufficient for automatic taps (Sydnor *et al.*, 2012). Berthelot *et al.* (2006) found that consecutive disinfection procedures (unspecified) were successful for plumbing work running up to the automatic tap, however *P. aeruginosa* was still eluted at levels above the alert limit for augmented care (≥10 CFU/100 mL; (Department of Health, 2013b)) from automatic taps. Similarly, Leprat *et al.* (2003) used a chlorine-based disinfection protocol but after six consecutive procedures only 11% of automatic taps had been successfully decontaminated. The flushing of taps (particularly those that are under-used) can be employed as a non-chemical microbial control strategy. The delivery of water increases the shear stress within the plumbing which can lead to sloughing and overall changes in biofilm stability and composition (Douterelo *et al.*, 2016). However, the water-saving

nature of automatic taps means that the effect of flushing as a microbial control mechanism is reduced (Yapicioglu *et al.*, 2012, Halabi *et al.*, 2001)

The design of automatic taps can play a role in contamination potential (Hargreaves *et al.*, 2001). It is therefore important to consider alternative designs (such as different SV material composition) before discounting automatic taps as a whole, especially considering the potential environmental and financial benefits of having automatic taps in place.

In response to reports of SV diaphragm colonisation, in particular the typical EPDM-based design (Moore *et al.*, 2015b), the clinical tap industry are investigating alternative materials for diaphragms. Typical alternatives to EPDM rubber include nitrile and silicone (Kilb *et al.*, 2003, Wagner and von Hoessle, 2003), however comparison of all three materials in a bioreactor model (Chapter 4.3.5) demonstrated the ability of both nitrile and silicone rubbers to become colonised, at levels significantly higher than EPDM. Manufacturers are also investigating antimicrobial compounds (e.g. silver impregnated rubbers) for which they provide limited evidence for antimicrobial efficacy and rely upon the reported properties of silver ions (Choi *et al.*, 2008). Silver ion-impregnated materials did not demonstrate antimicrobial effects in the bioreactor system (Chapter 4.3.5). As discussed in Chapter 4.4, *in vitro* studies do not always translate *in situ* due to the inherent variability of real-life water systems/situations. Thus, alternative materials to EPDM should be tested in the context of their product, and under conditions more representative of a real-life scenario.

5.2 Aims

The aim of this chapter was to investigate the colonisation of *P. aeruginosa* on different solenoid valves in a model more representative of an hospital water distribution system. The objectives were to:

- Install solenoid valves incorporating different materials onto an experimental water distribution system.
- Artificially inoculate individual tap assemblies with *P. aeruginosa* and monitor contamination of the water over time.
- Investigate biofilm formation on the solenoid valve diaphragms via culture and microscopy.
- Assess the effect of remedial measures including flushing and chlorination on water contamination and on contaminated solenoid valves.

5.3 Results

5.3.1 Investigating the contamination of water from taps with contaminated solenoid valves

After five days of stagnation, the median number of *P. aeruginosa* colonies recovered from water delivered via EPDM and nitrile solenoid valves (SVs) was $6x10^3$ CFU/ 100 mL (n=5) and $1.5x10^4$ CFU/100 mL (n=5) respectively. Twelve weeks after inoculation, a mean $\log_{(10)}$ reduction of 1.7 and 1.4 occurred in taps with EPDM and nitrile SVs respectively (Figure 5.5). In the first six weeks, there was a general reduction in *P. aeruginosa* for both SV designs (R^2=0.377 (EPDM) and R^2=0.6957 (nitrile)). Over the final six weeks the water contamination levels began to stabilise (R^2=0.007 (EPDM) and R^2=0.001 (nitrile)). There was no significant difference (p=0.71) in water contamination over the course of the 12-weeks between EPDM and nitrile SVs.

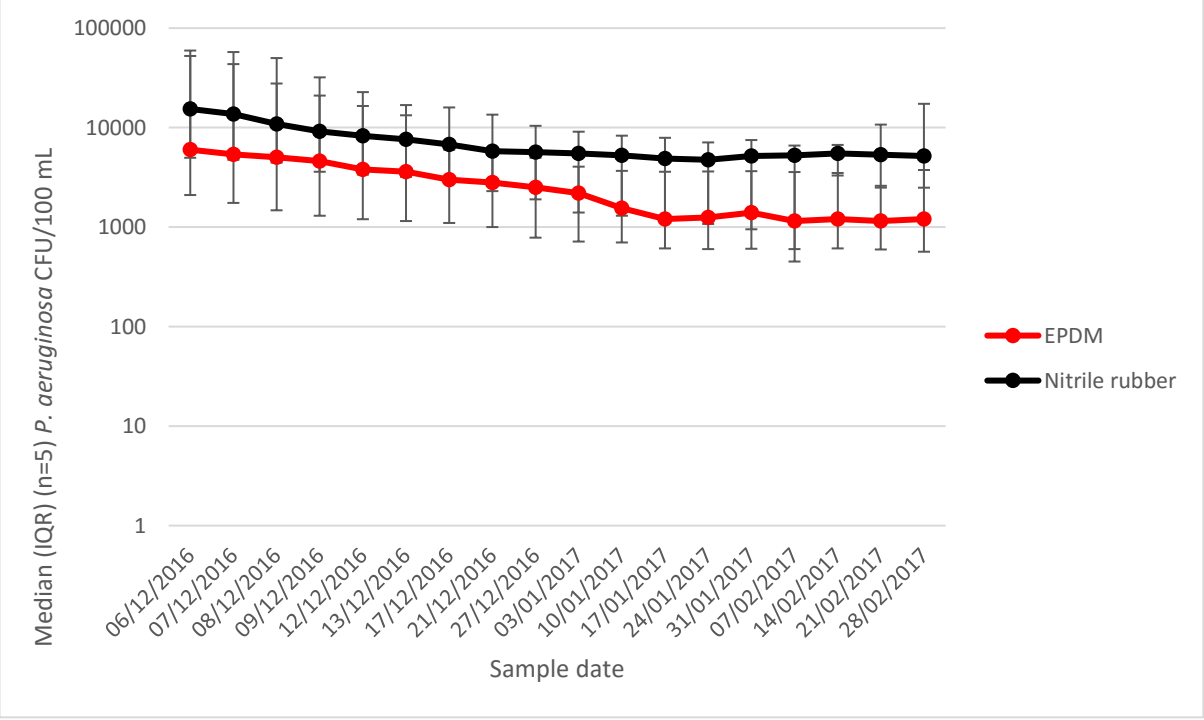


Figure 5.5 *P. aeruginosa* recovered from water collected from artificially inoculated tap assemblies. Tap assemblies incorporated EPDM (red) and nitrile rubber (black) solenoid valves flushed twice daily over a 12-week period (CFU/100 mL).

At the end of the 12-week experiment, removal of the SVs led to 7/10 taps becoming negative for *P. aeruginosa*. Water delivered from taps 2, 21 (EPDM) and 26 (nitrile) was contaminated at levels ranging from 4-28 CFU/ 100 mL. Removal of the Binder points (BPs) from these taps led to taps 21 and 26 turning negative for *P. aeruginosa*. Tap 2 contamination persisted at low levels for several

days after BP removal, but *P. aeruginosa* levels reduced to below detection (<1 CFU/100 mL) after 12 days. Outlet fittings taken from these three taps were negative for *P. aeruginosa* contamination.

5.3.2 Investigating biofilm formation on solenoid valves

5.3.2.1 Nitrile and EPDM solenoid valves

5.3.2.1.1 Culture-based analysis

The median *P. aeruginosa* recovered from the EPDM SV diaphragms (n=5) was $4.2x10^4$ CFU/diaphragm, ranging from $4.6x10^3$ to $1.6x10^6$ CFU/diaphragm. Nitrile SVs had a median *P. aeruginosa* recovered of 5.3x105 CFU/diaphragm, with recovery ranging from 4.2x103 to 6.7x105 CFU/diaphragm. No significant difference (p=0.55) was observed in *P. aeruginosa* recovered from diaphragms (Figure 5.6).

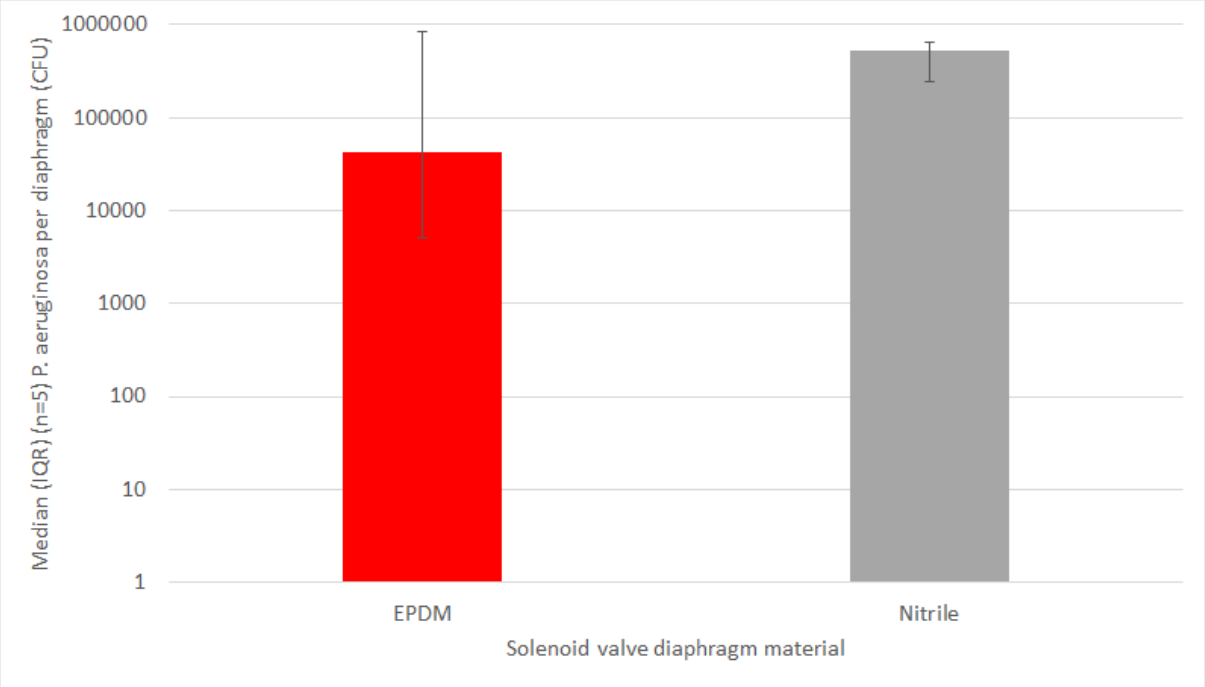


Figure 5.6 *P. aeruginosa* recovered from solenoid valve diaphragms after 12 weeks in the EWDS (n=5). Solenoid valve diaphragms (EPDM (red) and nitrile (black)) were extracted and *P. aeruginosa* recovered.

5.3.2.1.2 Microscopic analysis

Macroscopic contamination on both SV types was minimal. One nitrile diaphragm had visibly accumulated loosely attached, visible material (Figure 5.7 A and B) (tap 7), which was not present on any other diaphragms. There was some evidence of discolouration on one SV of each type of diaphragm (Figure 5.7 C and D) and white scale accumulation on another two EPDM SVs (Figure 5.7 E and F). Once the water used to rinse the diaphragms had air-dried, all diaphragms (EPDM and nitrile) had some evidence of limescale deposit which had not been apparent immediately after extraction from the SVs.

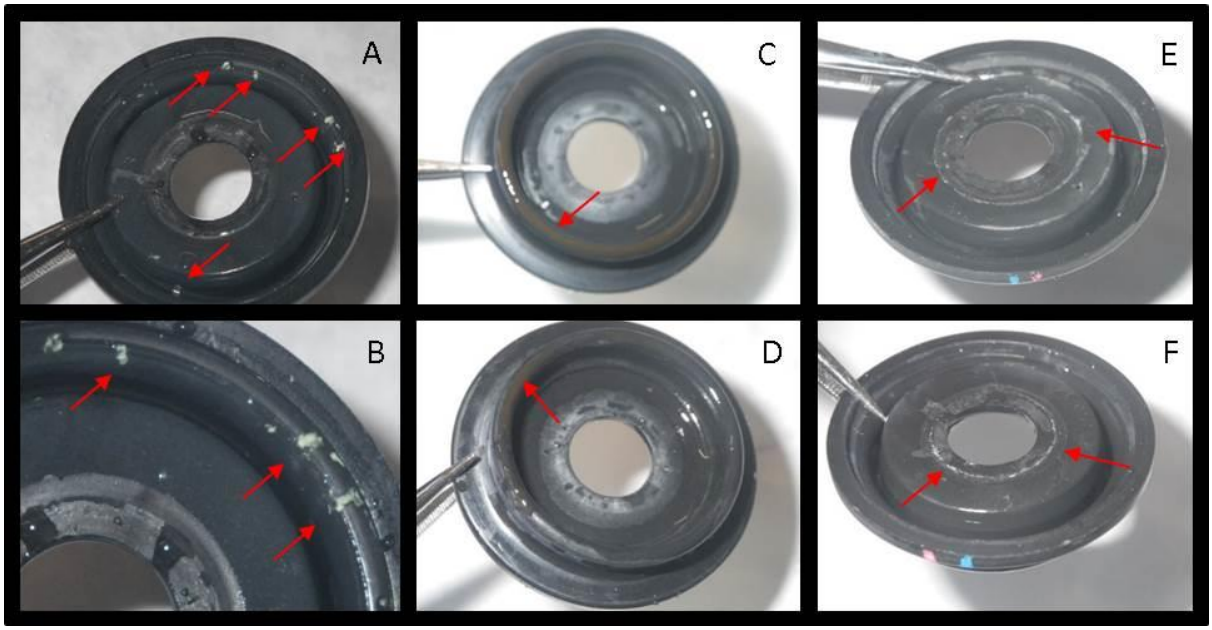


Figure 5.7 Photographs of solenoid valve (SV) diaphragm (EPDM and nitrile) after 12-weeks in the EWDS. A and B: Evidence of debris accumulation (red arrows) on a nitrile diaphragm; C: evidence of discolouration of a nitrile SV; D: evidence of discolouration; E and F: evidence of white, scale-like deposits on EPDM SVs.

Micrographs revealed heavy biofilm on all of the SV diaphragms. Figure 5.8 A and B (EPDM) SEM images demonstrate a webbing effect of the matrix of extracellular polymeric substances (EPS), entangling visible individual cells. The fluorescence microscopy images of the same diaphragm (Figure 5.8 E and F) reveal a cracked, scale like surface, possibly limescale deposits, with visible individual cells across the surface. Figure 5.8 C depicts heavy EPS that has coated, and is beginning to coat, individual cells from the left hand side to the right hand side of the image. In Figure 5.8 D, several groups of cells are seen clustering on the surface of the EPS. Both fluorescence images for these two diaphragms (Figure 5.8 G and H) revealed heavy contamination which appeared somewhat evenly spread on the surface of the biofilm.

SEM images of the nitrile diaphragms also show individual cells on the surface of a biofilm (Figure 5.9 A). However the EPS is far less webbed, and the scale-like sections are not apparent in the corresponding fluorescence microscopy image (Figure 5.9 D). Fig 5.9 B reveals heavy EPS (similar to that seen in Figure 5.8 C) with visible cells atop of the EPS, but also shows cells still visible engulfed in EPS slightly below the surface. In Figure 5.9 C, a large number of cells are visible on the surface of the EPS, with a bulbous area of heavy EPS on the right hand side of the image, which potentially could be the beginnings of the cloud-like EPS structures seen in Fig 5.9 B and thick EPS seen in more mature biofilms. The fluorescence microscopy images for nitrile SVs (Figure 5.9 D-F) highlight individual cells apparent on the surface of the biofilm. Unfocused areas of the image (such as those in Figure 5.9 F) are due to the surface not being flat, which has narrowed the plane of focus.

Both material types supported heavy biofilm, and whilst biofilm structure varied, more webbing was evident on EPDM diaphragms and more cloud-like, bulbous structures were evident on nitrile diaphragms. However, biofilm structures were variable across SVs of the same material.

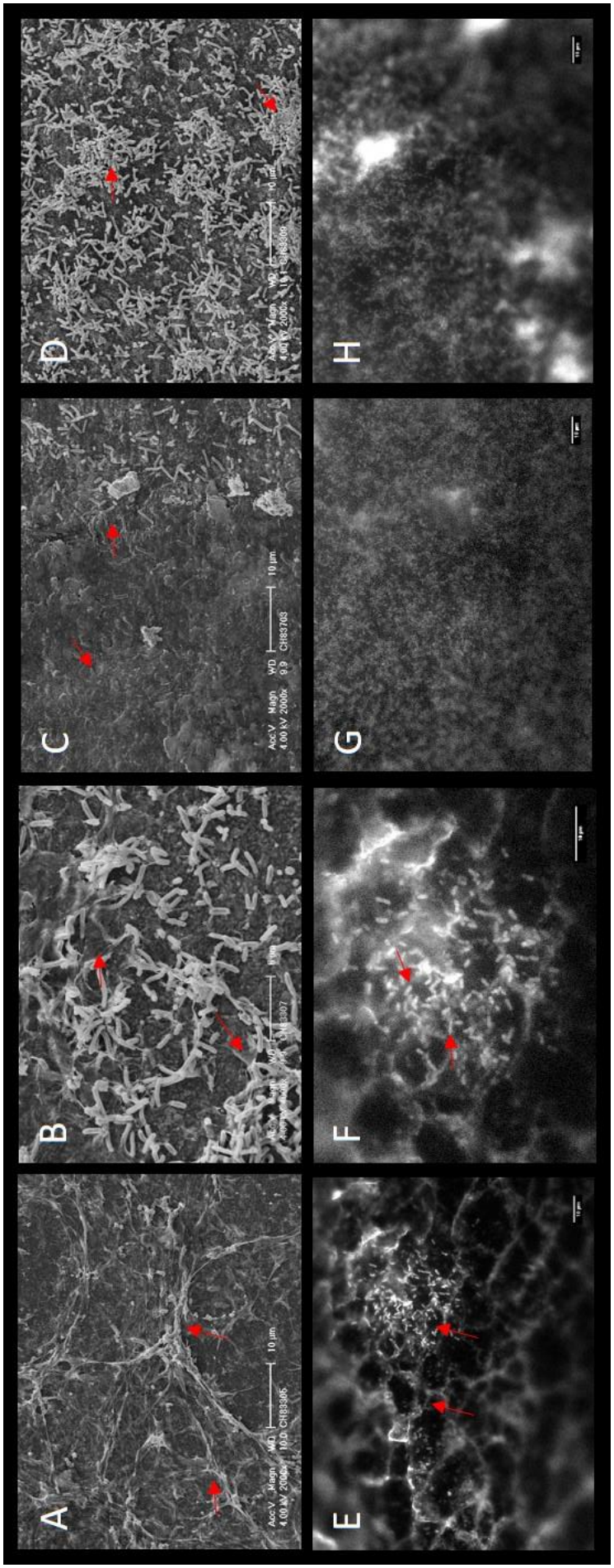

A
B
C
D
E
F
G
H

Figure 5.8 EPDM solenoid valve diaphragms after 12 weeks in the EWDS. Scanning electron and fluorescence microscopy were used to image biofilm. SEM images of SVs from taps 2 (A and B), 21 (C) and 4 (D); fluorescence microscopy images from taps 2 (E and F), 21 (G) and 4 (H). Arrows indicate: (A and B) EPS webbing; (C) heavy EPS has coated or is beginning to coat individual cells; (D) clustering of cell on the surface of heavy EPS of the underlying biofilm; (E) cracked/scale-like surface; (F) individual cells attached to the scale-like surface.

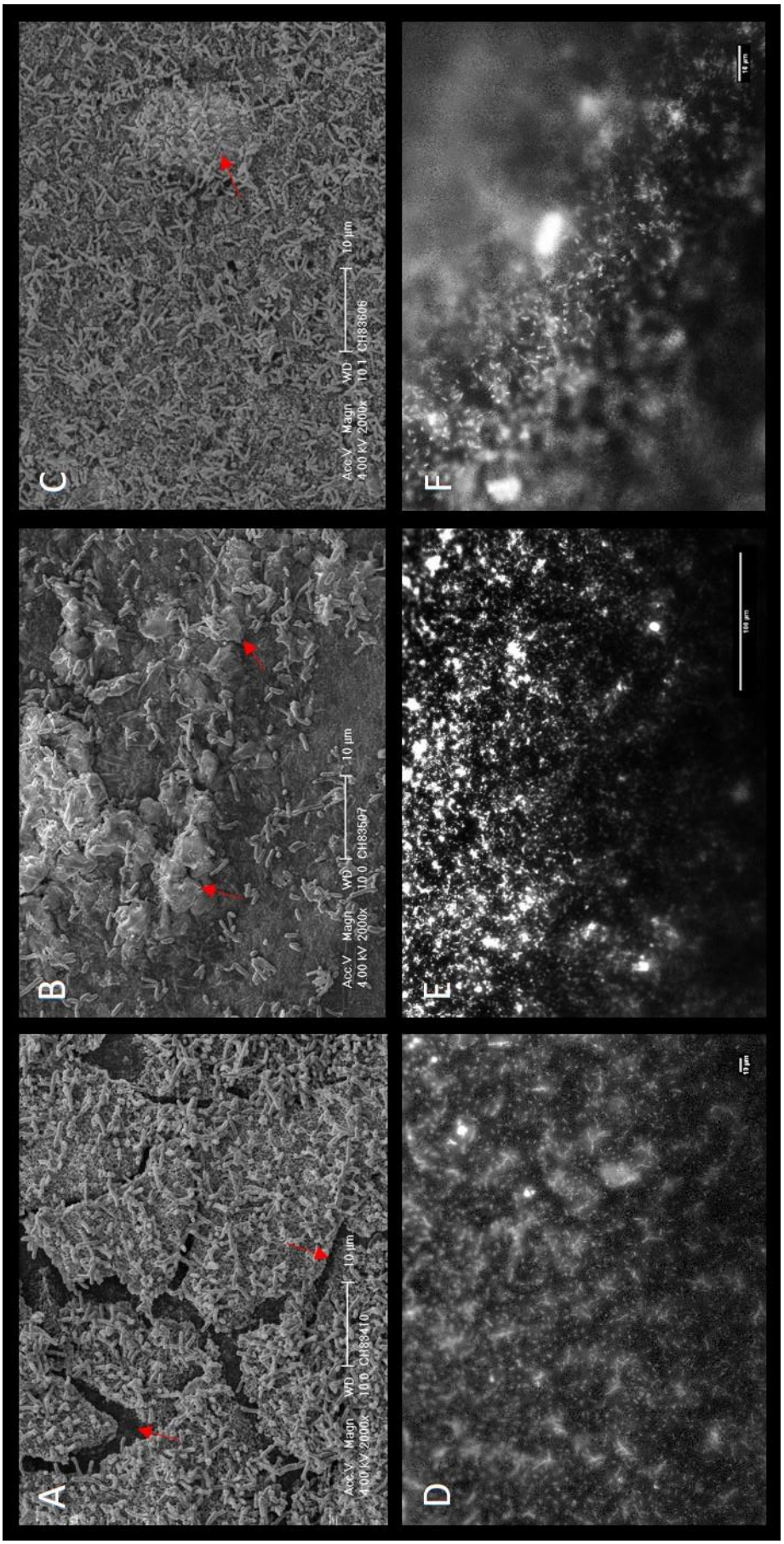


Figure 5.9 Nitrile solenoid valve diaphragms after 12 weeks in the EWDS. Scanning electron and fluorescence microscopy used to image biofilm. SEM images of SVs from taps 7 (A),

15 (B) and 19 (C); fluorescence microscopy images from taps 7 (D), 15 (E) and 19 (F). Arrows indicate: (A) cracking of the biofilm surface; (B) bulbous, cloud-like EPS structure; (C) bulbous area.

5.3.2.2 Silicone-based solenoid valves

5.3.2.2.1 Detection of leached silver ions

ICP-MS analysis of water samples from the EWDS hot and cold water tanks revealed a background level of silver-ions ranged from 0.02-0.07 ppb. Silver ion concentrations of the water tanks did not increase post-flush. Silver ion concentrations in water delivered by tap assemblies with silicone and EPDM SVs ranged between 0.008-0.04 ppb in the first sample of water collected, and 0.008-0.03 ppb post-flush. Seven out of eight water samples from the four taps with silver-ion impregnated silicone SVs had a range of between 0.01-0.05 ppb. One sample, a pre-flush sample from tap 19, had a silver ion content of 0.13 ppb, which was significantly higher (p<0.05) than the readings from any other sample.

NanoSight analysis was used to detect the presence of silver nanoparticles. A minimum number of 20 particles per frame indicates a positive result with an ideal range of 20-60 (Gardiner *et al.*, 2013). Silver nanoparticles were not detected in any of the water samples, i.e. silver leaching did not occur from silver-ion impregnated silicone diaphragms (Table 5.1).

Table 5.1 Nanoparticle detection in water delivered by SVs with and without silver-ion impregnation. Mean particles per frame detected by NanoSight.

SV diaphragm material	Tap assembly	Average number of particles per frame
Colloidal silver positive control	n/a	52.5
Silver-ion impregnated silicone rubber SV	Tap 6	1.4
Silver-ion impregnated silicone rubber SV	Tap 19	2.3
Silver-ion impregnated silicone rubber SV	Tap 21	None detected
Silicone rubber SV	Tap 22	2.6

SV diaphragm material	Tap assembly	Average number of particles per frame
Silicone rubber SV	Tap 1	1
Silicone rubber SV	Tap 10	2.6
EPDM SV	Tap 5	1.1

5.3.2.2.2 Detection of *P. aeruginosa* from silicone-based solenoid valves

Of the eight taps with silicone-based SVs installed, seven were positive for *P. aeruginosa* immediately after installation (i.e. prior to artificial inoculation). *P. aeruginosa* levels in water delivered from these taps ranged from 5 CFU/100 mL to $3.5x10^3$ CFU/100 mL. In contrast, all tap assemblies with new EPDM SVs (n=4) delivered water that was negative for *P. aeruginosa.* Taps were flushed over a two-week period in an attempt to remove any superficially attached *P. aeruginosa*, however contamination of the water persisted. Of the silicone-based SVs, after two weeks of consistent contamination of water, the most highly contaminated SV of each type (silver ion-impregnated or non) was selected for further investigations into remedial measures of flushing and/or chlorination.

New SVs (n=2) of each diaphragm material (silicone, silver ion-impregnated silicone or EPDM) were investigated for contamination without being installed onto the EWDS. SVs were dismantled and diaphragms halved as described. Half of each diaphragm was enriched in 25 mL nutrient broth (Oxoid) at 37 ˚C for 16-48 hours, with a broth-only negative control. 100 µL of the enrichment broths were plated onto TSA and CN agar at 24 and 48 hours. The remaining half diaphragms were halved again, and the resulting quarter was agitated in Dey-Engley broth with 5 glass beads and plated onto TSA and CN agar. No growth was observed from EPDM SVs; silicone-based SV suspensions were visibly turbid. Silicone and silver-ion impregnated silicone SVs (n=1) were positive for *P. aeruginosa*, with 156 CFU/diaphragm and 160 CFU/diaphragm respectively. The *P. aeruginosa* negative silicone-based SVs were both positive for *Bacillus* spp. (n=1) as confirmed by MALDI-TOF analysis. Wet patches were observed on the plastic casing surrounding the type silicone-based diaphragms, however the swab enrichments were negative after 24-hours incubation. No wet areas were observed in EPDM SVs.

5.3.2.3 Genomic analysis

The mean genome sequencing coverage was 21.6x, with a minimum and maximum coverage of 16x and 31.1x respectively (n=8). To investigate the natural rate of mutation of *P. aeruginosa* within the EWDS, an isolate of *P. aeruginosa* recovered from the EPDM diaphragm of a SV in tap 5 that had remained *in situ* for five years was sequenced and mapped against the whole genome sequence of the PA14 strain originally contaminating the EWDS (Moore *et al.*, 2015b). The two isolates had identical VNTR profiles (12,2,1,5,5,2,4,5,12). There were three single nucleotide polymorphisms (SNPs) between the two isolates.

Following the installation of nitrile and EPDM SVs, a phenotypically distinct strain, strain "E" became prominent in water samples from two nitrile and one EPDM tap, with a VNTR profile of 12,2,-,3,2,2,9,5,8.

Strain "E" was consistently bright peach, circular and convex, with no indication of green/blue pyocyanin production that was seen in all other strains tested (Chapter 2; Table 2.1; page 35). In contrast, strain "A" grew as either round and creamy-green colonies, or dark green, irregular colonies with undulating edges (uneven and spreading across the plate).

The water and diaphragm (biofilm) isolates from taps containing "old" SVs contaminated with PA14 (i.e. taps 5 and 11) had a consistent bright yellow/green pigment and irregular, undulating appearance.

Due to its distinct appearance, strain "E" was also identified from the biofilm culture of an additional nitrile SV diaphragm taken from a tap assembly that had not delivered water contaminated with strain "E".

P. aeruginosa isolates from water samples delivered by taps with silicone-based SVs as well as from the silicone-based SV diaphragms had VNTR profiles identical to PA14 (12,2,1,5,5,2,4,5,12), confirming that the isolates differed from the phenotypically distinct strain "E" (VNTR profile of 12,2,-,3,2,2,9,5,8). PA14 isolates were also present in water samples from some 'control' taps (taps 5, 14 and 27; Table 4.2). Therefore, to determine whether the PA14 isolated from new SVs was identical to the PA14 strain of the same VNTR profile in the EWDS, a comparison of the whole genome sequences of four silicone-based SV diaphragm isolates (one isolate from four SVs, of which two had not been installed onto the EWDS) revealed 21-22 SNPs in comparison to the PA14 isolate used to previously inoculate the control taps. The SNPs for all silicone-based SV *P. aeruginosa* isolates were at the same loci.

One water sample isolate taken at the end of the 12-week experiment (tap 1; nitrile SV) had a VNTR profile of a PA14 variant, with variation at the ninth locus (12,2,1,5,5,2,5,5,9) from the inoculating strain. WGS analysis and comparison to the inoculating strain revealed three SNPs between the two genomes.

5.3.3 Investigating remedial measures: the effect of flushing on water contamination levels from taps with *P. aeruginosa* biofilm present.

Silicone and silver ion-impregnated silicone SVs (n=1) and one EPDM SV with long-standing *P. aeruginosa* contamination (tap 5) were selected for comparison. The very first sample of water (flush 1 sample 1 (F1S1)) was consistently contaminated with a higher concentration of *P. aeruginosa* than any other water samples taken over the proceeding twenty-minute flushing period. The number of *P. aeruginosa* colonies recovered from each water sample was $\log_{(10)}$ transformed and subtracted from the F1S1 ($\log_{(10)}$) value. The $\log_{(10)}$ reduction (in relation to F1S1) observed over time is illustrated in Figure 5.10 As an example, in one instance, the $\log_{(10)}$-transformed colony count values associated with F1S1, F1S2, F1S3 and F2S1 were 3.79, 2.68, 2.58 and 3.22 respectively. All values were subtracted from F1S1 (3.79), resulting in values of 0, 1.11, 1.21 and 0.57 respectively. These values demonstrate that a single 30-second flush reduced the concentration of *P. aeruginosa* in the collected water by 1.21-$\log_{(10)}$ values. However, after 4.5-minutes of stagnation (i.e. in the absence of flushing), bacterial numbers had increased and in the first sample of water collected during flush 2 (F2S1), the concentration of *P. aeruginosa,* whilst lower than in F1S1, had, in comparison to F1S3, increased by 0.64-$\log_{(10)}$.

The 4.5-minute stagnation period between flushes (i.e. in samples F2S1, F3S1, F4S1 and F5S1) led to similar levels of *P. aeruginosa* in the water. The greatest reduction in *P. aeruginosa* in S1 samples across the five flushes was observed in silver ion- impregnated SVs, with a mean $\log_{(10)}$ reduction of 1.2 between F1S1 and F5S1, compared to 0.5 and 0.9 in EPDM and silicone SVs respectively. However, there was no significant difference ($p>0.05$) across F*x*S1 samples for any SV material type.

Regardless of diaphragm material, over the course of one flush, *P. aeruginosa* counts were lower in the second and third samples (i.e. F*x*S2 and –S3) than the first water samples (F*x*S1) (Figure 5.10).

EPDM and silicone SVs demonstrated the most exaggerated $\log_{(10)}$ reductions and across flushes, following a clear peak-and-trough pattern. Both material types had significant reductions ($p<0.05$) in *P. aeruginosa* between the first and third sample of water (F*x*S1 and -S3) per flush, for all five flushes for EPDM SVs, and the first four flushes of silicone SVs. Despite the difference between

F5S1 and F5S3 of silicone SVs not being significant (p>0.05), the reduction in *P. aeruginosa* across the flush followed the trend seen in flushes 1-4.

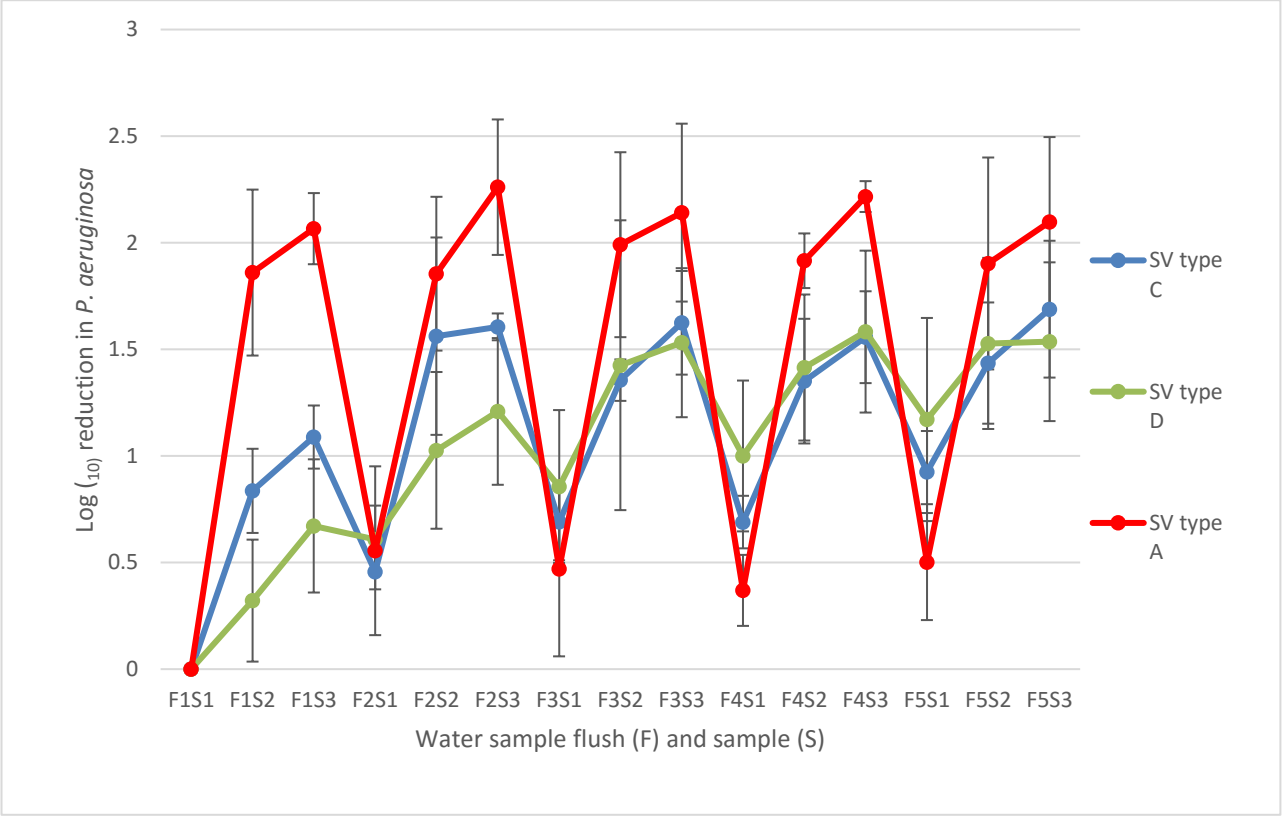


Figure 5.10 Mean (SD) log (10) reduction in *P. aeruginosa* recovered from water samples over a series of 30-second flushes. Silicone, silver-ion impregnated silicone and EPDM solenoid valves (colonised with *P. aeruginosa*) were flushed five times over a 20-minute period (F1-5), and the first, second and third samples of water collected (S1-3). *P. aeruginosa* counts were log (10) transformed and each value subtracted from the very first sample of water per assay (i.e. subtracted from F1S1) to provide log (10) reduction in *P. aeruginosa* over the course of the five flushes.

The silver ion-impregnated silicone SV, whilst following a peak and trough pattern in contamination levels, had a greater rate of decline in *P. aeruginosa* water levels (R^2= 0.72) compared to silicone (R^2= 0.3) and EPDM (R^2= 0.05).

5.3.3.1 Staff willingness to flush taps before use

The results of a mixed staff survey carried out as part of this study (Chapter 3) suggest that 55% (69/125) of staff do not believe that colleagues would run a tap for more than 30 seconds before using the water to wash their hands (Figure 5.11; page 170).

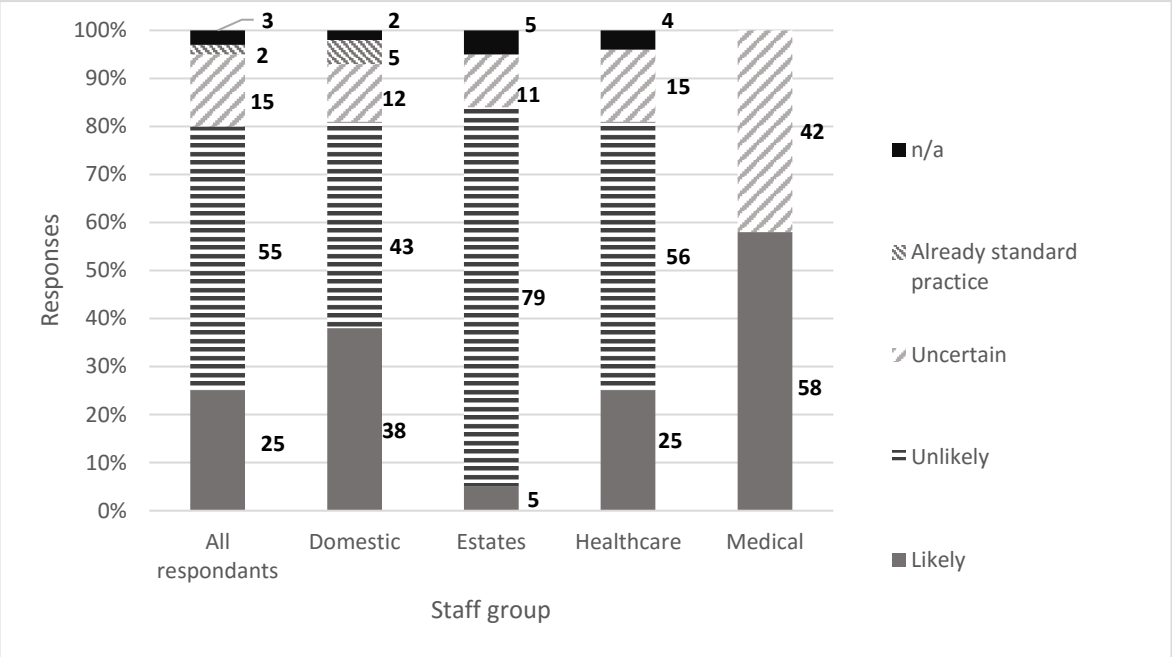


Figure 5.11 Staff survey responses by percentage. "In your opinion, are staff likely to carry out the following practices? *All staff to run a tap for more than 30 seconds before using the water to wash hands."* 125 total respondents (domestic (n=42); estates (n=19); healthcare (n=48); medical staff (n=12); other (n=4).

However, fewer staff (25%; 31/124) believed it unlikely that clinical (healthcare and medical) staff would not flush a tap prior to patient use. The only staff group to believe differently was the medical staff, with the majority (7/12 (58%); Figure 5.11) believing it likely they would allow a >30-second flush for their own hands and the minority (1/12 (8%); Figure 5.12; page 170) believing it likely that clinical staff would allow >30-second flush for water being collected for patient use.

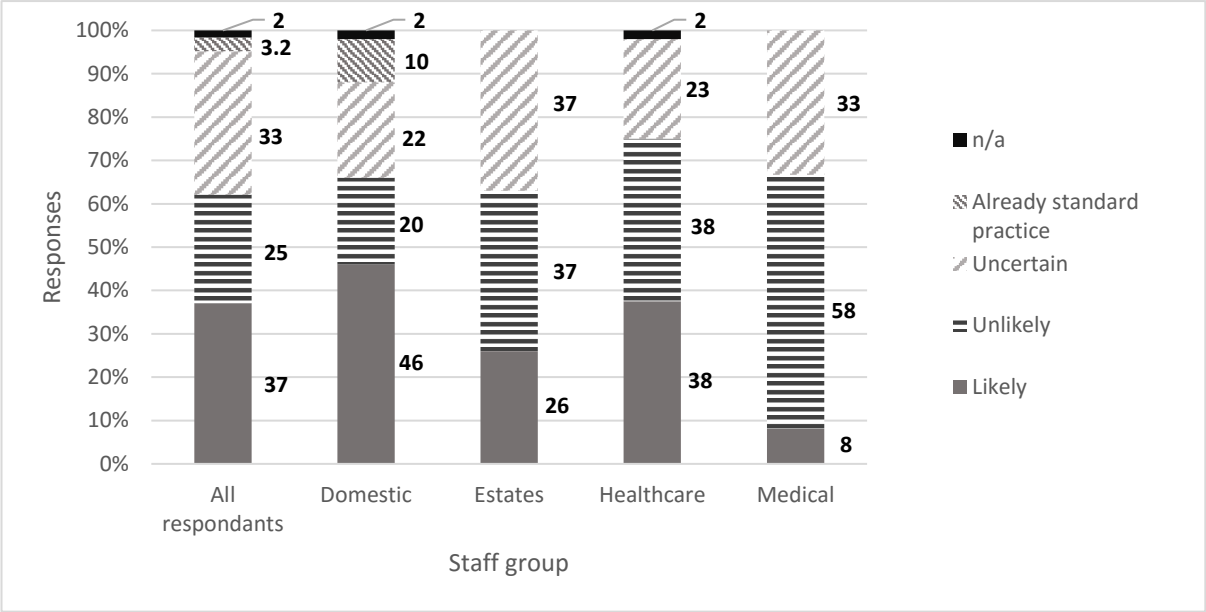


Figure 5.12 Staff survey responses by percentage. "In your opinion, are staff likely to carry out the following practices? *Clinical staff to run a tap for more than 30 seconds before collecting water for patient use."* 124 total respondents (domestic (n=41); estates (n=19); healthcare (n=48); medical staff (n=12); other (n=4).

5.3.4 Investigating remedial measures: the effect of shock-dosing with chlorine on water contamination levels

5.3.4.1 Two hours post-chlorination

Prior to chlorination, chlorine levels were 0.003 ppm in the source water and between 0-0.06 ppm at distal points (i.e. taps at the four corners of the EWDS system). After the tap assemblies were exposed to chlorine (63 ppm at the source water; 0.06-3.7 ppm at distal points) for two hours, *P. aeruginosa* was reduced to ≤1 CFU/100 mL in water delivered by SVs (n=4) and one tap dispensed water at 49 CFU/100 mL (above the alert limit for augmented care (≥10 CFU/100 mL (Department of Health, 2013b))). The total viable counts (TVCs) saw a mean $\log_{(10)}$ reduction of 2.7. Of the five SVs removed after 2-hours of exposure to chlorinated water, three associated diaphragms were positive for *P. aeruginosa* by culture at levels of 4 CFU/diaphragm (silver ion-impregnated silicone) and 1280 CFU/diaphragm and 2940 CFU/diaphragm in EPDM SVs that had remained *in situ* for five years (EPDM-old). *P. aeruginosa* was the predominant organism. No organisms were recovered from the other two SVs (one silicone and one new EPDM), however contamination was evident by SEM (Figure 5.8 E and F). Removal of the SVs reduced *P. aeruginosa* water counts to <1 CFU/100 mL.

5.3.4.2 Two weeks post-chlorination

Over the course of two weeks of twice-daily flushing with chlorinated water, 1/3 taps with SVs in place, and 1/5 taps with SVs removed were positive for *P. aeruginosa* on one occasion each, at levels of 1 and 3 CFU/100 mL respectively. No other taps were positive for *P. aeruginosa*. Chlorine levels ranged between 0.16-0.54 ppm in taps with SVs and 0.13-0.41 ppm in taps without SVs at the end of the two weeks. After two weeks, TVCs from taps with SVs in place had significantly higher counts (p=0.0007) than taps without SVs, with a mean $\log_{(10)}$ difference of 2.4. The removal of these three SVs lead to a significant reduction in TVCs (p=0.002), with a mean CFU $\log_{(10)}$ reduction of 3.7. One out of three of the SV diaphragms removed after two weeks was positive for *P. aeruginosa (*28 CFU/diaphragm; a silicone SV previously associated with *P. aeruginosa* contaminated water). The other two SV diaphragms had TVCs of 8 CFU/diaphragm (silver-ion impregnated silicone; previously associated with *P. aeruginosa* contaminated water) and 224 CFU/diaphragm (EPDM-new; not associated with *P. aeruginosa* water contamination at any stage). By comparison, over the same sampling periods, taps with no SVs had a small but significant mean CFU $\log_{(10)}$ reduction of 0.9 (p=0.022).

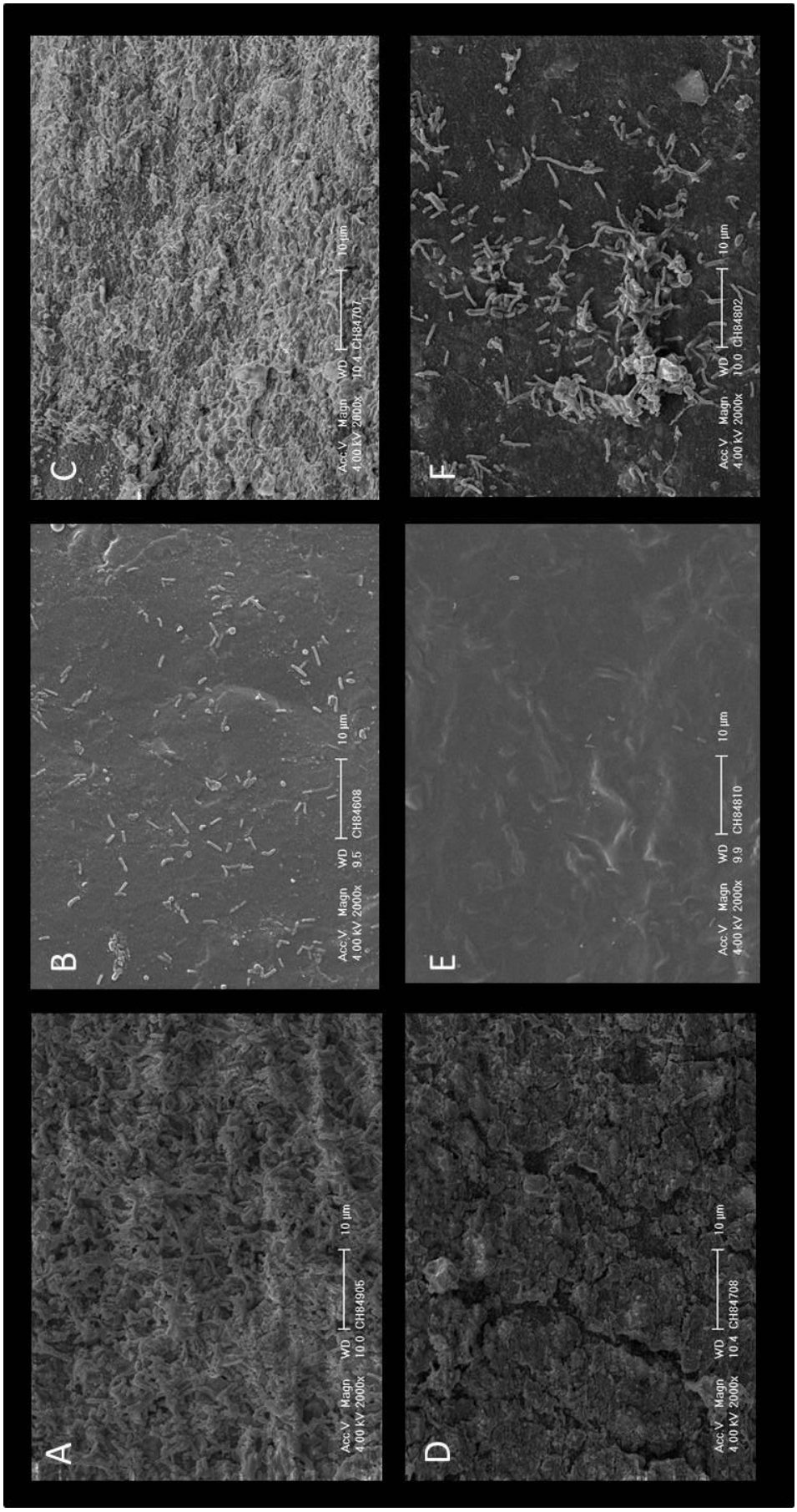


Figure 5.13 Solenoid valve diaphragms after two hours of exposure to chlorinated water. A-D SV diaphragms that were culture-positive for *P. aeruginosa;* E-F SV diaphragms with no

organisms recovered by culture. A, C and D are old EPDM SVs inoculated in previous studies (*in situ* for five years). B, E and F are new silicone-based SVs.

5.3.5 Staff opinion on the use of manual taps over automatic taps

A mixed-staff survey carried out as part of this study (Chapter 3) investigated staff attitudes/opinions regarding the risks associated with the use of manual and automatic taps. The majority (60%; 75/125) of staff believed using automatic taps instead of manual taps was unlikely to increase the risk of infection (Figure 5.14; page 166).

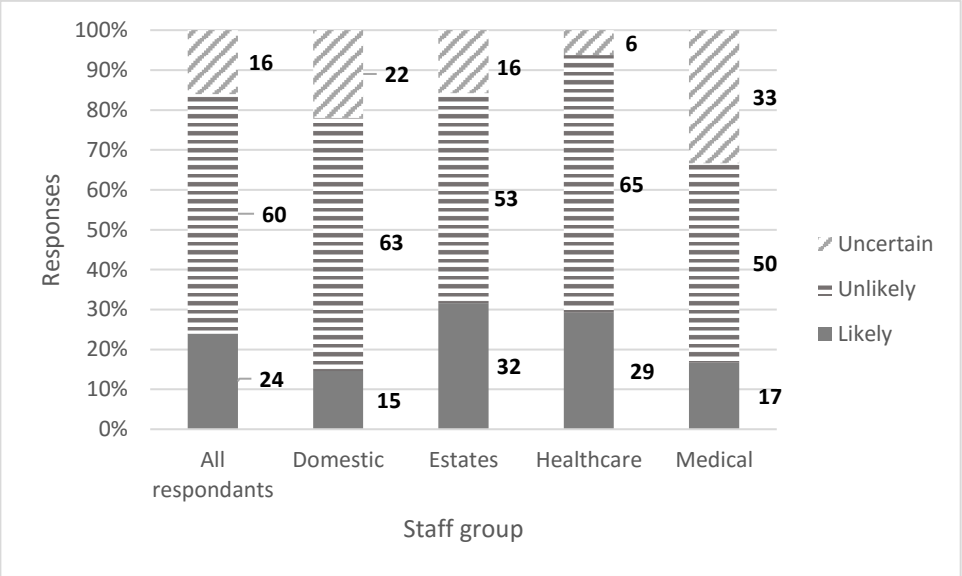


Figure 5.14 Staff survey responses by percentage. "In your opinion, what is the likelihood of the following increasing the risk of infection? *Staff use non-touch taps instead of taps with handles."* 125 total respondents (domestic (n=41); estates (n=19); healthcare (n=48); medical staff (n=12); other (n=5).

However, when presented with the risks of contamination from each tap type, only 31% (38/124) agreed that the risk of spreading infection through hand contact with tap handles (i.e. manual taps) was greater than the risk from contaminated water (i.e. automatic taps/SV presence) (Figure 5.15; page 171).

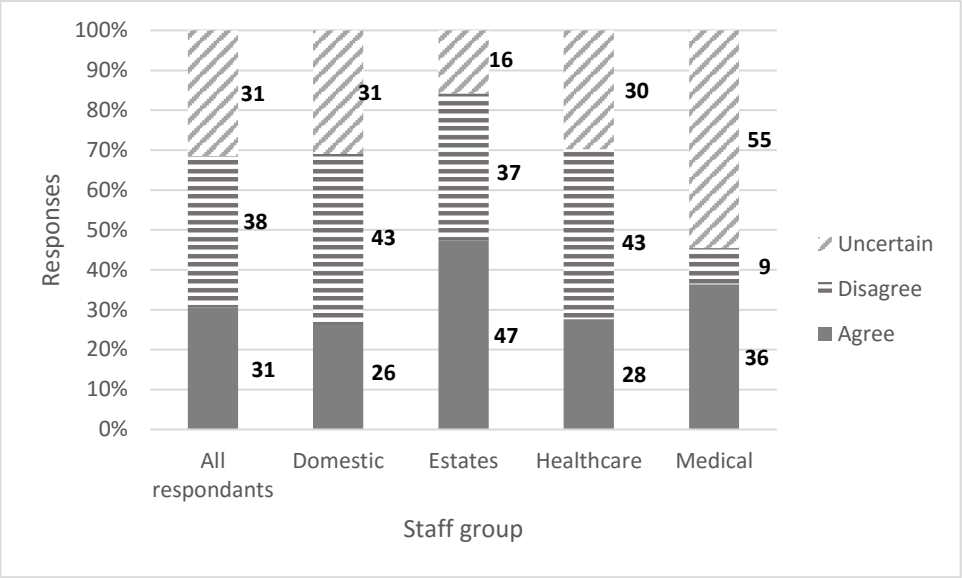


Figure 5.15 Staff survey responses by percentage. "Please indicate whether you agree with the following statements. *The risk of spreading infection after touching tap handles is greater than the risk of infection from washing hands in contaminated tap water."* 124 total respondents (domestic (n=42); estates (n=19); healthcare (n=47); medical staff (n=11); other (n=5).

5.4 Discussion

5.4.1 Biofilm formation on EPDM and nitrile valves

The colonisation of solenoid valves (SVs) containing EPDM diaphragms has been reported in real life scenarios (Kilb *et al.*, 2003) and investigated under controlled laboratory conditions (Moore *et al.*, 2015b), leading to the material's suitability for use in healthcare plumbing to come under question. As a result, manufacturers are looking to move away from the conventional EPDM diaphragms and use other materials that would be physically suitable for the task (i.e. would maintain mechanical properties after ageing events such as exposure to shear, heat and chemical stresses). Suitability with regards to minimising microbial growth is limited to the British Standards (BS6920) testing, "growth of aquatic organisms" (Chapter 1.2), which relies upon differences in dissolved oxygen uptake as a marker for microbial proliferation over time exposed to materials (British Standards Institution, 2014a), however this does not mean that materials that have passed the BS6920 will not become colonised and harbour biofilm; EPDM is such an approved material.

In Chapter 4, *in vitro* early biofilm comparisons between rubber materials demonstrated that nitrile could support significantly higher levels of *P. aeruginosa* biofilm than EPDM. A conclusion that could have been drawn was that EPDM is the most suitable of the rubbers tested and should be used favourably compared to other materials being considered. However, in the EWDS and in the context that the materials would be present in real hospital water distribution systems, no significant difference was observed either in contamination of water over the 12-week period or microbial load recovered from the nitrile and EPDM diaphragms.

Despite a twice-daily flushing regimen, *P. aeruginosa* was able to form a strong biofilm on the rubber diaphragms. Within six weeks of contamination, the biofilm matured to deliver stable levels of *P. aeruginosa* in the water, demonstrating its resilience against shear stress. The architecture of the biofilms was investigated by microscopy, which revealed complex, multi-layered structures on both nitrile and EPDM SV diaphragms.

Although *P. aeruginosa* was artificially introduced to the EWDS, these results demonstrate that a single-event spike in *P. aeruginosa* levels was sufficient to colonise EPDM and nitrile SVs and lead to persistent contamination of the water. In the 2013 DoH guidance, it was advised that a tap, if negative for *P. aeruginosa,* should be tested six-monthly (Department of Health, 2013b). However, if a systemic spike in *P. aeruginosa* occurred (e.g. due to a chlorination failure (Gallay *et al.*, 2006)) within the six month window, it could go undetected, lead to biofilm formation on tap components and stabilise into a mature, persistent biofilm. Similar results (i.e. a single

contamination event (albeit artificial) leading to prolonged water contamination) were demonstrated by Moore *et al.* (2015b). The updated DoH guidance no longer advises a six month wait between sampling *P. aeruginosa*-negative taps (Department of Health, 2016c).

Further investigations in this chapter demonstrated that the persistent shedding of *P. aeruginosa* into the water caused by biofilm was characterised by a 'peak and trough' pattern across flushes. Solenoid valves with biofilm that was the result of artificial contamination (and left *in situ* for five years) was comparable to a more realistic example of contaminated SVs (i.e. through the installation of SVs pre-seeded with *P. aeruginosa*).

5.4.2 The contamination of solenoid valves from source

Contamination of outlets in new or refurbished hospitals can occur should water in the pipework be allowed to stagnate (e.g. between installing the plumbing and handing over the building (British Standards Institution, 2006, Health and Safety Executive, 2000)). Alternatively, plumbing components may become contaminated prior to installation, i.e. at the point of manufacture (Quick *et al.*, 2014).

Plumbing components are often water-tested in factories, and factory disinfection procedures (if in place) may not be sufficient to reach niches within components. In this study, all four of the materials investigated (EPDM, nitrile, silicone and silver-ion impregnated silicone) were able to support *P. aeruginosa* colonisation and had evidence of source-contamination.

During a pilot study (data not presented), the installation of nitrile and EPDM SVs resulted in *P. aeruginosa* being recovered from water delivered from one of the 12 taps. Contamination levels were high ($8x10^4$ CFU/100 mL) and the strain (designated strain "E") was phenotypically and genotypically distinct from strains previously isolated from the EWDS. During the 12-week study presented in this chapter, the installation of nitrile and EPDM SVs led to the presence of strain "E", this time in water delivered from of 3/12 taps and strain "E" was isolated from an additional nitrile SV whilst culturing diaphragm biofilms.

The contamination of an un-inoculated control (tap 10; Table 2.4) provided further evidence that strain "E" could have been introduced from source. This tap had been inoculated with strain "A" during the pilot trial but was not inoculated for the 12-week experiment. After removal of the SV installed for the purposes of the pilot study, tap 10 remained positive for *P. aeruginosa*, confirmed to be the inoculating strain used in the pilot run. Investigations into the source of residual strain "A" contamination highlighted the potential for Binder point (point of inoculation) seals to facilitate biofilm, and all Binder points were replaced with new, IPA-treated Binder points.

After the five-day stagnation period, tap 10 remained positive for *P. aeruginosa,* however with strain "E". No evidence of strain "A" was present in tap 10 water samples over the course of 12-weeks, nor from the diaphragm at the end of the 12-weeks. Strain "E" was later recovered from biofilm on the diaphragm.

Determining and comparing genetic features of strains is a method that is increasingly being applied to identify sources of contamination (Quick *et al.*, 2014, Martin *et al.*, 2013). Techniques can vary in genetic resolution, with methods such as VNTR focusing on areas of tandem repeats (i.e. areas of a genome where there are consecutive repeated short sequence patterns), the number of which varies between strains, allowing some evolutionary insight (Frothingham and Meeker-O'Connell, 1998). VNTR does not provide the resolution that whole genome sequencing (WGS) offers, i.e. the ability to detect single nucleotide changes and differences amongst strains of the same VNTR sequence (den Bakker *et al.*, 2011, Read *et al.*, 2002).

Evidence of source contamination was also apparent following the installation of silicone-based SVs, immediately after which (and in the absence of inoculation), water dispensed from 7/8 taps was positive for *P. aeruginosa*. As water isolates had identical VNTR profiles to PA14 (a strain present within the EWDS) and differed from strain "A" by only one locus, WGS and dry investigations were conducted, which demonstrated that SVs were arriving pre-contaminated with *P. aeruginosa* (dry investigations were conducted under sterile conditions in an MSC Class II biological safety cabinet using IPA-treated tools and gloves as per Chapter 2.3.5). PA14 is a common strain (Martin *et al.*, 2013) that had been used to inoculate the EWDS in previous studies (Moore *et al.*, 2015b). Quick *et al.* (2014) report an average genetic distance of 4.1 mutations within a clone of *P. aeruginosa* commonly isolated from water. Natural mutations of strains endogenous to the EWDS led to mutations of 3 SNPs within a five year period. In contrast, the isolates recovered from four contaminating silicone-based SVs (two water isolates and two from dry investigation diaphragm cultures) differed from the PA14 endogenous to the EWDS by 21-22 SNPs, all at the same loci.

These results support the hypothesis that the SVs were contaminated at source and those of others who speculate that plumbing fittings could be "installed in hospitals 'pre-seeded' with *P. aeruginosa*" (Quick *et al.*, 2014). Water used in the testing of components during manufacture could be the source of contamination, but it is also possible that factories that do not manufacture particular constituents in-house take receipt of contaminated components.

5.4.2.1 Investigating the antimicrobial properties of silver ion-impregnated silicone diaphragms

The *P. aeruginosa* present on silver ion-impregnated silicone diaphragms was able to survive in both dry and wet conditions, and persist within the EWDS. This implied that the silver additive was not having an effect on *P. aeruginosa* and/or was not leaching from the materials. To investigate whether this was due to the contaminating *P. aeruginosa* strain being highly tolerant to silver ions, an isolate from a silver ion-impregnated silicone diaphragm was used to conduct minimum inhibitory- and minimum bactericidal concentration (MIC and MBC) investigations of silver nitrate as previously described (Chapter 2.2.7). These revealed an MIC and MBC of 0.04 ppb, i.e. the strains were not especially tolerant to silver presence in comparison to strain "A" (Chapter 4.3.3.3). The lack of leaching of both silver ions and nanoparticles from the 'antimicrobial' SVs was confirmed by ICP-MS and NanoSight.

It is not unheard of for authors to state a reduction of $<0.1\text{-log}_{(10)}$ demonstrates antimicrobial effects (Kaali *et al.*, 2010). Such conclusions are misleading for manufacturers who may not necessarily read and/or understand the microbiological aspects of studies claiming to have developed an antimicrobial material.

Where components are marketed as 'antimicrobial', there is a risk that hospitals may believe the product reduces contamination and/or is not able to become contaminated. It is likely that such products may be procured and used in areas where patients are at higher risk of infection (e.g. augmented care units). The implications of providing contaminated components, is that manufacturers are essentially supplying the problem they were designed to minimise, putting patients at risk.

5.4.3 Microbial control strategies for solenoid valve contamination

5.4.3.1 The effect of flushing

A basic water hygiene measure that is recognised by the British Standards Institute, WHO and Health and Safety Executive (HSE) is to prevent the stagnation of water within plumbing systems, both to minimise accumulation of chemicals such as lead, and to minimise the opportunity for microbial proliferation. In order to achieve this, the DoH guidance (2013) for augmented care units recommends flushing taps regularly, or at least once daily (Department of Health, 2013d).

DoH guidance also states that tap 'pre-flush' and 'post-flush' water samples are indicators of where the source of contamination may lie. 'Pre-flush' is defined as "the first water to be delivered from the outlet" and 'post-flush' defined as "the sample obtained after allowing water

to flow from an outlet" (Department of Health, 2013d). Taps with higher contamination levels pre-flush and reduced/undetected *P. aeruginosa* post-flush are likely to have local contamination (i.e. biofilm on the faucet or on a local plumbing component, as opposed to the water source).

This study has confirmed that the presence of biofilm inclose proximity to the outlet results in higher microbial counts in pre-flush water samples than in those taken post-flush. However, the results also demonstrated that if such a biofilm is presence, the impact of flushing as a microbial control measure can be superficial and/or ineffective, with microbial counts returning to pre-flush levels within minutes. These results highlighted the need for additional control measures, such as avoiding the first sample of water from a tap as rule of thumb, regardless of stagnation period.

It is highly unlikely that the results obtained were due proliferation of *P. aeruginosa* during the 4.5 minute stagnation. A more plausible explanation would be that the difference in shear force from stagnation to flow was enough to slough *P. aeruginosa* from the biofilm, but continued exposure to this level of shear force did not result in *P. aeruginosa* sloughing at the same rate, hence the reduction in counts by the second and third sample of each flush. These results are in agreement with the *in vitro* findings of Telgmann *et al.* (2004) and *in situ* findings of Charron *et al.* (2015), who also observed a ~2-$\log_{(10)}$ reduction after the first sample of water from taps in a hospital setting, however Charron's study did not control the stagnation time prior to water sampling.

The flushing time used in this study is in line with the findings of Suchomel *et al.* (2013) who observed the most effective reductions in microbial water counts after a pre-use flush of 30-seconds in comparison to a 2-second flush or 10-second post-use flush. Suchomel also postulated that hospital staff would not be willing to wait for a tap to flush in a clinical setting. Charron *et al.* (2015) also suggests that healthcare workers would wash their hands in the first sample of water delivered from a tap. However, neither Suchomel nor Charron investigated whether their speculations about staff behaviour were true.

5.4.3.2 Staff willingness to flush taps before use

Interestingly, staff belief in their colleagues' willingness to flush a tap for 30 seconds before use varied depending on the intended use of the water; hand washing or patient care. Whilst generally more staff believed taps would be flushed for patient use than handwashing, the medical staff produced an opposing opinion. Where the microbial content of water has recontaminated hands, there is further opportunity for disinfection, e.g. the use of alcohol gel. However, in the absence of expensive intervention strategies (e.g. point-of-use filters) tap water collected for patient use cannot be further sterilised and should arguably be seen as the priority for pre-use tap flushing over the two scenarios. Where water is intended for patient use but taps

are not pre-flushed, reducing the microbial content of water would be dependent upon decontamination strategies in place at source, e.g. chlorination.

5.4.3.3 Chlorination

Solenoid valves installed within the EWDS were contaminated either several years prior to this study (Moore *et al.*, 2015b), or at source (factory). Both were representative of biofilm situations that could arise in hospitals: long-standing contamination and the presence of very mature biofilm on plumbing components in areas that have not been refurbished for a number of years or; contamination resulting from the installation of a component colonised during manufacture.

This study highlighted a difficulty that can be faced during the hyperchlorination of water distribution systems: despite dosing the system with an appropriate concentration of chlorine, the levels are diluted out and reach distal points at variable levels even within a controlled model. The addition of surplus chlorine could have major effects on the integrity of pipework and plumbing materials, such as corrosion of the pipes, which can lead to plumbing/tubing failures (Atlas *et al.*, 1982). Corrosion of plumbing materials can further provide microbial niches, leading to greater protection of bacterial cells from residual chlorine in the water compared to non-corroding plumbing materials (Niquette *et al.*, 2000).

Short-term exposure to increased chlorine levels had a greater microbiological effect on SVs with contamination from factory than the SV with long-standing contamination. This could be beneficial for newly-furbished or refurbished hospitals considering tap components are not likely to be provided sterile, however further investigations would need to be undertaken to support this. SEM imaging revealed lower levels of biofilm on the new, silicone-based SVs than those *in situ* for >5 years (Figure 5.13). Evidence of thick biofilm on older components post-chlorination, as well as the reduced microbial counts in the water but recovery of viable *P. aeruginosa* from the biofilm, highlight the risk that water samples taken post-chlorination could provide false-negative microbial results, with intact biofilm containing viable cells remaining on plumbing components. Hospitals that perceive a chlorination event to be successful may mistakenly conclude that the recurrence of contaminated water is caused by a new source. It may be more appropriate to exchange plumbing components with (sterile/uncontaminated) replacements, rather than relying upon chlorination as a remedial measure for plumbing components with heavy biofilm contamination. Where possible, hospitals should therefore consider components that are autoclavable rather than trusting factory hygiene standards. Alternatively, hospitals could return to manual, elbow-operated taps instead of automatic taps, with the first sample of water lost during the opening of the lever and reducing the number of plumbing components available for microbial colonisation.

5.4.3.4 Staff opinion on the use of manual taps over automatic taps

A concern in maintaining hand-hygiene, particularly amongst healthcare staff, is the recontamination of hands after hand washing through contact with contaminated tap handles. The WHO recommends closing taps using paper towels (Pittet *et al.*, 2009), and theatre staff are encouraged to use elbows to operate taps (Widmer *et al.*, 2010). Tap handles have been shown to provide a surface on which bacteria can persist, even after cleaning (Griffith *et al.*, 2000). To minimise hand contact with surfaces that could potentially recontaminate hands, automated sensor taps have been installed into hospital hand-wash stations. However, this control measure does not take into account recontamination of hands via contaminated tap water.

The use of automatic taps instead of manual taps was generally not believed to increase the risk of infection. This is not in-keeping with the evidence presented both in this chapter and in previous studies, where conversion or replacement of a tap from manual to automatic via the installation of SVs has provided a microbial niche and led to persistent (and increased) *P. aeruginosa* presence in water dispensed from that tap (or vice versa) (Merrer *et al.*, 2005, Yapicioglu *et al.*, 2012).

However, when asked about the risks associated with each type of tap, staff opinion was divided on whether the risk associated with tap handles was greater than that of contaminated water. This contradicted in many cases to opinions given in the previous question (Figure 5.15), possibly indicating a lack of awareness of the risks associated with automatic taps.

5.5 Conclusions

The results of this chapter demonstrate that alternative materials to EPDM, currently available or in development, do not prevent the colonisation of SV diaphragms with *P. aeruginosa* and can lead to persistently contaminated water being dispensed from taps. The condition in which silicone-based SVs were received (i.e. contaminated) helped provide evidence that healthcare plumbing components can and are being distributed pre-seeded with *P. aeruginosa*.

Although results in this chapter raise questions as to the suitability of nitrile as a replacement for EPDM, they also demonstrate, in contrast to the *in vitro* study (Chapter 4), that EPDM does not have ‘superior’ microbial performance *in situ*. This finding demonstrated an advantage of model systems over *in vitro* models, allowing materials to be tested within the products and under conditions they are expected to perform in in real life. Use of the EWDS allowed mimicking of regular tap usage, investigations into a ‘worst-case scenario’ (i.e. highly contaminated plumbing

components) without the disruption that could be caused by field testing in a real hospital, as well as investigating decontamination strategies in a controlled environment.

This study also highlighted the dangers of labelling products as 'antimicrobial' on the basis of incorporation of agents reported to have antimicrobial effects in the literature. Studies such as those by Kaali *et al.* (2010) report antimicrobial effects by inference as opposed to statistics.

The shedding of bacteria from local biofilm (as a result of the shear stress caused by water movement when a tap is switched on) demonstrate that best-practice should perhaps require staff to avoid the first sample of water that is dispensed from taps. However, staff opinions as to whether colleagues would carry this out varied depending upon the how the water is to be used (e.g. for personal or patient use) and the staffing group. Any request for behaviour change may have to be supported by training/explanation, as staff also demonstrated a lack of awareness of the risk automatic taps pose to water hygiene.

This chapter also highlights the risks of hospitals deciding to purchase one product design over another on the basis that one design (e.g. EPDM SVs) has been criticised when the other (e.g. nitrile/silicone-based SVs) has not been tested. Where hospitals may not have the capacity to carry out their own *in situ* investigations and, with little/no evidence relating to the efficacy of alternative SVs, it may be a safer alternative to return to manual, elbow-operated taps.

Importantly, companies manufacturing tap components (or any product with the potential to impact public health) need to do more to ensure they are not providing hospitals with contaminated components; this is particularly dangerous where products are being marketed as antimicrobial in response to recent attention regarding *P. aeruginosa* contamination in augmented care. The implication of essentially buying-in contamination poses a major infection risk in a healthcare setting, as immunocompromised patients are more susceptible to infection from water contaminants (Department of Health, 2013b).

The aim of this study was to investigate SV contamination, which the EWDS model has allowed to be carried out in a relatively controlled manner, however, a limitation of the EWDS being used to investigate systemic contamination scenarios is that variables found in hospital environments that are exogenous to the water system were not accounted for. In this study and as previously reported, consistently high levels of systemic *P. aeruginosa* contamination did not result in outlet fitting contamination (Moore *et al.*, 2015b), which has been reported by several hospitals (Mäkinen *et al.*, 2013, Walker *et al.*, 2014, Wang *et al.*, 2009). It seems likely that for outlet fittings, the tap component most proximal and exposed to the ward environment, contamination is caused by exogenous factors (i.e. retrograde contamination (Vonberg *et al.*, 2005a)). As well as

SVs, manufacturers have been marketing new outlet fitting designs and incorporation of antimicrobial or anti-biofilm materials. These materials have been investigated using the bioreactor model (Chapter 4) but their performance *in situ* and in a model more realistic of the mode of contamination likely to affect them (retrograde contamination) has not been investigated; this will be the focus of Chapter 6.

Chapter 6 Investigating the attachment and persistence of *P. aeruginosa* on conventional and alternative outlet fittings

6.1 Introduction

One of the original and primary functions of outlet fittings (OFs), particularly those with aerating function, is to reduce water consumption. As previously discussed in Chapter 5.1, the NHS seeks to reduce its water usage (reported as 40 billion litres per annum) for financial and environmental reasons (Department of Health, 2013c). Aeration (the incorporation of air into a water flow) can produce a 'thickened' column of water from taps with low flow rates (i.e. a low volume of water dispensed per minute), whilst simultaneously reducing splashing (Bader and Abu-Hijleh, 2011, Chaplin, 1998).

An unintended consequence of incorporating OFs in taps is the facilitation of microbial survival and consequential contamination of tap water (Weber *et al.*, 1999). Outlet fittings have been identified as bacterial niches, including for *P. aeruginosa,* for almost half a century (Wilson *et al.*, 1961). Other hospitals have also reported the presence of biofilm on OFs (Weber *et al.*, 1999, Mäkinen *et al.*, 2013). It is unclear from hospital studies how biofilm comes to develop on OFs and whether biofilm development is dependent upon conditions exclusive to the hospital environment. The accumulation of debris and/or the retention of water within the OF have been suggested as contributing factors for biofilm formation at the outlet (Weber *et al.*, 1999, Kappstein *et al.*, 2000). Kappstein *et al.* (2000) attributed the control of an *Acinetobacter junii* outbreak with the removal of 'complex' OFs (i.e. those consisting of metal meshes) and their replacement with a relatively simplified OF design. However, removing layers and/or reducing the complexity of the rosette does not necessarily correlate with lower levels of water contamination (Moore *et al.*, 2015b).

In response to the incidents in Northern Ireland, the DoH advised that OFs should be removed (Department of Health, 2013b, Walker *et al.*, 2014). However, OF removal can lead to a turbulent flow of water from a tap that can result in splashing of the user and surrounding surfaces (Reuter *et al.*, 2002), including the floor. Where OFs have been installed as a secondary flow rate control measure (Bader and Abu-Hijleh, 2011) (i.e. installation correlates with a reduced volume of water dispensed per minute), OF removal can lead to an inappropriately high flow rate from the tap, which can also lead splashing and spillage onto the floor (Figure 6.1).

Figure 6.1 Effect of flow rate upon the dispersal of water droplets (splashing). Splashing is indicated by the darker patches on the absorbent material on the floor. A: tap with an outlet fitting (point of use filter) in place showing relatively limited splashing; B: the same tap with the outlet fitting removed showing a large amount of splashing onto the floor. Photos taken and provided by Jimmy Walker (Public Health England).

NHS trusts have reported that, regardless of flooring material, the largest cause of slips in hospitals is a wet floor or spillage (Healey and Scobie, 2007). Incidents caused by slipping on wet floors are categorised under 'slips, trips and falls' (Healey and Scobie, 2007). The implications of slips and falls do not only extend to the injured person; the financial repercussions of lost staff days, NHS treatment of the faller (reported to be £133,000,000 per year) as well as any legal compensation are substantial (Munn, 2017, Health and Safety Executive). A study investigating near misses and adverse incidents across 18 NHS hospitals over a nine-month period (n=28,998), concluded that slips, trips and falls were responsible for 41% of events (Shaw *et al.*, 2005). However, as this study was carried out on a voluntary reporting basis the real figures are likely to be higher (Healey *et al.*, 2008). It has been reported that the healthcare sector has the highest rate of days lost due to work-related illness and injury with approximately 1.9 days lost per person (Lipley, 2009). Thus, the impact of removing OFs from taps may extend beyond the control of water flow.

An OF designed to prevent systemic contaminants from leading to contaminated water being dispensed from the tap, is the point of use (POU) filter (Daeschlein *et al.*, 2007). POU filters can be large relative to the activity space available at a hand-wash station (i.e. the space between the water outlet point and the drain, available for hand washing or other activities), and so need to be installed where there is an appropriate amount of room to prevent hand contact with the filter (Department of Health, 2016a). POU filters are effective in reducing the microbial burden of the

water, often to below detection (Sheffer *et al.*, 2005, Warris *et al.*, 2010) and have been reported to successfully manage hospital water contamination scenarios that have caused outbreaks and endemic infections (Engelhart *et al.*, 2002, Trautmann *et al.*, 2008, Vianelli *et al.*, 2006). POU filters are disposable and the costs involved with their replacement have been reported to be substantial, with Garvey *et al.* (2016b) estimating an annual cost of £138,600 if every outlet in their hospital was to have POU filters installed (price based on a quote of £50/POU filter). Public Health England (PHE) was previously contacted by several Trusts in response to a request for tap manufacturer information. Feedback provided by one trust stated that, "*it would be cheaper for some Hospitals to replace a tap twice a year rather than fit filters as we feel they generate more problems than they solve*," and another stated, "*we find installing bacteria filters a bit a waste of time as the flow decreases dramatically and creates a further problem as now the Pseudomonas aeruginosa has a better breeding ground*." Misuse of POU filters by staff and patients has been reported by Florentin *et al.* (2016), who noted some patients grew so impatient with the reduced flow caused by POU filters that they removed them. Development of biofilm within filters has also been reported (Daeschlein *et al.*, 2007). Concerns were also raised by (Garvey *et al.*, 2016b), who noted visible soiling of the POU filters which, upon sampling, was identified as contamination by *P. aeruginosa,* highlighting the fact that POU filters are still susceptible to retrograde contamination (contamination exogenous to the water system), a reality that is indicated in the DoH guidance by recommending measures to minimise the chance of contamination of the filter by hand contact (Department of Health, 2016a). This is an example of an expensive remedial measure that has the potential to be made redundant by human behaviour.

In response to the highly contaminated OFs associated with the Northern Irish neonatal *P. aeruginosa* incidents (Walker *et al.*, 2014), as well as the impracticalities caused by removal of OFs and installation of POUs, the clinical tap industry has produced OFs marketed to reduce the risk of bacterial colonisation. Materials such as ion-impregnated plastics and antimicrobial metals were incorporated into designs to be marketed as 'antimicrobial'. OF designs have also been simplified from multi-layered rosettes to single-bore in an attempt to reduce surface area and water retention. Particular materials were also claimed to be 'anti-biofilm', based on the material's reduced lime scale and debris attachment properties (theoretically resulting in reduced microbial attachment).

Some of the novel OF designs would require the replacement of the entire tap as they cannot be securely retrofitted into taps of other manufacturers, which would increase the cost of their installation. If the OFs are unable minimise contamination potential from retrograde sources, especially in hospitals where retrograde contamination is a recognised infection control problem (Vonberg *et al.*, 2008), they could be an expense without merit. Vonberg *et al.* (2008) who

acknowledged retrograde contamination as a known problem, proposed that retrograde contamination of POU filters could occur by cloth contamination and contaminated hands, and also stated that regular training would be required to educate staff due to the likelihood of non-compliance over time by staff already trained. Vonberg also suggested that patients need to be instructed on proper use to minimise misuse and retrograde contamination (Vonberg *et al.*, 2008), in line with Florentin's observations of patient misuse (Florentin *et al.*, 2016).

As it is the hospital's priority to resolve outbreak situations and reduce the risk to patient and staff health, the nature of retrospective/outbreak studies is that several remedial measures are put in place at once and investigative work has few control measures (Kappstein *et al.*, 2000, Oliveira *et al.*, 2007, Vianelli *et al.*, 2006).

The individual materials used in OFs have demonstrated the ability to become colonised and support *P. aeruginosa* biofilm (Chapter 4), even those claiming to be 'antimicrobial' or 'anti-biofilm'. However, as previously discussed (Chapter 4.4), *in vitro* results do not always translate when applied *in situ*, so it is important to determine how alternative OFs perform in a scenario more representative of a hospital environment. The manufacturer of the OFs that were contaminated in the Northern Ireland *P. aeruginosa* incidents (Walker *et al.*, 2014) suggested that the conditions in which the OFs were kept was a key factor in their colonisation. It is not clear how the OFs in Northern Ireland became contaminated, however it was suggested by the manufacturers that poor staff practice was a likely cause; these claims were unsubstantiated but warranted controlled investigations into whether human behaviour could lead to contaminated OFs, survival and the contamination of water.

6.2 Aim and objectives

The aim of this study was to investigate the attachment and persistence of *P. aeruginosa* on conventional and alternative outlet fittings, and the consequences for water hygiene. The objectives were to:

- Confirm that conventional outlet fittings can support *P. aeruginosa* biofilm via culture and microscopy.
- Assess the survival and persistence of artificial *P. aeruginosa* biofilm on conventional outlet fittings and the impact on water hygiene using an experimental water distribution system.
- Develop a cloth-contamination model to simulate a hospital-style retrograde contamination scenario.

- Assess the survival and persistence of cloth-transferred *P. aeruginosa* on conventional and 'antimicrobial/anti-biofilm' outlet fittings.
- Assess the impact of flushing as a remedial measure for retrograde contamination of outlet fittings.

6.3 Results

6.3.1 The attachment and survival of *P. aeruginosa* on conventional outlet fittings

Conventional OFs immersed in a nutrient-rich *P. aeruginosa* culture were readily colonised. Fluorescence microscopy images (Figure 6.2) illustrate high numbers of irreversibly attached bacteria, which have attached within the five-day incubation period and withstood rinsing steps. Figure 6.2 C and D (48 hours *in* situ) are stained with propidium iodide at a concentration ten-fold higher [10 mg/mL] than propidium iodide present in *Bac*light® [1.1 mg/mL]. Propidium iodide is an indicator for non-viable or cells with damaged membranes, due to its inability to penetrate an intact cell membrane (Nocker *et al.*, 2007, Boulos *et al.*, 1999). However, there is evidence that propidium iodide can staining viable cells (Shi *et al.*, 2007) and if a stain is used at an excessive concentration, staining accuracy can be reduced (Boyd *et al.*, 2008), thus cells in Figure 6.2 C and D could represent some of the viable population of attached *P. aeruginosa;* survival beyond 48-hours *in situ* was demonstrated by culture.

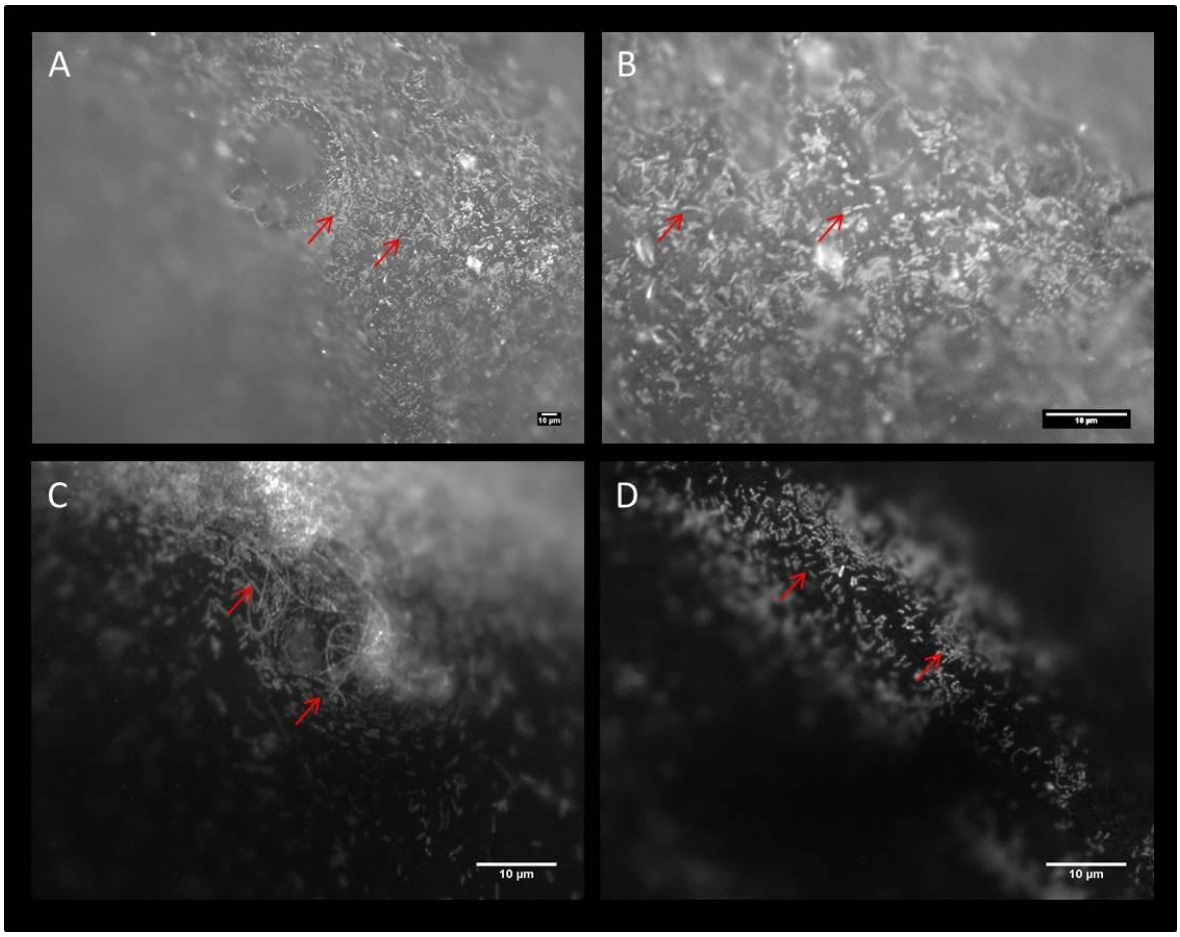


Figure 6.2 Fluorescence microscopy images showing biofilm on conventional outlet fittings. Red arrows indicate bacterial cells. Images A and B are of *Bac*light® stained segments after five days *in situ*. Images C and D are stained with propidium iodide [10 mg/mL] only after 48 hours *in situ*.

When cultured, the median number of *P. aeruginosa* recovered from conventional OFs contaminated via immersion was $1.5x10^7$ CFU per outlet fitting (CFU/OF; n=9). Once attached, *P. aeruginosa* survived on the OF surfaces for at least 10 days (Figure 6.3). However, numbers declined steadily over time with a significant $\log_{(10)}$ reduction of 1.8 after 24 hours (Figure 6.3). After remaining *in situ* for five days and after a single flush, the number of *P. aeruginosa* recovered was significantly lower than that recovered after 24 hours and equated to a further 2.1-$\log_{(10)}$ reduction. Between five and ten days, the loss in viability was less rapid and equated to just 0.8-$\log_{(10)}$ values. After 10 days *in situ* the median number of *P. aeruginosa* recovered from OFs was $5.5x10^2$ CFU/OF; overall a 4.7-$\log_{(10)}$ reduction over the 10-day experimental period. Nonetheless, water delivered from all OFs was contaminated at levels above the alert level for augmented care (Table 6.1).

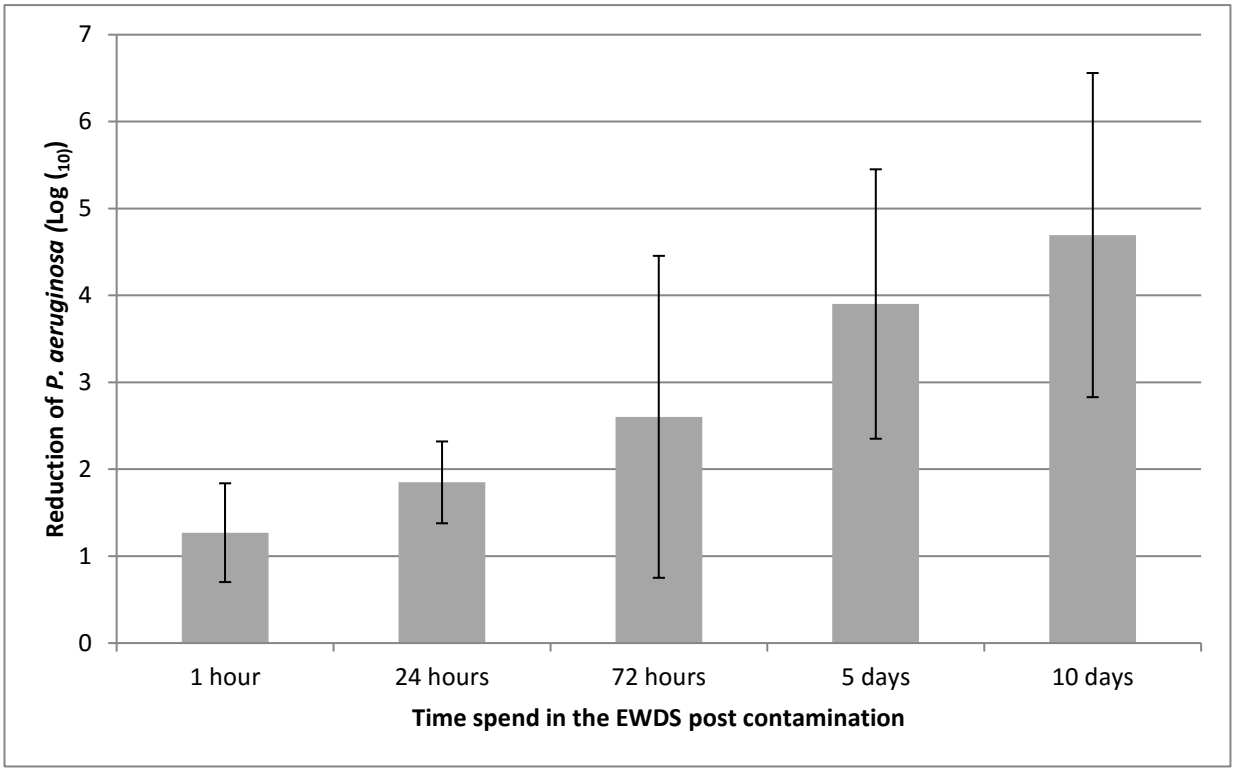


Figure 6.3 Mean (±SD) reduction ($\log_{(10)}$) of *P. aeruginosa* recovered from conventional outlet fittings with artificial biofilm post 30-second flush over a 10-day period *in situ* (n=9).

Table 6.1 Number of water samples delivered through outlet fittings contaminated with *P. aeruginosa* biofilm that contained *P. aeruginosa* at levels above (≥10 CFU/100 mL) or below (1-9 CFU/100 mL) the alert level for augmented care or below the detection limit of the assay (<1 CFU/100 mL) (n=9)

P. aeruginosa (CFU/100 mL)	1 hour	24 hours	72 hours	5 days	10 days
≥10	9	8	8	8	9
1-9	0	1	0	0	0
<1	0	0	1	1	0

6.3.2 Cloth-contamination of outlet fittings

P. aeruginosa was transferred to all OFs via contaminated microfibre cloths. The median number (n=12) of *P. aeruginosa* transferred was $5.7x10^5$CFU (OF-A), $1.9x10^6$CFU (OF-B), $1.4x10^5$CFU (OF-C) and $2.9x10^6$CFU (OF-D). Significantly fewer bacteria were transferred to OF-C than to any other OF (p<0.05). No other differences were significant.

6.3.2.1 Neutralisation of antimicrobial ions

Recovery of *P. aeruginosa* from OF-D was significantly reduced in thiosulphate Ringer's solution compared to D/E neutralising broth, with a mean $\log_{(10)}$ difference of 1.1 (n=6), compared to a mean $\log_{(10)}$ difference of 0.04 (OF-B; n=6) and 0.3 (OF-A; n=3) which were not significant. D/E broth was used for recovery from OF-D only throughout the experiments.

6.3.2.2 Cloth-transferred *P. aeruginosa* survival on conventional and alternative OFs

Figure 6.3 illustrates the reduction in viable *P. aeruginosa* recovered from the different OFs up to 24-hours after contamination. For all OF types, significant $\log_{(10)}$ reductions were observed by 15-minutes (p<0.05). There were also significant $\log_{(10)}$ reductions between one- and four-hours for all OF types, with reductions of 2.7 (OF-A), 3.7 (OF-B), 2.2 (OF-C) and 2.6 (OF-D). The rate of *P. aeruginosa* reduction decreased after the first four-hours, with significant $\log_{(10)}$ reductions of 1.8 (OF-A), 1.6 (OF-B), 1.3 (OF-C) and 2.6 (OF-D) over the following 20-hour period.

No significant difference in *P. aeruginosa* viability was observed after 24-hours between the conventional and 'antimicrobial/anti-biofilm' OFs. OF-B showed greater $\log_{(10)}$ reduction than OF-A at 12-hours (p<0.05), however this did not carry through to the 24-hour time point.

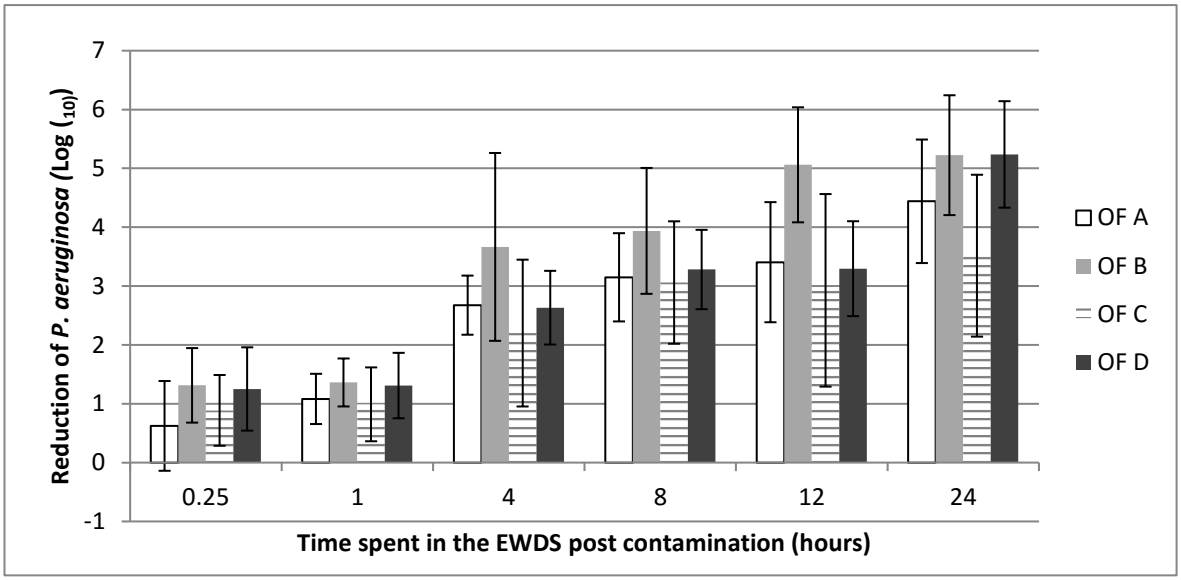


Figure 6.4 Mean (±SD) reduction ($\log_{(10)}$) of cloth-contaminated *P. aeruginosa* on outlet fitting types (n=12)

6.3.3 Contamination of water as a result of retrograde contamination

6.3.3.1 Contamination of water over a 24-hour period from cloth-contaminated conventional outlet fittings

Taps fitted with OF-A were flushed once to simulate an infrequently-used tap. All water samples (n=6) taken immediately after contamination of the OF were positive for *P. aeruginosa* at ≥10CFU/100mL (above the augmented care alert limit). The level of *P. aeruginosa* in all water collected at 15-minutes, one-, four- and 12-hours after contamination also exceeded the alert limit. *P. aeruginosa* persisted on the surface of the OF and 24-hours after contamination, 3/6 (50%) of water samples delivered through OF-A contained *P. aeruginosa* at levels ≥10CFU/100mL.

6.3.3.2 Flushing as a remedial measure for retrograde contamination

Regardless of OF design, the first sample of water dispensed from taps contaminated in a retrograde manner was contaminated at levels above the alert limit for augmented care. In all cases, increased usage of the tap correlated with reduced water contamination (Table I). X^2 -tests indicated that the level of contamination was not independent from the number of flushes that had occurred for all four OF types (i.e. there is an association between flushing and the observed reduction in *P. aeruginosa* counts) (n = 12; OF-A: X^2 (4) = 10.7, p <0.05; OF-B: X^2 (4) = 60.0, p<0.0001; OF-C: X^2 (4) = 46.2, p<0.0001; OF-D: X^2 (4) =49.3, p <0.0001).

Table 6.2 The number of water samples delivered through contaminated outlet fittings that contained *P. aeruginosa* at levels above (≥10CFU/100mL) or below (1-9CFU/100mL) the hospital alert limit for augmented care, or below the detection limit of the assay (<1CFU/100mL). Samples (n=12) were collected over five consecutive tap usages (i.e. five consecutive 30 second flushes).

OF type	*P. aeruginosa* (CFU/100mL)	Frequency of tap use				
		1st flush	2nd flush	3rd flush	4th flush	5th flush
OF-A	≥10	12	11	8	8	6
	1-9	0	1	2	1	2
	<1	0	0	2	3	4
OF-B	≥10	12	0	0	0	0
	1-9	0	0	0	0	0
	<1	0	12	12	12	12
OF-C	≥10	12	2	1	0	0
	1-9	0	0	0	1	0
	<1	0	10	11	11	12
OF-D	≥10	12	11	2	0	0
	1-9	0	1	4	4	2
	<1	0	0	6	8	10

Contamination of water delivered from OF-A persisted despite continued flushing (or usage) and 6/12 (50%) in the fifth flush contained *P. aeruginosa* at levels of ≥10CFU/100mL (Table 6.2). In contrast, by the second flush, the level of *P. aeruginosa* in water delivered through OF-B was always below the detection limit of the assay. Whilst two (17%) and one (8%) of the 12 water samples collected during the second and third use of taps fitted with OF-C respectively contained *P. aeruginosa* at ≥10CFU/100mL, the majority of samples (11/12, 92%) were below detection by

the fourth flush. *P. aeruginosa* was recovered from water delivered from OF-D at ≥10CFU/100mL until the fourth flush, by which point no *P. aeruginosa* was detected in 8/12 (67%) samples. In contrast to OF-B and –C, water dispensed by contaminated OF-D contained *P. aeruginosa* at detectable levels in 2/12 (17%) samples by the fifth flush.

6.3.4 Antimicrobial leaching from alternative outlet fittings

Water samples from the EWDS were collected from three different taps with and without OF-D (containing silver-ion impregnated hostaform) in place. There was no evidence of silver leaching in nanoparticle form, with a mean particle per frame reading of 0. The background level of silver ions ranged from 0.015-0.05 ppb. Installation of OF-D led to two of the three taps reading mean increases in silver presence by 0.07 ppb, however one of the three taps saw a decrease of 0.01 ppb. The differences in silver ion content from the same taps with or without outlet fittings in place were significant for all three taps.

OF-D was inserted into the EWDS, for which the variability of background copper within the EWDS ranged between 363.9 and 507.7 ppb with a mean of 421.1 ppb. Water collected after installation of OF-D had copper ion content ranging between 609.7 to 1098.4 ppb, with a mean of 777.1 ppb (a significant increase of 356 ppb) (n=3, $p<0.0001$).

The background copper ion level across three taps of the secondary EWDS (used to install OF-B) ranged between 641.6 and 951.3 ppb, with a mean reading of 833.8 ppb. After installing OF-B, the copper ion content of the water ranged between 615.4 and 782.2 ppb, with a mean reading of 686.1 ppb (a significant decrease of 147.7 ppb) (n=3, $p<0.0001$).

6.3.5 Staff opinions on cloth-contamination of hospital taps

Staff responses to a mixed staff survey included as part of this study (Chapter 3) demonstrated that the majority of staff (58%) believed that infection risk would increase if cloths were not changed between hand-wash stations (Figure 6.6).

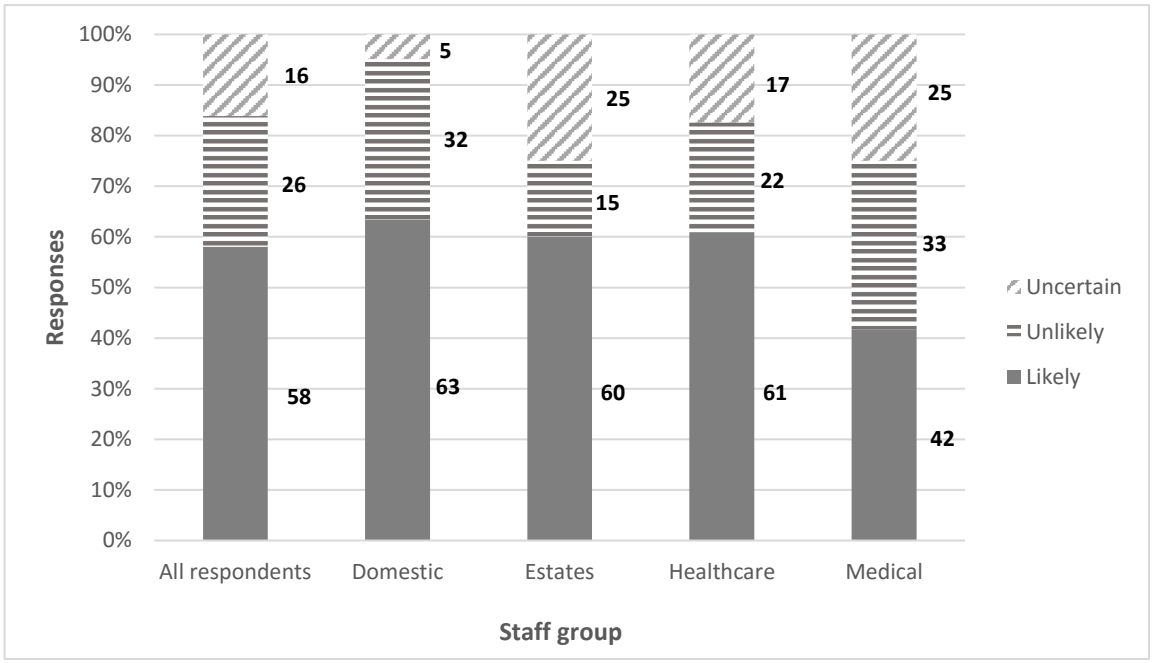


Figure 6.5 Staff survey responses by percentage. *"What is the likelihood of the following increasing the risk of infection? Cloths are not changed between hand-wash stations during cleaning."* 124 total respondents (domestic (n=41); estates (n=19); healthcare (n=48); medical staff (n=12); other (n=5)).

The majority of staff (53%) also believe that cleaning around the drain-hole and basin before wiping the tap spout could increase the risk of infection (Figure 6.6).

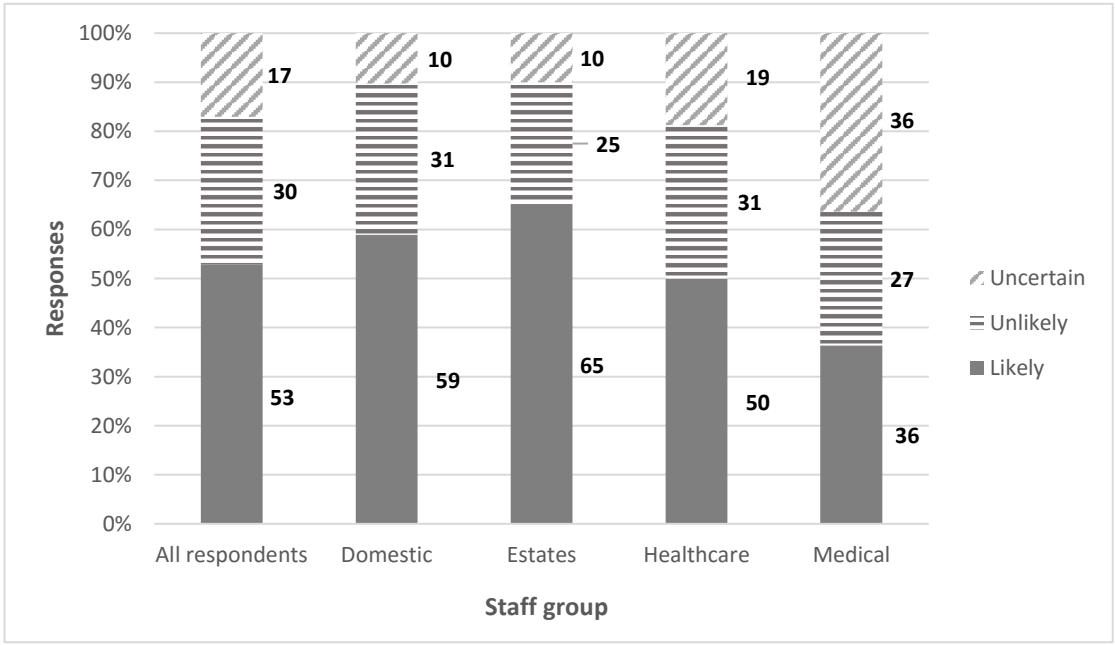


Figure 6.6 Staff survey responses by percentage. "*What is the likelihood of the following increasing the risk of infection? During cleaning, cloths are used to wipe around the drain-hole and basin before wiping the tap spout."* 123 total respondents (domestic (n=39); estates (n=20); healthcare (n=48); medical staff (n=11); other (n=5)).

When asked about the willingness to change cloths between hand-wash stations, only 38% (48/123) believed domestic staff would oblige (Figure 6.7; page 170). However, domestics themselves demonstrated higher confidence in their own staff group behaviours, with 71% (29/41) believing they would comply and only 5% uncertain (vs. a range of 32-58% of other staff groups showing uncertainty). Eleven percent (13/123) of respondents stated that this was standard practice in their hospital and the majority of these (11/13) were domestic staff (Figure 6.9).

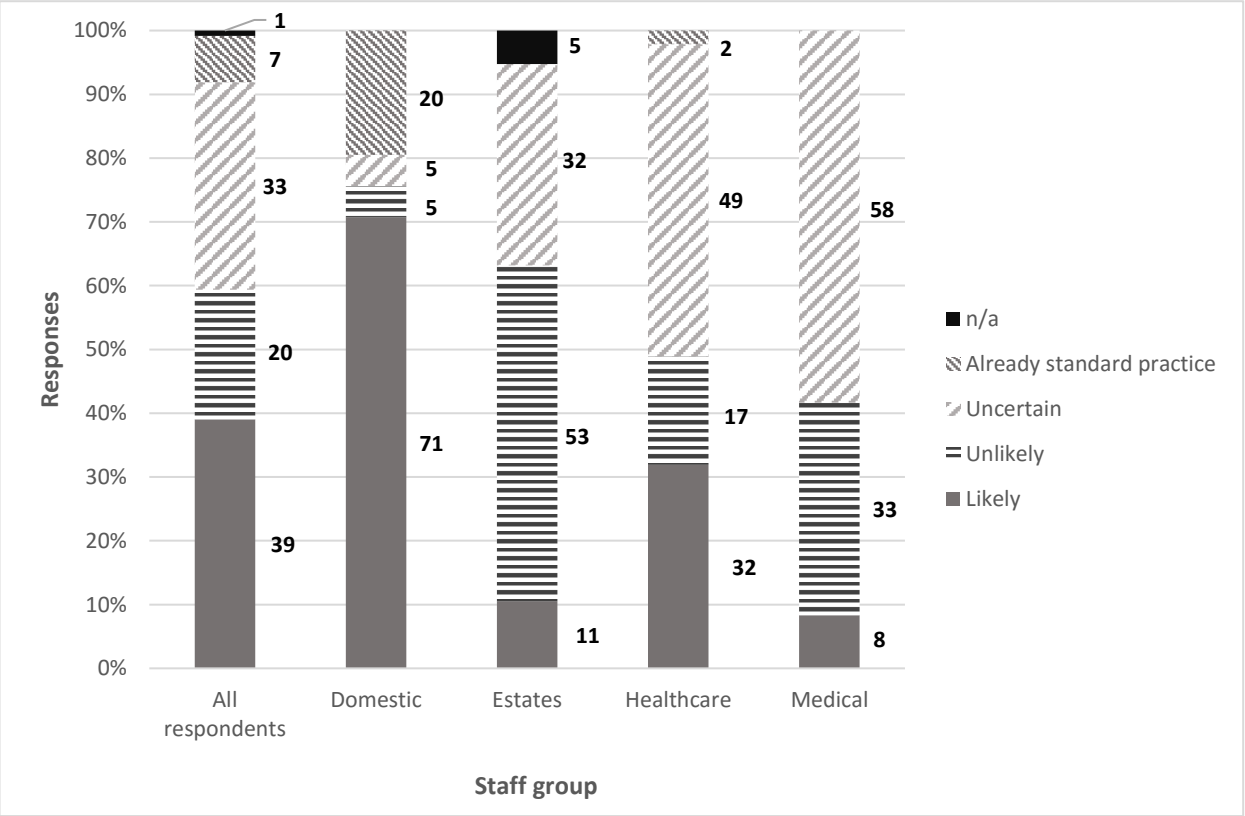


Figure 6.7 Staff survey responses by percentage. *"Are staff likely to carry out the following practices? Domestic staff to change cloths between every tap when cleaning."* 123 total respondents (domestic (n=41); estates (n=19); healthcare (n=47); medical staff (n=12); other (n=4)).

6.4 Discussion

6.4.1 The contamination of conventional outlet fittings

The contamination of outlet fittings (OFs) in hospital taps, associated with biofilm formation on the components, has been widely reported and linked to several outbreaks of waterborne disease (Kappstein *et al.*, 2000, Weber *et al.*, 1999, Walker *et al.*, 2014).

A crucial stage in biofilm formation is irreversible attachment, and organisms that are unable to irreversibly attach to a surface are unable to progress into a biofilm (Hinsa *et al.*, 2003). In the current study, the immersion of OFs in a nutrient-rich medium resulted in the irreversible attachment of *P. aeruginosa* to conventional OFs, demonstrated by both microscopy (Figure 6.2) and culture (Figure 6.3); a strong indicator of their ability to support biofilm. Furthermore, *P. aeruginosa* was able to survive on these OFs within an EWDS without introduction of additional nutrients or water for at least ten days, and attachment was strong enough to endure the shear force induced by flushing. Bacterial recovery did reduce over time. This may have been due to losses in viability and/or a conversion to a viable-but-non-culturable state as a result of stressful conditions such as low nutrient availability (Klančnik *et al.*, 2009) and/or weakening attachment (thus greater detachment during flushing). Regardless, water delivered by taps defined by the Health and Safety Executive as being "under-used" (i.e. a tap not used for ≥7 days) (Health and Safety Executive, 2000) was still contaminated at levels above the hospital alert limit.

There is debate in the literature as to how OFs become contaminated, with some studies drawing links to staff practices (Reuter *et al.*, 2002) whilst others postulate that colonisation occurs as a result of stagnation of water containing low-level contamination within the OF (Weber *et al.*, 1999). Previous studies exposing OFs to water contaminated with *P. aeruginosa* did not result in contaminated OFs (Chapter 5) (Moore *et al.*, 2015b). Moore speculated that sources exogenous to the water system may have an important role in OF contamination (i.e. retrograde contamination), an hypothesis that has been proposed by many others investigating hospital tap contamination (Florentin *et al.*, 2016, Balm *et al.*, 2013, Reuter *et al.*, 2002). Potential methods of retrograde contamination were reported by Florentin *et al.* (2016), who observed physical contact between the OFs (in this case, point of use filters) with patient hands, as well as cleaning practices that could lead to cross contamination from the sink to the OF.

The contamination of cleaning cloths and their ability to spread bacteria has been reported widely during food production/preparation (Tebbutt, 1986, Scott and Bloomfield, 1990b, Scott and Bloomfield, 1993). It has also been demonstrated in the hospital environment (Bergen *et al.*, 2009) and linked to *P. aeruginosa* outbreaks (Engelhart *et al.*, 2002). Microfibre cleaning cloths,

commonly used in the hospital setting (Smith *et al.*, 2011), have been shown to enhance cleaning efficiency compared to conventional cloths (Nilsen *et al.*, 2002). However, their ability to recontaminate surfaces as well as the variability of performance across brands have also been demonstrated (Moore and Griffith, 2006).

This is the first published study investigating the transfer of *P. aeruginosa* from contaminated microfibre cloths to OFs and the consequences on water hygiene (Hutchins *et al.*, 2017). The high load of *P. aeruginosa* used to inoculate the microfibre cloths was comparable to contamination levels found in cleaning cloths in previous studies (Yepiz-Gomez *et al.*, 2006), and resulted in OF contamination at levels comparable to those recovered from the OFs implicated in the Northern Ireland incidents ($1.8x10^5$ CFU) (Walker *et al.*, 2014). This contamination persisted on the surface of conventional OFs over a 24-hour period and led to water unsuitable for use in augmented care, even after several flushes (Table 6.2) and/or when low levels of *P. aeruginosa* remained on the OFs (median of 68 CFU/OF at 24 hours; n=12).

6.4.2 Cloth-contamination of alternative 'antimicrobial/anti-biofilm' outlet fittings

6.4.2.1 Transfer of *P. aeruginosa* to alternative outlet fittings

It has been suggested that retrograde contamination has the ability to out-do (or make redundant) engineering solutions to contamination, as demonstrated by one hospital's silver-impregnated point of use filters eluting contaminated water only seven days after installation (Vonberg *et al.*, 2008).

In response to the findings in Northern Ireland and the 2013 DoH guidance on *P. aeruginosa* control in augmented care, manufacturers started producing 'antimicrobial/anti-biofilm' alternative OFs. The antimicrobial/anti-biofilm efficacies of materials incorporated into alternative OFs were investigated in Chapter 4, with copper-based metals demonstrating antimicrobial and anti-biofilm effects. However, silver-impregnated plastics, and 'anti-biofilm' plastics did not reduce microbial colonisation and/or survival.

All three alternative OFs were able to become contaminated using a cloth-contamination model. In comparison to conventional OFs, OFs incorporating antimicrobial materials (copper and silver) did not demonstrate a significant bactericidal effect over 24-hours. The only OF to demonstrate a greater $\log_{(10)}$ reduction compared to conventional OFs was OF-B (i.e. a single-bore OF with a copper (100%) interior lining), but only at the eight hour time point; there was little evidence that copper based components reduced bacterial survival, as has been reported for dry healthcare surfaces (Warnes *et al.*, 2010).

Whilst this study only investigated the transfer of *P. aeruginosa* from microfibre cloths to outlet fittings, it is important to remember that this may be reflective of the spread of other organisms, as microfibre cloths have been associated with environmental cross-contamination previously (Moore *et al.*, 2008, Bergen *et al.*, 2009).

6.4.2.2 Leaching of antimicrobial agents from alternative outlet fittings

Recovery of *P. aeruginosa* from the surface of OF-D required the OF to be transferred to D/E neutralising broth, suggesting that, in a liquid medium, OF-D may release antimicrobial ions. However, the antimicrobial surface materials of OF-D had no significant effect upon bacterial survival *in situ* compared to conventional OFs (Figure 6.3). In contrast, significant differences in silver ion levels were recorded in water collected from the EWDS with and without OF-D *in situ*. However, the concentrations detected were extremely low and ~1000-fold below the MIC or MBC of *P. aeruginosa* for silver ions (Chapter 4.3.3.3). When silver-ion impregnated hostaform (incorporated in OF-D) coupons were exposed to *P. aeruginosa* within a bioreactor (i.e. when being assessed for their ability to support biofilm; Chapter 4.3.5), no increase in silver ions or nanoparticles were detected in the bioreactor medium, suggesting it was unlikely that the changes in silver ion levels detected in water samples collected from the EWDS were due to the presence of OF-D.

Installation of OF-D into taps on the EWDS correlated with an increase in copper ion levels in the water dispensed from affected taps. However, the use of ICP-MS to draw conclusions on the effects of OFs on copper levels in the water may not be appropriate. There was a substantial amount of copper piping both within the two EWDSs used for this study as well as throughout the building plumbing system leading up to the laboratories. It is possible that there were other confounding factors from within the water system that were responsible for fluctuations in copper levels seen in water from the EWDSs that have not been elucidated.

6.4.3 Effect of flushing on cloth-contaminated outlet fittings

As previously discussed (Chapter 5.4.3), flushing of taps is widely recommended to minimise microbial contamination of taps, prevent accumulation of chemicals and to introduce fresh antimicrobial agents such as chlorine from the water source to the distal points.

This study demonstrated that increased tap use (i.e. an increase in the number of flushes) correlated with a reduction in the number of *P. aeruginosa* recovered from the surface of conventional OFs. In comparison, all three ‘antimicrobial’ OFs demonstrated superior microbial clearing performance and after a single contamination event, there was no evidence of superficial

reductions in water contamination levels between flushes, as was the case for local biofilm (Chapter 5). OFs of single-bore designs (OF-B and –C) were more efficient at reducing contamination than OFs incorporating a multi-layered rosette. It is possible that this was, in part, due to the more turbulent flow of water delivered from the single-bore designs (Figure 6.8).

The incorporation of OFs in healthcare settings is primarily to regulate the flow of water from taps, minimising splashing (Department of Health, 2016a, Reuter *et al.*, 2002). However, during this study, installing certain OFs into taps of a different manufacturer, notably OF-C, resulted in visible splashing. These observations were fed back to the manufacturers. Manufacturers of OF-C have since retracted the claim that the OF, "can be installed on any TMV3 tap to ensure a good flow with no splashing," and have amended it to, "can only be installed on a mixer/tap with a restricted flow rate to ensure a good flow with no splashing." OF-B, which also caused a less controlled flow of water than OF-A and –D, was the most consistent OF to clear residual retrograde contamination.

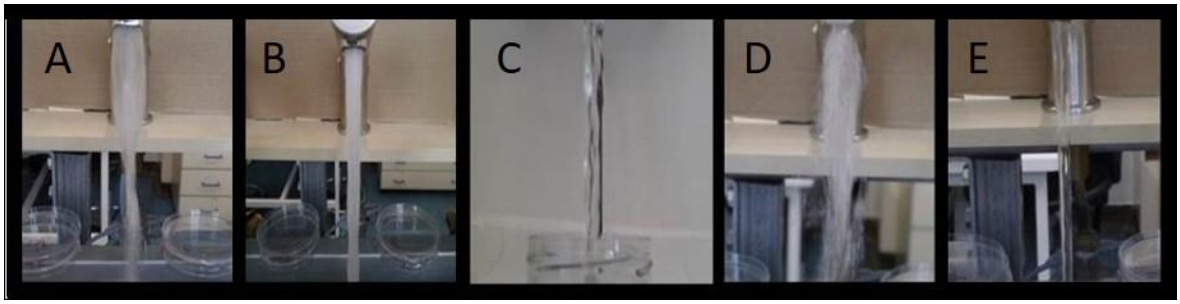


Figure 6.8 Photographs of flow regulation dependent upon outlet fitting present. A: no outlet fitting showing a turbulent flow; B: OF-A showing a more controlled column of water; C: OF-B with a turbulent flow of water; C: OF-C with a turbulent flow of water; E: OF-D with a controlled column of water and reduced flow. [Figure cropped to remove identifiable images]

6.4.4 Staff opinions on cloth-contamination of hospital taps

The results of a mixed staff survey as part of this study indicated that staff had an appreciation for the risk of cross-contamination by cleaning cloths both between taps and within a hand-wash station (e.g. wiping from the drain to the spout).

The risks of infection resulting from a 'dirty-to-clean' method are highlighted in DoH guidance (2013) (Department of Health, 2013b). The staff group in highest agreement with regards to drain-to-tap cross contamination was the estates, stating there could be a negative impact on infection control. It is possible that the estates staff, who are responsible for addressing sink

blockages, would have potentially seen the internal surfaces of sink drainage pipes, where biofilm can be visible by eye (Figure 6.9) (Inglis *et al.*, 2010).

Figure 6.9 Visible biofilm in hospital sink waste traps. Red arrows indicate accumulation of biofilm. Traps (n=3) were sent to Public Health England, Porton Down. Photographs taken and provided by Ginny Moore (Public Health England).

The spread of contamination around hand-wash stations during cleaning has been demonstrated by Garvey *et al.* (2016a), who used ultraviolet cream as a marker for potential microbial dispersal. Garvey suggested a fold-and-refold method, a concept which has been criticised by Bergen *et al.* (2009), who demonstrated a reduction in microbial counts but simultaneous dissemination of the contamination over the area in contact with the cloth. However, unlike Bergen's method, Garvey suggests replacement of the cloth in between key areas, reducing the risk of contamination between the tap, sink/drain and surrounding surfaces.

When asked whether staff believed their colleagues would change cloths between each tap whilst cleaning, the only staff group with a majority believing it likely were the domestics themselves. Some respondents stated that this was standard practice in their hospital and the majority of these were domestic staff, highlighting mixed practices across and within hospitals.

Domestic staff should be aware of the risks associated with contaminating their cleaning cloths in the sink/drain area and spreading it to hospital taps (Florentin *et al.*, 2016)/other hand wash stations, a risk that standard NHS cleaning protocols does not address (Anonymous, 2009) but novel methods in place in some Trusts do (Garvey *et al.*, 2016a). Although the majority of staff demonstrated an appreciation for the risks associated with residual contamination of cloths/cross contamination between taps (Figure 6.5), nearly a third of domestics did not. Use of detergents on cloths between hand wash stations may not be effective (Barker *et al.*, 2004, Scott and Bloomfield, 1990a). The implication of this is that even if a 'clean-to-dirty' method were used (i.e. cloth used to wipe the tap before the drain), continued use of the cloth could spread drain contaminants to the tap of another hand wash station.

6.5 Conclusions

This study reproducibly demonstrated that OFs are able to support a *P. aeruginosa* biofilm and this leads to water contaminated at levels inappropriate for patient use after periods of stagnation (Department of Health, 2013b), something that had not previously been shown. In addition, a method of retrograde contamination that could have a role in the development of *P. aeruginosa* biofilm on OFs has been investigated, demonstrating the potential risks in a cleaning cloth cross-contamination scenario. 'Antimicrobial/anti-biofilm' OFs did not reduce the likelihood of OFs becoming contaminated with *P. aeruginosa* in a retrograde manner, nor reduce the ability of bacteria to persist and contaminate water at levels above the alert limit for augmented care after the contamination event. Alternative designs did, however, allow for more efficient reduction in retrograde contamination over a series of 30-second flushes than conventional OFs, highlighting the importance of regular tap flushing/usage. In contrast to previous findings (that flushing leads to artificially reduced levels of *P. aeruginosa* when sourced from biofilm *(*Chapter 5.5.3)*,* flushing can be a longer-lasting, effective remedial measure for retrograde contamination. These findings also support the idea of regular flushing of taps for a minimum period of 30-seconds (Suchomel *et al.*, 2013) to help reduce retrograde contamination on OFs that could occur throughout the day (Balm *et al.*, 2013). The results presented in this chapter demonstrate that recent retrograde contamination can be reduced and potentially cleared through flushing.

The importance of the role of human behaviour plays in tap maintenance was highlighted. As discussed in Chapter 5.4.3.2, the willingness of staff to flush a tap for 30-seconds before use varied depending upon both staff group and what the water was intended for, however the evidence presented here supports the practice of pre-use flushing and water hygiene training. Staff awareness of the risks associated with a cross-contamination during cleaning to water hygiene was variable, however the domestic staff who would be responsible for introducing cloths to OFs showed a willingness to change practice. It should, however, be noted that cloth contamination is not the only method of retrograde contamination and staff awareness of the risk their actions can play to water hygiene (and thus patient health) should be encouraged and increased through water hygiene training.

Chapter 7 Conclusions and recommendations

HCAIs are estimated to cost the National Health Service (NHS) £900 million per year (Plowman *et al.*, 2001). In the UK, *P. aeruginosa* is responsible for a at least 6% of HCAIs, including 28% of pneumonias, 11% of surgical site infections, 9% of urinary tract infections and 5% of bacteraemias (Health Protection Agency, 2012). Therefore, despite *P. aeruginosa* being ubiquitous in the environment, its ability to cause fatal infections (particularly in immunocompromised patients) means that within a healthcare setting, its presence must be controlled (Mena and Gerba, 2009, Selezska *et al.*, 2012). However, the control of waterborne pathogens can be difficult due to their ability to survive and persist in biofilms (Lehtola *et al.*, 2004, Yu *et al.*, 2010) and to cause infection through numerous routes of transmission including inhalation, ingestion, direct and indirect (fomite) contact (Keller *et al.*, 1996, Döring *et al.*, 1991, Braeye *et al.*, 2015, Petrini, 2006, Osho *et al.*, 2013, Oie *et al.*, 2012).

During 2011/12, 25 babies admitted to neonatal intensive care units acquired *P. aeruginosa* (Troop, 2012). These incidents prompted the DoH to produce guidance on *P. aeruginosa* control in augmented care units, for which there was none prior to 2013 (Department of Health, 2013b, Department of Health, 2016c). A particular focus of this guidance is the potential for tap components, for example thermostatic mixing valves, solenoid valves and outlet fittings to become colonised, leading to the contamination of hospital tap water. In response, the clinical tap industry has been attempting to 'engineer-out' *P. aeruginosa* contamination via material choice and design. However, the 'anti-biofilm' claims made by many manufacturers are based on the findings of others rather than their own investigations, particularly where materials with known antimicrobial properties (e.g. copper) have been incorporated. Furthermore, the performance of the products *in situ* (as opposed to individual materials) is rarely investigated.

The use of automatic (sensor) taps controls water usage, offering financial and environmental benefits and ensures bacteria are not transferred from tap handles onto clean hands (Merrer *et al.*, 2005, Department of Health, 2013c). However, an unintended consequence of their installation is the ready colonisation of the tap components, specifically the ethylene propylene diene monomer (EPDM) rubber diaphragm within the associated solenoid valve (SV) and subsequent contamination of tap water (Moore *et al.*, 2015b, Berthelot *et al.*, 2006). Alternative SVs are available, yet replacing the EPDM diaphragm with nitrile- or silicone-based alternatives has the potential to exacerbate the problem with both materials supporting significantly higher levels of biofilm *in vitro* (Chapter 4).

Results of laboratory-based assays do not necessarily translate to real-life, highlighting the importance of testing a product within a model system and obtaining results that may be more realistic of a hospital setting (Muraca *et al.*, 1987). When installed within an EWDS, SVs incorporating a nitrile rubber diaphragm supported higher levels of biofilm and resulted in higher levels of water contamination than SVs incorporating an EPDM diaphragm (Chapter 5). Although this increase was not significant, the results demonstrate that "an alternative to EPDM" does not necessarily mean a "suitable alternative". Future studies should include multispecies models which represent the microbial diversity found in hospital water and water systems.

During this investigation, the controlled (*in situ*) testing of alternative solenoid valves was hindered by products arriving contaminated from source. The apotheosis of this was the installation of 'antimicrobial' SVs which were provided contaminated with the very organism they were designed to engineer-out (Chapter 5). It has been previously hypothesised that contamination of plumbing components could originate from the manufacturer (Berthelot *et al.*, 2006). However, this is the first study to demonstrate, in the absence of confounding factors, that this can occur and was supported by molecular typing and whole genome sequencing (Chapter 5).

There is a risk that products aimed towards the healthcare sector and marketed as 'antimicrobial', could be relied upon by hospitals to remediate existing contamination problems and/or prevent contamination entirely. In this thesis, the efficacy of 'antimicrobial' outlet fittings was investigated. When compared to the original material, the addition of silver ions did not significantly reduce *P. aeruginosa* colonisation (Chapter 4) and no antimicrobial effects were observed in molybdenum trioxide treated material. Furthermore, outlet fittings incorporating materials that did demonstrate an antimicrobial effect *in vitro* (primarily copper) were, *in situ*, no more effective than conventional outlet fittings in preventing contamination (Chapter 6) demonstrating that even when a product incorporates materials with known antimicrobial properties, contamination of components and tap water can still occur.

Evidence suggests that the colonisation of hospital taps is a multifactorial issue (Williams *et al.*, 2013a). However, owing to the nature of hospital-based studies, the impact of individual contributory factors and the effects of individual remedial measures are rarely conclusive. This is often due to investigations lacking controls and/or applying multiple remedial strategies simultaneously (Garvey et al., 2017, Vianelli et al., 2006, Walker et al., 2014). Furthermore, bacteria dislodged from a biofilm can readily attach and colonise surfaces elsewhere within a water system, leading to multi-component contamination (Chapter 5). Component removal or replacement does not guarantee a *P. aeruginosa* free tap.

Use of existing engineering solutions (e.g. point of use filters) can be expensive but effective, but if the/a cause of contamination is exogenous to the water system, there is evidence to suggest that such products can also become contaminated and lead to contaminated water (Garvey *et al.*, 2016b, Garvey *et al.*, 2016c). Biofilm formation and outlet contamination has been attributed to retrograde contamination (i.e. the introduction of contaminants from the ward environment) (Reuter *et al.*, 2002). As part of this PhD, the potential for human behaviour-derived retrograde contamination has been demonstrated (i.e. cross contamination from cleaning cloths) and was shown to cause contamination of conventional and 'antimicrobial' OFs and, as a consequence, tap water (Chapter 6). It was also demonstrated that *P. aeruginosa,* given the opportunity to form biofilm on OFs, can survive over an extended period (≥10 days) within infrequently or under-used taps, and withstand the shear forces from flushing.

There are numerous methods by which retrograde contamination could occur, but during cleaning and through the misuse of hand wash stations are considered most likely. Misuse of hand wash stations is not limited to the actions of healthcare and domestic staff; the majority of staff, visitors and patients interact with hospital taps. The mixed-staff group questionnaire (Chapter 3) revealed that most staff appreciate how the misuse of hand wash stations could negatively impact infection control. However, when provided with specific examples of misuse (e.g. disposal of patient fluids), there was a lower appreciation of risk and fewer participants believed staff would comply with suggested preventative infection control measures. There was also a general lack of awareness of how waterborne pathogens could reach and/or infect patients and the importance of maintaining tap- and water hygiene. A training programme should be developed and delivered to all staff (regardless of role). This should address awareness of waterborne opportunistic pathogens and transmission routes, and how behaviours and actions can impact tap- and water hygiene. Training should also raise awareness of the role of the water safety group.

In addition, observational studies could be conducted to investigate misuse of hospital hand-wash stations and potential routes of retrograde contamination. Focus groups involving hospital staff would allow a more detailed understanding of the factors that influence staff behaviour. This would help inform appropriate engineering solutions and more realistic laboratory-based models, for example, how to assess the risk of cross-contamination during routine plumbing/maintenance. Standard protocols for water hygiene as well as a non-role specific training programme could ensure that all staff are informed of the risks associated with microbial contamination of taps and tap water, thus providing them with the capability and motivation to comply with water safety group recommendations and reduce non-compliance from ignorance or a perceived lack of evidence (Erasmus *et al.*, 2009).

It is recommended that taps are regularly flushed to minimise stagnation of water, introduce antimicrobial agents (such as chlorine) from source of disinfection (storage tanks) to distal outlets and introduce shear force to any contaminants at the outlet resulting in the sloughing of bacteria (Department of Health, 2016a, Douterelo *et al.*, 2014). Evidence from this PhD demonstrates that the efficacy of flushing, as a remedial measure for tap-associated *P. aeruginosa*, was dependent upon the type of contamination present. When the source of contamination was established biofilm (e.g. the colonisation of a solenoid valve), flushing led to a superficial reduction in *P. aeruginosa*. The number of bacteria recovered from water dispensed from the outlet reduced over the course of a single 30-second flush but the concentration of *P. aeruginosa* returned to similar levels within five minutes (Chapter 5). In contrast, when the contamination could perhaps be considered more transient (e.g. resulting from retrograde cross-contamination), flushing had a longer-lasting effect (Chapter 6). After *P. aeruginosa* was transferred to the surface of an OF, its efficient clearance (through flushing) was influenced by the design of the OF, with single-bore OFs demonstrating a more efficient reduction in *P. aeruginosa* than a conventional OFs. However the simplification of OF design can be at the detriment of flow regulation - the primary reason for OF installation.

Regardless of how a tap becomes contaminated, the water initially dispensed after any period of stagnation should be discarded as it is likely to be more highly contaminated in comparison to the water that follows for the duration of the flush. Results presented in Chapters 5 and 6 provide evidence for a >30 second pre-use flush, particularly when collecting water for patient use. Staff provided a mixed response as to whether they would be willing to flush a tap for 30 seconds before use, highlighting disparity both within and across staff groups. Healthcare and medical staff were divided as to whether it was likely, depending upon the intended use of the water, with medical staff very sceptical that water for patient use would be collected post-flush. It has been suggested that the time taken to turn the handle of a manual tap would ensure that the first sample of water (eluted whilst still using the lever) would not be used; an alternative solution for Trusts where staff compliance is an issue and/or flushing is impracticable (Charron *et al.*, 2015). Whilst not removing the potential for retrograde contamination, reintroducing manual (ideally elbow operated) taps would also remove the need for SVs, thus, eliminating a potential bacterial reservoir and source of contamination.

The work presented in this thesis provides evidence to support the hypothesis that the colonisation of hospital taps is a multifactorial issue for which there is no single solution (Williams *et al.*, 2013a). To maintain water quality, human behaviour and tap engineering should complement each other; neither can entirely prevent or resolve contamination facilitated by the other, but both have the potential to exacerbate the effect (or problem) caused by the other.

Hospitals cannot rely upon a product marketed as 'antimicrobial', 'bio-safe' or 'suitable alternative' without evidence of that product's efficacy. Such claims can be made simply by incorporating a particular feature or material into the design rather than proving functional capabilities. The clinical tap industry should take greater responsibility for ensuring their products are not contaminated at source and are able to perform as theorised and/or marketed.

Immunocompromised patients do not only attend augmented care wards and outbreaks of waterborne opportunistic pathogens have been linked to non-clinical (staff room) taps (Kappstein *et al.*, 2000, McKenzie *et al.*, 2011). Therefore, it should not be considered that the findings from this PhD are only applicable to augmented care units, despite that being the focus of *P. aeruginosa* guidance (Department of Health, 2013d). Similarly, engineering solutions and/or behaviour change should also impact other waterborne organisms (e.g. *S. maltophilia* (Brooke, 2012)). A standardised water safety training programme for all hospital staff would help ensure that good infection control practices stretch beyond augmented care units and raise awareness of the role everyone plays in maintaining water hygiene.

7.1 Summary

This thesis has:

1) Explored the knowledge, attitudes and beliefs/opinions of mixed groups of hospital staff on issues surrounding water hygiene. The results provided evidence for a standardised water hygiene training programme for all staff regardless of role, and outlined key areas that should be addressed in such a training programme.
2) Investigated the ability of *P. aeruginosa* to attach to and/or form biofilm on individual tap component materials, either currently in use or in development. Results highlighted that whilst some antimicrobial materials could be effective, other materials (chosen either as alternatives to criticised materials, or incorporating antimicrobial ions) did not prevent *P. aeruginosa* biofilm formation, nor reduce planktonic *P. aeruginosa*.
3) Investigated *P. aeruginosa* contamination and biofilm formation on conventional and alternative plumbing components of automatic taps (solenoid valves), and the impact on water hygiene over time. This PhD also investigated the possibility that plumbing components could be 'pre-seeded' with *P. aeruginosa*, highlighting the risks associated with installing contaminated components. The effect of remedial measures such as flushing and chlorination on long-standing, and newly installed, contaminated

components was also investigated, providing evidence to support the practice of discarding pre-flush tap water regardless of stagnation period.

4) Investigated the transfer and persistence of *P. aeruginosa* from cleaning-cloths in a retrograde-contamination model, and consequences for water hygiene. This thesis highlighted that incorporation of antimicrobial materials does not guarantee an antimicrobial product. It was demonstrated that whilst simplification of product design appeared to have a positive influence in the clearance of retrograde contamination, this could be to the detriment of the product's original function. Regardless, the reduction of cloth-transferred *P. aeruginosa* over a series of tap usages, demonstrated in this thesis, also provided evidence to support the practice of discarding pre-flush tap water.
5) Investigated staff attitudes towards changing practices (for which this thesis has provided evidence to support) that could be adopted by hospitals and addressed in either staff-wide or role-specific training e.g. flushing taps prior to collecting water for patient use and minimising the risk of retrograde contamination during cleaning.
6) Demonstrated that engineering and human behavioural factors have the potential to influence each other's impact on water safety, thus highlighting the need for a holistic approach to maintain water safety. This PhD emphasised that there is a responsibility for the clinical tap industry to provide products that perform as theorised and are not contaminated at the point of manufacture, as well as a responsibility for hospitals to increase staff awareness of, and compliance with, good water hygiene practices.

7.2 Recommendations

The evidence provided in this thesis leads to the following recommendations:

1. A standardised water hygiene training programme for all hospital staff should be in place in addition to mandatory and statutory infection control training already in place.
2. Nitrile rubber should not be considered an 'hygienic' alternative to EPDM.
3. Hospitals should not trust antimicrobial solutions such as the impregnation of materials with metal ions unless companies provide evidence of the product's efficacy.
4. Tap component manufacturers should undertake regular microbial testing of product batches and/or make all products autoclavable for hospitals to sterilise pre-installation.
5. The first sample of water dispensed from a tap (ideally the first 30 seconds of water flow) should be avoided in all cases. Where staff compliance is a problem, hospitals should consider reinstating manual taps so that the first sample of water is dispensed whilst the user is still turning the manual lever.

7.2.1 Future research

Key areas for future research include:

1. Implementation of a water hygiene training programme including observational studies before and after the programme to determine whether good water hygiene practice increases and is maintained.
2. Investigations on the long-term effects of silicone rubber SVs in comparison to EPDM on biofilm and water contamination.
3. Continued investigations of plumbing materials that have been developed further e.g. silver-ion impregnated silicone SVs with higher concentrations of the active agent.
4. Investigations into other material surfaces, perhaps looking to enhance those already established as physically suitable plumbing, e.g. by using material coatings such as anti-adhesive polymer coatings (Yu *et al.*, 2017), photocatalytic particles for areas exposed to visible light (Verdier *et al.*, 2014).
5. Investigations into other components involved in hand-wash stations such as basin material and design which have the potential to become contaminated with biofilm and cause further contamination through splashing.
6. Knowledge, attitudes and beliefs/opinions of hospital staff on issues surrounding other water outlets in the hospital environment such as showers, ice machines and domestic-style (kitchen/staff room) taps.

Appendix A Questionnaire and responses

A.1 The questionnaire

1) Please indicate which of the following employers you belong to by marking an ‘x’ in the appropriate box:

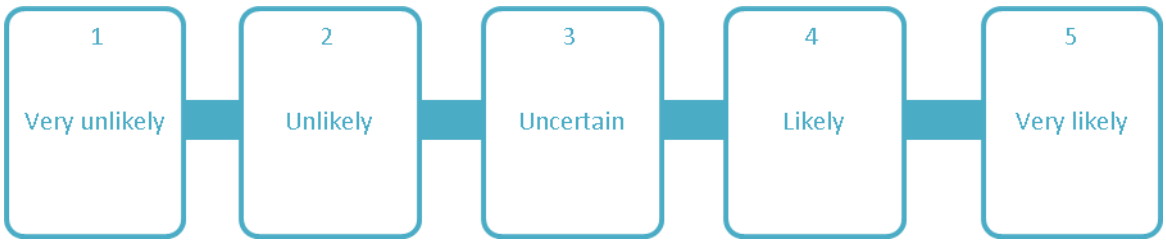
	NHS (including NHS bank)
	Non-NHS (including agency, subcontractors, locum)
	Other (please specify).....

2) What is your job title?

_____

3) In **your opinion**, what is the likelihood of the following increasing the risk of infection?

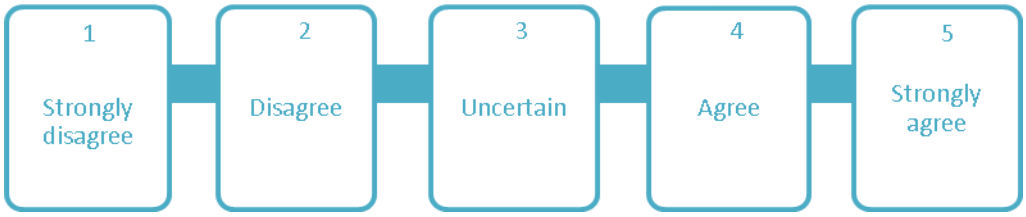
Please mark your answer with an 'x' in the appropriate box.



	1	2	3	4	5
Staff dispose of patient fluids down hand-wash basins.					
Staff do not follow NHS handwashing procedure whilst in the clinical environment.					
Staff use non-touch taps instead of taps with handles.					
Cloths are not changed between hand-wash stations during cleaning.					
During cleaning, cloths are used to wipe around the drain-hole and basin before wiping the tap spout.					
Point-of-use filters attached to taps are wiped during cleaning.					
Plumbing tools are not disinfected between jobs/ wards.					
Patients use clinical hand-wash stations as they would at home e.g. pouring foods down the drain-hole, hanging flannels/ sponges over the taps.					

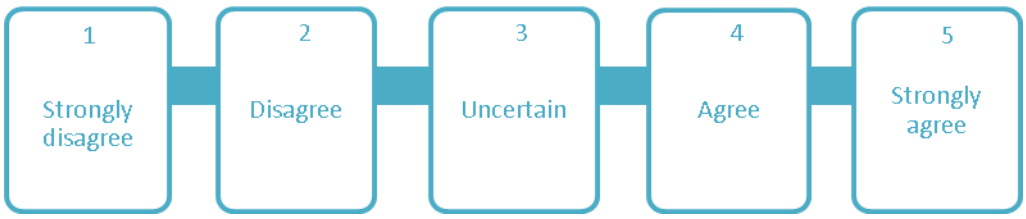
Hand-wash basin drain-holes are blocked in order to fill the basins.					
Not all taps are used regularly.					
Problems within the taps themselves: certain tap designs are harder to clean or become contaminated (have bacteria introduced) more easily.					

4) Please indicate whether you agree with the following statements by marking an ‘x’ in the appropriate box.



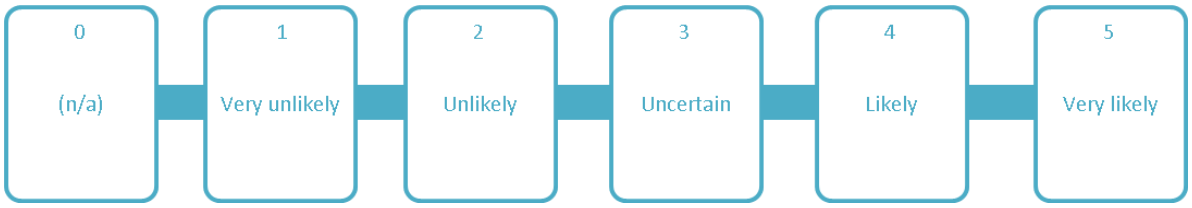
	1	2	3	4	5
Hospital tap water can spread infection.					
Splashing water from the tap onto medical equipment is not an infection risk.					
Poor maintenance, misuse or inappropriate cleaning of taps increases the risk of infection from tap water.					
Tap water is only a risk of infection if spray/aerosols (water particles and droplets in the air) are inhaled.					
Because water to clinical taps undergoes disinfection treatment, it is safer than the tap water in my home.					

5) Please indicate whether you agree with the following statements by marking an 'x' in the appropriate box.



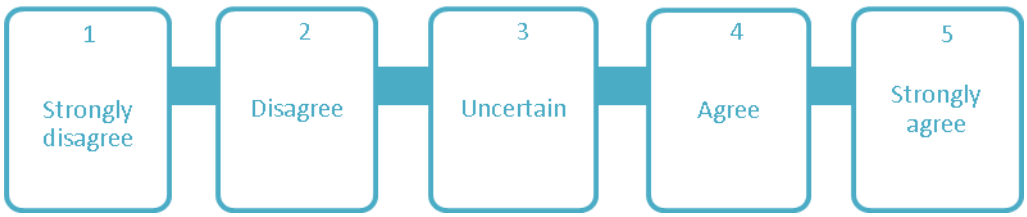
	1	2	3	4	5
Some taps are easier to clean than others.					
Some taps accumulate dirt and grime more than others.					
Taps are more likely to become contaminated by bacteria from the ward than from the water supply.					
On the wards I work on, there are taps which do not get used very often.					
When a tap is taken out of use, the reasons are explained to me.					
My hospital has given me water hygiene training.					

6) In **your opinion**, are staff likely to carry out the following practices?



	0	1	2	3	4	5	Already standard practice
All staff to disinfect hands with alcohol gel after using tap water.							
All staff to run a tap for more than 30 seconds before using the water to wash hands.							
Domestic staff to change cloths between every tap when cleaning.							
Estates staff to disinfect their tools between each task.							
Clinical staff to run a tap for 30 seconds before collecting water for patient use.							
Clinical staff to dispose of human waste in the sluice room only.							
Clinical staff to use a thermometer to measure the temperature of water for patient use instead of relying upon taps with a set temperature.							

7) Please indicate whether you agree with the following statements by marking an 'x' in the appropriate box.



	1	2	3	4	5
The risk of scalding (from hot water) is greater than the risk of bacterial infection from contaminated water.					
The risk of spreading infection after touching tap handles is greater than the risk of infection from washing hands in contaminated water.					
Slips and falls associated with splashing water are a greater risk to patients and staff than contact with contaminated water.					

Please make use of the space below if you have any comments regarding this survey or subject.

Thank you for your time.

A.2 Responses

Table 7.1 Staff appreciation of water as a vector for infection

Question /Statement	Staff group	Number of responses (n)	Disagree-strongly disagree (%)	Agree- strongly agree (%)	Uncertain (%)
Hospital tap water can spread infection	All staff	128	17	59	24
	Domestics	43	19	60	21
	Estates	20	25	60	15
	Healthcare	48	13	54	33
	Medics	12	16	67	17
Because water to clinical taps undergoes disinfection treatment, it is safer than the tap water in my home.	All staff	127	35	21	44
	Domestics	43	30	16	54
	Estates	19	26	48	26
	Healthcare	48	44	17	39
	Medics	12	34	8	58
Tap water is only a risk of infection if spray/aerosols (water particles and droplets in the air) are inhaled.	All staff	127	52	20	28
	Domestics	43	33	21	46
	Estates	19	47	42	11
	Healthcare	48	60	17	23
	Medics	12	92	0	8
Splashing water from the tap onto medical equipment is	All staff	128	59	15	26
	Domestics	43	53	14	33
	Estates	20	75	20	5
	Healthcare	48	50	19	31

Question /Statement	Staff group	Number of responses (n)	Disagree-strongly disagree (%)	Agree- strongly agree (%)	Uncertain (%)
not an infection risk.	Medics	12	83	0	17
My hospital has given me water hygiene training.	All staff	122	35	53	12
	Domestics	41	12	73	15
	Estates	18	0	94	6
	Healthcare	47	64	23	13
	Medics	12	33	50	17

Table 7.2 Staff opinion on whether retrograde or systemic contamination of water outlets is more likely.

Question /Statement	Staff group	Number of responses (n)	Disagree-strongly disagree (%)	Agree- strongly agree (%)	Uncertain (%)
Taps are more likely to become contaminated by bacteria from the ward than from the water supply.	All staff	124	14	57	29
	Domestics	41	17	51	32
	Estates	19	10	79	11
	Healthcare	47	17	44	39
	Medics	12	0	83	17

Appendix A

Table 7.3 Staff opinions regarding the misuse of hand-wash stations and behaviour change

Question /Statement	**Staff group**	**Number of responses (n)**	**Disagree-strongly disagree / Unlikely-very unlikely (%)**	**Agree- strongly agree/ likely- very likely (%)**	**Uncertain (%)**
Poor maintenance, misuse or inappropriate cleaning of taps increases the risk of infection from tap water.	All staff	127	3	89	8
	Domestics	43	5	86	9
	Estates	19	0	100	0
	Healthcare	48	4	90	6
	Medics	12	0	75	25
(*What is the likelihood of the following increasing the risk of infection*) **Staff dispose of patient fluids down hand-wash basins.**	All staff	127	28	66	6
	Domestics	42	33	64	3
	Estates	20	20	65	15
	Healthcare	48	29	67	4
	Medics	12	17	75	8
(*What is the likelihood of the following increasing the risk of infection*) **Plumbing tools are not disinfected between jobs/wards.**	All staff	127	16	47	37
	Domestics	42	22	33	45
	Estates	20	10	75	15
	Healthcare	48	10	52	38
	Medics	12	33	34	33

Question /Statement	Staff group	Number of responses (n)	Disagree-strongly disagree / Unlikely-very unlikely (%)	Agree- strongly agree/ likely- very likely (%)	Uncertain (%)
(*What is the likelihood of the following increasing the risk of infection*) **Patients use clinical hand-wash stations as they would at home e.g. pouring foods down the drain-hole, hanging flannels/sponges over the taps.**	All staff	127	27	60	13
	Domestics	42	38	52	10
	Estates	20	20	65	15
	Healthcare	48	23	62	15
	Medics	12	17	75	8
(*What is the likelihood of the following increasing the risk of infection*) **Hand-wash basin drain-holes are blocked in order to fill the basins.**	All staff	127	32	48	20
	Domestics	42	26	52	22
	Estates	16	35	45	20
	Healthcare	48	37	48	17
	Medics	12	25	50	25

Table 7.4 (Are staff likely to carry out the following practices) Clinical staff to dispose of human waste in the sluice room only

Staff group	**Number of responses (n)**	**Response option**	**Responses (%)**
Overall	124	Likely-very likely	69
		Uncertain	7
		Unlikely-very unlikely	7
		Already standard practice (*A: total; B: selected as only response; C: selected alongside another response)*	A: 20 B: 15 C: 5
		Not applicable	2
Domestics	41	Likely-very likely	71
		Uncertain	5
		Unlikely-very unlikely	5
		Already standard practice (*A: total; B: selected as only response; C: selected alongside another response)*	A: 27 B: 17 C: 10
		Not applicable	2
Estates	19	Likely-very likely	74
		Uncertain	5
		Unlikely-very unlikely	10

Staff group	**Number of responses (n)**	**Response option**	**Responses (%)**
		Already standard practice (*A: total; B: selected as only response; C: selected alongside another response)* Not applicable	A: 21 B: 10.5 C: 10.5 0
Healthcare	48	Likely-very likely Uncertain Unlikely-very unlikely Already standard practice (*A: total; B: selected as only response; C: selected alongside another response)* Not applicable	75 2 8 A: 15 B: 15 C: 0 0
Medics	12	Likely-very likely Uncertain Unlikely-very unlikely Already standard practice (*A: total; B: selected as only response; C: selected alongside another response)* Not applicable	34 33 8 A: 33 B: 25 C: 8 0

Table 7.5 (Are staff likely to carry out the following practices) Estates staff to disinfect their tools between each task.

Staff group	**Number of responses (n)**	**Response option**	**Responses (%)**
Overall	124	Likely-very likely	37
		Uncertain	33
		Unlikely-very unlikely	25
		Already standard practice (*A: total; B: selected as only response; C: selected alongside another response)*	A: 4 B: 2 C: 2
		Not applicable	3
Domestics	41	Likely-very likely	29
		Uncertain	49
		Unlikely-very unlikely	15
		Already standard practice (*A: total; B: selected as only response; C: selected alongside another response)*	A: 7 B: 2 C: 5
		Not applicable	5
Estates	19	Likely-very likely	37
		Uncertain	11

Staff group	Number of responses (n)	Response option	Responses (%)
		Unlikely-very unlikely Already standard practice (*A: total; B: selected as only response; C: selected alongside another response)* Not applicable	47 A: 5 B: 5 C: 0 0
Healthcare	48	Likely-very likely Uncertain Unlikely-very unlikely Already standard practice (*A: total; B: selected as only response; C: selected alongside another response)* Not applicable	27 56 15 A: 0 2
Medics	12	Likely-very likely Uncertain Unlikely-very unlikely Already standard practice (*A: total; B: selected as only response; C: selected alongside another response)* Not applicable	8 75 17 A: 0 0

Table 7.6 Infrequently used taps

Question /Statement	**Staff group**	**Number of responses (n)**	**Disagree- strongly disagree / Unlikely-very unlikely (%)**	**Agree- strongly agree/ likely-very likely (%)**	**Uncertain (%)**
(*What is the likelihood of the following increasing the risk of infection*) **Not all taps are used regularly.**	All staff	126	34	50	16
	Domestics	42	36	43	21
	Estates	20	40	60	0
	Healthcare	47	36	49	15
	Medics	12	25	42	33
On the wards I work on, there are taps which do not get used very often.	All staff	122	31	44	25
	Domestics	41	39	39	22
	Estates	19	21	42	37
	Healthcare	48	35	48	17
	Medics	10	0	40	60
Some taps accumulate dirt and grime more than others.	All staff	124	6	81	6
	Domestics	41	10	80	10
	Estates	19	0	89	11
	Healthcare	48	4	72	21
	Medics	12	0	83	17
(*What is the likelihood of the following*	All staff	126	11	65	24
	Domestics	42	9	74	17

Question /Statement	Staff group	Number of responses (n)	Disagree- strongly disagree / Unlikely-very unlikely (%)	Agree- strongly agree/ likely- very likely (%)	Uncertain (%)
increasing the risk of infection) **Problems with the taps themselves: certain tap designs are harder to clean or become contaminated (have bacteria introduced) more easily.**	Estates	20	25	50	25
	Healthcare	48	36	56	8
	Medics	11	9	82	9
(*What is the likelihood of the following increasing the risk of infection*) **Staff do not follow NHS hand washing procedure whilst in the clinical environment.**	All staff	126	38	56	6
	Domestics	41	37	58	5
	Estates	20	45	45	10
	Healthcare	48	38	60	2
	Medics	12	25	58	17

Appendix A

Table 7.7 (Are staff likely to carry out the following practices) All staff to disinfect hands with alcohol gel after using tap water.

Staff group	**Number of responses (n)**	**Response option**	**Responses (%)**
Overall	125	Likely-very likely	38
		Uncertain	18
		Unlikely-very unlikely	36
		Already standard practice (*A: total; B: selected as only response; C: selected alongside another response)*	A: 14; B: 6; C: 8
		Not applicable	2
Domestics	42	Likely-very likely	45
		Uncertain	22
		Unlikely-very unlikely	24
		Already standard practice (*A: total; B: selected as only response; C: selected alongside another response)*	A: 17; B: 7; C: 10
		Not applicable	2
Estates	19	Likely-very likely	31
		Uncertain	32
		Unlikely-very unlikely	37
			A: 0

Staff group	**Number of responses (n)**	**Response option**	**Responses (%)**
		Already standard practice (*A: total; B: selected as only response; C: selected alongside another response)* Not applicable	 0
Healthcare	48	Likely-very likely Uncertain Unlikely-very unlikely Already standard practice (*A: total; B: selected as only response; C: selected alongside another response)* Not applicable	38 8 46 A: 12.5; B: 4.2; C: 8.3 4
Medics	12	Likely-very likely Uncertain Unlikely-very unlikely Already standard practice (*A: total; B: selected as only response; C: selected alongside another response)* Not applicable	25 17 33 A:42; B:25; C: 17 0

Table 7.8 Staff risk assessment of physical injury (immediate consequences) vs. infection risk (delayed consequences)

Question /Statement	**Staff group**	**Number of responses (n)**	**Disagree- strongly disagree**	**Agree- strongly agree**	**Uncertain (%)**
The risk of scalding (from hot water) is greater than the risk of bacterial infection from contaminated water.	All staff	124	39	19	42
	Domestics	42	40	17	43
	Estates	19	63	11	26
	Healthcare	47	32	19	49
	Medics	11	27	36	36
Slips and falls associated with splashing water are a greater risk to patients and staff than contact with contaminated water.	All staff	124	35	26	39
	Domestics	42	26	33	41
	Estates	19	63	16	21
	Healthcare	47	34	23	43
	Medics	11	18	27	55

Table 7.9 (Are staff likely to carry out the following practices) Clinical staff to use a thermometer to measure the temperature of water for patient use instead of relying upon taps with a set temperature.

Staff group	**Number of responses (n)**	**Response option**	**Responses (%)**
Overall	124	Likely-very likely	15
		Uncertain	28
		Unlikely-very unlikely	48
		Already standard practice (*A: total; B: selected as only response; C: selected alongside another response)*	A: 0
		Not applicable	9
Domestics	41	Likely-very likely	17
		Uncertain	37
		Unlikely-very unlikely	44
		Already standard practice (*A: total; B: selected as only response; C: selected alongside another response)*	A: 0
		Not applicable	2
Estates	19	Likely-very likely	16
		Uncertain	32
		Unlikely-very unlikely	47

Appendix A

Staff group	**Number of responses (n)**	**Response option**	**Responses (%)**
		Already standard practice (*A: total; B: selected as only response; C: selected alongside another response)*	A: 0
		Not applicable	5
Healthcare	48	Likely-very likely	12
		Uncertain	19
		Unlikely-very unlikely	54
		Already standard practice (*A: total; B: selected as only response; C: selected alongside another response)*	A: 0
		Not applicable	15
Medics	12	Likely-very likely	17
		Uncertain	42
		Unlikely-very unlikely	41
		Already standard practice (*A: total; B: selected as only response; C: selected alongside another response)*	A: 0
		Not applicable	0

Appendix B Permissions

B.1 Permission to reprint Figure 1.1 (biofilm life cycle stages)

Reprinted from: STOODLEY, P., SAUER, K., DAVIES, D. G. & COSTERTON, J. W. 2002b. Biofilms as complex differentiated communities. Annual Review of Microbiology, 56, 187-209.

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B.3 Permission to reprint Figure 5.4 (Biofilm on a solenoid valve diaphragm)

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12 June 2018

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Appendix C - List of publications and presentations on work presented in this thesis

Date	Organisation	Category	Description
07/09/2015	International Biodeterioration Biodegradation Society	Poster presentation	"An investigation into the role of retrograde contamination in *Pseudomonas aeruginosa* colonisation of hospital taps."
14/09/2015	Public Health England	Poster presentation	"An investigation into the role of retrograde contamination in *Pseudomonas aeruginosa* colonisation of hospital taps."
24/09/2015	University of Southampton	Internal seminar	"An investigation into the role of retrograde contamination in *Pseudomonas aeruginosa* colonisation of hospital taps."
13/10/2015	Society for Applied Microbiology	Oral presentation	"The survival of *Pseudomonas aeruginosa* on tap outlet fittings and the contamination of hospital tap water."
01/07/2016	University of Southampton	Poster Presentation	"Survival of *Pseudomonas aeruginosa* on tap outlet fittings and contamination of hospital tap water."
12/09/2016	Public Health England	Oral presentation	"The survival of *Pseudomonas aeruginosa* on tap outlet fittings and the contamination of hospital tap water."

Appendix C

Date	Organisation	Category	Description
11/10/2016	Public Health England	Internal seminar	"Engineering, environmental and human behavioural factors influencing the colonisation of hospital taps by *Pseudomonas aeruginosa"*
06/11/2016	Federation Infection Societies/ Healthcare Infection Society	Poster presentation	"*Pseudomonas aeruginosa*: contamination of tap outlet fittings and consequential contamination of tap water."
06/11/2016	Federation Infection Societies/ Healthcare Infection Society	Oral presentation	"The survival of *Pseudomonas aeruginosa* on tap outlet fittings and the contamination of hospital tap water."
18/03/2017	Public Health England	Oral presentation	"Contamination of hospital tap water: the survival and persistence of *Pseudomonas aeruginosa* on conventional and 'antimicrobial' outlet fittings"
October 2017	Journal of Hospital Infection	Journal article	Hutchins *et al*. (2017) Contamination of hospital tap water: the survival and persistence of *Pseudomonas aeruginosa* on conventional and 'antimicrobial' outlet fittings. Journal of Hospital Infection, **97**, 156-161.

Glossary of Terms

Term	Definition
Augmented care units	Hospital units that are designated for high-risk patients (e.g. those with immunocompromised status or under-developed immune systems) such as intensive care units, neonatal wards and burns units.
Colony forming units (CFU)	A unit of measurement of viable cells; the number of colonies grown on an agar plate represents the number of viable cells present in a sample.
Department of Health (DoH)	An United Kingdom government department responsible for policy and legislation on health and care.
Drinking Water Inspectorate (DWI)	A UK body that checks water companies in England and Wales supply safe drinking water in line with British legal requirements.
Health Protection Agency (HPA)	A UK organization that provided evidence-based scientific support with regards to public health between 2003-2013, now incorporated into Public Health England.
Health Research Authority (HRA)	A research approvals board that ensures research conducted within the NHS is ethically reviewed and/or appropriate.
Healthcare associated infection (HCAI)	Infections that occur as a result of healthcare provided in either the hospital or community environment.
Immunocompromised	A status in which a person's immune system does not function either at full capacity (is impaired) or to a normal level (i.e. an individual is more susceptable to infection than normal).
Integrated Research Application System (IRAS)	A system for applying for HRA approval to conduct health, social and community care research in the UK.
National Health Service (NHS)	The United Kingdom's healthcare system, providing free service to UK residents and EU nationals.
Opportunistic pathogen	Microorganisms that do not normally cause infection, under particular circumstances become pathogenic, e.g. within an immunocomprosied host.
Public Health England (PHE)	An executive agency of the Department of Health and Social Care, providing evidence-based scientific support.

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