Automated Decontamination of Multiple Washbasin U-bends in a Hospital Setting Using Electrochemically Activated Solutions: Exploring the Role U-bends and the Wastewater Network Plays in the Trafficking of Potentially Pathogenic Bacteria in a Healthcare Facility

Elaine Mary Moloney

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Microbiology Research Unit,
Division of Oral Biosciences,
Dublin Dental University Hospital,
Trinity College Dublin

Supervisor

Professor David Coleman

Declaration

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Summary

Hand washing is a vital component of any hand hygiene strategy and essential for reducing nosocomial infections. Paradoxically, hand washbasins have been identified as reservoirs and disseminators of infection in the healthcare setting. The washbasin U-bend or trap is a pipe fixture located directly below the drain outlet designed to retain water that acts as a seal preventing sewer gases entering buildings from wastewater pipes. However, the water in U-bends frequently stagnates, facilitating the formation of biofilms. This reservoir of infection is increasingly being associated directly or indirectly with hospital outbreaks of infection, including those caused by *Pseudomonas aeruginosa.* To date, all previous approaches to decontaminate U-bends have been ineffective in the long-term, potentially hazardous to staff, toxic to the downstream environment and/or incur high running costs.

The primary aim of the research described in this thesis was to develop an automated system for simultaneously decontaminating multiple washbasin U-bends and associated proximal wastewater pipes in the setting of the Dublin Dental University Hospital (DDUH) Accident & Emergency Department (A&E) by sequential treatment with electrochemically activated solutions (ECAs) generated from brine. Proof of concept of this approach was demonstrated previously using two ECAs (catholyte and anolyte) with a single washbasin located in a staff bathroom in DDUH. Catholyte, which is predominantly composed of sodium hydroxide and has detergent properties, was used first to disrupt and loosen organic material. Secondly, anolyte, which is predominantly composed of hypochlorous acid and is a powerful disinfectant, was used to decontaminate washbasin pipework. A programmable system was developed whereby 10 identical washbasins, U-bends and associated pipework in A&E underwent automated sequential 10 min treatments with the ECAs. The incorporation of a valve downstream of the U-bends permitted retro-filling and retention of the solutions upstream of the valve into each washbasin, increasing the active contact area. Six untreated washbasins were selected as controls from non-clinical areas of DDUH fitted with identical U-bends to those in A&E. This initial phase of the study (Chapter 3) investigated the efficacy of the ECA decontamination approach on the bacterial bioburden in A&E U-bends, monitored over a period 5 months and 62 decontamination cycles, both immediately following and 24 h after treatment. Bacterial counts in U-bends were determined by swab sampling of treated and untreated U-bends, facilitated by sampling ports incorporated in the U-bends. Quantitative bacterial counts were determined on Columbia blood agar (CBA), Reasoner's 2A agar (R2A) and *Pseudomonas aeruginosa* Selective Agar (PSCN). The average bacterial densities recovered from samples (n = 372) from the six-untreated control U-bends during the study period on CBA, R2A and PSCN were 2×10^5 ($\pm 4 \times 10^5$), 3.3×10^5 ($\pm 1.1 \times 10^6$) and 2.7×10^4 ($\pm 1.2 \times 10^5$) colony forming units (CFU)/swab, respectively. The corresponding average bacterial counts from samples (n = 620) from the 10 ECA-treated A&E U-bends immediately after decontamination were significantly lower at 73.4 (± 258.2), 122.5 (± 371.3), and 15.3 (± 184.5) CFU/swab, respectively. The average bacterial counts from ECA-treated U-bends compared to the control U-bends displayed a >3 log difference and were highly significant (P <0.0001). The microbial populations present in ECA-treated and control U-bends were also monitored, where *P. aeruginosa* predominated in each case. The average *P. aeruginosa* density within control U-bends during the study period was significantly (P <0.0001) greater (2.7×10^4 verses 15.3 CFU/swab, respectively) than treated U-bends immediately after decontamination.

Following the success of the first phase of research (Chapter 3), the next phase of research (Chapter 4) investigated the efficacy of the ECA decontamination approach in the A&E over a longer period (52 weeks and 156 decontamination cycles), immediately following-, 24 h after- and 48 h after- ECA treatment. In contrast to the initial study phase, the 10-control washbasin U-bends used in the second study phase were located in Clinic 2 in DDUH. The washbasins, U-bends and wastewater pipework in Clinic 2 were identical to A&E and both clinics had similar daily use. In this second phase study, the average bacterial counts from the 10 ECA-treated U-bends following treatment relative to the 10 control U-bends showed a >4.4 log, >4.1 log and a >3.3 log reduction in bacterial counts on all media immediately after-, 24 h after- and 48 h after-ECA treatment, respectively. The results of both studies demonstrated

that automated decontamination of washbasin U-bends with ECA solution was consistently effective at minimising bacterial contamination in the long-term.

The second aim of this thesis was to investigate the bacterial communities present in washbasin U-bends and the associated wastewater pipe network in DDUH. These communities were investigated using a culture-based approach (selection on CBA agar and identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry [MALDI-TOF-MS] analysis) and a culture independent approach (Illumina high throughput 16S rRNA amplicon sequencing). Representative colonies, based on colony morphology, were recovered from the 10 treated A&E U-bends over a five-week period during the second phase of ECA decontamination (Chapter 4) and from the 10 non-treated Clinic 2 U-bends over two time points. MALDI-TOF-MS analysis revealed that *P. aeruginosa* predominated in both ECA-treated and control U-bends. Using the culture independent approach, the bacterial communities in non-treated U-bends (n = 14) and wastewater pipe locations (n = 2) were investigated, where a median number of 421 genera (range 291 – 573) were identified per sample. The family *Pseudomonadaceae* and the genus *Pseudomonas* was present in every location sampled, the latter accounting for >10% of the relative genera in 5/16 samples. A large variance in the relative abundance of the genus *Pseudomonas* throughout the wastewater system (0.03% – 50.4%) was observed. These data showed that more diverse bacterial populations exist in the wastewater network than are evident by conventional culture-based approaches.

The third aim of the thesis (Chapter 5) was to investigate trafficking of potentially pathogenic bacteria via U-bends and the wastewater pipe network in DDUH. *Pseudomonas aeruginosa* was used as a marker organism for this work as it is ubiquitous in U-bends. The relatedness of *P. aeruginosa* isolates from U-bends and wastewater pipe locations throughout DDUH was investigated by whole genome sequencing (WGS) and subsequent single nucleotide variant (SNV) and whole genome multi-locus sequencing typing (wgMLST) analyses. A total of 99 *P. aeruginosa* isolates were investigated from 25 U-bends in five locations (10 in Clinic 2, 10 in A&E and five in three other locations) and two wastewater pipe locations. Eleven sequence types (STs) were identified: ST179, ST560, ST298, ST308, ST27, ST252, ST773, ST296, ST253, ST309, and ST606. Twenty-one additional isolates were included as comparators including isolates from U-bends in two other Irish hospitals, dental chair water reservoirs, dental suction systems, and two reference strains. Isolates belonging to ST560 (n = 27) and ST179 (n = 34) predominated throughout the wastewater network in DDUH and isolates within each of these STs were highly related regardless of source. ST179 isolates were recovered from Clinic 2, A&E, a separate clinic (Clinic 1) and a third-floor staff bathroom between January 2017 – November 2019. ST560 isolates were recovered from Clinic 2, A&E, another non clinical location in DDUH and the point of discharge wastewater pipe common to A&E, Clinic 1 and Clinic 2 between May 2017 – May 2019. Two ST560 clusters were identified; isolates within Cluster I (25/27) and Cluster II (2/27) exhibited average allelic differences and SNVs of three and zero, and two and five, respectively. Cluster II was differentiated from Cluster I by 59 SNVs. ST179 isolates exhibited average allelic difference and SNVs of three and 10, respectively. Washbasin tap samples (n = 80) and mains and washbasin tap water samples (n = 72) consistently failed to yield *P. aeruginosa* whereas *P. aeruginosa* was recovered from all DDUH U-bends included in the study at some time point. These findings showed the presence of closely related *P. aeruginosa* isolates in multiple U-bends and wastewater pipes in diverse locations in DDUH and that they were most likely spread by trafficking via the wastewater network.

The research presented in this thesis highlights the role washbasin U-bends play in the spread of potential pathogens in a hospital wastewater network and emphasises the need for effective decontamination approaches. The ECA decontamination approach developed here effectively and consistently decontaminated washbasin U-bends and could be scaled to automatically treat hundreds of washbasins. The system would provide most benefit in hospital units housing vulnerable patient groups. Finally, the results of the study highlight the power of WGS analysis in revealing hitherto unknown routes transmission of potential pathogens in the healthcare setting.

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Abbreviations

ABS	Acrylonitrile-butadiene-styrene
AMR	Antimicrobial resistance
ATTCC	American Type Culture Collection
A&E	Accident and Emergency Department
bp	Base pair
BLT	Bead Linked Transposome
CA	California
CBA	Columbian blood agar
CFU	Colony forming unit
CFU/ml	Colony forming unit per millilitre
CFU/swab	Colony forming unit per swab
cgMLST	Core genome MLST
CHCA	Alpha-Cyano-4-Hydroxycinnamic Acid
cm	centimetre
CPO	Carbapenemase-producing organisms
CPE	Carbapenemase-producing *Enterobacteriaceae*
CRE	Carbapenem-resistant *Enterobacteriaceae*
CRO	Carbapenem resistant organisms
CSSD	Central Sterile Services Department
C-390	9-chloro-9-(4-diethylaminophenyl)-10-phenylacridan
DC	Direct current
DDUH	Dublin Dental University Hospital
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
ddNTPs	Dideoxyribonucleotide triphosphate
DP	Domestic pattern
ECAs	Electrochemically activated solutions
e.g	*Exempli graita*; for example
EDTA	Ethylenediaminetetraacetic acid
EHR	Epidemic high-risk
EPS	Extracellular polysaccharides
ESBL	Extended spectrum beta-lactamases
et. al.	*Et alia*; and others

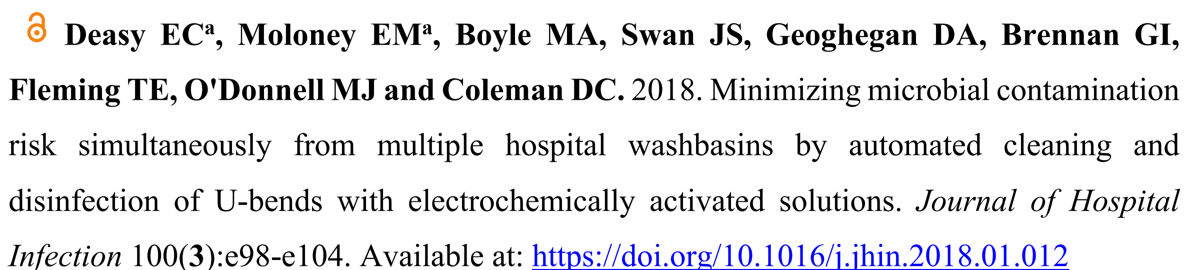
etc.	*Et cetera*; and the rest
EPM	Enhanced PCR Mix
EUCAST	European Committee of Antimicrobial Susceptibility Testing
F.A.C	Free available chlorine
FEM	Flow-through electrolytic module
g	Gravitational force
Gb	Gigabase
GC	Guanine-Cytosine
h	Hour
HAI	Healthcare acquired infection
HDPE	High-density polyethylene
HGT	Horizontal gene transfer
HP	Hospital pattern
HOCl	Hypochlorous acid
HT1	Hybridization buffer
I	Intermediate
ICU	Intensive care unit
IPC	Infection prevention and control
in situ	On site; performing a given procedure exactly in place where it occurs
in vitro	Within the glass; performing a given procedure in a controlled environment outside of a living organism
kb/kbp	Kilo-base pair
L	Litre
LPS	Lipopolysaccharide
m / M	Metre / Molar
mM	Millimolar
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization Time of Flight
MBL	Metallo-β-lactamases
MDR	Multidrug-resistant
mg	Milligram
mg/ml	Milligram per millilitre
MIC	Minimum inhibitory concentration
min	Minute
ml	Millilitre
MLST	Multilocus sequence type

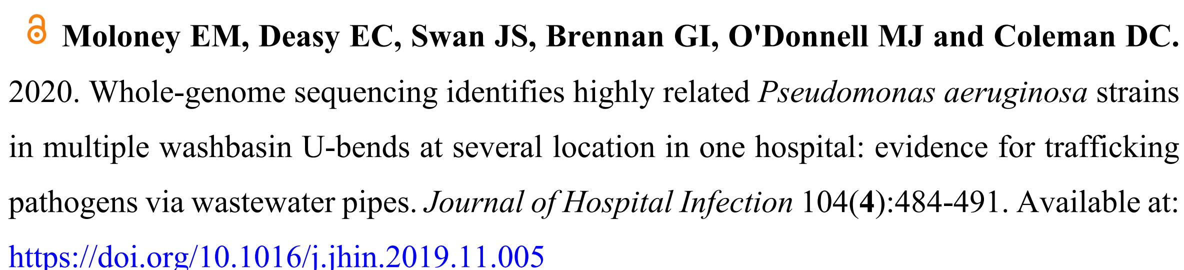
mM	Milli molar
MS	Mass spectrometry
MST	Minimum spanning tree
mV	Millivolt
n	Number (sample size)
n	Nano
N	Newton / Normal
N/A	Not available
Nm	Nanometre
NaCl	Sodium chloride
NaOH	Sodium hydroxide
N/A	Not applicable
NGS	Next generation sequencing
ONT	Oxford Nanopore technologies
ORP	Oxidation-reduction potential
PA	*Pseudomonas* selective agar
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
PGFE	Pulsed-field gel electrophoresis
pH	Potential Hydrogen; quantitative measure of the acidity or basicity of aqueous or other liquids solutions
p.p.m.	Parts per million
PVC	Polyvinylchloride
PSCN	*Pseudomonas aeruginosa* selective agar
QAC	Quaternary ammonium compounds
R	Resistant
R2A	Reasoners 2A agar
RAPD	Random amplified polymorphic DNA
rpm	Rotations per minute
rRNA	Ribosomal ribonucleic acid
RSB	Resuspension Buffer
s	Second
S	Susceptible
SEM	Scanning electron microscope
SMRT	Single molecule real-time

SNV	Single nucleotide variants
SPB	Sample Purification Beads
ST	Sequence type
TBE	Tris/Borate/EDTA
TB1	Tagmentation Buffer
TCD	Trinity College Dublin
TE	Tris/EDTA
Tris	Tris (hydroxymethyl) aminoethane
TSB	Tagmentation Stop Buffer
TWB	Tagmentation Wash Buffer
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
v	version
VNTR	Variable number tandem repeat
WC	West Clinic
w/v	Weight over volume
wgMLST	Whole genome multi-locus sequence type
WGS	Whole genome sequencing
WI	Wisconsin
16S	Sixteen Svedberg units
µg	Microgram
µg/ml	Microgram per millilitre
µl	Microlitre
%	Percentage
°C	Degrees Celsius
′	Prime
×	Times
>	Greater than
<	Less than
≥	Greater than or equal to
≤	Less than or equal to

Publications

Some of the original work presented in this thesis has been published in an international peer-review journal or in an edited international specialist academic textbook. Copies of the open access publications are included in the Appendix.


Deasy EC[a], Moloney EM[a], Boyle MA, Swan JS, Geoghegan DA, Brennan GI, Fleming TE, O'Donnell MJ and Coleman DC. 2018. Minimizing microbial contamination risk simultaneously from multiple hospital washbasins by automated cleaning and disinfection of U-bends with electrochemically activated solutions. *Journal of Hospital Infection* 100(**3**):e98-e104. Available at: https://doi.org/10.1016/j.jhin.2018.01.012


Moloney EM, Deasy EC, Swan JS, Brennan GI, O'Donnell MJ and Coleman DC. 2020. Whole-genome sequencing identifies highly related *Pseudomonas aeruginosa* strains in multiple washbasin U-bends at several location in one hospital: evidence for trafficking pathogens via wastewater pipes. *Journal of Hospital Infection* 104(**4**):484-491. Available at: https://doi.org/10.1016/j.jhin.2019.11.005

Coleman DC, Deasy EC, Moloney EM, Swan JS and O'Donnell MJ. 2020. Decontamination of hand washbasins and traps in hospitals. *In* (Walker J, Editor) *Decontamination in Hospital and Healthcare*, Edition 2, Chapter 7, Woodhead Publishing (Elsevier), Oxford, UK, pp 135-162.

[a] Co-first authors

Chapter 1

General Introduction

1.1 Water networks in healthcare facilities

Water networks are an integral part of any healthcare facility. However, over the past three decades water networks have been increasingly identified as significant reservoirs of microbial contamination responsible for nosocomial infections (Doring *et al.*, 1991; Anaissie *et al.*, 2002; Denton *et al.*, 2003; Cholley *et al.*, 2008; Decker and Palmore, 2013; Blom, 2015; Bloomfield *et al.*, 2015). In Ireland, Irish Water is a state-owned entity responsible for providing potable, or drinkable, water to the majority of urban and rural areas, including the water network in Dublin Dental University Hospital (DDUH). Once potable water passes a building's perimeter, the quality of water at outlets is affected by a variety of factors including the condition and cleanliness of the water distribution network, water flow rates and water stagnation, amongst others. With this, the proprietors of public buildings are required to maintain the water distribution network to minimise risks to public health (Environmental Protection Agency Ireland, 2014). At no point in the water distribution network in healthcare facilities is the water sterile, hence neither are all three sections comprising the network including: the water supply, water storage and consumption, and wastewater (Figure 1.1) (Hanlin and Myers, 2018).

1.1.1 Water supply

Water in healthcare facilities is generally considered to be safe due to the assumption that water delivered to the facilities is of an acceptable quality. Large quantities of safe water are required by healthcare institutions such as hospitals. This water is required for drinking, hand washing, routine cleaning, flushing toilets, showers, and for special purposes such as steam sterilisation, washer-disinfection, hydrotherapy, haemodialysis, amongst others. Some aspects of the supply water require special treatment, including treatment of water by reverse osmosis for water provided to steam sterilisers and water used in dialysis clinics owing to the potential presence of bacterial endotoxins that may cause pyrogenic reactions in humans (Centers for Disease Control and Prevention, 2003). Direct connections from municipal mains supplies are required to provide drinking water of potable standards. Potable water is water deemed suitable for human consumption based on set microbiological, chemical and physical quality parameters (European Union Drinking Water Regulations 2014, SI 2014/122). These include biological limits of 0 colonies per 100 ml detection of *Clostridium perfringens*, *Escherichia coli*, *Enterococci spp*., and any detection of *Cryptosporidium* and total coliforms in a drinking water sample is regarded as an exceedance of the acceptable limit.

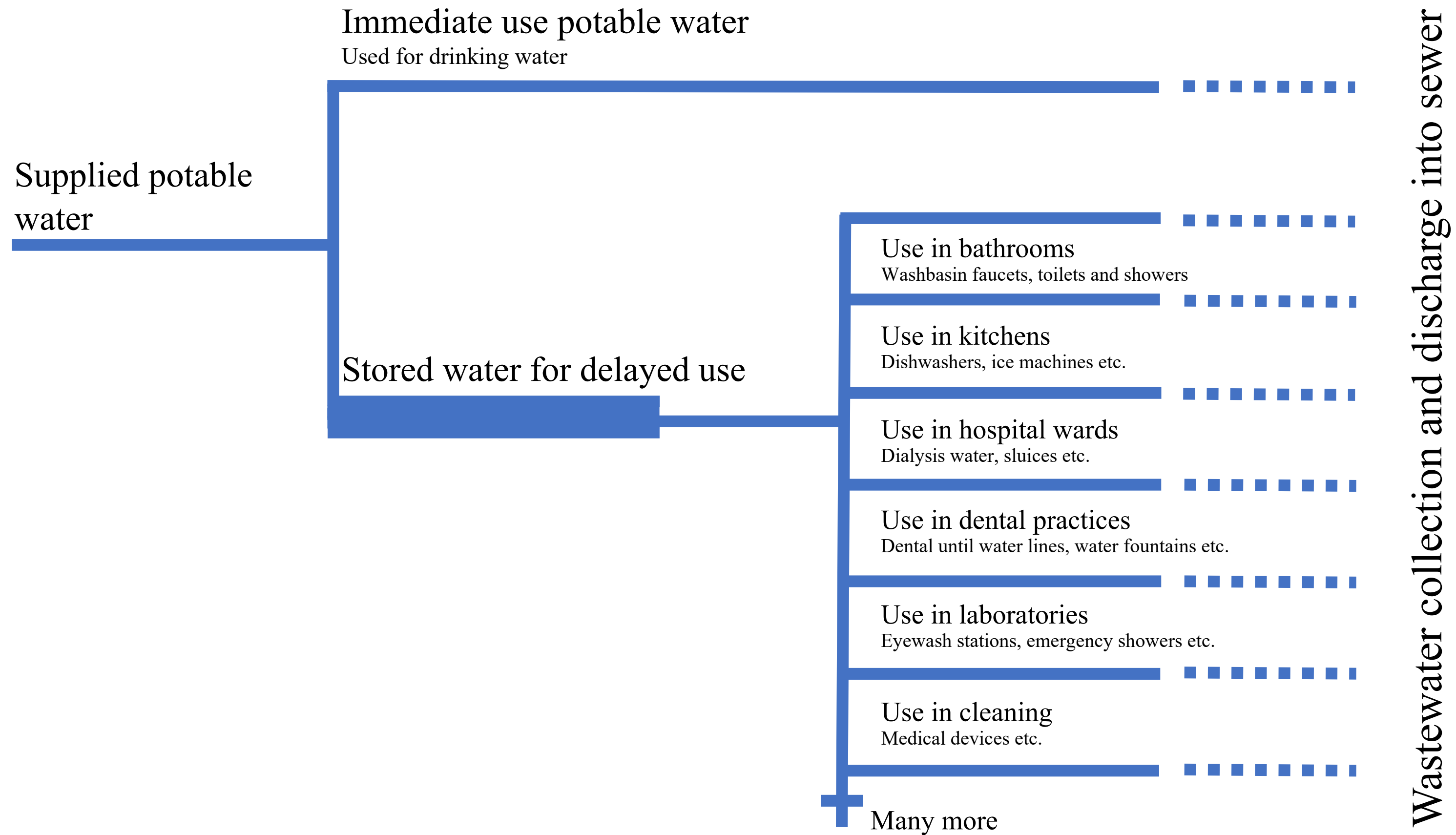


Figure 1.1 A schematic representation of the water networks in healthcare facilities. The water supplied to the healthcare facility is used either for immediate or for delayed use, with all the wastewater collected at communal sites for discharge into the municipal sewer system.

The water distribution networks of healthcare facilities are usually composed of a complex and lengthy system of water supply pipes (both hot and cold), water storage tanks, calorifers, pumps and valves (Blom, 2015; Bloomfield *et al.,* 2015; Bédard *et al.,* 2016). A number of preventive measures for minimising microbial proliferation in plumbing systems include the eradication of sections of pipe that promote the stagnation of water, also known as dead legs,adequate thermal controls on hot and cold water supply networks (i.e., cold water should be circulated at <20°C and hot water at approximately 55°C) and reducing opportunities for water stagnation (Health Protection Surveillance Centre, 2009, 2015). In 2012, it was estimated that approximately 40,000 infections per annum, costing $970 million, in the United States were caused by bacterial pathogens prevalent in building's plumbing networks (Collier *et al.*, 2012). One of the main ways to mitigate the risk of acquiring nosocomial infections from the water supply to high-risk populations (i.e., the immunocompromised, neonates and high-dependency patients) in healthcare facilities is to use water supplied from local public mains water only (Bloomfield *et al.,* 2015). Local public mains water is regularly monitored and the results are reported to governing bodies and external auditors.

While water treatment plants can operate slightly differently, there are eight basic steps that are generally used to produce potable water: abstraction of water mainly from surface water locations, screening, coagulation, flocculation, settlement, filtration, chlorine disinfection, and finally storage (http://www.dublincity.ie/main-menu-services-water-waste-and-environment-your-drinking-water-managing-our-water-supplies/how-1). The characteristics for water supply distributed from the municipal source are based on the reliability of the source, source characteristics, water pressure and quantity, temperature, pH, hardness, total organic carbon and dissolved organic carbon, trihalomethanes, nitrates, turbidity, and chlorine residuals (Health Protection Surveillance Centre, 2015)

1.1.2 Water storage and consumption

Water in healthcare facilities is essential for care activities and consumption. The water used in hospitals has been estimated to account for 7% of the total water use in commercial and institutional facilities in the United States (Enviornmental Protection Agency United States, 2012). In Ireland, the Health Service Executive (HSE) is one of the largest users of water spending €8 million per annum to supply water to over 2500 sites (GreenHealthcare, 2016). Water is supplied not only for immediate use, but also for storage. The water supply to toilets, showers, sinks and hand washbasins in hospitals usually comes from water storage tanks. Water storage is necessary in every healthcare facility to reliably meet the demands

for water at high-use times such as the morning hours or lunchtime hours. Likewise, water storage is necessary to serve the needs of the communities regardless of water interruptions or outages. The zoning of water distribution networks in healthcare facilities can mitigate the risk of complete facility outages in incidences such as when pipes breakages occur. Underutilisation of stored water and in situations where the tank storage capacity exceeds demand, stagnation occurs providing ideal conditions for microbial proliferation (Decker and Palmore, 2013; Bloomfield *et al.,* 2015).

1.1.3 Wastewater

All buildings including hospitals and other healthcare facilities are equipped with a wastewater network that collects water from a variety of fixtures including sinks, washbasins, showers, baths and toilets. This wastewater network discharges wastewater into the municipal sewerage system. Larger buildings, such as hospitals, usually contain large, complex and intricate wastewater networks. These wastewater pipes are constantly moist and often contain sludge and sediment, which are conditions that are ideal for microbial proliferation (Dancer, 2014). The wastewater pipe networks are exposed to the hospital environment in areas utilised by both patients and staff throughout healthcare facilities at hand washbasins, sink drains, and shower drains. Hospital wastewater pipework in particular, often consists of a lattice of many different types of pipe fittings and materials, modified over time to service the ever-changing nature of the healthcare environment. Most of the wastewater infrastructure is concealed behind walls, ceilings, and floors and once installed is generally considered safe and not requiring routine maintenance (Blom, 2015; Bloomfield *et al.*, 2015).

Wastewater networks of most healthcare facilities discharge wastewater into the municipal sewerage systems. As shown in Figure 1.1, water is distributed at multiple sites throughout the healthcare facility but all discharge through the same wastewater pipe network. In hospitals, these include wastewater pipes from a wide variety of locations and specialities including bathrooms, kitchens wards, cleaning areas, wards etc.. The concealed wastewater pipe network are open conduits to the hospital patient environment, which can remain underutilised, damp and containing residual discharged organic matter ideal for microbial proliferation. When wastewater is generated in healthcare facilities, the wastewater pipes deliver the effluent into a wastewater sewerage system to be transferred to wastewater treatment plants. It is important to note that surface water and storm water runoff are not included in this effluent. While rural establishments may utilise septic tanks for wastewater

treatment, most urban facilities utilise wastewater treatment plants. However, this approach is not always sufficient; a report by the Environmental Protection Agency Ireland in 2017 reported wastewater treatment at 28 of Ireland's 179 large urban areas failed to meet European Union Standards (Environmental Protection Agency Ireland, 2017). Likewise, in low-income countries it has been estimated that more than 90% of all wastewater is discharged untreated directly into waterways (Corcoran, 2010; World Health Organization, 2014). The discharged water from healthcare facilities often contains large quantities of active pharmaceuticals such as antibiotics, antimicrobial agents and/or disinfectants released into the wastewater system by human excretion or disposal (Fuentefria *et al.*, 2011; Slekovec *et al.*, 2012; Mir-Tutusaus *et al.*, 2017). Untreated or partially treated wastewater constitutes a risk to human health due to contamination of treated systems and/or the release of antimicrobial agent resistant organisms into the environment. Increased levels of resistant organisms in the environment may lead to the uptake of resistance genes and increase the prevalence of multidrug resistant potential pathogens. For example, the Gram-negative environmental bacterium *Pseudomonas aeruginosa* has been detected in higher concentrations in hospital wastewater than in urban wastewater and as a consequence, so has antibiotic resistant *P. aeruginosa* (Hocquet *et al.,* 2016). With this knowledge, there is a responsibility on healthcare facilities to effectively manage, monitor and reduce the microbial burden in hospital wastewater prior to discharge into the municipal sewers.

1.2 Washbasin U-bends as reservoirs of microbial contamination

1.2.1 Handwash stations

Hand hygiene is recognised as the single most important intervention for preventing infection in healthcare facilities (Dancer, 2014). Hand washing is a vital component of effective infection prevention and control (IPC) strategies, and hand washbasins are usually incorporated into almost every hospital ward and patient room. Healthcare associated infections (HAIs) can be caused by the dispersal of potentially pathogenic bacteria from washbasin drain outlets if the tap water flow directly impacts the drain outlet causing splash back and/or aerosolisation of microorganisms (Kramer *et al.,* 2006; Starlander and Melhus, 2012). The splash zone relates to the surfaces around the sink drain outlet, which may become contaminated by droplets dispersed by the impact of water from the faucet (Kotay *et al.,* 2019; Franco *et al.,* 2020). Surface contamination of washbasins stemming from the washbasin drain outlets is a public health problem, especially due to the fact that HAIs are a significant cause of morbidity and mortality worldwide (Kizny-Gordon *et al.,* 2017;

Montagna *et al.,* 2019). The principal routes of infection by waterborne microorganisms are by inhalation of contaminated aerosols, by aspiration of contamination water into the lungs and skin contact when hand washing, bathing in showers and baths, and in the use of toilets (World Health Organization, 2012). The transmission of contaminated water from washbasins within handwash stations can occur by the contamination of the upstream water supply, contamination linked to biofilm formation within tap fittings, contamination from the washbasin bowl or splash back from contaminated drains and associated pipework.

During the last decade, IPC strategies in healthcare facilities have focused attention on the design of hospital handwash stations (Department of Health UK, 2013). Washbasins used in modern hospitals generally differ from the domestic pattern (DP) washbasins that are in general use elsewhere (Figure 1.2). In DP washbasins, the drain outlet is frequently located directly below the tap water flow. DP washbasins usually are fitted with a stopper or plug in the floor of the washbasin to retain water and also usually have an overflow outlet located in the back wall of the washbasin. The overflow outlet is connected to the U-bend below the washbasin by a ceramic tube. This area is concealed from washbasin decontamination approaches, is usually damp and therefore routinely harbours biofilm. In hospital pattern (HP) washbasins, the drain outlet is offset so that it is not directly impacted by tap water when water is flowing. Furthermore, HP washbasins do not have an overflow outlet and are not fitted with plugs or stoppers. HP design reduces the incidence of splash back from drain outlets and the removal of overflows reduces the surface area where biofilm can develop. The water in U-bend traps located directly beneath washbasin drain outlets is frequently stagnant encouraging the formation of microbial biofilm (Dancer, 2014). Washbasin U-bend biofilms are usually composed of a wide variety of bacterial species, but Gram-negative species such as *P. aeruginosa* and related species are of particular importance (Hota *et al.*, 2009; Breathnach *et al.*, 2012; Leitner *et al.*, 2015; Stjarne Aspelund *et al.*, 2016).

1.2.2 Recognition of washbasins as reservoirs of infection

Handwash station were internationally acknowledged as reservoirs of infection following an outbreak of *P. aeruginosa* from contaminated taps in Northern Irish neonatal units in 2011, which resulted in several infant fatalities (Regulation and Quality Improvement Authority, 2012; Wise, 2012; Walker *et al.,* 2014). Prior to this event, washbasins were often overlooked as potential reservoirs of contamination responsible for nosocomial infection. Investigation into the incident identified complex flow straighteners on taps as a source of

(a)

(b)

Figure 1.2 Photographs showing examples of a DP and a HP washbasin in DDUH. Panel **(a)** shows an example of a HP washbasin located in Clinic 2. The washbasin lacks an overflow outlet, has an offset drain outlet and the tap is operated by a motion sensor indicated by the red arrow. The tap is equipped with a thermostatic mixing valve and is configured to provide output water at an average temperature of 38°C to prevent scalding. Panel **(b)** shows an example of a DP washbasin located in West Clinic. The tap water flow directly impacts the drain outlet and the washbasin has an overflow outlet. The washbasin is equipped with a manual mixer tap. Tork Extra Mild Liquid Soap (SCA Hygiene Products Ltd., Bedfordshire, UK) (non-medicated) is the only soap used for handwashing in DDUH.

P. aeruginosa biofilm, and these were identified as the likely source of infection (Walker *et al.,* 2014). Flow straighteners are incorporated into washbasin taps to regulate and narrow the flow of water from the tap outlet into the washbasin. The use of variable number tandem repeat (VNTR) analysis indicated commonality between the *P. aeruginosa* samples in the flow straighteners with the isolates recovered from the neonates. Since 2012, numerous reports of nosocomial outbreaks associated with contaminated washbasin U-bends in particular have been reported (Breathnach *et al.*, 2012; Stjarne Aspelund *et al.,* 2016; Kossow *et al.,* 2017).

1.2.3 Washbasin U-bends

Washbasin U-bends, also known as traps, are a fundamental part of plumbing fixtures including washbasins, sinks, baths, showers and toilets. U-bends function by preventing sewer gas entering buildings from wastewater pipes. Washbasin and sink U-bends are situated directly below the drain outlet and consist of shaped pipework designed to retain a volume of water that acts as a seal against sewer gas (Figure 1.3) (Deasy *et al.,* 2018). Washbasin U-bends come in a variety of different shapes including S-traps, bottle traps and the most commonly used P-trap. Washbasin U-bends collect not only the water waste washed down the drain outlet but any debris, hair, small items and sometimes incorrectly disposed of antibiotics and other fluids. This liquid waste stagnates in the U-bends when the plumbing fixtures are not in use, encouraging the growth of biofilm within and above the retained water section. The microorganisms present in the biofilm in the wastewater U-bend, drain and associated pipework can contaminate the washbasin and the surrounding environment, particularly if washbasin tap water flow directly impacts the drain outlet resulting in splashing and aerosol formation (Breathnach *et al.*, 2012; Kotay *et al.,* 2017; Deasy *et al.*, 2018). The first report of transmission of *P. aeruginosa* from a handwash station was in 1967 (Ferroni *et al.*, 1998). The isolates were pyocin typed, serotyped and phage-typed and the same strain was identified in a patient and a washbasin trap. While these typing methods might be considered poorly discriminatory today, more recent reports of infections associated with contaminated U-bends and drains investigated by more informative typing approaches have supported the findings of this outbreak.

1.2.4 Antimicrobial agent resistance and biofilm formation in water networks

1.2.4.1 Antimicrobial resistance (AMR)

Antimicrobial resistance is the ability of a microorganism to impede the effects of an

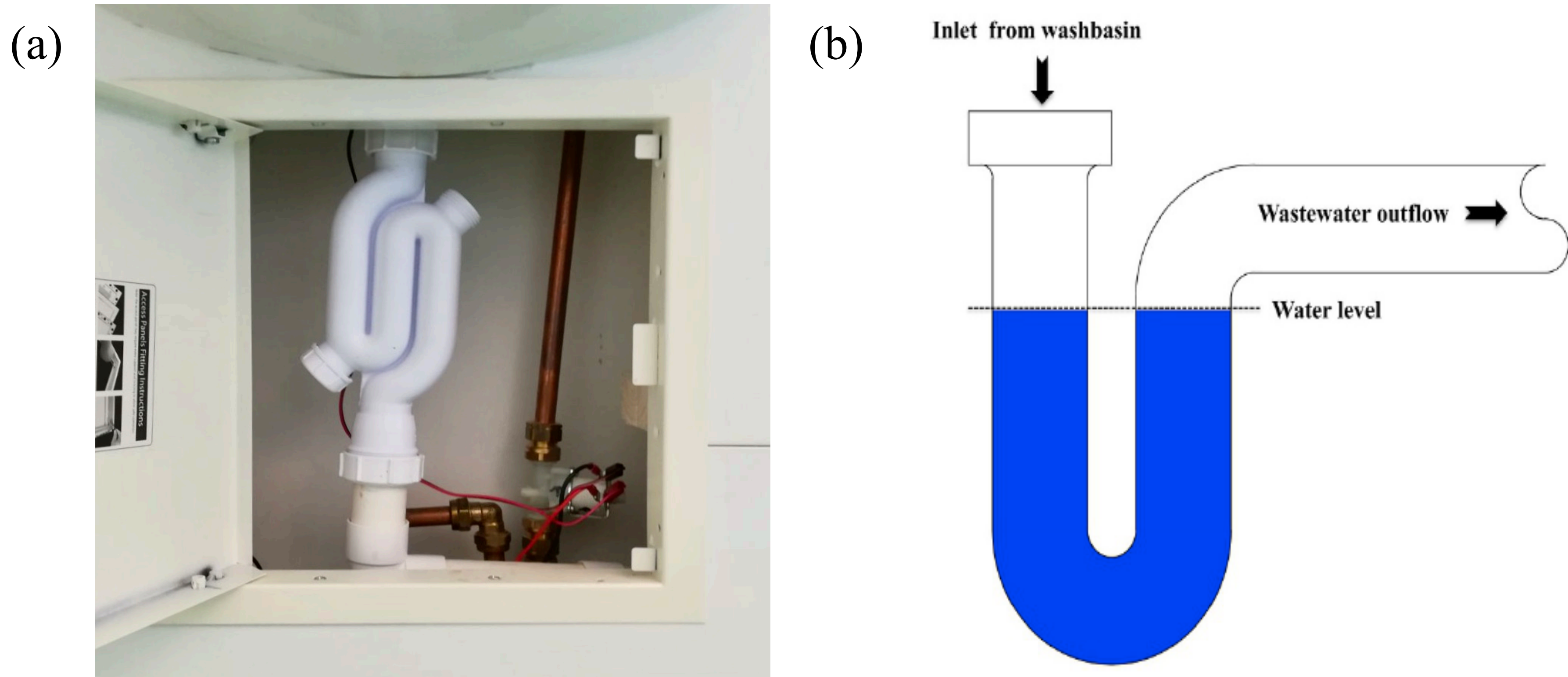


Figure 1.3 A photograph and schematic of a washbasin U-bend. Panel **(a)** shows a washbasin U-bend located directly below and connected to the underside of the washbasin drain outlet. This particular U-bend model is equipped with two sampling ports (McAlpine Plumbing Products, Glasgow, Scotland). Panel **(b)** shows a schematic diagram of a cross section of a washbasin U-bend (Coleman *et al*., 2020). The horizontal dashed line indicates the junction of the retained water section of the U-bend and air. Microbial biofilm can form at any part of the washbasin U-bend while *P. aeruginosa* biofilm primarily forms at the air-water interface.

antimicrobial agent (such as antibiotics, antivirals, antimalarials and disinfectants) exhibiting its antimicrobial effect on the microorganism (Prestinaci *et al.,* 2015). Bacterial species can be intrinsically resistant to antibiotics, can develop resistance by mutation, and/or can become resistant following the acquisition of antibiotic resistance genes. Bacterial species can acquire resistance to antimicrobial agents due to selective pressures. Exposure to antimicrobials at sublethal strengths is a good example of selective pressure than can affect microorganisms. The reduced functionality or complete ineffectiveness of therapeutic antimicrobial agents facilitates the progression of infection in treated populations and increases the risk of transmission throughout healthcare facilities. Washbasin U-bends are relatively protected locations of high bacterial densities that are ideal for horizontal gene transfer (HGT) (Sorensen *et al.,* 2005). HGT is the movement of genetic material between organisms of unrelated generations and can be divided into the following processes: transduction, transformation and plasmid conjugation or mobilisation. HGT of resistance genes is central to the ongoing crisis of antimicrobial resistance in clinically important bacteria (Mathers *et al.,* 2019). While the development of AMR is an evolutionary process in microorganisms, this process is accelerated by the selective pressures mediated by the widespread use of antimicrobial agents (World Health Organization, 2018). The spread of antimicrobial resistance is not limited to healthcare facilities, however owing to the high use of antimicrobial agents in healthcare facilities, these are areas of high selective pressure for AMR.

1.2.4.2 Biofilm structure

In nature, microbial survival is dependent upon the ability of microorganisms to persist in biofilms. Biofilms are complex structures where either homogeneous or heterogeneous microbial communities (i.e., planktonic bacteria, fungi, protozoa and/or amoebae) produce a complex, sticky, highly hydrated matrix of extracellular polysaccharides (EPS) that facilities attachment to surfaces (i.e., teeth, intestinal mucosae, rocks, soil, washbasin U-bends). Biofilms form readily in moist, damp, and relatively protected environments commencing with the irreversible attachment of microorganisms to a surface. Due to the protective nature of biofilms, microorganisms found in biofilms exhibit decreased susceptibility to antimicrobial treatments (e.g. disinfectants, antibiotics etc.) relative to their planktonic counterparts (Stewart and Costerton, 2001). Biofilms offer protection from environmental stresses, such as drying and the presence of antimicrobial compounds and disinfectants, which penetrate poorly into biofilms. In general, Gram-negative bacteria are adapted to surviving in low osmolarity environments such as washbasin U-bends. This is due in part to the structure and components of the Gram-negative cell wall containing both

lipopolysaccharides and porins (Anwar and Choi, 2014). While Gram-positive bacteria are less adapted to surviving in washbasin U-bends by virtue of the peptidoglycan rich strong cell wall. Similarly, most fungi colonise drier environments compared to bacteria.

1.4.2.3 Biofilms in washbasin U-bends

Biofilms present in wastewater pipes have been shown to be difficult to eradicate (Cole and Talmadge, 2019). U-bends are concealed, wet, humid and nutrient rich segments of pipework where stagnation occurs and routine inspection is limited or non-existent due either to the inaccessibility of parts for cleaning, or that these areas are perceived as safe. For this reason, washbasin U-bends and wastewater networks are frequently coated in dense biofilms. These biofilms consist of organic and inorganic materials that are derived from both the supply water, the materials and liquids that have been washed down the drain outlet, and from microorganisms originating in pipework downstream of the washbasin U-bends. Wastewater pipes are an ideal location for dispersal of mature biofilm, portions of which can be detached by the impact and flow of wastewater. Biofilm also form rapidly on old pipework due to the effects of aging on the pipes. Aged pipes are often roughened or textured, increasing the surface area for biofilm to form. U-bend biofilms are usually heterogenous communities consisting of a range of opportunistic bacterial pathogens, which are mainly Gram-negative. These include *Pseudomonas* spp., *Acinetobacter* spp., *Klebsiella* spp. and *Enterobacter* spp., all of which can exhibit resistance to the major classes of clinically relevant antibiotics (Hota *et al.,* 2009; Breathnach *et al.,* 2012; Leitner *et al.*, 2015; Stjarne Aspelund *et al.,* 2016). Formation of biofilms allows these diverse microbial communities to resist disinfection and to survive cleaning processes (Muzslay *et al.,* 2017). A range of microorganisms associated with healthcare facility outbreaks recovered from washbasin U-bends are summarised in Table 1.1.

<u>1.3 Transmission of microorganisms from wastewater systems</u>

HAIs are infections acquired by patients while being treated in healthcare facilities. HAIs are an aspect of healthcare services that should be avoided or at least minimised if good working practices are implemented. While numerous protocols, guidelines and standards have been established to reduce the occurrence of HAIs, wastewater pipes themselves have only recently been identified as a contributor to nosocomial infections (Cholley *et al.,* 2008; Parkes and Hota, 2018; Regev-Yochay *et al.,* 2018). Healthcare facilities include hospitals, outpatient primary health clinics, dental practices, and long-term care facilities, amongst

Table 1.1 A range of antimicrobial resistant microorganisms recovered from contaminated washbasin U-bends that are associated with healthcare facility outbreaks

Organism[1]	**Molecular methods used for analysis**[2]	**Antimicrobial agent resistance status**[3]	**Reference**
Acinetobacter baumani	N/A	MDR	(Landelle *et al.*, 2013)
Klebsiella oxytoca	PFGE	MDR	(Vergara-López *et al.,* 2013)
Klebsiella pneumoniae	PFGE	CRE	(Clarivet *et al.,* 2016)
Klebsiella pneumoniae	PCR	CRO	(Mathers *et al.,* 2018)
Serratia marcescens	PFGE	CPE	(Regev-Yochay *et al.*, 2018)
Polymicrobial CPE	PFGE	CPE	(De Geyter *et al.*, 2017)
Polymicrobial GNB	N/A	MDR	(Shaw *et al.,* 2018)
Pseudomonas aeruginosa	VNTR	MDR	(Guleri *et al.*, 2012)
Pseudomonas aeruginosa	RAPD-PCR; microarray	MBL	(Schneider *et al.*, 2012)
Pseudomonas aeruginosa	PFGE	MDR	(Gbaguidi-Haore *et al.,* 2018)
Pseudomonas aeruginosa	N/A	MDR	(Kossow *et al.,* 2017)
Pseudomonas aeruginosa	PCR	MBL	(Wendel *et al.,* 2015)
Pseudomonas aeruginosa	PFGE	N/A	(Zhou *et al.*, 2016)
Pseudomonas aeruginosa	PFGE	MDR	(Varin *et al.,* 2017)

[1] GNB, Gram-negative bacteria, CPE, carbapenemase-producing *Enterobacteriaceae.*

[2] N/A, not available; PFGE, pulse-field gel electrophoresis; PCR, Polymerase chain reaction; RAPD-PCR, random amplification of palindromic DNA; VNTR, variable number tandem repeat analysis.

[3] MDR, multidrug drug-resistant; CRO, carbapenem resistant organisms, MBL, metallo-β-lactamases.

others. The mode of transmission of potentially pathogenic microorganisms from the wastewater network to hospital staff and patients include: contact, ingestion, aspiration and inhalation of aerosols (Chinn and Sehulster, 2003). Inadequate cleaning of washbasins can lead to contact of patients and equipment with microorganisms emerging from the wastewater drain outlets (Decker and Palmore, 2013; Blom, 2015). Ironically, while handwashing is the single most effective preventative measure for reducing nosocomial infections, washing hands in contaminated washbasins may lead to further spread of microorganisms. Currently, the spread of microorganisms in water is well understood, however, the spread of potentially pathogenic bacteria from and within plumbing systems has been less well studied. The movement of microorganisms in wastewater is facilitated by three main factors: (i) mobility of the microorganism by means of pili and flagella, (ii) the carriage of microorganism within water being washed down the pipes, and the carriage of microorganism against gravity due to pressure differences in pipe networks, (iii) air flow in pipes and the formation of partial vacuums behind discharged water in wastewater pipes (Gormley *et al.*, 2017). Water flow in pipework is irregular and causes unsteady and turbulent pressure differences, which can carry microorganisms both up and down the pipework (Gormley *et al.,* 2017). The water retained in the washbasin U-bend acts as a barrier preventing microorganisms within the wastewater pipe being carried by air currents directly into the hospital environment.

1.4 Nosocomial infections associated with washbasin U-bends

A marked increase of reports over the last twenty years have described outbreaks of hospital infection predominantly caused by Gram-negative bacterial species, associated directly or indirectly with contaminated washbasin and sink wastewater drain outlets (Hota *et al.,* 2009; Breathnach *et al.,* 2012; Kizny-Gordon *et al.,* 2017; Hopman *et al.*, 2019; Mombini *et al.*, 2019; Snitkin, 2019). Many recent reports have highlighted the importance of washbasin and sink drains as a reservoir for nosocomial transmission of *P. aeruginosa* and extended-spectrum beta-lactamase (ESBL) producing and carbapenem-resistant organisms (Kizny-Gordon *et al.*, 2017), the latter an emerging global health threat.

1.4.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, aerobic and non-fermenting bacterium that is commonly found in soil and water. *Pseudomonas aeruginosa* exhibits nutritional versatility and its ability to form biofilms plays a pivotal role in its clinical relevance as a pathogen (Moradali *et al*., 2017; Valentini *et al.*, 2018). In humans *P. aeruginosa* is not considered a

commensal and exhibits low rates of carriage (Bertrand *et al.,* 2001). However, *P. aeruginosa* is described as an opportunistic pathogen causing mild to severe infections especially in cystic fibrosis patients (Moradali *et al*., 2017).

The ability of *P. aeruginosa* to persist for long periods in biofilms may explain its high rate of colonisation of wastewater networks (Walker *et al.,* 2014). *Pseudomonas aeruginosa* commonly colonises taps, in particular flow straighteners, alongside the washbasin U-bend and drain outlet. This is due to the fact that these environments are nutrient rich and highly oxygenated, and biofilms within washbasin U-bends are relatively free from disruption, due to the nature of the fittings. *Pseudomonas aeruginosa* thrives primarily in the distal water/air barrier in wastewater systems, areas such as the washbasin drain outlet extending towards the washbasin U-bend (Figure 1.2) (Bédard *et al*., 2016; Hutchins *et al.,* 2017).

1.4.1.1 Pseudomonas aeruginosa *genome and population structure*

Pseudomonas aeruginosa has a relatively large genome size ranging between 5.2 – 7 Mb and exhibiting a high GC content of 65% (Tagini and Greub, 2017). The large GC rich bacterial genome is common for microorganisms that inhabit soil. *Pseudomonas aeruginosa* is a naturally competent organism capable of acquiring both genomic and plasmid DNA (Nolan *et al*., 2019). Likewise, *P. aeruginosa* exhibits a remarkable level of genome plasticity and diversity that is facilitated by both HGT and genome-wide homologous recombination. Owing to this, multidrug resistant *P. aeruginosa* has recently been identified as a serious threat by the Centers for Disease Control and Prevention (Centers for Disease Control and Prevention, 2019).

The population structure of *P. aeruginosa* has been revisited many times since the organism was first described in 1882 (Pirnay *et al*., 2009). In the early 1990s, *P. aeruginosa* was first described as having a panmictic population structure, meaning no genetic mating restrictions upon the population where all recombination events are equally possible to occur. However, by the 2000s a study of *P. aeruginosa* from strains isolated from keratitis and its environment suggested an epidemic population of highly conserved sequence types worldwide (Lomholt *et al.*, 2001). By 2004, this classification was revisited, and the population structure was re-defined as a non-clonal epidemic population, meaning that clinical and environmental isolates were indistinguishable with no specific clones associated with a specific disease or habitat selection. However, recent studies have suggested the existence of dominant epidemic high-risk (EHR) clones (Mulet *et al.,* 2013; Willmann *et al.,* 2014; Oliver *et al.,*

2015; Abdouchakour *et al.,* 2018; Slekovec *et al.,* 2019). The reclassification of the population structure is indicative of the refinement of bacterial typing methods adopted.

1.4.1.2 Pseudomonas aeruginosa *resistance profile*

Pseudomonas aeruginosa contains one of the largest bacterial genomes, which encodes an array of intrinsic resistance mechanisms and can exhibit a diversity of acquired resistance mechanisms. *Pseudomonas aeruginosa* exhibits a high level of intrinsic resistance to antibiotics with the number of multidrug resistant strains increasing worldwide (Pachori *et al.*, 2019). Intrinsic resistance to antibiotics is due to a wide range of resistance mechanisms. The three major intrinsic resistance mechanisms include: (a) the loss of porins (such as OprD2) due to exposure to antibiotics reducing outer membrane permeability, (b) the expression of AmpC an inducible broad-spectrum beta-lactamase, and (c) the extensive efflux system (Jayaraman *et al.,* 2010). Due to these mechanisms, *P. aeruginosa* has been shown to be intrinsically resistant to beta-lactam and penem group antibiotics (Jayaraman *et al.,* 2010). The ability of *P. aeruginosa* to acquire resistance mechanisms through HGT and mutation has resulted in its developing and/or acquiring resistance to aminoglycosides and fluoroquinolones (Pachori *et al*., 2019). Finally, *P. aeruginosa* can develop resistance to antibiotics and disinfectants due to continuous exposure to the antimicrobial agents concerned at sublethal concentrations and overexposure to environmental stresses (Pachori *et al*., 2019).

1.4.2 Extended spectrum beta-lactamases (ESBL) and carbapenem resistant organisms (CROs)

Beta-lactamases are a group of enzymes found in a diversity of bacterial sources that can enzymatically hydrolyse the beta-lactam ring of beta-lactam antibiotics. These enzymes can be divided into two categories, those which can perform hydrolyses by the formation of acyl enzymes with an active-site serine, or can perform hydrolyses with one or two essential zinc ions in active sites of metallo-β-lactamases (MBLs) (Bush, 2018). Washbasins in intensive care units (ICUs) have been associated with ESBL-producing *Enterobacteriaceae* outbreaks. A study by Roux *et al.* (2013) found that 30% of the 13 ICUs tested contained washbasins that were contaminated with ESBL-producing *Enterobacteriaceae*. The treatment of severe infections caused by ESBL-producing *Enterobacteriaceae* has primarily focused on the administration of carbapenems (Shaikh *et al.,* 2015). Recent reports have increasingly highlighted the importance of wastewater pipework as a reservoir for the

nosocomial transmission of ESBLs and CROs as an emerging global health threat (Roux *et al.*, 2013; Kizny-Gordon *et al.*, 2017).

CROs are a group of microorganisms that are increasingly associated with high mortality and morbidity particularly in healthcare facilities (Goodman *et al.,* 2019). CROs encompass both Gram-negative bacterial groups of non-fermenters of lactose (e.g. *P. aeruginosa* and *Acinetobacter baumannii*) and *Enterobacteriaceae* (e.g. *Klebsiella pneumoniae* and *Escherichia coli*), with inherent and acquired resistance (e.g. KPC and NDM type carbapenemases, or production of ESBL, or AmpC with porin loss in *Enterobacteriaceae*). CROs can be divided into a further three categories based on bacterial family and/or acquisition of acquired resistance elements such as carbapenemase-producing organisms (CPOs), carbapenem-resistant *Enterobacteriaceae* (CRE) and carbapenemase-producing *Enterobacteriaceae* (CPE). Carbapenems are a group of beta-lactams that contain a structural beta-lactam ring in their molecular structure. Carbapenemases are a class of beta-lactamases, which are enzymes that confer resistance to the majority of beta-lactam antibiotics. These antibiotics are used to treat multi-drug resistant bacterial infections and primarily dispensed in hospitals. CROs have emerged relatively recently as a major health threat in hospitals and the community in many countries, and only a few antimicrobial agents remain active against these microorganisms (Meletis, 2016). High administration rates of carbapenems have led to an increase in carbapenem resistance emerging in Gram-negative bacilli (World Health Organization, 2017).

1.5 Previous approaches to minimise microbial contamination risks from wastewater networks in healthcare facilities

The establishment of effective decontamination practises is essential for the reduction of wastewater system associated with HAIs (Hanlin and Myers, 2018). Chlorine-based disinfection has historically been the most widely adopted decontamination approach for water and wastewater systems. One of the first documented uses of chlorine disinfection was in 1897 in the treatment of water facilities in England (Tulchinsky, 2018). However, a range of disinfectants have been used for wastewater decontamination resulting in varying levels of success over the last 100 years. Recently there have been growing fears over the potential toxicity to workers handling these solutions, stability over time, and the downstream effect on the environment when washed through the system. Concerns surrounding the use of

biocides in the environment are based on potential long-lasting residual effects in the environment.

To date, decontamination strategies for contaminated washbasins and washbasin U-bends have focused around six main approaches: (i) washbasins designed to reduced release of microorganisms from drain outlets, (ii) conventional disinfection of washbasin and sink drains, (iii) self-disinfecting sink drains, (iv) 'water-free' patient care, (v) the use of antimicrobial materials, and (vi) replacement of washbasins and U-bends (Schwartz *et al*., 1998; Department of Health, 2013; Fusch *et al.*, 2015; Clarivet *et al.*, 2016; Salm *et al.*, 2016; De Geyter *et al.*, 2017; Livingston *et al.*, 2018). Currently, no single decontamination approach has proved to be effective in the long term and cost efficient.

1.6 Electrochemically activated solutions (ECAs)

ECAs were first developed in the 1970s by Professor Vitold Bakhir (Bakhir, 1992). ECAs are produced when diluted saline solution is activated by electrolysis within specially designed electrolytic cells, whereby a process of chemical change is triggered by an electric current passing through the solution resulting in the migration of electrolyte ions towards the negative and positive electrodes (Huang *et al.*, 2008). The input saline solutions are electrochemically activated by pumping dilute salt solution, otherwise known as brine at 0.5 to 2% (w/v) sodium chloride (NaCl), at a steady rate through the electrolytic cell (Su *et al.,* 2007). Sodium chloride is the most widely adopted electrolyte in water disinfection and the electrolysis of sodium chloride solution can produce chlorine, hypochlorous acid, hydrochloric acid, hypochlorite, chlorine oxide, and chlorate (Cal, 2005).

The flow-through electrolytic module (FEM) cell was first described in 1989. The FEM-3 is one of the most recent versions that produces ECAs of consistent composition and generates anolyte at neutral pH (Bakhir and Zadorozhny, 1996). The FEM-3 consists of a set of electrodes separated by a semi-porous membrane (Figure 1.4). During electrolysis, an electric current is passed across the cell, which is also provided with dilute brine. The electrode connects to the positive terminal of the generator known as the anode, and at the negative terminal known as the cathode. When a current is applied to the cell, the anode attracts the negative charged ions (anions), and the cathode attracts the positive charged ions (cations). In keeping with the electrode at which they are produced, the two solutions generated by this

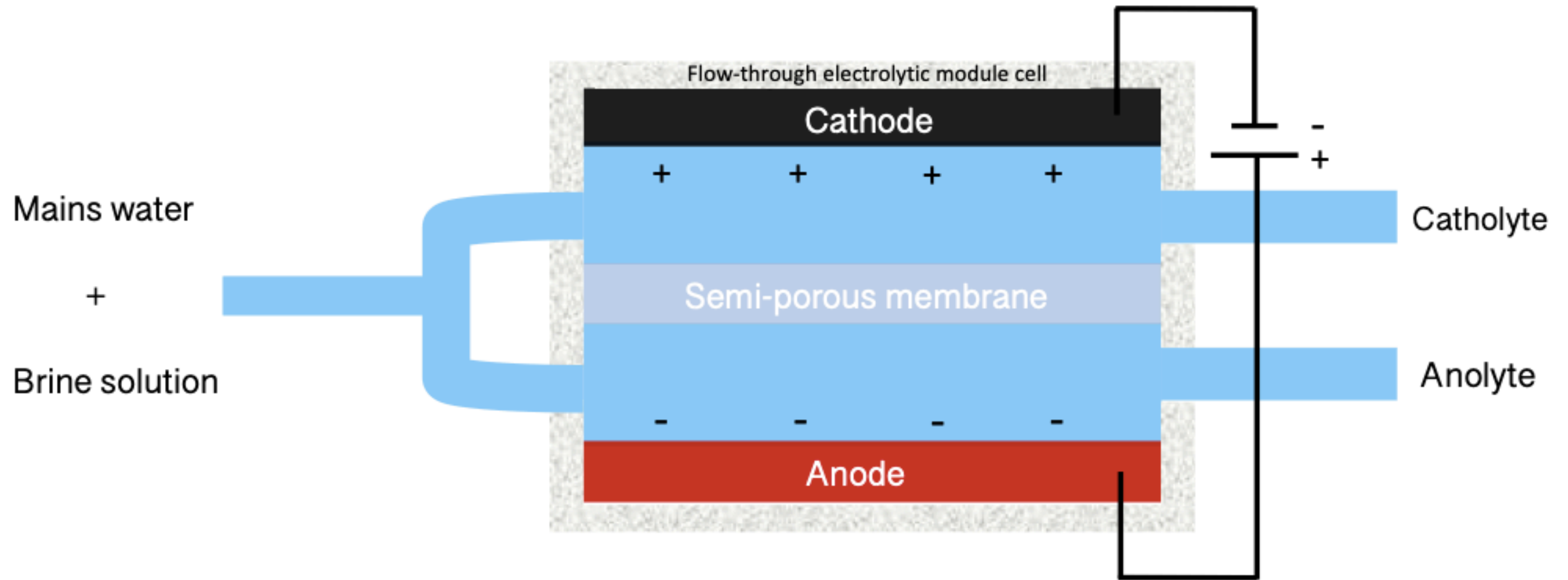


Figure 1.4 A schematic of an electrolytic cell**.** A mixture of water and dilute 0.2% sodium chloride solution are passed through an electrolytic cell. The cell is composed of a set of electrodes separated by a semi-porous membrane and is provided with electrolytic solution for electrolysis. During electrolysis, negative ions migrate to the anode and the predominant product formed is hypochlorous acid, while positive ions migrate to the cathode and the predominant product formed is sodium hydroxide. In keeping with the electrode at which they are produced, the two metastable solutions generated by this process are called anolyte and catholyte. The figure is adapted from Swan, 2017 and the Envirolyte website (http://www.envirolyte.com/electrochemistry-and-electrolyzed-water.html).

process are called anolyte and catholyte. These solutions are considered to be produced in a metastable state where the solutions are in a state of excitation or activation. The semi-porous membrane separating the electrodes is utilised to inhibit interaction of catholyte and anolyte that would otherwise interact, severely reducing the efficiency of the anolyte as an oxidant and the catholyte as an antioxidant.

The production of the anolyte and catholyte solutions is dependent on the configuration of the semi-porous membrane in the electrolytic cells and the pH at which the solutions are generated. The catholyte produced in DDUH is an alkaline solution (pH 12) composed primarily of sodium hydroxide (NaOH). Sodium hydroxide has been used as a detergent for cleaning and has also been utilised for flocculation, coagulation and neutralising the toxicity of heavy metals (Bakhir, 1992). The catholyte generated in this study was produced in a modern ECA generator known as the Qlean-Genie UL-75a ECA generator (Qlean Tech Enterprises, Mendota Heights, MI, USA) at an oxidation-reduction potential (ORP) of approximately of –1000 mV. The anolyte solution generated by the Qlean-Genie UL-75a ECA generator configured as described above has an ORP of approximately +880 mV when the generator is configured to produce anolyte measured at 800 parts per million (ppm) free available chlorine (FAC). FAC is defined as the portion of the total residual chlorine not bound to other compounds (e.g. ammonia) that exists mainly as hypochlorous acid or hypochlorite ion (OCl^-) (Taharaguchi *et al.,* 2014). Hypochlorous acid and hypochlorite act as residual disinfectants, where hypochlorous acid is one of the most effective disinfects derived from chlorine (Taharaguchi *et al.,* 2014). Hypochlorous acid can penetrate the bacterial cell wall, interact with key bacterial enzymes interfering with respiration processes and is naturally produced by neutrophils in the human body to kill a wide range of pathogens (Pullar *et al.,* 2000; Su *et al.*, 2007; Taharaguchi *et al.,* 2014). Hypochlorous acid was first employed during World War I to treat infection and was only surpassed as an antimicrobial agent by the introduction of antibiotics (Armstrong *et al.,* 2015). The anolyte produced in this study consists of approximately 632 ppm hypochlorous acid (79%) and 162 ppm hypochlorite (20.2%) at pH 7.0 (Deasy *et al.,* 2018). The ratios of hypochlorous acid to hypochlorite within the solution is due to the production of anolyte at a neutral pH.

ECAs, in particular anolyte, have been referred to by many names include electro-chemically activated waters, electrochemically activated solutions, electrolyzed oxidising (EO) water, acidic electrolysed water (AEW, AcEW), mixed oxidant (MIOX) solutions, super oxidized water and redox water (Hata *et al.*, 1996; Selkon *et al.*, 1999; Solovyeva and Dummer, 2000;

Robinson *et al.,* 2012; Eftekharizadeh *et al.*, 2016; Deasy *et al*., 2018; Tenzin *et al*., 2019). However, it is important to note that ECA solutions can be generated using a range of equipment containing electrolytic cells, sometimes referred to as ECA solution generators, and depending on the configuration, the ECAs produced may vary considerably in composition between different generators. Early ECA generators often produced highly acidic anolyte, which can be corrosive (O'Donnell *et al*., 2009). Despite the variance in the properties of ECA solutions, the production of ECAs is based on the same principle of electrolysis of brine in an electrolytic cell.

1.6.1 Application of ECAs

ECAs have been used in three main areas: (i) in the healthcare sector for decontaminating medical devices, treating infection and dental treatment, (ii) in agricultural sector decontamination, and (iii) in environmental decontamination. Anolyte has been shown to display broad-spectrum microbiocidal activity against *Staphylococcus aureus*, *P. aeruginosa*, and vancomycin resistant *Enterococcus faecalis*, hepatitis B virus (HBV), human immunodeficiency viruses (HIV), *Bacillus atrophaeus* spores and *Clostridium difficile* spores (Morita *et al.,* 2000; Tagawa *et al.,* 2000; Robinson *et al.,* 2010). In medicine and dentistry, ECAs have previously been used for cleaning medical devices and surfaces, in wound infection prevention and for hand washing (Thorn *et al.,* 2012; Eftekharizadeh *et al.,* 2016). This is due to the fact ECAs, in particular hypochlorous acid, is microbiocidal but non-toxic to human tissue (Solovyeva and Dummer, 2000; Boyle *et al*., 2010; Kamil, 2014). ECAs have been used for decontaminating root canals, dental implant decontamination, and in the prevention/treatment of gum disease (Solovyeva and Dummer, 2000; Lata *et al.,* 2016; McReynolds, 2018; Vo *et al.*, 2019). ECAs have also been used to disinfect wounds, burns and diabetic foot ulcers (Hadi *et al.*, 2007; Martínez-De Jesús *et al.,* 2007). Secondly, ECAs have also been used in the agricultural and farming sectors ranging from decontamination of fruit and vegetables, hydrating fur skins and to remove soluble proteins, carbohydrates and fatty substances to environmental decontamination on pig farms (Liato *et al.*, 2015; Danylkovych *et al.*, 2016; Liato *et al.*, 2017; Tenzin *et al.*, 2019). Thirdly, ECAs have been used successfully in DDUH for decontaminating dental unit waterlines (DUWLs), associated water networks and water tanks and water provided to hand washbasins and washbasin taps for over ten years (O'Donnell *et al.*, 2009; Boyle *et al.*, 2010; Boyle *et al.*, 2012).

Following the long-term success of residual anolyte treatment of the water networks in DDUH, interest developed in harnessing both anolyte and catholyte solutions as an enhanced decontamination approach for heavily bio-fouled pipework. As stated previously, wastewater networks are not sterile environments, containing both organic and inorganic materials derived from supply water, from the materials and liquids that have been washed down the drain outlet, and from microorganisms originating in pipework downstream of the washbasin U-bends. The use of disinfectants alone is dramatically impeded by high levels of organic materials in the pipework, known to interfere with disinfection efficacy (Gélinas and Goulet, 1983). An approach was developed in DDUH to sequentially treat washbasin, U-bends and associated pipework with catholyte solution to clean the wastewater pipework by lifting the organic material, and to subsequently use anolyte solution to effectively disinfect the pipework. This decontamination approach also incorporated valves downstream of the test washbasins to enable increased contact times between the solutions and the pipework.

This decontamination approach was first tested in a staff bathroom in DDUH. Swan (2017) incorporated a manual ball valve in the wastewater pipe 50 cm downstream of the test washbasin. Catholyte solution was manually poured down the washbasin drain outlet and the ball valve was closed slowly, releasing any trapped air (Swan, 2017). The solution was held *in situ* for 5 min and then voided, by manually opening the ball valve (Swan, 2017). Once voided, the anolyte solution was poured down the washbasin drain outlet, held *in situ* for 5 min, and then voided, completing the decontamination cycle. The biofilms within the test washbasin and comparator untreated U-bends were monitored by removing the washbasin U-bends and swab sampling the internal sections. Over a five-week period, the average bacterial densities on two medias tested (Colombia blood agar [CBA] and Reasoners 2A agar [R2A]) between the control and test washbasin U-bends demonstrated a ≥4.3 log reduction in bacterial counts.

Following the proof of principle, Swan *et al.* (2016) aimed to automate the decontamination system. The previous test washbasin and associated wastewater network was modified to include an electronically controlled valve, replacing the manual valve, fitted to the wastewater pipe downstream of the U-bend (Figure 1.5) (Swan *et al.,* 2016). Two tanks containing anolyte and catholyte solutions were connected to the wastewater pipe downstream of the U-bend and upstream of the valve by additional pipework to which dosing pumps were connected (Figure 1.5). A programmable automated controller managed the

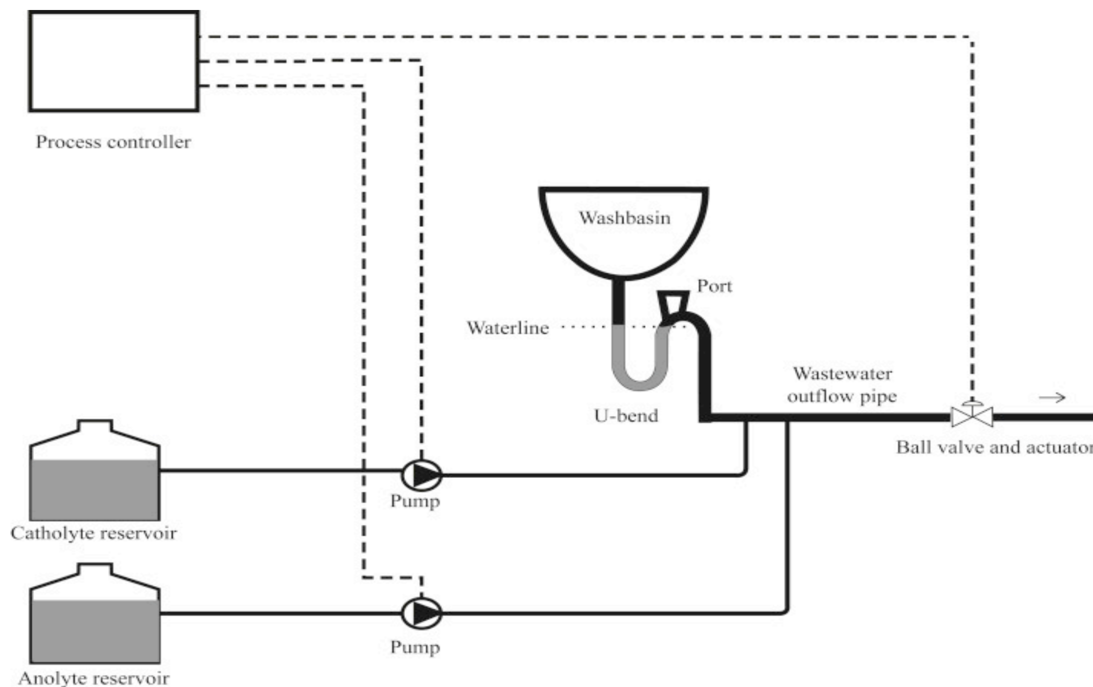


Figure 1.5 Schematic diagram of the prototype automated washbasin U-bend decontamination system based on sequential treatment with the ECA solutions catholyte followed by anolyte developed by Swan *et al*., 2016. Treatment cycles were initiated by the programmable process controller. At the start of each cycle the actuator closed the valve on the wastewater outflow pipe. After a 20 s delay, catholyte was pumped into the pipework below the washbasin U-bend until the pipework and U-bend were completely filled to a level a few centimetres above the washbasin wastewater outlet. After 5 min the valve opened and the catholyte was voided into the wastewater stream. Then the valve closed and after a 20 s delay anolyte was pumped into the pipework and U-bend and the cycle proceeded as for catholyte dosing. After 5 min the anolyte was voided into the wastewater stream completing the decontamination cycle.

closing and opening of the downstream valve. In contrast to the manual system developed, the ECAs were backfilled sequentially into the upstream wastewater pipes just covering the drain outlet in the washbasins. Backfilling of the ECAs permitted total removal of air pockets, previously limiting the surface exposure of the ECAs to the internal pipework. The decontamination approach began with the automated controller closing the downstream valve and initiating dosing of catholyte from the storage tank up through the system and stopping once the solution was held a few cm above the washbasin drain outlet. The catholyte solution was held *in situ* for 5 min and then voided, and the processes was repeated with anolyte solutions (Figure 1.5). This system was studied over three months, where the distal wastewater network was decontaminated three times weekly for a total of 35 cycles (Swan *et al.,* 2016). The biofilms within the test washbasin and comparator untreated U-bends were monitored by swab sampling the internal sections through a sampling panel incorporated into the washbasin U-bends. Swab samples were taken immediately following decontamination and 24 h following decontamination from the test and untreated control washbasin U-bends. The swab samples were processed and plated on four selected media (CBA, R2A, *P. aeruginosa* selective agar [PSCN], and *Pseudomonas* selective agar [PA]). The study found the average bacterial density from the three untreated U-bends was >1 × 10^5 CFU/swab on all media, with *P. aeruginosa* isolates accounting for up to 50% of the counts (Swan *et al.,* 2016). The average bacterial bioburden was significantly reduced following the decontamination cycle, with average counts from the 35 cycles on CBA, R2A, PSCN and PA of 2.1 ± 4.5 ($P < 0.0001$), 13.1 ± 30.1 ($P < 0.05$), 0.7 ± 2.8 ($P < 0.001$), and 0 ($P < 0.05$) CFU/swab, respectively (Swan *et al.,* 2016). Following the proof of principle and development of the automated system, the work carried out in this thesis investigated and developed a large-scale automated decontamination system in an active healthcare facility capable of simultaneously decontaminating multiple washbasin U-bends.

1.6.2 The advantages of washbasin U-bend decontamination by automated treatment with ECAs

One of the main advantages of adopting ECAs to decontaminate washbasin U-bends is safety. Utilisation of an automated system reduces opportunities for human error, but also reduces potential adverse effects on cleaning staff. Previously, electrolytic cells produced anolyte solutions at inconsistent levels of hypochlorous acid and varying pH, alongside the production of free radicals from electrochemical activation of brine solutions with potentially produce harmful effects (O'Donnell *et al.,* 2009). However, the FEM-3 used throughout this project consistently produced anolyte at neutral pH with 79% hypochlorous

acid and 20.2% hypochlorite, with no recorded toxicity to humans (Boyle *et al*., 2010). Likewise, no other harsh chemicals are used, reducing the risk of adverse effects following accidental contact. There are no toxic waste streams and no special requirements for disposal as ECA solutions are environmentally friendly and inactivated readily following discharge in wastewater (Thorn *et al.,* 2012).

Other advantages of the system are that backfilling of the ECAs from the downstream wastewater pipe into the washbasin reduces the likelihood of air being trapped and ensures all surfaces are contacted by the solutions during decontamination, that there is no need for staff so the system can be programmed to activate when washbasins are not in use (i.e. late at night), and that the electronic valve used to seal the wastewater outflow pipe can be located at a distance downstream of the washbasins ensuring that distal wastewater pipework is disinfected, thus further reducing opportunities for re-colonization of U-bends and drain outlets.

1.6.3 Current limitations associated with ECAs

The main limitation of ECA solutions is the short half-life of anolyte once generated. Anolyte deteriorates at high temperatures or following exposure to sunlight and cannot be stored for long periods. Studies have shown anolyte deactivates when exposed to air from anywhere between 24 – 72 h (Cai, 2005; Robinson *et al.,* 2010). Other studies report activity of anolyte up to 6 days after activation if stored in cool air-tight conditions (Robinson *et al.*, 2012). Owing to this, the use of ECA solutions is reliant on purchasing and maintaining an ECA generator. While there is an obvious limitation with an upfront cost, the limited active lifespan of anolyte solution reduces potential harmful effects to the downstream environment and reduces bulk storage of chemicals in healthcare facilities. The short time period between generation and usage reduces the volume of solutions held for long-term storage.

1.7 Conventional and molecular methods for bacterial identification

Three main bacterial identification methods were adopted throughout the course of this work. Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was utilised in this study to identify bacterial isolates following the culture on specific media. The two culture-independent approaches used were whole genome sequencing (WGS) and Illumina 16S rRNA gene sequencing. Each approach offers their

own advantages and disadvantages, and the selection of typing methods was based on the desired data outcome.

1.7.1 MALDI-TOF-MS identification

MALDI-TOF-MS technology is a bacterial identification method recognised as the current clinical laboratory standard for microbial identification in many countries. MALDI-TOF-MS is an analytical technique that ionises samples into charged molecules and measures the ratio of mass-to-charge of the ions. In the biological sciences, the main use of MALDI-TOF-MS is the definitive identification of bacterial species based on ribosomal protein separation into charged molecules and the ratio of their mass-to-charge (Duncan *et al.,* 2008).

MALDI-TOF-MS relies on three steps: ionization, ion separation/isolation, and detection. This approach begins with placing a single bacterial colony on the target plate and mixing with matrix crystallization solution (Figure 1.6). The matrix solution used depends on the application of the technique. The most common compound used for the detection of peptides, smaller proteins, and numerous other compounds is alpha-cyano-4-hydroxycinnamic acid (Gross, 2006). Matrix crystallization solutions are used in MALDI-TOF-MS for the dilution of the samples in the initial stages and as a mediator for energy absolution in later stages. After the matrix solution-sample is dry, an ultraviolet laser heats the crystallized matrix solution-sample and the proteins within the matrix solution-sample became ionized (Duncan *et al*., 2008). These ions are accelerated by a potential difference that pass through a high-vacuum time of flight (TOF) tube towards the detector (Figure 1.6). The ions are separated by mass-to-charge and the size of the ions is determined by the time it takes the ions to hit the detector (Boesl, 2017). The spectral representation of the ions formed is used to generate an MS profile, which is used for identification of the test organism by reference to validated libraries. Accepted species are identified based on confidence values of >99.9 % (Boesl, 2017). The advantages of using MALDI-TOF-MS analysis include the relatively low costs, ease of interpretation of data, and the relatively fast bacterial identification times. However, the disadvantages of this technology are that only microorganisms that can be cultured in the laboratory can be used as the current MALDI-TOF-MS reference databases are biased towards clinically important microorganisms.

1.7.2 Whole-genome sequencing (WGS)

1.7.2.1 The three generations of WGS technologies

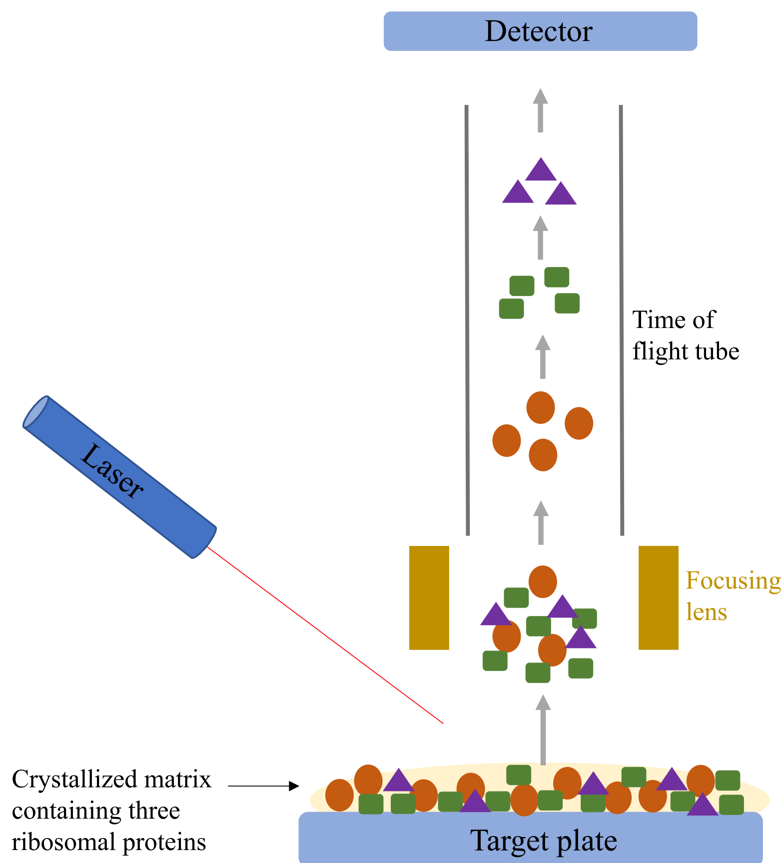


Figure 1.6 A schematic diagram of bacterial identification by MALDI-TOF-MS. The bacterial colonies are mixed with the matrix solution on the target plate. The three shapes (red, green, and purple) represent ribosomal proteins within the matrix solution. Once dry, a laser heats the solution causing ionization of the proteins, which are accelerated through the high-vacuum TOF tube towards the detector. The ionized proteins are accelerated by the potential difference created by the focusing lenses. The ions are separated by mass-to-charge and the size of the ions is determined by the time it takes the ions to hit the detector resulting in the generation of MS spectra. Spectra are compared with the validated reference libraries to achieve identification. The schematic was adapter from Lavigne *et al.* (2013).

WGS is the process of determining the complete DNA sequence of an organism at a single time point. Genomic sequencing has come a long way since the first widely adopted method was described by Maxam-Gilbert in 1977 (Maxam and Gilbert, 1977). WGS platforms are currently categorised in three groupings: first generation sequencing that includes Sanger and Maxam-Gilbert technologies; second generation sequencing or next generation sequencing (NGS) which includes Illumina, Ion Torrent, SOLiD and Roche 454 sequencing platforms; and third generation sequencing including Pacific Biosciences single molecule real-time (SMRT) technology and Oxford Nanopore technologies (ONTs).

First generation sequencing were based on either Sanger or Maxam-Gilbert technologies (Maxam and Gilbert, 1977; Sanger *et al.*, 1977). While both advanced technologies for their time, only Sanger sequencing is still used in part today. In this technique, a complementary strand of DNA is produced from the input template DNA using fluorescently-labelled deoxynucleotides (dNTPs), including dideoxynucleotide triphosphates (ddNTPs) that are nucleotides lacking a 3'-OH group needed for cDNA elongation (Churko *et al.*, 2013). Once a ddNTP is added to an elongating DNA strand, the elongation is terminated and generates DNA fragments of all lengths, and ensures termination of synthesis for every position (Sanger *et al.*, 1977). The fragments are electrophoresed and a chromatogram is produced revealing coloured peaks linked to the different nucleotide bases in the DNA sequence (Churko *et al.,* 2013). Sanger technologies are highly accurate, relatively inexpensive and yield read sequences of an average of 800 bp (Sanger *et al.*, 1977; Churko *et al.*, 2013; Quainoo *et al.*, 2017).

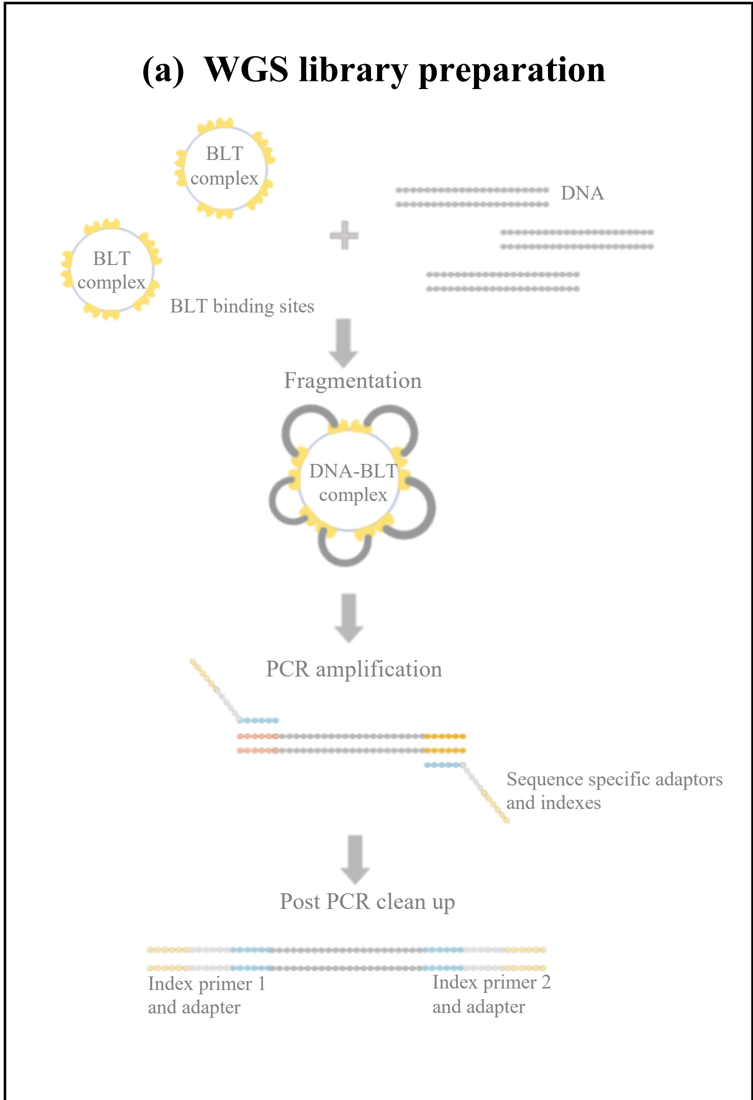
Second generation sequencing is currently the most widely used sequencing technology due to its reduced costs and preparation times, which has facilitated its use in epidemiological investigations and for the real-time analysis of outbreaks (International Human Genome Sequencing Consortium, 2004). Illumina second generation sequencing is the most widely used NGS technology, which will be described below in detail. This technology yields short read sequences up to 2 × 300 bp at the lowest cost per-base (Roumpeka *et al.*, 2017). Second generation sequencing technologies are considered high-throughput sequencing as they can sequence multiple DNA molecules in parallel at the same time (Churko *et al.,* 2013).

Third generation sequencing platforms are sequencing technologies capable of sequencing single molecule DNA. These technologies produce long read sequences of an average median length of approximately 10 – 20 kbp, but have been recorded as large as 50 kbp

(Quainoo *et al.,* 2017; Giani *et al.*, 2020). The two main platforms are Pacific Biosciences single molecule real-time (SMRT) technology and Oxford Nanopore technologies (ONTs). SMRT technology sequencing ligates the strand DNA of the sample to hairpin adaptors, which in turn bind to an immobilised DNA polymerase on a SMRT cell (Rhoads and Au, 2015). During sequencing fluorescently labelled nucleotides are added to a new DNA strand and as the strand extends the labels are cleaved emitting a fluorescent signal. This signal is recorded by a laser beam determining the nucleotide sequence (Rhoads and Au, 2015). ONTs, in comparison, is referred to as real-time sequencing. ONT measures the changes in electric current once a single DNA molecule traverses pores in a flow cell (McGinn and Gut, 2013). Current limitations of the third generation sequencing platforms include either the associated high costs with Pacific BioSciences or the high error rates associated with ONT.

1.7.2.2 The Illumina WGS workflow

The Illumina WGS workflow is based around four basic steps: library preparation, cluster generation, sequencing, and alignment and data analysis. The library preparation kit used throughout the research chapters of this thesis was selected for its ability to sequence genomes of varying sizes and bacterial species. The library preparation kit incorporates sample DNA into a solution containing bead linked transposome (DNA-BLT) complexes, where DNA saturates binding sites (Figure 1.7(a)). This DNA-BLT approach fragments DNA into consistently uniform sizes and adds adaptor tag sequences, a step referred to as tagmentation (Illumina, 2017). A subsequent PCR step ligates specific index adaptors to both ends of the DNA fragments enabling sequencing of multiple samples simultaneously (Figure 1.7(a)). A bead based clean-up prepares samples for library pooling, denaturing and loading onto reagent cartridges initiating cluster generation and sequencing on the Illumina MiSeq sequencing platform (Illumina, 2017). Cluster generation occurs on the surface of the experimental flow cell. The experimental flow cell is placed within the Illumina MiSeq sequencing platform, where single stranded DNA fragments within the pooled library can bind to the primers that coat the surface of the flow cell. The primers are complementary to the adapters ligated to the DNA fragments (Figure 1.8) (Mardis, 2008). Each ligated fragment folds over and hybridizes to complementary primers bound to the surface of the flow cell and is amplified into distinct clonal clusters known as bridge amplification (Mardis, 2008). Once the clusters are generated, the template DNA is ready for sequencing. DNA is sequenced on the flow cell utilising Illumina's sequencing-by-synthesis technology. DNA polymerase incorporates fluorescently labelled dNTPs into the DNA template strand


(a) WGS library preparation
BLT complex
BLT complex
DNA
BLT binding sites
Fragmentation
DNA-BLT complex
PCR amplification
Sequence specific adaptors and indexes
Post PCR clean up
Index primer 1 and adapter
Index primer 2 and adapter

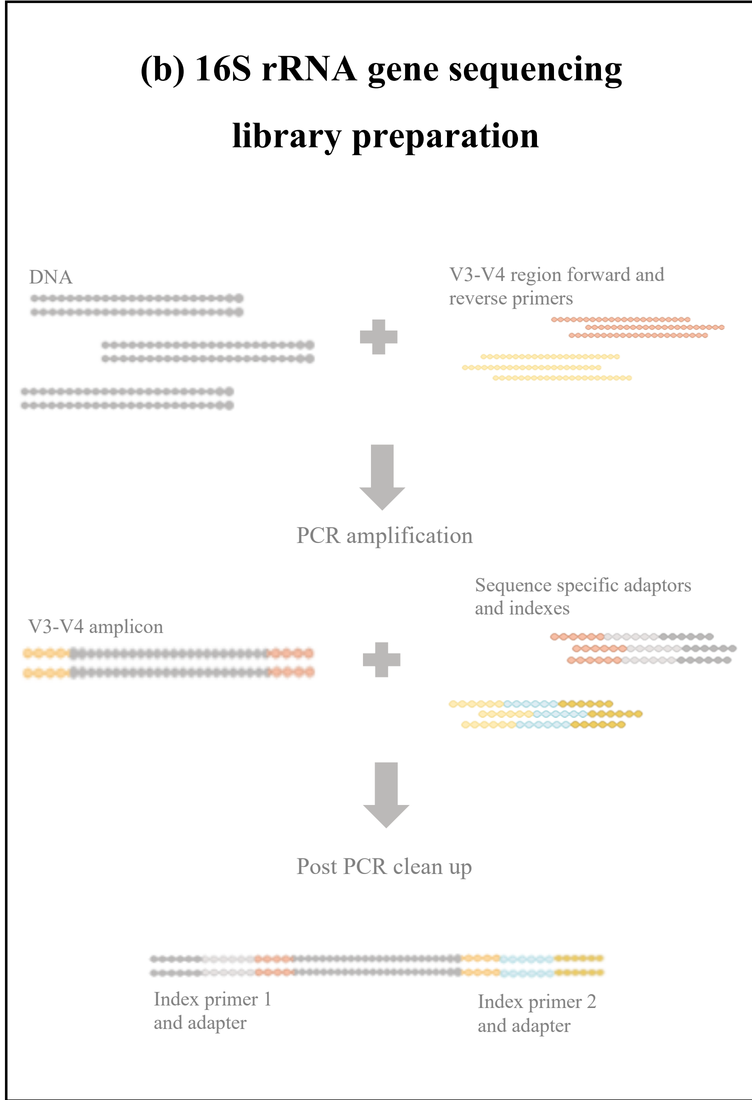

(b) 16S rRNA gene sequencing library preparation
DNA
V3-V4 region forward and reverse primers
PCR amplification
Sequence specific adaptors and indexes
V3-V4 amplicon
Post PCR clean up
Index primer 1 and adapter
Index primer 2 and adapter

Figure 1.7 Schematic diagram of the two Illumina based library preparation approaches used in this work. Panel **(a)** depicts the WGS library preparation approach. DNA is fragmented into consistently uniform sizes and adapters and indexes are ligated by a subsequent PCR step. The fragments are cleaned, and the prepared DNA fragments are ready for pooling, denaturing and loading onto reagent cartridges, which are then placed in an Illumina MiSeq sequencer. Panel **(b)** depicts the 16S rRNA library preparation approach. The sample DNA is incorporated into an initial PCR that targets the V3-V4 regions of the 16S rRNA genes within the sample and amplifies the segment. This step is followed by a clean-up that removes any solutions that may interfere with sequencing downstream. The second PCR ligates specific index adaptors to both ends of the DNA fragments. The fragments are cleaned by a bead based approach and the prepared DNA fragments are ready for pooling, denaturing and loading onto reagent cartridges for loading onto Illumina sequencing platforms. This image is adapted from 'An Introduction Next-Generation Sequencing Technology' document by Illumina, 2017.

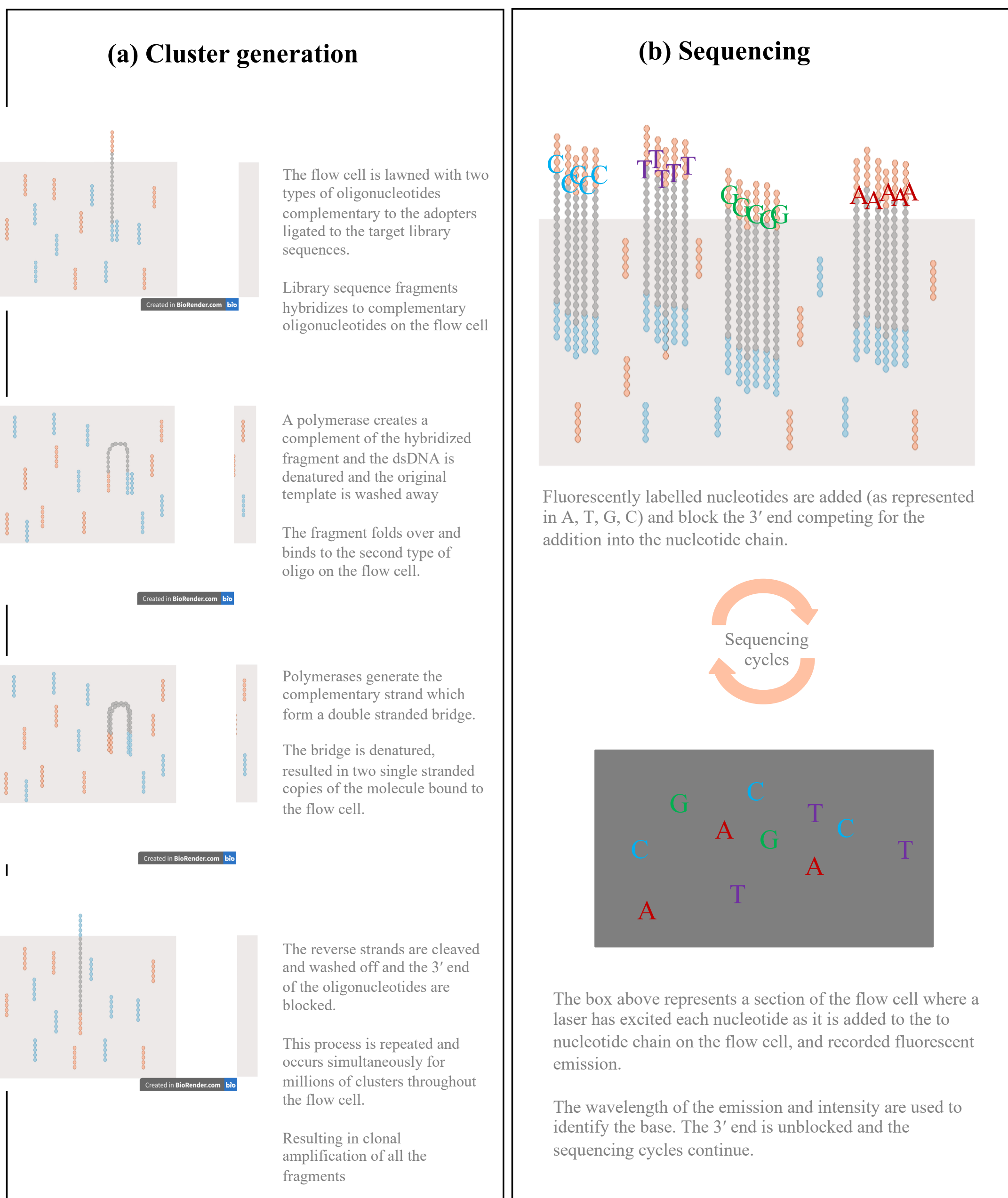


Figure 1.8 Schematic diagram of cluster generation and sequencing steps within the Illumina NGS platform. Panel **(a)** depicts the hybridization of a prepared library fragment to the MiSeq flow cell. The dsDNA fragment is denatured, the template DNA washed away, and the complementary strand forms a double stranded bridge. The bridge is denatured, resulting in two ssDNA copies. This process occurs repeatedly throughout the flow cell and for millions of clusters resulting in clonal amplification of all fragments. Panel **(b)** depicts the addition of fluorescently labelled nucleotides into the nucleotide chain. With each sequencing round, a laser excites the tagged nucleotides emitting a light corresponding to a base. This image is adapted from An Introduction Next-Generation Sequencing Technology by Illumina, 2017 and Mardis (2008).

throughout sequential cycles of DNA synthesis (Mardis, 2008). All four dNTPs are present during each cycle reducing incorporation bias. The fluorescently labelled dNTPs are identified when a characteristic emission caused by laser excitation of the nucleotide when generating single-end sequencing reads. Paired-end sequencing reads are generated by repeating this process on the opposite end of the template strand (Mardis, 2008). Throughout the sequencing cycles, internal sequence read quality metrics are recorded with error rates within base calling scored using an inbuilt Illumina quality scoring system. The average quality score for fragments is between Q30 - Q40 which indicates an error rate of 1 in 1,000 and 1 in 10,000 base calls, respectively. While the error rates in Illumina data are relatively low, the quality scores decrease towards the ends of sequence reads (Quainoo *et al.*, 2017). Following the completion of the sequencing cycles, the generated sequence read data are analysed utilising a number of approaches. Data analysis is undertaken using terminal command line pipelines and/or the commercially available Applied Maths BioNumerics suite of software programmes (Applied Maths, Sint-Martens-Latem, Belgium). Data analysis tools and commands are subject to the desired data output: (i) raw data can undergo assembly-free allele calling for whole genome multi-locus sequence typing (wgMLST), (ii) quality controls include sequence read trimming where the sequence reads can be mapped for single nucleotide variation (SNV) analysis, (iii) the trimmed reads can be assembled into contiguous DNA fragments or contigs (sets of overlapping DNA segments that together form a consensus region of DNA) using *de novo* assemblers that can be used for assembly-based allele calling for wgMLST, (iv) traditional multi-locus sequence typing (MLST) and (v) resistance gene finder tools, to name a few.

1.7.3 16S rRNA gene sequencing

16S rRNA gene sequencing is a molecular approach used to detect bacterial communities and their relative abundances. The move from culture dependent approaches to sequence-based approaches permit the detection of microorganism that cannot be cultured or are difficult to culture. Likewise, high-throughput sequencing facilitates the simultaneous detection of multiple taxa often down to the species level. The 16S rRNA gene is a 1,500bp sequence which forms a small subunit of the 30S subunit of the prokaryotic ribosome. Ribosomes are ubiquitous organelles utilised across the domains of life for the translation of mRNA into polypeptide chains during protein synthesis. The 16S rRNA gene contains nine hypervariable regions (V1 – V9), which are flanked by conserved sequences that can be used for designing PCR primers to investigate the variable regions (Fuks *et al.,* 2018). Sequence variations within the nine hypervariable regions can be used to track the evolution of

microorganisms (Chakravorty *et al.,* 2007; Kim *et al.,* 2012).

Initially, the 16S rRNA gene was selected as a potential phytogenic marker for characterising bacterial communities due to the depth of research previously carried out (Fox *et al.,* 1977). However, further research identified the 16S rRNA gene as an ideal phytogenic marker for a number of reasons: (i) the 16S rRNA gene is present in almost all bacteria, (ii) the function of the gene has not changed over time and (iii) the 16S rRNA gene contains conserved and variable regions of sequence that evolve at different rates enabling the resolution of microorganism at the species and genus levels (Srinivasan *et al.,* 2015). The advent of NGS technologies developed targeted sequencing of hypervariable regions of the 16S rRNA gene enabling cost-efficient high throughput data analysis. While no single hypervariable region is able to distinguish bacterial taxa, combination sequencing methods have been developed (Chakravorty *et al.,* 2007). In the case of the Illumina sequencing platform, the entire 16S rRNA gene cannot be sequenced accurately due to the generation of short read fragments. This results in sequencing subsets of the 16S rRNA gene typically ranging between 16 – 22 % of the total gene length (Fuks *et al.*, 2018). One subset largely adopted is the selection of the V3-V4 region for Illumina paired-end sequencing. The V3-V4 16S rRNA gene primers display increased universality for bacterial selection while within the constraints of the short read length available by the Illumina MiSeq platform (Fuks *et al.,* 2018). However, resolution, especially to the species taxon is limited, as some bacteria share the same selected amplified regions (Fuks *et al.,* 2018).

1.7.3.1 The 16S rRNA amplicon sequencing workflow

The Illumina 16S rRNA amplicon sequencing workflow is based on the same four basic steps as the WGS workflow: library preparation, cluster generation, sequencing, and alignment and data analysis. This library preparation workflow targets the V3 and V4 regions of the 16S rRNA gene target within the samples (Figure 1.7(b)) (Klindworth *et al.*, 2013). The preparation is divided into two PCR steps: the first PCR step amplifies the targeted V3-V4 regions of the 16S rRNA genes within the sample, while the second PCR step ligates specific index adaptors to both ends of the DNA fragments enabling sequencing of multiple samples simultaneously (Figure 1.7(b)). Following each PCR, a bead based clean-up removes any solutions that may interfere with sequencing. Following the final clean-up step all samples are pooled, denatured and loaded onto reagent cartridges initiating cluster generation and sequencing on the Illumina MiSeq sequencing platform (Illumina, 2017). Cluster generation and sequencing follow the same steps described in Figure 1.8.

Like the sequencing data generated from Illumina WGS, Illumina high throughput 16S rRNA amplicon sequencing data can be analysed using multiple approaches. A number of pipelines can be used to cluster sequences into operational taxonomic units (OTU) based on similar sequence variants. Representatives from these OTUs are defined into bacterial taxa depending on the similarity of sequence thresholds. The characterisation of the bacterial communities is reliant on dedicated 16S rRNA gene databases. The most widely utilised databases are EzBioCloud, Ribosomal Database Project, SILVA and GreenGenes (Balvočiute and Huson, 2017; Yoon *et al.,* 2017).

1.8 Aims of the study

The initial aim of this Ph.D. thesis was to develop an automated system that simultaneously decontaminates multiple washbasin U-bends using sequential treatment with catholyte followed by anolyte solutions. However, throughout the course of this work a number of research avenues arose to further investigate the role washbasin U-bends and the wastewater network may play in the spread of potentially pathogenic microorganisms in healthcare facilities. The three research chapters (Chapters 3-5) specifically aimed to:

- Develop a large-scale automated system to decontaminate multiple washbasin U-bends by sequential treatment with catholyte followed by anolyte. The first research chapter (Chapter 3) aimed to test the efficacy of routine decontamination of 10 washbasin U-bends in the A&E *in situ* over a period of five months with 62 decontamination cycles, with little to no disruption to routine clinical activities. Throughout this proof of principle test period, the impact of routine ECA-decontamination on the bacterial populations colonising U-bends and drains was monitored relative to the non-decontaminated U-bends.

- Determine the long-term efficacy of the novel system developed in Chapter 3, to determine the relative bacterial recovery rates using the swab sampling technique adopted in these studies and to investigate the predominant and total bacterial communities throughout the wastewater network in DDUH. The second research chapter (Chapter 4) aimed to study the effectiveness of ECA decontamination over a period of 52 weeks. The characterisation of bacterial communities were determined by MALDI-TOF-MS and Illumina high throughput 16S rRNA amplicon sequencing.

MALDI-TOF-MS analysis was used to investigate the predominant population structure of culturable bacteria within A&E washbasin U-bends exposed to routine decontamination by ECAs, and isolates from the control washbasin U-bends. While, 16S rRNA amplicon sequencing was used to investigate the total bacterial communities present in 16 non-ECA treated distinct locations within the wastewater pipe network. This research was conducted to achieve a more accurate indication of the total bacterial communities compared to culture-dependent approaches.

- Investigate the potential trafficking of bacteria between washbasin U-bends via the wastewater pipe network throughout DDUH. The aim of the third research chapter (Chapter 5) was to use WGS as a technique to investigate the relatedness of *P. aeruginosa* isolates recovered from the wastewater network in DDUH. The *P. aeruginosa* isolates were compared using a range of data analysis tools including: MLST analysis, wgMLST analysis, SNV analyses, and resistance gene finding tools. A range of isolates were selected to determine phenotypic resistance of isolates recovered from DDUH. Isolates from other Irish healthcare facilities and from DDUH dental unit suction systems were used as comparators.

Chapter 2

General Materials & Methods

2.1 General microbiology methods

2.1.1 Chemicals, purified water and buffers

All chemicals, molecular biology grade water and reagents, unless otherwise specified, were of analytical or molecular biology grade and were purchased from Sigma-Aldrich Aldrich Limited (Wicklow, Ireland). The water used to prepare buffers was ultra-purified using the Milli Q Biocel system (Millipore Ireland, Cork, Ireland). Molecular biology grade water was used for all PCR reactions, DNA dilutions and elution steps.

Swab samples taken from hand washbasin U-bends were processed by suspension and subsequent dilution in phosphate buffered solution (PBS). Phosphate buffered saline solution tablets (Thermo Fisher Scientific Oxoid, United Kingdom) were dissolved in ultra-purified water and sterilised by autoclaving in a Tomy SX-500E autoclave (Tomy Kogyo Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions.

The lysis buffer used for extraction of DNA from *P. aeruginosa* isolates by disrupting the cell wall structure consisted of 0.02 mg/ml lysozyme solution prepared in 1 ml Tris-EDTA (TE) buffer [10 mM Tris-HCl, pH 7.5 and 1 mM ethylenediaminetetraacetic acid (EDTA) pH 8]. The Tris-borate/EDTA (TBE) buffer used for conventional agarose gel electrophoresis was prepared at 10 X concentration and consisted of 0.45 M Trizma base, 0.45 M boric acid and 0.01 M EDTA, pH 8. This was diluted using Millipore water to 0.5 X concentration for use. TBE was used for both preparing agarose gels and as the running buffer for agarose gel electrophoresis.

2.1.2 General disposable laboratory plasticware

The general disposable laboratory plasticware used throughout the course of this study are listed in Table 2.1.

2.1.3 Oligonucleotides

The oligonucleotides used in this study were custom synthesised by Sigma-Aldrich, unless otherwise specified, and were stored at a stock solution of 10 mM at -20°C.

2.1.4 Agarose gel electrophoresis

Agarose gels were prepared at a concentration of 0.8% (w/v) and 2% (w/v) by dissolving

Table 2.1 General disposable laboratory plasticware used in this study

Plasticware	**Manufacturer**	**Size / Volume**
Sterile Petri dishes (triple vented)	Greiner bio-one (Kremsmünster, Austria)	90 mm diameter
Sterile individually wrapped pipettes	Cellstar, Greiner bio-one (Kremsmünster, Austria)	5 ml, 10 ml and 25 ml
Pipette tips	StarLab Ltd. (Milton Keynes, UK)	10 µl, 200 µl, 1000 µl
Sterile filter pipette tips	StarLab Ltd (Milton Keynes, UK)	10 µl, 200 µl, 1000 µl
Microcentrifuge tubes	Eppendorf (Hamburg, Germany)	1.5 ml
Micro tubes (Screw cap)	Sarstedt (Hildesheim, Germany)	2 ml
Sterile tubes (13 ml)	Sarstedt (Hildesheim, Germany)	13 ml
Sterile conical base centrifuge tubes	Greiner bio-one (Kremsmünster, Austria)	50 ml
Sterile PCR tubes	Fisherbrand (Fisher Scientific, Dublin, Ireland)	0.2 ml
Sterile plastic inoculation loops	Greiner bio-one (Kremsmünster, Austria)	1 µl
Sterile L-shaped cell spreaders	Greiner bio-one (Kremsmünster, Austria)	Not applicable

agarose powder (Type I, low EEO; Sigma-Aldrich) in 0.5X TBE buffer. Agarose gels were cast in Galileo Bioscience electrophoresis system gel boxes (Cambridge, MA, USA) on 10 x 8 x 3 cm trays with 10-well sample combs (1.5 mm depth) (Genesee Scientific, CA, USA). Agarose gel electrophoresis was conducted using a Consort power pack model EV222 (B-2300 Turnhout, Belgium) at a voltage of 100 V for 50 min. Following electrophoresis, gels were visualised under ultraviolet light (312 nm) using an Alpha Innotech UV transilluminator model AVT26V (Protein Simple, San Jose, CA, USA).

Molecular weight markers (100 bp) for use as size reference markers in agarose gels were purchased from the Promega Corporation (Madison, WI, USA). The GelRed nucleic acid stain loading buffer was purchased from Biotium (Frement, CA, USA) and was used according to the manufacturer's instructions. DNA loading dye for agarose gel electrophoresis was purchased from Promega.

2.1.5 Bacterial storage and reactivation

Bacterial isolates were stored at -80°C on plastic cryogenic beads in individual preserver vials (Microbank cryovials, Prolab Diagnostics, Cheshire, UK). Isolates were reactivated by removing a single bead using a sterile forceps and the bead was streaked onto a CBA plate using a sterile wire loop and incubating overnight in a static incubator (Gallenkamp, Leceister, UK) at 37°C.

2.2 Identification of bacterial isolates

2.2.1 Bacterial isolation

All bacterial isolates investigated during this study originated from the wastewater network servicing hand washbasins in DDUH unless otherwise stated. Three agar culture media were used for bacterial isolation throughout the study including Columbia Blood Agar (CBA; Lip Diagnostic Services, Galway, Ireland), Reasoner's 2A agar (R2A; Lip) and *Pseudomonas* selective agar containing cetrimide (200 μg/ml) sodium nalidixate (15 μg/ml) (PSCN; Oxoid, Thermo Scientific, Hampshire, UK). These culture media were selected to maximise the range of bacteria recovered from environmental sites and water. CBA was selected for the recovery of bacteria that can lyse haem and are potentially pathogenic. R2A was selected for the recovery of slow-growing bacteria commonly found in water and aqueous environments. The sodium pyruvate added to R2A facilitates the recovery of chlorine-stressed bacteria isolated from water (Boyle *et al.*, 2010). PSCN was used for enhanced recovery of *P.*

aeruginosa. This medium facilitates pigmentation of *P. aeruginosa* colonies and suppresses the growth of other organisms commonly recovered from aqueous sources such as *Klebsiella*, *Proteus* and *Providencia* spp.. *Pseudomonas aeruginosa* colonies frequently exhibit blue-green or brown pigmentation on PSCN agar. The bacterial growth characteristics of some of the bacterial species capable of growth on PSCN agar are outlined in Table 2.2. None of these three media used for recovery of bacterial isolates can be used for the definitive identification of bacterial species.

CBA agar plates were incubated at 37°C for 48 h, R2A agar plates were incubated at 20°C for 10 days and PSCN agar plates were incubated at 32°C for 48 h (Figure 2.1). Following incubation, colonies on agar plates were counted using a Stuart™ Scientific colony counter model SC5 (Sigma-Aldrich). Colony counts were recorded as colony forming units (CFUs) per swab (Swan *et al*., 2016) unless otherwise specified.

2.2.2 C-390 diatabs

C-390 diatabs were utilised in this study to assist in the identification of potential *P. aeruginosa* isolates. C-390 diatabs contain 40 μg of the antimicrobial agent 9-chloro-9-(4-diethylaminophenyl)-10-phenylacridan (C-390), which is selective for *P. aeruginosa* (Davis *et al*., 1983). In this study, suspect *P. aeruginosa* isolates were tested for susceptibility to C-390 by placing a 9 mm C-390 diatab (Rosco Diagnostica, Taastrup, Denmark) in the centre of a CBA agar plate the entire surface of which had been streak-inoculated with a single purified colony and incubated overnight at 37°C. Following incubation, the plates were examined for the presence of a zone of inhibition around the C-390 diatab. The absence of a zone of inhibition on plates exhibiting heavy or confluent bateria growth is indicative of *P. aeruginosa* (Figure 2.2). The presence of a zone of inhibition measuring <12 mm on plates exhibiting semi-confluent growth may also be indicative of *P. aeruginosa.* Zones >12 mm on plates exhibiting confluent or semi-confluent growth indicates that the organism being tested is not *P. aeruginosa* (Figure 2.2). However, it should be noted that this method is not a definitive method for *P. aeruginosa* identification as other *Pseudomonas spp*. and non-fermenters may sometimes fail to yield zones of inhibition in the presence of C-390 diatabs.

2.2.3 Polymerase chain reaction (PCR) for *P. aeruginosa* identification

The identification of *P. aeruginosa* was undertaken by PCR amplification of a segment of the *oprL* gene as described previously (Jami Al-Ahmadi and Zahmatkesh Roodsari, 2016) using the primers listed in Table 2.3. The *oprL* gene encodes a peptidoglycan-associated

Table 2.2 The relative growth characteristics of some environmental bacterial species on PSCN agar[1]

Bacterial species	Amount of growth on PSCN plate
Pseudomonas aeruginosa	+++
Pseudomonas fluorescens	+++
Pseudomonas putida	++
Burkholderia cepacia ATCC 25416	+
Burkholderia cepacia ATCC 17759	±
Proteus hauseri ATCC 13315	-

[1] Taken from Oxoid, Thermo Scientific (http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0559&c=UK&lang=EN). The plus and minus signs represent the relative level of growth of the organisms on PSCN agar.

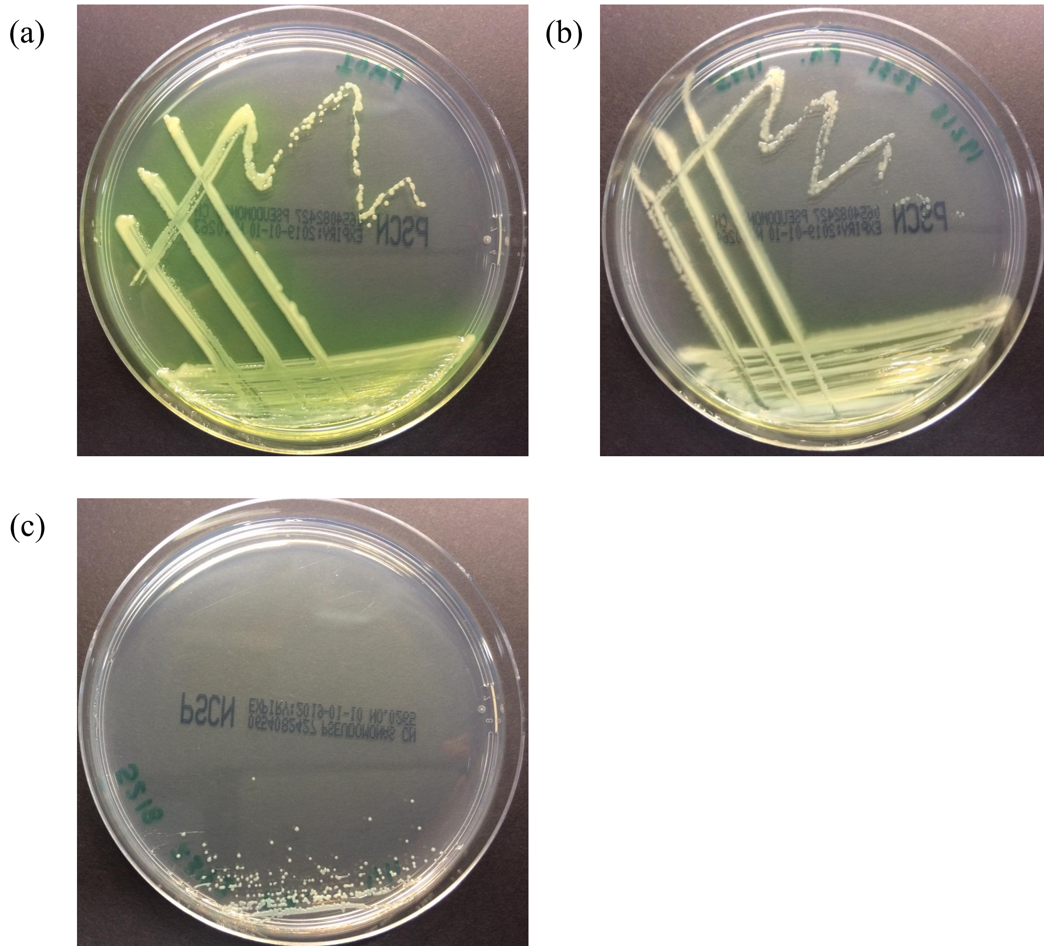


Figure 2.1 Photographs showing some examples of the growth characteristics of *P. aeruginosa* samples on PSCN after incubation at 32°C for 24 h. The three photographs show differences in colony morphology and pigmentation exhibited by three separate *P. aeruginosa* isolates, which were definitively identified by MALDI-TOF-MS analysis.

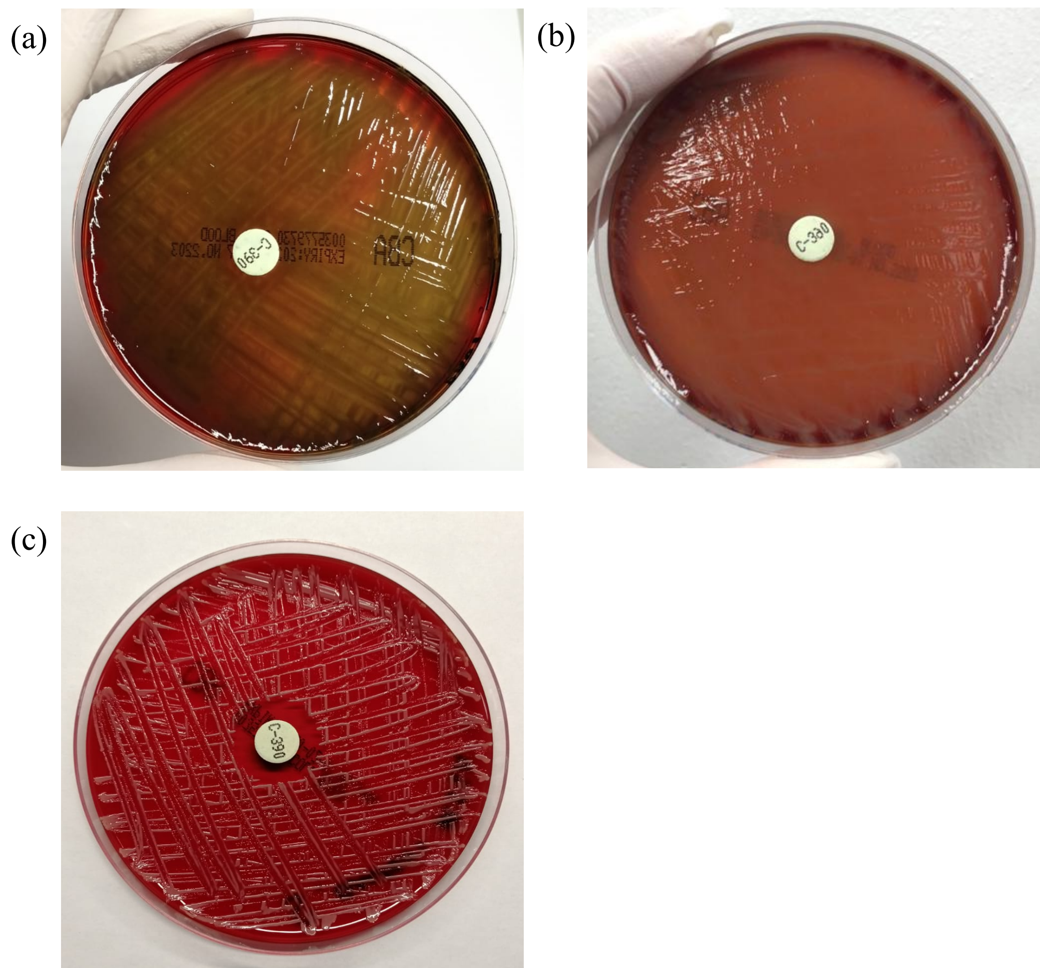


Figure 2.2 Photographs showing the use of C-390 diatabs on CBA plates streaked-inoculated with **(a)** *P. aeruginosa* (PAO1), **(b)** *P. aeruginosa* (ATCC 15442), and a quality control strain of **(c)** *Staphylococcus aureus* (ATCC 6538). In photographs **(a)** and **(b)** no zone of inhibition is evident for the *P. aeruginosa* strains, however the *S. aureus* strain shown in panel **(c)** exhibits a zone of inhibition >12 mm. Confluent or semi-confluent bacterial growth on plates with zones of inhibition >12 mm indicates that the organism in question is not *P. aeruginosa*.

Table 2.3 Primers used for amplification of the *P. aeruginosa oprL* gene

Primer	5' – sequence – 3'	Expected product length (bp)	Reference
OprL F	ATGGAAATGCTGAAATCCGGC	504	(De Vos *et al*., 1997; Jami Al-Ahmadi and Zahmatkesh Roodsari, 2016)
OprL R	CTTCTTCAGCTCGACGCGACG		

lipoprotein, OprL, which is specific to *P. aeruginosa* and responsible for inherent resistance to antibiotics and antiseptics (Jami Al-Ahmadi and Zahmatkesh Roodsari, 2016). Aliquots of 1 µl DNA extracted from *P. aeruginosa* (as described in Section 2.3 below) were used for amplification in 50 µl reaction volumes. Each reaction consisted of 10 µM of the *oprL* gene primer pair (Table 2.3), 1.5 mM magnesium chloride ($MgCl_2$; Promega), 5X green GoTaq flexi buffer (Promega), sterile water, 200 µM dNTPs (Promega) and 2.5 U of GoTaq DNA polymerase (Promega). All PCRs amplification steps were carried out using either a Kyratec Thermocycler model SC200 (Kyratec, Mansfield, Australia) or a G-Storm GSI Thermocycler (G-Storm, Somerset, UK). The cycle conditions consisted of an initial denaturation step at 96°C for 5 min, followed by 40 cycles of 96°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and ending with an elongation step at 72°C for 10 min. PCR amplimers were visualised using agarose gel electrophoresis as described in Section 2.1.2 above.

2.2.4 MALDI-TOF MS

MALDI-TOF-MS was used for the definitive identification of bacterial isolates recovered on CBA, R2A and PSCN agar plates using the Vitek MALDI-TOF MS system (bioMérieux Marcy l'Etoile, France) according to the manufacturer's instructions. All MALDI-TOF MS analyses was undertaken at the National Methicillin-resistant *Staphylococcus aureus* Reference Laboratory (NMRSARL) at St. James's Hospital, Dublin, Ireland. All isolates for MALDI-TOF MS testing were subcultured on CBA and incubated for 24 h. Following incubation, bacterial growth from a single colony was placed on a MALDI-TOF-MS target plate with matrix crystallization solution (Alpha-Cyano-4-hydroxycinnamic acid [CHCA]). The matrix solution was added to the bacteria to improve the spatial resolution for data analysis. Once dry, a pulsed UV laser causes the proteins in the sample to ionize toward a detector that differentiates mass based on time of flight. The mass spectra are generated and compared to validated libraries. Accepted species identification was based on confidence values of $\geq$ 99.9%.

2.3 Extraction of genomic DNA

Genomic DNA was extracted from *P. aeruginosa* isolates using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Crawley, West Sussex, UK). Isolates were reactivated from storage by streaking a single stored cryogenic bead on a PSCN agar plate and incubating overnight at 32°C in a stationary incubator. After 18 h incubation, a sterile inoculation loop was used

to select a single bacterial colony, which was used to inoculate the entire surface of a CBA plate followed by incubation overnight (18 h) at 37°C. Following incubation, an approximate 4 cm^2 area of bacterial growth was collected using a sterile inoculation loop and added to 200 µl lysis buffer (Section 2.1.1) using a Gilson pipette (Gilson Inc., WI, USA) in a 1.5 ml microfuge tube, vortexed for 30 s at max speed on a IKA Vortex shaker version 3 (IKA Works GmbH & Co, Staufen, Germany) and incubated in a shaking incubator at 350 rpm for 2 h. Following incubation, 25 µl proteinase K (20 mg/µl) and 200 µl buffer AL (both provided with the Qiagen's DNeasy Blood and Tissue Kit) were added to each tube for degradation of proteins and nucleases. The tubes were then incubated on a stationary heating block (Grant Instruments model QBD2, Royston, United Kingdom) at 70°C for 30 min followed by the addition of 200 µl 100% (v/v) ethanol to each tube. The contents of each individual tube were then transferred separately to individual 1 ml Qiagen mini-columns placed inside of a 2 ml collection tube (both provided with the Qiagen's DNeasy Blood and Tissue Kit). All columns were then centrifuged at 13, 000 × *g* for 1 min using a Eppendorf 5430 bench top centrifuge (Eppendorf, Hamburg, Germany) with a 9.5 cm rotor FA-45-30-11 (Eppendorf, Hamburg, Germany). Following centrifugation, the supernatant collected in a 2 ml collection tube was discarded and the mini-column was placed inside of a fresh 2 ml collection tube. A total of 500 µl of AW1 buffer (provided with the Qiagen's DNeasy Blood and Tissue Kit) was added to each mini-column and centrifuged at 13, 000 × *g* for 1 min. The supernatant collected in a 2 ml collection tube was discarded and the mini-column was placed inside of a fresh 2 ml collection tube. A total of 500 µl of AW2 buffer (provided with the Qiagen's DNeasy Blood and Tissue Kit) was added to each mini-column and centrifuged at 21,000 × *g* for 3 min. The supernatant collected in each 2 ml collection tube was discarded and the mini-column was placed inside of a fresh 2 ml collection tube, and was centrifuged at 21,000 × *g* for 3 min. Then the 2 ml collection tube were discarded and the mini-column was placed inside a fresh 1.5 ml microcentrifuge tube. A total of 50 µl AE buffer (provided with the Qiagen's DNeasy Blood and Tissue Kit) was added to each mini-column and incubated statically at room temperature for 5 min and then centrifuged at 13, 000 × *g* for 1 min. The DNA eluted into the 1.5 ml microcentrifuge tube were stored at 4°C for 4 – 6 weeks, or at -20°C for long term storage.

A nanodrop 2000c spectrophotometer (ThermoScientific, MA, USA) was used to determine the quality and concentration of DNA in each sample. The quality was determined by an A260:280 reading between 1.8 – 2, and an A260:230 reading between 2 – 2.2. An A260:280 reading of 1.8 is accepted as 'pure' DNA, while an A260:230 reading outside the 2 – 2.2

limit many be a due to other contaminants. For high sensitivity DNA readings, a Qubit Fluorometer 3.0 (Fisher Scientific, Dublin, Ireland) was used according to manufacturers' instructions.

2.4 Generation of electrochemically activated solutions (ECAs)

Anolyte and catholyte solutions were produced by electrochemical activation of 0.2% NaCl solution using a Qlean-Genie™ Model UL-75a ECA generator (Qlean Tech Enterprises, MN, USA). The anolyte solution is a positively charged solution, predominantly composed of hypochlorous acid with disinfectant properties as described in Chapter 1, Section 1.6. The generator produces anolyte measured at 800 parts per million (ppm) free available chlorine (FAC) at pH 7.0, having an oxidation-reduction potential (ORP) of +880 mV and consisting of approximately 632 ppm HOCl (79%) and 162 ppm OCl^- (20.2%). Other constituents in the anolyte solution include ozone, chlorine dioxide, chloric and chlorous acid, all of which together constitute the final 0.8% of the activated solution.

The catholyte solution is a negatively charged solution with detergent properties, comprised predominantly of sodium hydroxide. Catholyte was configured to a pH 12.5 with an ORP of approximately -1000 mV, consisting of approximately 400 ppm NaOH. The catholyte solution is an amphoteric surfactant with a surface tension of 63 mN force. For U-bend treatment freshly generated anolyte was used undiluted. Freshly generated catholyte was diluted 1:5 with heated mains water immediately prior to use with a temperature after dilution of approximately 33°C. The anolyte was used undiluted.

2.5 Quality indicators of ECA solutions

2.5.1 Measurement of free available chlorine

The free available chlorine (FAC) levels in anolyte were measured using a Hach Pocket Colorimeter II (Hach Company, Iowa, USA) according to the manufacturer's instructions.

2.5.2 Measurement of pH

The pH of the anolyte was measured using an Orion Star™ LogR Meter (Thermo Scientific).

2.5.3 Measurement of the redox potential

The reduction-oxidation potential was determined on the Qlean-Genie™ Model UL-75a ECA generator (Qlean Tech Enterprises, MN, USA).

2.5.4 Temperature of ECA solutions

Freshly generated catholyte was diluted 1:5 with heated mains water, and the temperature was measured using a Fisherbrand™ Traceable™ Digital Thermometers with short sensors (Fisher Scientific, NH, USA).

2.6 Hand washbasins, U-bends and wastewater pipes

All hospital pattern and the domestic pattern ceramic washbasins used in this study were manufactured by Armitage Shanks (Stoke-on-Trent, UK). As discussed in Chapter 1, HP washbasins contain an offset drain outlet in the back wall of the basin, whereas DP washbasins contain an overflow outlet and tap water flow directly impacts the drain outlet in the floor of the washbasin. Examples of each type of washbasin are shown in Figure 1.2 (Chapter 1). Each hospital pattern washbasin faucet had a thermostatic mixing valve set to provide water at 38ºC. The DP washbasin had a manual mixer tap. All washbasins were used for hand washing only with Tork Extra Mild Liquid Soap (SCA Hygiene Products Ltd., Bedfordshire, UK) and were in frequent use Monday-Friday each week.

All washbasins investigated in the present study were located across four floors in DDUH. These include staff bathrooms 5 & 6 on the third floor, staff bathrooms 3 & 4 as well as Clinic 2 located on the second floor, staff bathroom 1 & 2, Clinic 1 and the Central Sterile Services Department (CSSD) located on the first floor, and both the Accident and Emergency (A&E) Department and West Clinic (WC) on the ground floor. A schematic outlining the floor plan of DDUH is shown in Figure 2.3.

The washbasin U-bends used in this study were manufactured from polypropylene (McAlpine Plumbing Products, Glasgow, Scotland) and equipped with two sampling ports (Figure 2.4). The U-bends used in this study are similar to those found throughout other healthcare facilities, however they included two sampling ports for the purpose of sampling in this study. The wastewater pipes and fittings servicing all washbasins in DDUH were made of polyvinylchloride (PVC) or acrylonitrile-butadiene-styrene (ABS), both of which are compatible with long-term exposure to anolyte and catholyte (Deasy e*t al.*, 2018).

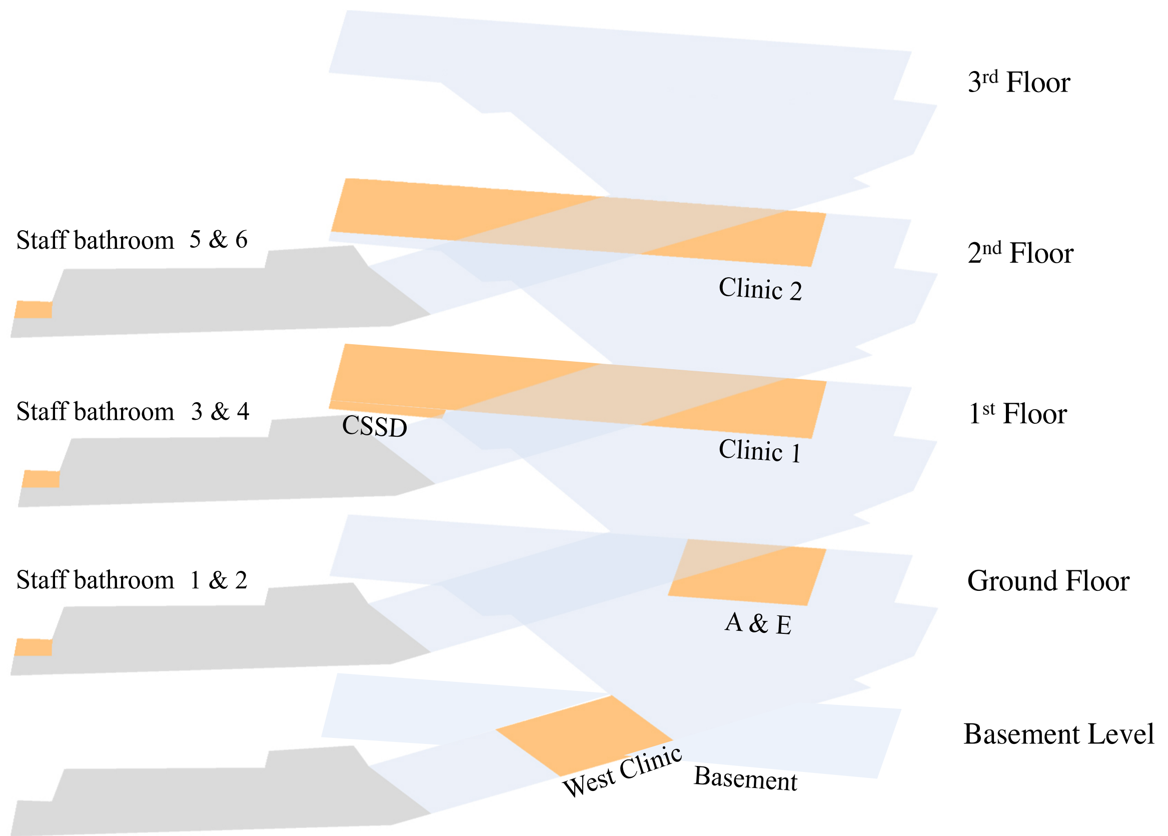


Figure 2.3 Schematic diagram of the DDUH floor plan showing the relative locations of clinics and bathrooms in which washbasins were investigated. Over the course of this study, the following washbasin, U-bends and drains were sampled in the following locations: the staff bathroom 5 & 6 on the third floor, the staff bathrooms 3 & 4 as well as Clinic 2 located on the second floor, the staff bathrooms 1 & 2, Clinic 1 and Central Sterile Services Department (CSSD) located on the first floor, both the Accident and Emergency (A&E) Department and West Clinic (WC) on the ground floor.

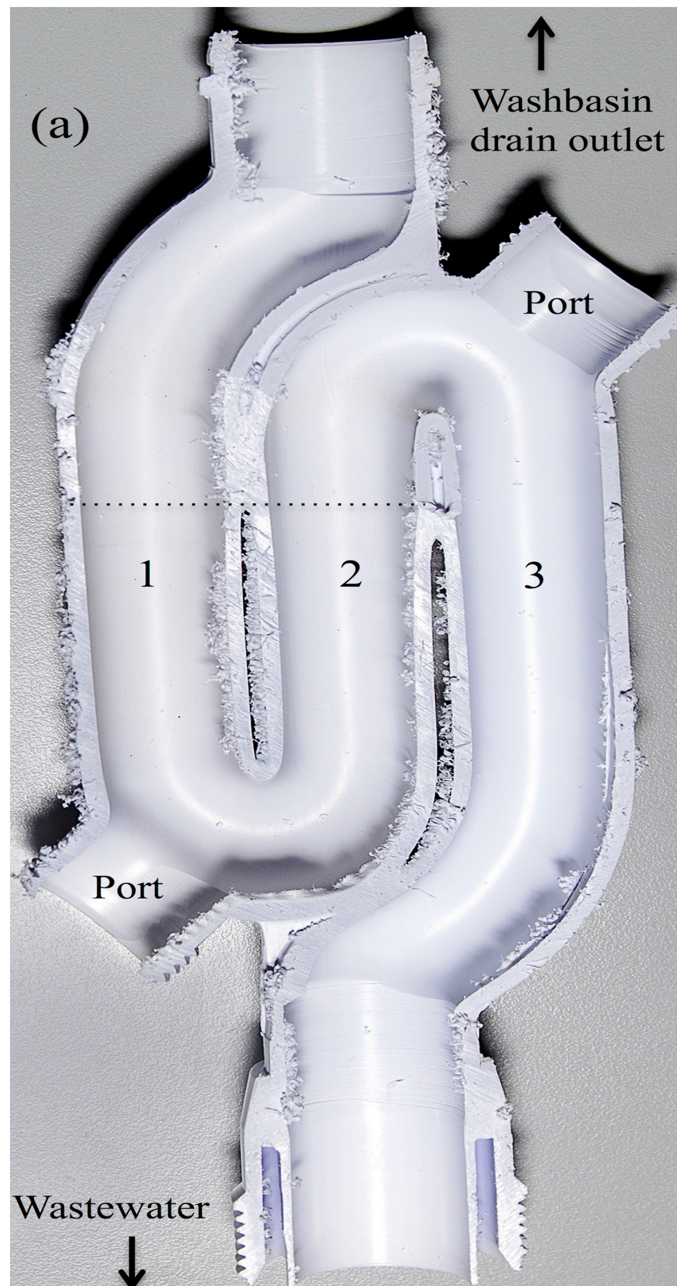


Figure 2.4 Photograph showing a cross-section of one of the polypropylene U-bends fitted to DDUH washbasins. The washbasin U-bend is located immediately below the washbasin and is connected directly to the washbasin drain outlet. The lower part of the U-bend is connected to a vertical wastewater pipe that discharges into a common wastewater collection pipe that discharges wastewater to the municipal sewerage system. Swab sampling of the internal sections of the U-bend were facilitated by the incorporation of two sampling ports. The dashed lines indicate the water level within the U-bends. Six internal sampling sites within the washbasin U-bends were sampled in rotation. Three of these (labelled 1 – 3) are shown in the panel (explained fully in Chapter 3, Figure 3.6). The additional three sites were located on the other, mirror image half of the U-bend.

All pipes and connections (except for U-bends) were permanently sealed by chemical welding to reduce the potential for leaks (Deasy *et al*., 2018).

2.6.1 Testing of washbasin faucets and water for *P. aeruginosa*

Cold water to washbasin faucets was provided from a 15,000-L tank supplied with mains water, which also supplied a calorifier providing faucet hot water. Hot and cold water supplied to DDUH washbasins is treated with residual anolyte (2.5 ppm), an electrochemically activated disinfectant solution composed predominately of hypochlorous acid (Boyle *et al*., 2012). One litre water samples from washbasin faucets were taken in sterile glass bottles, neutralised with 0.5 % sodium thiosulfate ($Na_2S_sO_3$) (0.5g sodium thiosulfate per 100 ml ultra-purified water) and filtered vacuum filtered through 0.45 µm Biosart microbial filtration units (Sartorius Stedim Biotech GmbH, Goettingen, Germany). Following filtration, filters were removed using a sterile forceps and placed on a PSCN agar plate. The plates were incubated upside down in a static incubator at 30°C for 48 h, after which they were visually inspected for growth on the PSCN agar plate and colony counts were recorded.

2.6.2 Testing of the drain outlet for *P. aeruginosa*

All investigated washbasin drain outlets were sampled using sterile viscose transport swabs (Sarstedt, Nümbrecht**,** Germany). If the drains had been treated with ECA solutions, the swabs were dipped into a neutralising solution of 0.5% sodium thiosulfate prior to sampling. The swabs were processed by cutting off swab tips and placing them into individual microcentrifuge tubes containing 1 ml of sterile PBS. The tubes were vortexed (IKA) for 1 min, serially diluted and 100 µl aliquots spread in duplicate on CBA, R2A and PSCN agar plates. The plates were incubated as detailed in Section 2.2.1 above. Following incubation, the colonies were counted using a Stuart™ Scientific colony counter and the colony counts were recorded in CFU per swab.

2.6.3 Testing of washbasin U-bends for *P. aeruginosa*

All washbasins used in the study were equipped with U-bends containing two integrated sampling ports. Six internal sampling sites were identified within the washbasin U-bends (Figure 2.4). This permitted six selected sites to be sampled in rotation to reduce the mechanical removal of biofilm from washbasin U-bends. Samples were recovered from washbasin U-bends by swab sampling of the interior surface of the U-bend through the sampling ports using sterile viscose transport swabs (Sarstedt) dipped in a neutralising

solution of 0.5% sodium thiosulfate. The swabs were rotated 360° three times within the sample ports, covering an average swab contact surface area of 3 cm x 1 cm. Prior to sampling, all sampled U-bends were flushed with tap water. Once collected, the swab tip was cut off, suspended in 1 ml of sterile PBS, vortexed, serially diluted and 100 µl aliquots spread in duplicate on CBA, R2A and PSCN. The plates were incubated as stated in Section 2.2.1. Following incubation, the colonies were counted using a Stuart™ Scientific colony counter and the colony counts were recorded in CFU per swab.

2.7 Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, San Diego, USA). Statistical significance of one set of data was determined using an unpaired, two-tailed Student's t-test with 95% confidence interval (C.I.).

Chapter 3

Minimising microbial contamination risk simultaneously from multiple hospital washbasins by automated cleaning and disinfection of U-bends with electrochemically activated solutions

3.1 Introduction

Over the last two decades many studies have reported hospital outbreaks, caused mainly by Gram-negative bacilli (GNB), associated directly or indirectly with contaminated washbasins and sink drains (Cholley *et al.,* 2008; Hota *et al.,* 2009; La Forgia *et al.,* 2010; Breathnach *et al.,* 2012; Vergara-López *et al.,* 2013; Leitner *et al.,* 2015; Chapuis *et al.,* 2016; Jung *et al.,* 2020; Tracy *et al.,* 2020). As described in Chapter 1, U-bends are pieces of shaped pipework fitted beneath washbasins that retain a volume of water, creating a seal preventing sewer gas from entering buildings from the downstream pipework. This water may stagnate for considerable periods, encouraging the development of biofilms. The spread of microorganism from U-bends has been observed with biofilm growth upwards towards the washbasin drain, which can contaminate the washbasin and surrounding areas (Swan *et al*., 2016; Kotay *et al*., 2017). The mode of transmission of these potentially pathogenic microorganisms from the wastewater drain outlet into the hospital environment can include contact, ingestion, or inhalation. These transmission events may occur by either direct contact or interaction with microorganisms spread due to the generation of aerosols or spread of droplets from tap water impacting drain outlets (Chinn and Sehulster, 2003; Kotay *et al.,* 2019). The establishment of effective decontamination practises, in particular washbasins U-bends and drains, is vital for the reduction of HAIs associated with wastewater networks (Hanlin and Myers, 2018).

A range of approaches have been developed to minimise the risk of infection from washbasins, sink drains and U-bends in the hospital setting with varying degrees of success (Table 3.1). As discussed briefly in Chapter 1, previous approaches have revolved around one or more of the following: (i) washbasins designed to reduce release of microorganisms from drain outlets, (ii) conventional disinfection of washbasin and sink drains, (iii) self-disinfecting sink drains, (iv) 'water-free' patient care, (v) the use of antimicrobial materials, and (vi) replacement of washbasins and U-bends. The quantification of bacterial burden within washbasins, drains and U-bends has primarily been determined by the enumeration of microorganism that readily grow on the selected media (Cundell, 2015). The predominant measurement for estimating the viable number of microbial cells in a sample is recorded in colony forming units (CFUs). The unit for measuring bacterial densities recovered from swab sampling, where colonies may represent one or more cells, is determined in colony forming units per swab (CFU/swab).

Table 3.1 Previous approaches for minimising the risk of infection from washbasin U-bends

Intervention	Advantage	Disadvantage	Reference
Use of disinfectants	Effective at minimising contamination	Requires regular use. Negative effects on the environment. Labour intensive. Organic material can affect disinfection efficiency. Reduced contact time of disinfectant with pipework and incomplete contact with all wastewater pipe surfaces.	(Clarivet *et al.*, 2016; Stjarne Aspelund *et al.,* 2016; Swan *et al.*, 2016; Kossow *et al.*, 2017; Parkes and Hota, 2018; Buchan *et al.*, 2019)
HP washbasins	Prevents generation and dispersal of aerosols and droplets	Washbasin U-bends and drain outlets remain heavily contaminated.	(Department of Health UK, 2013)
'Water-free' patient care	The absence of washbasins eliminates the risk of infection from U-bends and drains	Water is necessary for all aspects of healthcare. Need to store water supplies. Could discourage hand hygiene and frequency of hand hygiene.	(Salm *et al.,* 2016; Hopman *et al.*, 2017, 2019; Mathers *et al.,* 2018; Shaw *et al.,* 2018)
Self-disinfecting drains	Automated system effective at minimising biofilm in U-bends	Necessity for regular heating to high temperatures resulting in high energy costs. Potential to release foul odours from drains.	(Döring *et al.,* 1991; Fusch *et al.,* 2015; Cole and Talmadge, 2019; de Jonge *et al.,* 2019)

Table 3.1 continued overleaf

Table 3.1 (continued)

Modified washbasin drain covers	Water flow does not impact drain outlets, reducing dispersal of contaminated aerosols and droplets	Not proven in long-term. Made from materials on which biofilms form readily, which could lead to further contamination.	(Livingston *et al.,* 2018)
Antimicrobial materials for wastewater fixtures and fittings	Use of materials such as copper that exhibit significant antimicrobial effects.	Copper pipes and associated fittings are expensive to purchase and expensive to install. The development of oxidation layers over time reduces antimicrobial effects.	(Schwartz *et al.,* 1998; Lehtola *et al.*, 2004; Waines *et al.,* 2011)
Replacement of wastewater network fixtures and fittings	Eradication of a reservoirs of contamination.	A short-term solution only as replaced pipes and U-bends can rapidly become recolonised from downstream wastewater pipes.	(Breathnach *et al.,* 2012; Starlander and Melhus, 2012; De Geyter *et al.,* 2017).

In recent years an array of washbasins design adaptions have been explored to reduce the release of microorganisms from drain outlets. As described in Chapter 1, the use of HP washbasins with offset drain outlets reduces the incidence of aerosolisation of microorganisms from drain outlets by eliminating direct impact of water flow with the drain. This also reduces splashback of material and microorganisms from drains. Likewise, HP washbasins do not have overflows, plug stoppers and chains, removing a protected opening from the wastewater network into the hospital environment and reducing the overall surface area for biofilm formation. Where HP washbasins have not been adopted, a number of studies have developed drain covers to prevent the aerosolisation and spread of microorganisms from within the U-bend and proximal drain outlet by the impact of the running water (Livingston *et al.,* 2018; Mather*s et al.,* 2018). Livingston *et al.* (2018) utilised plastic dome-shaped drain covers on washbasins in an ICU. In the facility 97% of sink drain outlets were colonised by GNB, but within the two week test period where the drain covers were incorporated, no dispersal of GNBs were detected by swab sampling of the sink bowl, adjacent surfaces or the top of the drain cover (Livingston *et al.,* 2018). However, the efficacy of the a plastic drain cover over a long time period has not been determined.

The use of conventional disinfection of washbasin and sink drains has been a universally adopted approach for minimising the risk of infection from washbasin U-bends. Disinfection is defined as the process of reducing the number of microorganisms to acceptable safe levels but not necessary all microorganisms (Hawley and Kozlovac, 2005). The use of disinfectants, such as chlorine, has been widely adopted for their antimicrobial properties for over 100 years (Cochran *et al.,* 2000). A 2017 study of a French hospital concluded that metallo-β-lactamase IMP-19–producing *P. aeruginosa* were endemic in the hospital drains and pipework, which were not readily accessible for decontamination (Amoureux *et al.,* 2017). Disinfectants may exhibit a range of antimicrobial effects including reversible or irreversible damage to the bacterial cell wall, or may act on nucleic acids, or by inhibiting enzymes or cell growth (Montagna *et al.*, 2019). Previously, liquid and foam disinfectants used to decontaminate washbasin drains and U-bends have included sodium hypochlorite solution (also known as bleach), hypochlorous acid, hydrogen-peroxide, quaternary ammonium compounds (QACs) and acetic acid (Clarivet *et al.*, 2016; Stjarne Aspelund *et al.*, 2016; Swan *et al.,* 2016; Kossow *et al.,* 2017; Parkes and Hota, 2018; Buchan *et al.,* 2019; Jones *et al.,* 2020). A study by Stjärne Aspelund *et al*. used a combination of acetic acid treatment and sink replacement to control a metallo-β-lactamase-producing *P.*

aeruginosa outbreak associated with contaminated sink drains, yet the wastewater pipes remained colonised after treatment (Stjarne Aspelund *et al.,* 2016).

While widely used, there are a number of limitations associated with a disinfectant-only decontamination approach for washbasin drains and U-bends. Disinfectants have diminished efficacy against dense biofilms present in U-bends and whereas they can temporarily reduce bioburden, they have to be applied regularly (Hota *et al*., 2009; La Forgia *et al.*, 2010; Stjarne Aspelund *et al.*, 2016). Inadequate decontamination of U-bends using chemical disinfectants is associated with neutralisation of disinfectants by heavy organic loads, failure to penetrate dense biofilm matrix and inadequate contact time. Effective decontamination of any device or system first requires a cleaning process to reduce the amount of organic material and other deposits present, followed by disinfection to reduce the number of residual microorganisms (Dancer, 2014). However, manually pouring disinfectants down washbasin drains is not an effective approach to decontaminating U-bends and drains, as it has previously shown transient results (Fusch *et al.,* 2015; Cadnum *et al.*, 2019). This is due to the short contact times between the disinfectant and the biofilm present in drains and U-bends. Washbasin and sink U-bends are seldom completely filled or flooded with disinfectant solution as wastewater networks are designed to rapidly transport wastewater to the point of discharge into the municipal sewerage system. In frequently used washbasins and sinks, disinfectant in the U-bend is displaced by wastewater discharged down drains. Vergara-Lopez *et al.* installed manual shut off valves into sink drainage pipes to increase contact time between the disinfectant and wastewater pipes in an attempt to control a *Klebsiella oxytoca* hospital outbreak in an ICU (Vergara-López *et al.*, 2013). Once the valves were shut, a 30 min treatment with Biguanid, a surface disinfectant based on quaternary ammonium compounds was implemented, followed by flushing with hot water. This approach was successful in terminating the hospital ICU outbreak. However, owing to the manual operation of the stop valves, air bubbles may be incorporated into the system and reduce overall contact of the disinfectant with the pipework (Coleman *et al.*, 2020).

Self-disinfecting sink drains were first proposed in 1991 to reduce the risk of infection from wastewater networks. Döring *et al.* (1991) demonstrated the effective elimination of detectable *P. aeruginosa* in aerosols generated by the impact of water flow on drain outlets. This was achieved following the introduction of a heating element into U-bends to decontaminate drain water and the drain by heating to 70°C overnight (Döring *et al.*, 1991). In 2015, this concept was again shown to be effective with self-disinfecting sinks that

contained a heating element in the U-bend that heated the water in the drains to ≥ 85°C, followed by 5 min vibration cleaning (Fusch *et al.*, 2015). Over the 13-month study period, the self-disinfecting sink significantly reduced of the prevalence of *P. aeruginosa* in aerosols generated by the impact of water flow on the drain. A 2019 study, engineered an experimental sink combining UV light to kill any bacteria within the basin, a hood to contain aerosols by establishing a negative pressure environment to control aerosols generated, ozonated water and a specific spray and flush system to regularly spray the basin behind the sink hood and to flush the drain and U-bend (Cole and Talmadge, 2019). Settle plates were used to recover aerosolised microorganisms resulting from the impact of water flow on the drain outlet. This study showed reduced bacterial recovery compared to the aerosols generated by water flow with the control DP washbasins. A major limitation of self-disinfecting sinks has been the costs of the units themselves and the high energy costs incurred with their use.

Removing washbasins from patient areas in hospitals has been explored as a means of reducing HAIs associated with washbasin U-bends and drains. A study by Hopman *et al.* (2017) reported that removal of sinks in an ICU of a large tertiary care medical centre in the Netherlands led to a reduction in the rate of GNB colonisation rates. The removal of washbasins in clinical areas near patient rooms showed a drop in overall GNB colonisation rate 26.3 to 21.6 GNB/1000 ICU admission days (Hopman *et al.,* 2017). Similarly, over a study period of six years, Shaw *et al.* (2018) demonstrated that reducing patient sink use reduced the incidence of GNB in surrounding environments (Shaw *et al.,* 2018). Interestingly, in this study these interventions had a greater impact in reducing the number of new cases of *Klebsiella pneumoniae* rather than *P. aeruginosa*. The authors suggest that this finding may reflect *P. aeruginosa* spread down the wastewater pipes, while *K. pneumoniae* remained in the proximal drains. A 2020 outbreak study demonstrated that the cessation of the routine practice of bathing infants in washbasins and the subsequent restriction of sink use, rapidly decreased the number of infants colonised by MDR GNB (Tracy *et al.,* 2020). These approaches have reduced incidences of colonisation of patients and health-care workers, but do not completely eliminate patients or health-care workers interacting with hospital water systems. A key limitation to this approach is the increased demand for stored water facilities, which can increase the potential for contamination as discussed in Chapter 1. The guideline quantities for water need per patient per day in a healthcare facility is 5 litres per out-patient and 40–60 litres per in-patient (WHO, 2013).

Furthermore, the absence of washbasins is likely to discourage good hand hygiene practices in the hospital setting.

The use of antimicrobial materials in washbasin U-bend design has also been considered for reducing the formation of biofilms. Most modern washbasin U-bends are manufactured from polyvinyl chloride (PVC), and wastewater pipes are commonly manufactured from PVC, polyethylene (PE), or high-density polyethylene (HDPE). Schwartz *et al*. (1998) used coupons of PE, PVC and stainless steel, as examples of materials commonly used for water pipes, to determine their relative ability to support microbial biofilm (Schwartz *et al.,* 1998). PE coupons were able to support more biofilm than PVC coupons. Furthermore, PE and PVC coupons were colonised rapidly in significantly higher densities than either steel or copper coupons (Schwartz *et al.,* 1998). In a subsequent study over 200 days, biofilm formation on copper pipes and PE pipes showed no significant differences (Lehtola *et al.,* 2004). The widespread use of copper and steel as antimicrobial materials is limited. This is due to materials selection primarily focusing on factors such as the cost of the material, the ease of installation, and the durability of the material. Also the antimicrobial activity of copper pipes reduces over time due to the formation of oxidation layers, which significantly reduces the release of copper ions and thus reduces antimicrobial effects (Waines *et al.,* 2011).

The final approach used to reduce the risk of outbreaks of infections from washbasin and sink drains and U-bends has been the removal and replacement of wastewater network components (Breathnach *et al.,* 2012; Starlander and Melhus, 2012; De Geyter *et al.,* 2017). While this approach is effective in the short term, there are a number of limitations. The removal and replacement of washbasins and associated pipework is an expensive solution, which also causes disruptions to service in healthcare facilities. Furthermore, the mobility of microorganisms commonly found in U-bends and wastewater pipes causes the replaced components to become rapidly recolonised from the contaminated downstream wastewater pipe network (Swan *et al.,* 2016; Kotay *et al.,* 2017). Replacement of sinks and U-bends has been shown to stop outbreaks in numerous studies, however the approach is not effective in the long-term due to recolonisation (Lowe *et al.,* 2012; Vergara-López *et al.,* 2013; Salm *et al.,* 2016; Stjarne Aspelund *et al.,* 2016; Kizny Gordon *et al.,* 2017). Replacing wastewater network components (e.g. U-bends) is usually used as a last resort approach to eradicate a significant reservoir of contamination associated with a protracted outbreak.

This chapter focuses on establishing proof of concept for a large-scale system developed for the simultaneous automated decontamination of multiple washbasin U-bends in an active healthcare facility using ECA solutions. The ECA solution anolyte has been used successfully for many years to efficiently minimise microbial contamination of dental unit waterlines in DDUH (O'Donnell *et al*., 2009; Boyle *et al*., 2010). Residual treatment of supply water with anolyte has also been used successfully to consistently minimise microbial contamination of washbasin hot (average aerobic bacterial counts 1 ± 4 CFU/ml) and cold water (average aerobic bacterial counts 2 ± 4 CFU/ml), and washbasin faucets (Boyle *et al*., 2012). The system used in the present study involves automated sequential treatment of multiple washbasin U-bends with the ECA solutions catholyte followed by anolyte. Catholyte solution exhibits detergent properties and was selected to clean washbasin U-bends and drains, whereas anolyte solution has disinfectant properties and was used to disinfect U-bends and drains following catholyte treatment. The system permits the complete filling of U-bends, common wastewater pipes connecting U-bends and washbasin drain outlets with ECA solutions by retro-filling from below. This is facilitated by closing an electronic ball valve on the downstream wastewater pipe that collects wastewater from multiple washbasins. The automated U-bend decontamination system described in this chapter is based on a smaller prototype programmable automated system developed in DDUH with a single washbasin U-bend that was shown be effective at minimising U-bend contamination over 35 decontamination cycles with a >4 log reduction in bacterial counts on a variety of culture media compared to controls (Swan *et al*., 2016).

3.2 Objectives

The purpose of this study was to develop and test the effectiveness of a large-scale programmable automated system for the simultaneous decontamination of multiple washbasin U-bends using sequential treatment of U-bends, associated wastewater pipework and washbasin drains with catholyte followed by anolyte. The DDUH Accident and Emergency Department (A&E) was selected as the test site for this work.

The specific aims of this study were:

- To develop a large-scale automated ECA treatment system capable of decontaminating 10 HP washbasin U-bends and drains simultaneously in a busy hospital clinic with no disruption to service.

- To assess the efficacy of ECA solutions as a means of decontamination of washbasin U-bends over a period of 62 cycles, while also investigating potential residual effects of ECA treatment by sampling the U-bends 24 h after each decontamination cycle.

- To identify the microbial populations present in ECA-treated and control U-bends during a sustained period (i.e. 62 decontamination cycles) of recurrent ECA decontamination of test U-bends.

- To monitor the effects of ECA decontamination on biofilm formation in washbasin U-bends following 62 decontamination cycles, visually and using electron microscope examination.

- To determine the impact of ECA decontamination on reducing microbial contamination on the drain outlets of ECA-treated washbasins versus controls.

- To monitor washbasin wastewater network components for potential adverse effects following repeated cycles of ECA decontamination.

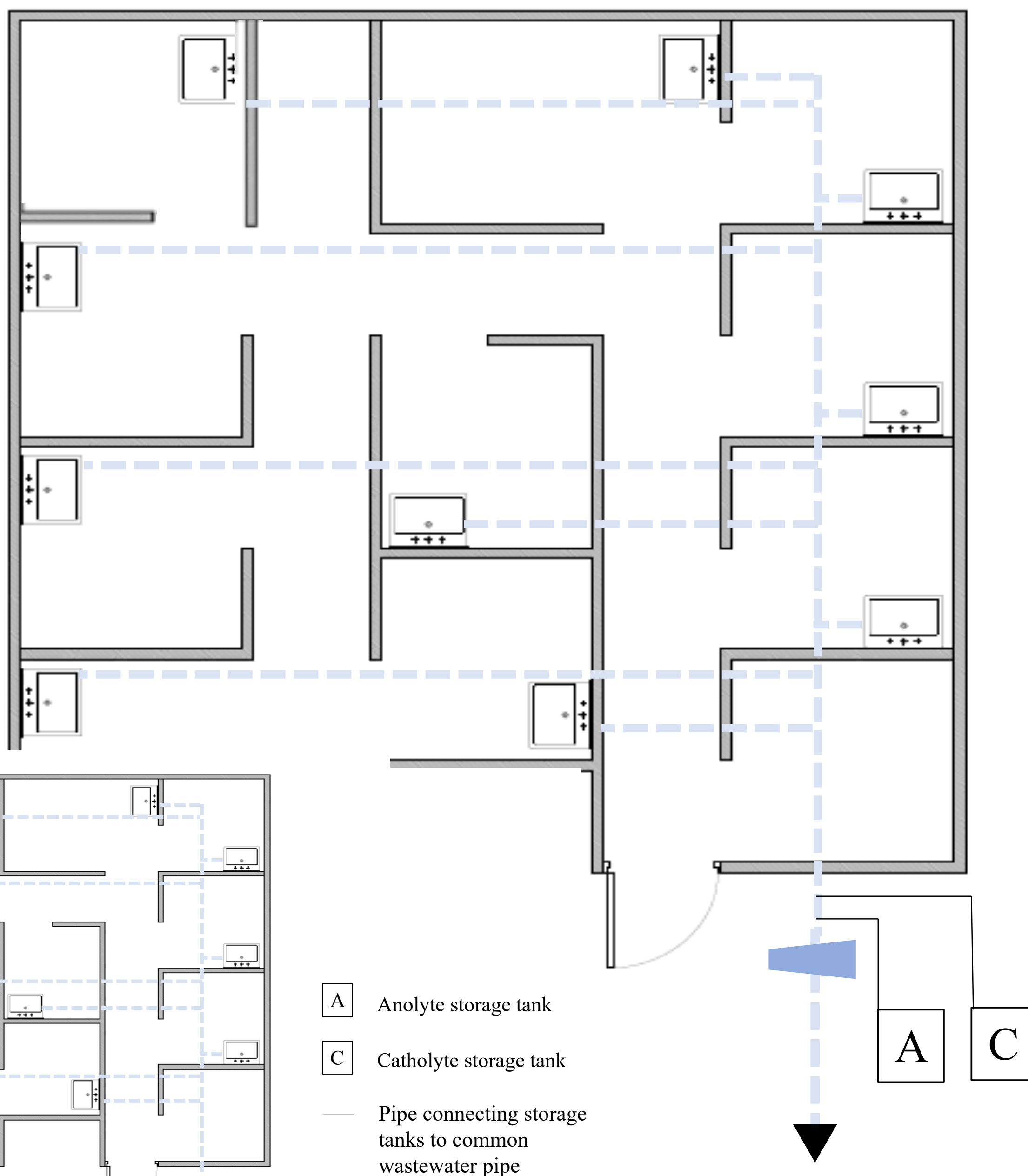
3.3 Materials & Methods

3.3.1 Test and control washbasins

The Accident and Emergency Department (A&E) of the DDUH was selected as the test site for large-scale automated decontamination of washbasins, U-bends and associated wastewater pipes. This square shaped clinic consists of 11 separate clinical treatment bays, each equipped with identical new ceramic HP washbasins (Armitage Shanks, Staffordshire, United Kingdom; compliant with the Department of Health United Kingdom Health Building Note 00-10 Part C: Sanitary assemblies (Department of Health UK, 2013)). The U-bends of 10 of these washbasins were sampled throughout this study (Figure 3.1). All washbasins were fitted with new polypropylene U-bends (McAlpine Plumbing Products, Glasgow, Scotland), which had two access ports, one above and one below the U-bend water line (Chapter 2, Figure 2.4). The U-bends of each of the 10 washbasins were connected by a vertical 1 metre pipe that discharged into a common horizontal wastewater collection pipe, that was located directly beneath the A&E Department in the DDUH basement (Figure 3.1). Washbasin faucets were fitted with a thermostatic mixing valve and provided output water at an average temperature of 38ºC. Hot and cold water supplied to washbasins at DDUH has been treated with residual anolyte (2.5 ppm) for several years (Boyle *et al.,* 2012). The A&E Department operates seven days a week and washbasins are in frequent daily use.

A number of additional washbasins were used as controls. These included one HP washbasin identical to the A&E washbasins located in the DDUH Central Sterile Supplies Department (CSSD) and five ceramic DP washbasins located in five DDUH staff bathrooms (2 – 5). All six control washbasins were fitted with polypropylene U-bends (McAlpine Plumbing Products) identical to those fitted to A&E washbasins. None of the control washbasin U-bends were decontaminated with ECA solutions during the study (Chapter 2, Figure 2.3). All of the control washbasins were in frequent daily use Monday-Friday, and located in non-clinical settings.

A secondary control group was selected to investigate the effects of ECA decontamination on washbasin drain outlets, consisting of six HP washbasins from Clinic 1 (Chapter 2, Figure 2.3). The control group was selected as the primary control group consisted of one HP and five DP washbasins, while all the washbasins from A&E were HP design (Figure 3.2). No other clinics were available for sampling washbasin U-bends due to the use of washbasins without sampling ports, HP washbasins were in wide use throughout clinics in DDUH.



ɩUH Accident & Emergency Department
are located in separate clinical treatment
by a 1-m vertical pipe to a common
ection pipe located directly beneath A&E in the DDUH basement.
used to seal the common wastewater collection outflow pipe to
lectrochemically activated solutions (ECAs) is indicated. The
ɔrage tanks were each connected to a separate dosing pump
connected to the common wastewater pipe located in the basement of DDUH.

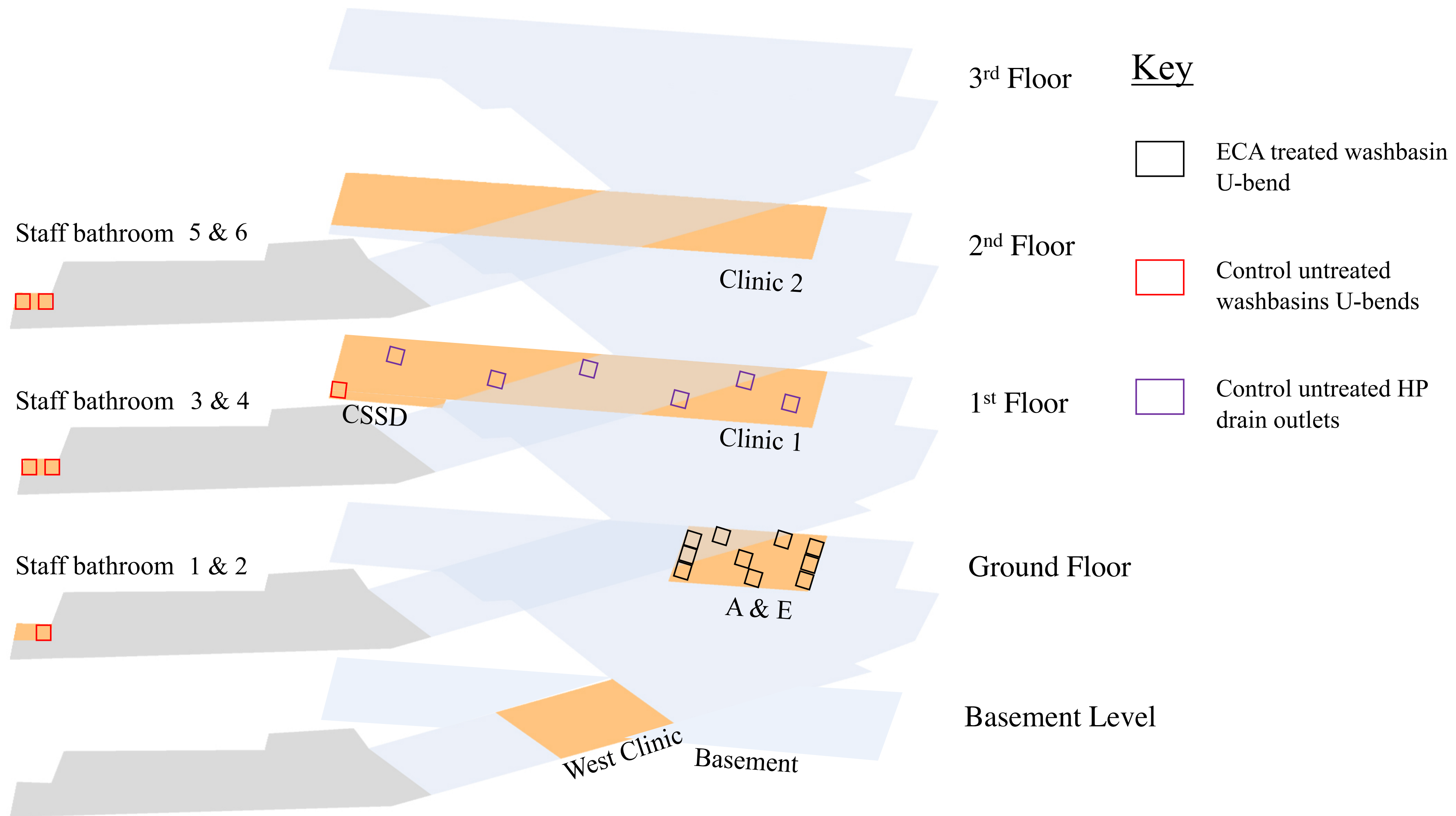


Figure 3.2 DDUH floorplan indicating the 10 sampled ECA-treated washbasin U-bends in the A&E (in black), the five sampled untreated U-bends sampled as comparators (in red) and the five untreated hospital HP drain outlets in Clinic 1 (in purple).

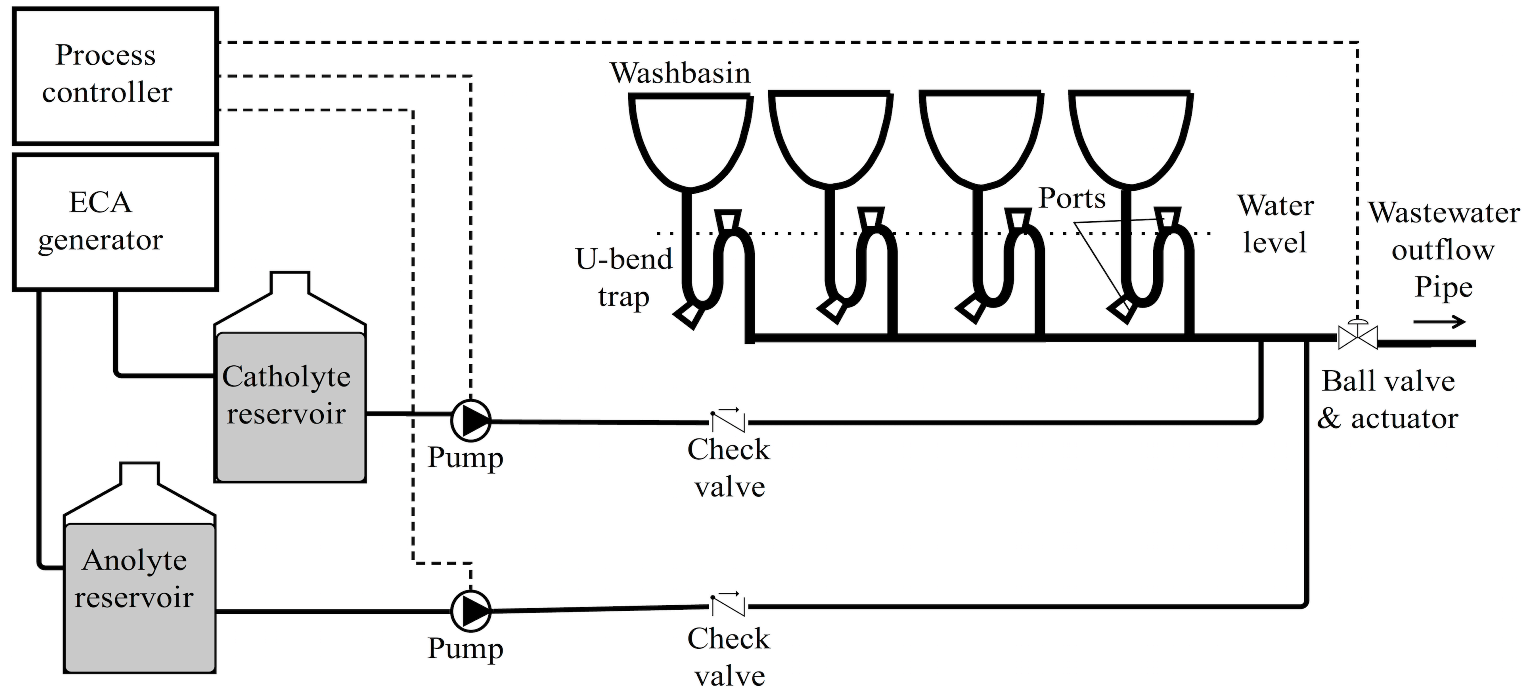
Washbasins were used for hand washing only. Tork Extra Mild Liquid Soap (SCA Hygiene Products Ltd., Bedfordshire, United Kingdom) was used for hand washing with all washbasins. Cold water supplied to test and control washbasin taps was provided from a 15,000 L tank supplied with potable quality mains water. This tank also supplied the calorifier, which provided hot water to all the washbasin taps. Automatic temperature recording was fitted on the out and return legs of the hot water network. Hot and cold water supplied to washbasins at DDUH has been treated with residual anolyte (2.5 ppm) for several years. Previous studies over 54 weeks showed average bacterial densities in hot and cold tap water of 1(± 4) and 2(± 4) CFU/ml, respectively (Boyle *et al*., 2012).

3.3.2 Electrochemically activated solutions

The anolyte and catholyte solutions used in this part of the present study are described in detail in Chapter 2, Section 2.4.

3.3.3 Design of the automated ECA decontamination system for U-bends

Anolyte and catholyte solutions were generated by electrochemical activation of a brine solution (Figure 3.3). The generation of the ECA solutions is described in Chapter 2, Section 2.4. The ECAs were generated, and stored in the basement of DDUH, directly below the A&E Department. The solutions were stored next to the generator in medium density UV-stabilised linear polyethylene tanks and were each connected to a dosing pump (Grundfos, Bjerringbro, Denmark) (Figure 3.4). A 25 mm diameter acrylonitrile butadiene styrene (ABS) rigid pipe from each dosing pump connected to the common wastewater pipe servicing all A&E washbasins. A 100 mm diameter Praher unplasticized-PVC S4 two-way ball valve (Schwertberg, Austria) was used in this study to allow for the retro-filling of the ECAs and holding of the solutions in the system (Figure 3.5). The common wastewater pipe and ball valve were accessible for visual inspection in the basement of DDUH, directly below the A&E Department. A H-004 24 V DC electric actuator was used to operate the opening and closing of the ball valve (Actuated Solutions Ltd., Bognor Regis, United Kingdom). The timing, sequence of activation and duration of activation of the actuator-controlled valve, dosing pumps and ECA reservoir outlet valves was managed by a programmable electronic process controller (Open System Solutions Ltd, Southampton, UK).


(a)
Process controller
ECA generator
Catholyte reservoir
Anolyte reservoir
Pump
Pump
Check valve
Check valve
Washbasin
U-bend trap
Ports
Water level
Wastewater outflow Pipe
Ball valve & actuator

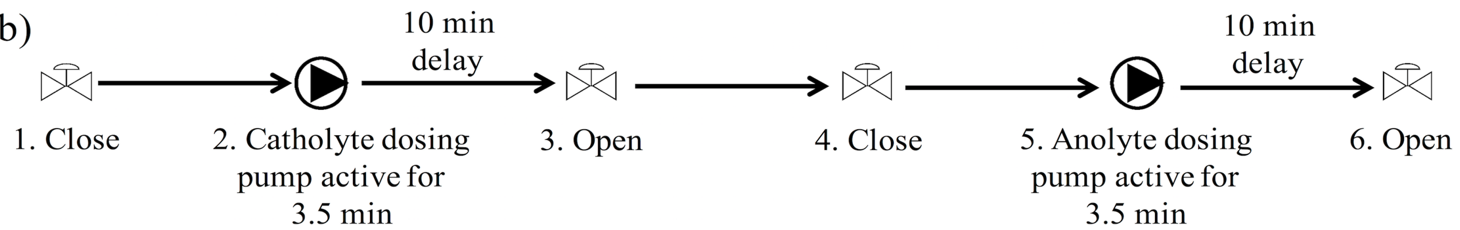
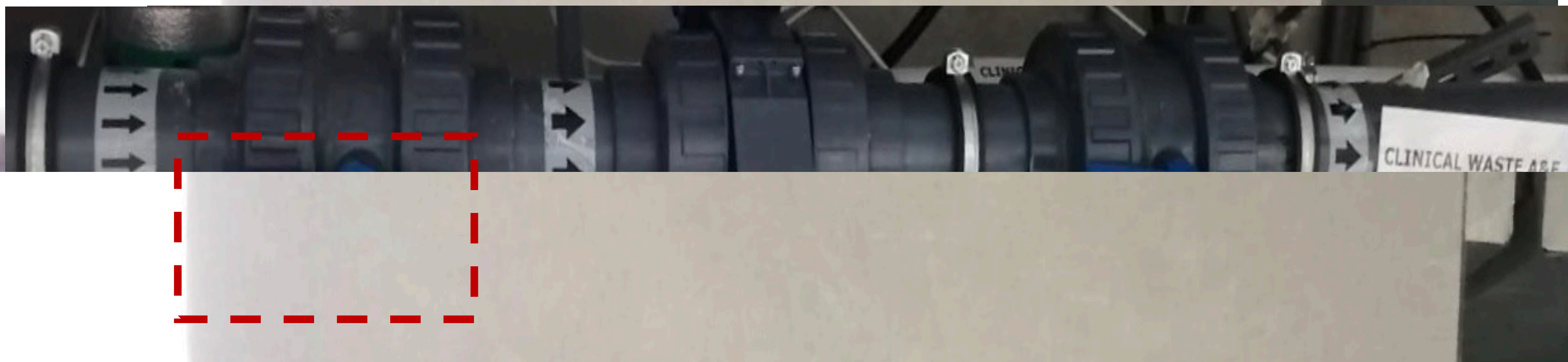

(b)
10 min delay
10 min delay
1. Close
2. Catholyte dosing pump active for 3.5 min
3. Open
4. Close
5. Anolyte dosing pump active for 3.5 min
6. Open

Figure 3.3 Schematic of the automated ECA decontamination system used in this study. Panel **(a)** Diagram of the automated system for the simultaneous decontamination of 10 A&E washbasin U-bends, drain outlets and associated wastewater pipes by sequential treatment with catholyte followed by anolyte throughout the study. Only four washbasins are shown for clarity. Each U-bend had two ports to facilitate sampling. Panel **(b)** Process control schematic for automated decontamination. The programmable process controller initiates treatment cycles. At the start of each cycle the process controller sends a signal to the actuator to close the ball valve on the wastewater outflow pipe. After a 20 s delay, a signal is sent that activates the catholyte dosing pump for 3.5 min and catholyte is pumped into the pipework below the washbasin U-bends until the pipework and U-bends are completely filled to a level a few cm above the washbasin drain outlets. Catholyte is left *in situ* for 10 min, after which time a signal from the process controller opens the ball valve voiding catholyte to the wastewater stream. The ball valve is then closed and after a 20 s delay another signal from the process controller activates the anolyte dosing pump for 3.5 min and the cycle proceeds as per catholyte dosing. After 10 min the anolyte is voided to the wastewater stream completing the cycle.

Figure 3.4 A photograph showing the ECA generator and anolyte and catholyte storage tanks used in this study. All were located in the basement of DDUH under the A&E Department. The anolyte (A) and catholyte (B) storage tanks, the ECA generator (C) and the brine solution storage tank (D) are indicated.


CLINICAL WASTE A&E

3.3.4 Automated ECA solutions decontamination cycle

The ECA decontamination system was programmed to automatically begin the cleaning and decontamination cycle for 13.00 h every Monday, Wednesday and Friday. The clinic was not in use during these times. The decontamination cycles began with the process controller activating the actuator and closing the valve on the common wastewater pipe (Figure 3.2). Following a 30 s delay, the catholyte-dosing pump was activated and began retro-filling catholyte into the wastewater pipe towards the ten washbasins and washbasin drain outlets over a 3.5 min period. The common wastewater pipe, vertical 1-m pipes, U-bends, and washbasins were retro-filled with 220 L of catholyte. Each washbasin was filled to a level of 5-cm above the drain outlet. Once filled, the catholyte-dosing pump was signalled to stop and the catholyte was held *in situ* for 10 min. Following the 10 min contact time, the solution was voided to waste by the opening of the valve on the common wastewater pipe (Figure 3.2). Following a further 30 s delay, the process was repeated with the anolyte solution. Upon completion, the anolyte was discharged to the sewerage system. This completed the automated ECA decontamination cycle. Control washbasins were flushed with mains water instead of ECA.

3.3.5 Microbiological culture

U-bend decontamination efficacy was determined by quantitative microbiological culture of U-bend samples (n = 620) immediately following each decontamination cycle for a total of 62 treatment cycles over the five-month study period in 2017 (3 treatment cycles per week). Additional samples (n = 420) were taken 24 h post-treatment for 42 cycles to assess microbial recovery following decontamination. Contemporaneous samples were also taken from the control U-bends (n = 372) following each cycle of decontamination of the test washbasin U-bends. After completion of the decontamination cycle, U-bends were flushed with tap water prior to sampling to void any retained residual anolyte. The interior surface of the U-bends from the test and control washbasins were sampled through the two sampling ports using sterile viscose transport swabs (Sarstedt, Nümbrecht, Germany) dipped in a neutralising solution of sodium thiosulphate (0.5% w/v) (Boyle *et al*., 2010; Boyle *et al*., 2012). The swabs were rotated 360° three times within the sampling ports, covering an average swab contact surface area of 3-cm x 1-cm. To avoid continually sampling the same part of each U-bend, six internal sampling sites were selected and sampled in rotation (Chapter 2, Figure 2.4). Only one site was sampled following each ECA treatment cycle and swabs were processed immediately. The tip of each swab was processed as described in Chapter 2, Section 2.6.3.

3.3.6 Identification of bacterial isolates

Definitive identification of bacterial isolates recovered on CBA, R2A and PSCN media was undertaken using MALDI-TOF-MS analysis as described in Chapter 2, Section 2.2.4.

3.3.7 Electron microscopy

At the end of the five-month study period of ECA decontamination of U-bends, one ECA-treated U-bend and one untreated U-bend were removed, cut longitudinally and the interior surfaces examined for the presence of microbial biofilm. Small sections of each U-bend were also examined for the presence of biofilm, without prior fixation, by electron microscopy using a Zeiss Supra 35 variable pressure field emission scanning electron microscope (SEM; Zeiss Microscopy, Jena, Germany). The SEM analyses were conducted and carried out under the supervision of the TCD Centre for Microbiology and Analysis.

3.3.8 Statistical Analysis

Statistical analyses were performed as described in Chapter 2, Section 2.7.

3.4 Results

3.4.1 Automated U-bend decontamination with ECA solutions

An automated decontamination system was developed and installed at the DDUH A&E Department that permitted the U-bends, drains and associated wastewater pipes of 10 identical hand washbasins to be completely filled sequentially with the ECA solutions catholyte followed by anolyte. The U-bends were exposed to three weekly decontamination treatment cycles (Monday, Wednesday and Friday) over a five-month period (62 cycles in total). Six additional washbasins located elsewhere in DDUH were used as controls. Swab samples were taken from the internal surfaces of the U-bends and quantitative bacterial counts recovered on CBA, R2A and PSCN agar media were determined. The average bacterial densities recovered from the six-untreated control U-bends during the study period on CBA, R2A and PSCN were 2×10^5 ($\pm 4 \times 10^5$), 3.3×10^5 ($\pm 1.1 \times 10^6$) and 2.7×10^4 ($\pm 1.2 \times 10^5$) CFU/swab, respectively (Table 3.2). The average bacterial densities recovered from the10 ECA-treated U-bends immediately after ECA-treatment (62 cycles) on CBA, R2A and PSCN were 73.4 (± 258.2), 122.5 (± 371.3) and 15.3 (± 184.5) per swab, respectively (Table 3.2). The average bacterial densities of culturable bacteria from ECA-treated U-bends compared to the control washbasins displayed a >3 log difference. Differences in the average bacterial densities recorded from treated U-bends on all media relative to the corresponding counts from untreated U-bends were highly significant ($P < 0.0001$) (Table 3.2). Additional U-bend samples were taken from all 10 treated U-bends 24 h after ECA treatment for 42 of the 62 decontamination cycles and no significant increase ($P > 0.05$) in bacterial counts recovered was observed (Table 3.2).

3.4.1.1 Bacterial count recovered in different U-bend sampling sites

The washbasin U-bends used in this study contain two sampling ports that enabled six sites to be swab sampled in rotation. Figure 3.5 displays a U-bend cut longitudinally showing the six U-bend sampling sites used in this study. For the purposes of this study, the U-bend was divided into two segments: the U-shaped segment that retains water, containing the water entry and exit sections, and the overflow segment (Figure 3.6). The U-bend was situated directly below the offset washbasin drain outlet, which retained water creating the seal within the U-shaped segment of the U-bend (Figure 3.6). The U-bend overflow connected the U-shaped segment to the downstream wastewater drainage pipe (Figure 3.6).

Table 3.2 The average quantitative bacterial counts from ten washbasin U-bends subjected to automated treatment with ECA solutions and the corresponding counts from six untreated U-bends

Agar medium	U-bend	Average bacterial counts in CFU/swab from ECA-treated (*n* = 62 cycles, 620 swabs) and control (*n* = 372 swabs) U-bends	SD	Range of bacterial counts in CFU/swab	*P* value
CBA	Treated	73.4	258.2	0 – 4.6 x 10^3	<0.0001
	Untreated	2 x 10^5	4 x 10^5	0 – 4 x 10^6	
R2A	Treated	122.5	371.3	0 – 5.8 x 10^3	<0.0001
	Untreated	3.3 x 10^5	1.1 x 10^6	0 – 1.8 x 10^7	
PSCN	Treated	15.3	184.5	0 – 3.4 x 10^3	<0.0001
	Untreated	2.7 x 10^4	1.2 x 10^5	0 –1.4 x 10^6	
		Average bacterial counts in CFU/swab 24 h after ECA treatment (*n* = 42 cycles, 420 swabs) and control (*n* = 252 swabs) U-bends[a]			
CBA	Treated[a]	53.2	127.6	0 – 1 x 10^3	<0.0001
	Untreated	2.1 x 10^5	4.3 x 10^5	500 – 3.2 x 10^6	
R2A	Treated[a]	91.7	277.6	0 – 3.5 x 10^3	<0.0001
	Untreated	2.9 x 10^5	6.1 x 10^5	1.3 x 10^3 – 5 x 10^6	
PSCN	Treated[a]	15.6	119	0 –1.7 x 10^3	<0.0001
	Untreated	2.6 x 10^4	1.1 x 10^5	0 – 1.4 x 10^6	

[a] The average bacterial counts in CFU/swab were determined for the 10 ECA-treated U-bends and the 6 untreated U-bends 24 h after treatment for 42/62 ECA treatment cycles. Abbreviations: ECA, electrochemically activated; CBA, Columbia blood agar; R2A, R2A agar; PSCN, *P. aeruginosa* selective agar; SD, standard deviation.

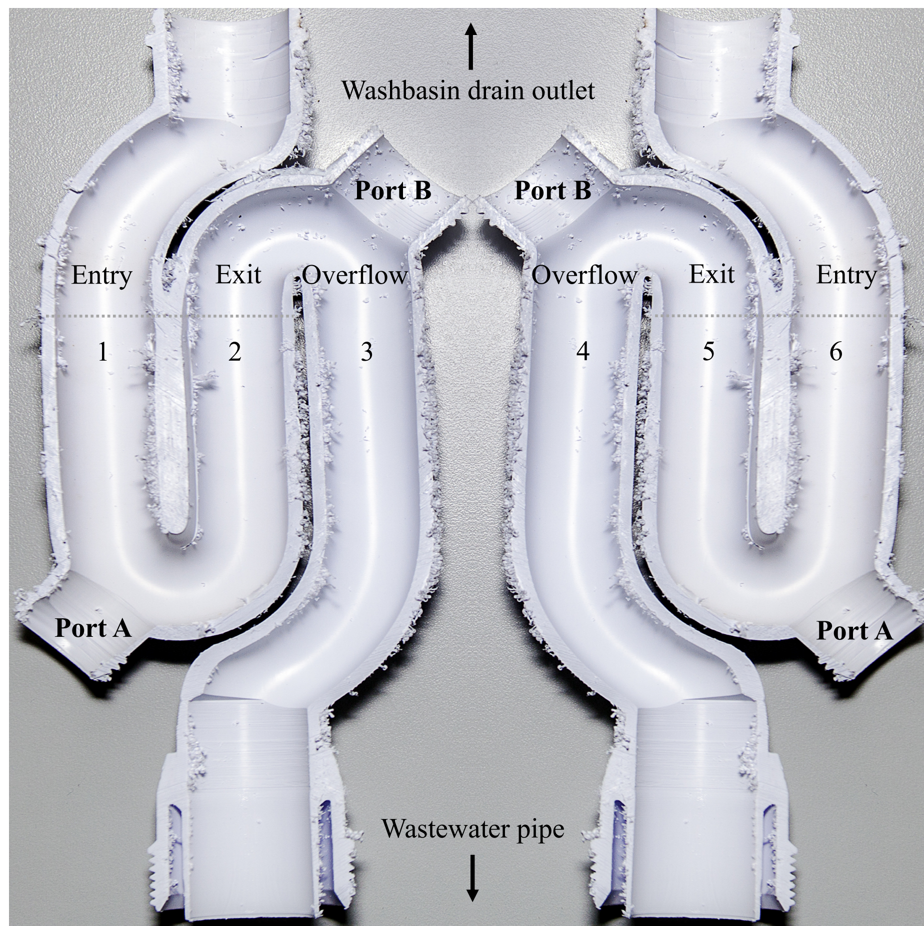


Figure 3.6 An example of one of the washbasin U-bends used in this study cut longitudinally to show the six U-bend sampling used. For the purposes of this study, the U-bend was divided into two segments: the U-shaped segment, containing the water entry and exit sections, and the overflow segment. The U-bend is situated directly below the offset washbasin drain outlet, which retains water creating the seal within the U-shaped segment of the U-bend. The dashed line represents the air-water junction, the level to which water in the seal is filled. The U-bend overflow connects the U-shaped segment to the downstream drainage wastewater pipe. Swab samples were taken in rotation from the two sampling ports. Port A is positioned at the bottom of the U-shaped segment of the U-bend and Port B is positioned at the U-bend overflow. Port A facilitated sampling of the water entry section of the U-shaped segment of the U-bend, with sampling sites labelled 1 and 6. The longitudinally cut washbasin U-bend demonstrates the two sampling sites on opposite sides of each section. Port B facilitated sampling of the water exit section of the U-bend segment, with sampling sites labelled 2 and 5. This U-bend segment was labelled 'exit' as the water exited the U-bend via the overflow towards the sewer network. Port B also facilitated sampling of the 'overflow' segment of the U-bend with sampling sites labelled 3 and 4.

Swab samples were taken in rotation from the two sampling ports. One sampling port (Port A) was positioned at the bottom of the U-shaped segment of the U-bend (Figure 3.5). The other sampling port (Port B) was positioned at the U-bend overflow (Figure 3.5). Port A facilitated sampling of the water entry section of the U-shaped segment of the U-bend (see sampling sites 1 and 6 in Figure 3.5). This section was labelled 'entry' as the water entered the U-bend from the drain outlet. The longitudinally cut washbasin U-bend in Figure 3.5 displays the two sampling sites on opposite sides of each section. Port B facilitated sampling of the water exit section of the U-shaped segment of the U-bend (see sampling sites 2 and 5 in Figure 3.5). This segment was labelled 'exit' as the water exited the U-bend via the overflow towards the sewer network. Port B also facilitated sampling of the 'overflow' segment of the U-bend (see sampling sites 3 and 4 in Figure 3.5). The bacterial densities recorded from sample sites 1 to 6 were amalgamated under the three section labels of the U-bend: entry, exit and overflow (Table 3.3).

On all three media the 'entry' section of the U-shaped segment of the U-bend, which allowed recovery of bacterial within the water retained section at the air-water interface, exhibited a higher average recovered bacterial density from ECA treated U-bends than both the exit and overflow sections (Table 3.3).

3.4.2 Bacterial species identified from ECA-decontaminated and control U-bends

The range of Gram-positive and Gram-negative bacterial species identified from ECA-treated and control U-bends throughout the study is shown in Table 3.4. Although the bacterial density in ECA-treated U-bends was consistently much lower than the control U-bends, the diversity of species identified was greater. This was due to a greater number of Gram-positive bacterial species identified comprising several species of staphylococci (Table 3.4). The array of Gram-negative bacterial species identified from treated and control U-bends were similar. *Pseudomonas aeruginosa* was recovered from all 10 treated and all six control U-bends during the course of the study and was recovered from 78% (290/372) of the control and 12% (74/620) of the ECA-treated U-bends samples investigated, respectively.

3.4.2.1 Bacterial species identified from ECA-decontaminated U-bends immediately following decontamination.

A narrower range of Gram-positive and Gram-negative bacterial species was identified from ECA-decontaminated washbasin U-bends immediately after decontamination (Table 3.5).

Table 3.3 The average bacterial counts from the 10 ECA treated washbasin U-bends and six non-treated U-bends, based on swab sampling of the different internal sampling sites (*n* = 33/U-bend location)

	U-bend location[a]	Agar medium	Average bacterial counts in CFU/swab	SD	Range of CFU/swab
ECA-treated	Outflow (Locations 3 and 4)[b]	CBA	47.93	104	0 – 509
		R2A	95.77	146.5	0 – 600.5
		PSCN	15.29	52.04	0 – 268
	Exit (Locations 2 and 5)[b]	CBA	57.46	58.06	0 – 208.9
		R2A	83.98	117.4	0 – 660
		PSCN	8.93	29.9	0 – 171.5
	Entry (Locations 1 and 6)[b]	CBA	98.38	98.67	0 – 335.5
		R2A	171.2	206.5	2 – 821.5
		PSCN	23.35	65.06	0 – 346
Untreated	Outflow (Locations 3 and 4)[b]	CBA	1×10^5	4.2×10^4	$3.89 \times 10^4 – 2.03 \times 10^5$
		R2A	1.65×10^5	1.06×10^5	$1.8 \times 10^4 – 4.9 \times 10^5$
		PSCN	1.83×10^4	3.17×10^4	$352 – 1.27 \times 10^5$
	Exit (Locations 2 and 5)[b]	CBA	1.51×10^5	1.12×10^5	$1.68 \times 10^4 – 5.84 \times 10^5$
		R2A	2×10^5	1.49×10^5	$2.79 \times 10^4 – 8.43\ 10^5$
		PSCN	1.26×10^4	1.36×10^5	$1.9 \times 10^3 – 7.5\ 10^4$
	Entry (Locations 1 and 6)[b]	CBA	1.59×10^5	7.91×10^4	$4.35\ 10^4 – 3.88 \times 10^5$
		R2A	2.33×10^5	2.73×10^5	$4.54 \times 10^4 – 1.65 \times 10^6$
		PSCN	1.67×10^4	2.47×10^5	$1.14 \times 10^3 – 1.28 \times 10^5$

[a] The three U-bend segments entry, exit and overflow refer to the segments within the U-bends used in DDUH.

[b] The locations of these sites in relation to Figure 3.5.

Abbreviations: ECA, electrochemically activated; CBA, Columbia blood agar; R2A, R2A agar; PSCN, *P. aeruginosa* selective agar; SD, standard deviation; CFU/swab, colony forming unit per swab.

Table 3.4 Bacterial species recovered from ECA-treated and control U-bends during the study

Bacterial species identified in ECA-treated U-bends	Bacterial species identified in ECA-treated and non-ECA treated U-bends	Bacterial species identified in non-ECA-treated U-bends
	Gram Positive	
Aerococcus viridans	*Micrococcus luteus*	*Brevibacterium casei*
Bacillus cereus		
Bacillus pumilus		
Bacillus simplex		
Staphylococcus aureus		
Staphylococcus capitis		
Staphylococcus cohnii		
Staphylococcus epidermidis		
Staphylococcus haemolyticus		
Staphylococcus hominis		
Staphylococcus saprophyticus		
Staphylococcus warneri		
	Gram-negatives	
Acinetobacter johnsonii	*Acinetobacter ursingii*	*Acinetobacter junii*
Acinetobacter radioresistens	*Aeromonas hydrophila*	*Enterobacter hormaechei*
Brevundimonas diminuta	*Citrobacter freundii*	*Rhizobium radiobacter*
Chryseobacterium indologenes	*Cupriavidus pauculus*	
Enterobacter cloacae	*Delftia acidovorans*	
Klebsiella oxytoca	*Hafnia alvei*	
Pseudomonas fluorescens	*Raoultella ornithinolytica*	
Raoultella planticola	*Stenotrophomonas maltophilia*	
	Pseudomonas aeruginosa	
	Pseudomonas putida	

Abbreviations: ECA, electrochemically activated solutions

Table 3.5 Bacterial species recovered from ECA-treated washbasin U-bends recovered immediately following decontamination

	Bacterial species
Gram-positive bacteria	*Micrococcus luteus*
	Staphylococcus saprophyticus
	Staphylococcus haemolyticus
	Bacillus cereus
	Bacillus pumilus
Gram-negative bacteria	*Pseudomonas aeruginosa*
	Stenotrophomonas maltophilia
	Cupriavidus pauculus
	Acinetobacter ursingii
	Citrobacter freundii
	Raoultella planticola
	Raoultella ornithinolytica
	Enterobacter cloacae
	Klebsiella oxytoca
	Hafnia alvei

Of the 13 Gram-positive species recovered from ECA-treated U-bends at both time points, only five Gram-positive species were recovered from ECA-treated U-bends immediately following decontamination. Likewise for the Gram-negative group, 18 species were recovered from ECA-treated U-bends at both times points, with 10 species recovered from ECA-treated U-bends immediately following decontamination.

3.4.3 Visual and electron microscope examination of an ECA-treated and a control U-bend

Immediately following the completion of the ECA treatment period of the present study, one ECA-treated U-bend and one control U-bend were removed and cut in longitudinal sections. Direct visual examination of the control U-bend revealed the ECA-treated U-bend was visually free from biofilm (Figure 3.7 (a)). In contrast, the presence of patchy, dense slimy biofilm on the inner surface of the untreated U-bend were visible. This biofilm extended towards the region of pipework connecting to the washbasin drain outlet (Figure 3.7 (b)). Scanning electron microscopy of several sections of the inner surfaces of the control U-bend confirmed the presence of dense biofilm and its virtual absence in the ECA-treated U-bend (Figure 3.8).

3.4.4 Biofilm on washbasin drain outlet surfaces

The washbasins used in this study were entirely cast as single ceramic units. These units included the offset drain outlet, washbasin bowl and were contiguous with the vertical back wall of the washbasins. At the end of the study period a visual examination of the drain outlets from the ECA-treated washbasins in A&E and six non-ECA treated identical HP washbasins from Clinic 1 were investigated. The washbasin drain outlet from Clinic 1 was chosen for comparison as the HP washbasin was identical to the HP washbasins in the A&E Department, and both clinics had similar use. Biofilm was clearly evident within the drain outlet of all the Clinic 1 control washbasins, but no visible biofilm was evident in the corresponding ECA-treated drain outlets from A&E (Figure 3.9). Neutralised swab samples taken at this point from the drain outlets of six ECA-treated washbasins yielded average quantitative bacterial densities on CBA agar of 1 CFU/swab (range 0 – 5). No bacteria were recovered on PSCN agar. The corresponding average bacterial densities recovered from six untreated washbasin drain outlets were 4.1×10^3 (range $120 – 5.6 \times 10^3$) on CBA and 874.2 (range $5 – 2.7 \times 10^3$) CFU/swab on PSCN. Additional swab samples were taken from the surface of each washbasin immediately adjacent to the drain outlets. No bacteria were

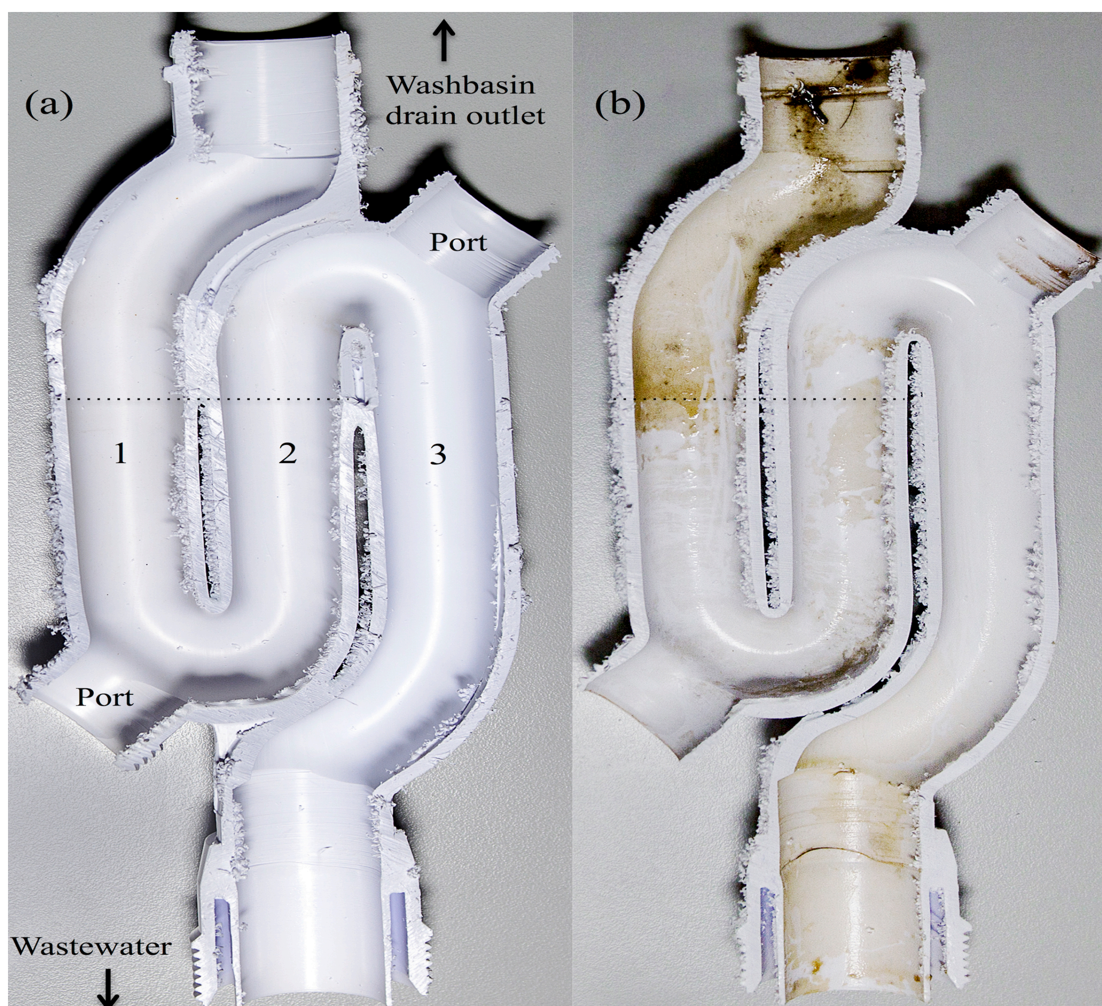


Figure 3.7 A photographic comparison of an ECAs treated and untreated U-bends. Panel **(a)** A longitudinal section of a U-bend following 62 cycles of ECA treatment over a five-month period. Panel **(b)** A longitudinal section of a control U-bend at the end of the study. Both U-bends were installed at the same time. The dashed lines indicate the water level within the U-bends. Following each ECA treatment cycle, treated and control U-bends were swab sampled through the ports indicated. To avoid sampling the same part of each U-bend continually, six internal sampling sites were selected and sampled in rotation. Three of these (labelled 1–3) are shown in **(a)**. The additional three sites were located on the other, mirror image half of the U-bend. The treated U-bend is noticeably free from visible biofilm, whereas the control U-bend contains slimy biofilm, especially above the waterline and at the junctions connecting to the washbasin drain outlet andwastewater discharge outlets.

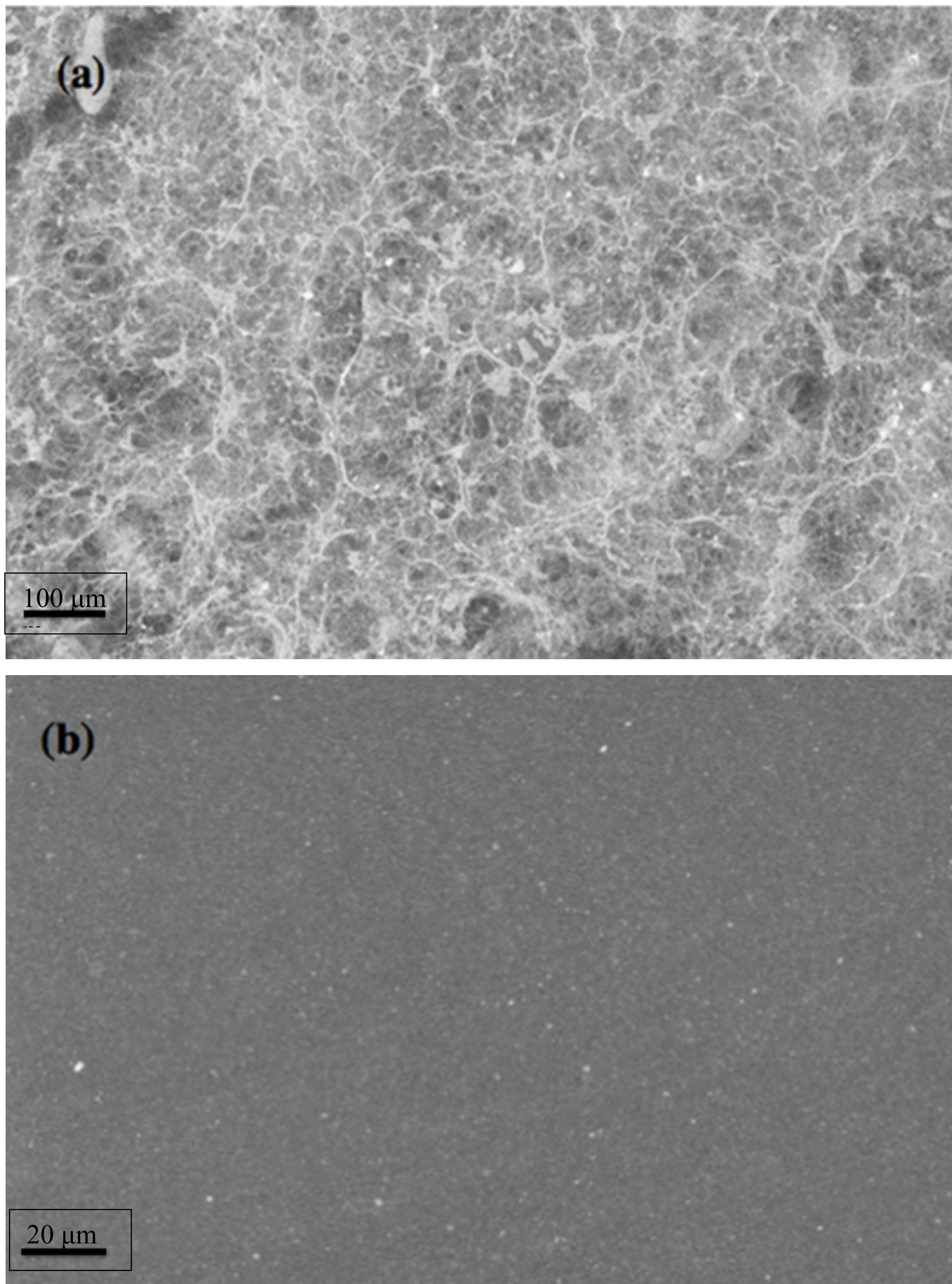


Figure 3.8 Electron microscope images of sections of the internal surfaces of **(a)** an untreated U-bend **(b)** an ECA-treated U-bend. The untreated section harbours dense biofilm, whereas the ECA treated section is totally free of biofilm. Both sections were taken from the U-bend cross sections shown in Figure 3.5 from the areas immediately above the waterline of sampling surface. The black line represents a distance of **(a)** 100 µm at 100x magnification and **(b)** 20 µm at 500x magnification.

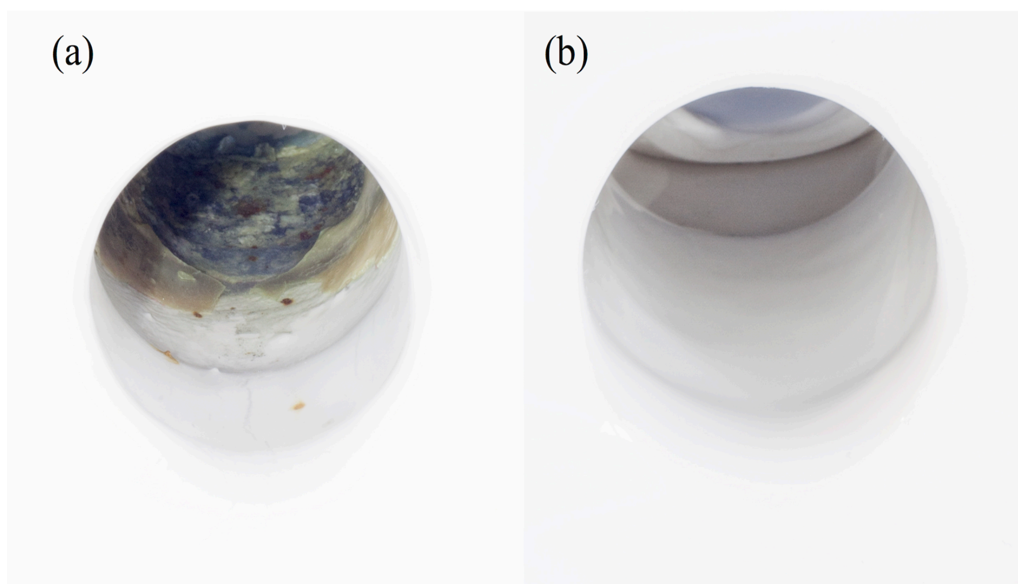


Figure 3.9 Photographs of **(a)** a control and **(b)** an ECA-treated HP washbasin drain outlet at the end of the study. The U-bend and drain outlet of the treated washbasin were subjected to 62 cycles of ECA treatment over five months. The treated drain outlet is noticeably free from visible biofilm, whereas the control drain outlet contains visible biofilm.

recovered from samples from the six test washbasins on CBA or PSCN media. In contrast from the control washbasin surface samples 3.6×10^3 (range $30 – 8.6 \times 10^3$) CFU/swab was recovered on CBA and 1.2×10^3 (range $0 – 6.2 \times 10^3$) on PSCN media (Figure 3.2).

3.4.5 Adverse effects on washbasin wastewater network

No adverse effects were observed following regular visual inspection of the washbasins, U-bends or associated wastewater pipework during and at the end of the study. All fitting incorporated into this system were ECA compliant.

3.5 Discussion

Hand washbasins, drain outlets and U-bends have been increasingly identified as significant environmental reservoirs of contamination in hospitals, predominantly with GNB. This chapter details the development of an effective approach for the automated simultaneous decontamination of multiple hand washbasins U-bend using ECA solutions. This research adopted the two-prong decontamination approach previously utilised by Swan *et al*. (2016) to reduce the bioburden of the biofilm present in washbasin U-bends by harnessing the detergent properties of catholyte to clean followed by disinfection with anolyte. This study builds on previous work conducted in DDUH that demonstrated that bacterial contamination of a single ECA-treated washbasin U-bend and drain outlet was virtually eliminated over the three-month study period relative to controls (Swan *et al.,* 2016). This chapter demonstrated the effective and consistent decontamination of the washbasin U-bends in an active healthcare facility where all previous single approaches to developing IPC protocols for decontaminating washbasin U-bend reservoirs have proved ineffective, ineffective in the long-term or effective but expensive.

This study reports the successful development, installation and testing of a large-scale system, based on the original prototype, for automated simultaneous decontamination of 10 washbasin U-bends, drain outlets and associated pipework. The decontamination system was field tested successfully in a busy hospital clinic with no disruption to daily clinical activities (Figure 3.3). The adaption of the prototype system for use in the A&E Department of DDUH required the establishment of the decontamination system parameters. The parameters known to affect the efficacy of decontamination processes include the concentration and potency of the disinfectants and detergents used, the physical and chemical factors including heat and pH, the presence of organic and inorganic matter, and the duration of treatment with the disinfectants and detergents (Rutala *et al.,* 2008).

Empirical experiments were undertaken with the system to determine the optimal concentrations of each ECA for effective decontamination of the 10 U-bends in a relatively short time period. The pilot study by Swan *et al.* (2016) used anolyte at a concentration of 450 ppm and catholyte at a concentration of 40 ppm, while for the A&E washbasin U-bend decontamination system, this was increased to 800 ppm anolyte and 80 ppm of catholyte. The increased strength of the solutions used with the larger system was necessary due the size of the wastewater network to be decontaminated. To increase the initial cleaning

efficiency of the catholyte solution, freshly generated catholyte was diluted 1:5 with heated mains water immediately prior to use with a temperature after dilution of approximately 33°C. Heating was included as a process in the decontamination system to increase cleaning efficacy of catholyte.

The contact time between the solutions and the pipework was increased from 5 min to 10 min. As previously discussed, a key feature of this system was the use of a ball valve in the wastewater pipe to hold the ECAs that were retro-filled into the wastewater pipework up into the washbasin bowl. All points of the wastewater system above the ball valve were therefore in contact with the ECAs. This was in contrast to the other studies that have used manual valves as a method to increase contact time of disinfectants with washbasin U-bends and associated pipework. As discussed in the introduction, Vergara-Lopez *et al.* (2013) installed manual shut off valves into sink drainage pipes in order to control a *K. oxytoca* hospital outbreak. The 30 min treatment with Biguanid, and the subsequent flushing with hot water for 5 min, resulted in an effective approach to cease the hospital outbreak, however this approach had its limitations. Each sink selected for disinfection was installed with individual valves that were manually operated. This manual approach and filling of the wastewater system through the drain outlet may lead to the trapping of air in the pipework shielding some areas from disinfection. More recently, Cadnum *et al.* (2019) tested the efficacy of multiple disinfectants with and without the use of stop valves in the drainage pipes of a single washbasin. A stop valve was installed below the U-bend of a washbasin known to be colonised with *P. aeruginosa*. Three interventions were tested (i) pouring 500 ml of 5 % acetic acid directly down the drain over a period of 1 min, (ii) pouring 500 ml of diluted household bleach (1:10) directly down the drain over a period of 1 min, and finally (iii) pouring the same quantity and concentration of diluted household bleach directly down the drain over a period of 1 min once the stop valve had been manually closed. When closed, the manual stop valve held the diluted household bleach to a level just above the drain outlet of the washbasin and also filled the associated wastewater pipework above the stop valve for a contact time of 1 h (Cadnum *et al.*, 2019). The effects of the disinfection were quantified using swab sampling a 2.5-cm area below the drain outlet both before the experiment was carried out and at five time points over 15 days. Each experiment was repeated a total of two times. Pouring bleach or acetic acid down the drain alone displayed only transient suppression of recovered bacterial drain colonisation. The third approach, where bleach was poured down the drain outlet and held in the wastewater pipework for 1 h, displayed lasting suppression of bacterial counts recovered by swab samples for up to 10

days post treatment (Cadnum *et al.*, 2019). Both studies were carried out over short time periods (3 months and 15 days) thus limiting the investigation of the long-term effects these approaches over time.

Both the pilot system and the system described in this study adopted three decontamination cycles times per week (Monday, Wednesday and Friday). The automated system developed during this study does not require direct staff involvement in U-bend decontamination and ECA solutions were generated on demand. A recent study by Ramos-Castaneda *et al.* (2020) aimed to investigate the ideal frequency of sink-drain decontamination using two hydrogen peroxide-based disinfectants. The CFU reductions observed following treatment, utilising swab sampling of the sink drains immediately following disinfection, returned to baseline five days post-disinfection with both disinfectants (Ramos-Castaneda *et al.,* 2020). This study failed to determine the ideal frequency of drain decontamination and one limitation of the study was that the swabs used for sampling the drain sites were not neutralised, thus significantly reducing the reliability of the results. In the design of the present study, the possibility of mechanical removal of U-bend biofilm by continuous swab sampling was taken into account. Compared to the Swan *et al.* (2016) study that utilised a single access port U-bend, the U-bends in the A&E Department all included two access sampling ports. This permitted six selected sites (Figure 3.6) to be sampled in rotation to reduce the mechanical removal of biofilm by sampling. The ECA-treated U-bends were sampled a total of 1040 times during the course of the study (Table 3.2). Apart from the number of sampling ports, the U-bends used in both studies were identical. The six sampling locations included sampling of the sections of washbasin U-bend containing the water seal as well as at the water-air interface, and the overflow section of the U-bend (Figure 3.6).

The results of this study demonstrated that the large scale ECA treatment system has a comparable decontamination efficacy to the pilot system as both resulted in a >3 log reduction in bacterial counts in U-bends relative to untreated controls (P <0.0001) (Table 3.2). The pilot study encompassed a total of 35 decontamination cycles and this current study investigated 62 decontamination cycles. The bacterial recovery data immediately following ECA-treatment and 24 h after ECA-treatment was similar on all media tested (Table 3.2), which demonstrated that biofilm within the pipework did not recover rapidly from ECA treatment. The study also investigated the bioburden within the internal sections of the U-bends sampled (Figure 3.6). The internal section of the U-bend was separated into the following segments: the water retention segment of the U-bend (both entry and exit

sections), and the overflow (where the water is flushed towards the sewer). On all three media, the 'entry' section of the ECA-treated U-bends, which allowed recovery of potential pathogens within the seal and at the air-water interface, demonstrated higher average bacterial densities than both the exit and overflow sections. The photograph of the control washbasin U-bend shown in Figure 3.6 shows visibly denser biofilm present in the entry section of the U-bend. One explanation for the observed biofilm, both visually and quantitatively at the entry section of the U-bend, is the ability of *P. aeruginosa* to readily form biofilm at an air-water interface (Abraham *et al.,* 2008).

In the pilot study, *P. aeruginosa* was not recovered from the ECA-treated U-bend. In the current study, the ECA-treated A&E washbasins yielded an average *P. aeruginosa* count from U-bends of 15 ± 185 CFU/swab (n = 620 samples), however, only 12% of samples plated on agar selective for *P. aeruginosa* yielded growth, and of these only 2% yielded >10 CFU/swab. In contrast, 78% of swab samples (n = 372) from the 6 control U-bends yielded growth on the same selective agar and of these, 58% yielded >1000 CFU/swab. The finding of low densities of *P. aeruginosa* in some U-bends following ECA treatment with the larger system relative to the pilot system is not surprising as it is a much larger network of pipes servicing the 10 washbasins and U-bends. Kizny Gordon *et al.* (2017) carried out a systematic review of 32 reports where CROs were recovered from hospital water reservoirs causing HAIs. *P. aeruginosa* was found to be the most frequent organism detected among the papers, detected in 41 % of all the studies as well as being isolated in all water reservoirs (Kizny Gordon *et al.*, 2017). In accordance with previous results, *P. aeruginosa* is the predominant species found in wastewater pipe biofilms (Roux *et al.,* 2013). A study by Cholley *et al*. (2008) sampled 28 U-bends over 8 weeks found that all U-bends were colonized at least once by *P. aeruginosa*. Likewise, Varin and colleagues reported that 79.3% (69/87) of U-bends tested harboured *P. aeruginosa* at least once but sampling was only performed over a two-week period (Varin *et al.,* 2017).

A wide variety of Gram-negative bacterial species other than *P. aeruginosa* were identified in both ECA-treated and control U-bends (Table 3.3). Some of those identified have been previously implicated in hospital outbreaks such as *Citrobacter freundii*, *Acinetobacter junii*, and *Stenotrophomonas maltophilia*, the latter of which is intrinsically carbapenem resistant (Kappstein *et al.,* 2000; Denton *et al.,* 2003; Hammerum *et al.,* 2016; Meletis, 2016). Other than *Stenotrophomonas maltophilia*, no observed carbapenem-resistant or carbapenemase-producing bacteria were recovered in the study. This is unsurprising, as this

project was undertaken in an environment of low antibiotic administration in a DDUH A&E Department. However, antimicrobial resistant bacteria are becoming more prominent in washbasin U-bends due to the draining of fluid containing antibiotics in hand washbasins (De Geyter *et al.,* 2017). The increased abundance of antibiotics promotes the selection of resistant bacterial strains. In contrast to the Gram-negative species recovered, a greater range of Gram-positive bacterial species was identified from treated U-bends. This may be due to the recovery of several staphylococcal species not identified in control U-bends. Staphylococci are common skin commensals that inevitably get transferred into washbasin U-bends during hand washing. The recovery of staphylococci from treated U-bends, albeit in low numbers, could be due to their presence being masked by the high densities of other bacteria, especially GNB within the control samples. However, further investigation of the array of species recovered immediately following decontamination, showed that only 5 Gram-positive species were recovered (Table 3.4). The reduced number of Gram-positives may be indicative of the decontamination process removal of species incorporated in the wastewater network by handwashing, which do not adapt readily to the aquatic environments. *P. aeruginosa* was the most prominent single species identified in the untreated U-bends. This finding was in accordance with the literature, as *P. aeruginosa* is well adapted to this environment and can produce a range of antimicrobial substances enabling it to compete against other bacteria and fungi within its environment (Bédard *et al.,* 2016; Gionco *et al.,* 2017).

The presence of Gram-negative bacteria in washbasin wastewater pipework constitutes a greater risk due to their motility and ability to grow upwards, against the water flow, towards the drain outlet. Kotay *et al*. (2017) used green fluorescent protein tagged *E. coli* found that bacteria inoculated into a U-bend supplied with nutrients reached the drain outlet in a week. In the present study, an ECA-treated washbasin U-bend and untreated U-bend were removed and cut longitudinally to visually inspect the biofilms. Both U-bends were installed at the same time. In Figure 3.7, the ECA treated U-bend following 62 ECA decontamination cycles was visually free of biofilm. In comparison, the cut control U-bend contains slimy biofilm, especially above the air-water interface extending towards the drain outlet and at the junctions connecting to the washbasin drain outlet and wastewater discharge outlet pipes. Utilising electron microscopy, the untreated section harbours dense biofilm, whereas the ECA treated section is totally free of biofilm (Figure 3.8).

In the present study, visual inspection of untreated offset drain outlets in HP washbasins indicated that bacteria are not entirely confined to the wastewater pipe network (Figure 3.9). Sampling from ECA-treated drain outlets recovered average bacterial counts of 1 CFU/swab (range 0 – 5) on CBA and no bacterial recovery on PSCN agar. Whereas untreated drain outlets recovered 4.1×10^3 (range 120 – 5.6×10^3) on CBA and 874.2 (range 5 – 2.7×10^3) CFU/swab on PSCN. This observed 3-log reduction in bacterial colonisation reduces the potential of transmission of potentially pathogenic bacteria. Previous studies have demonstrated the risk of bacterial biofilm at drain outlets. Starlander and Melhus (2012) identified four patients, over a seven-month period, becoming colonised by an ESBL producing strain of *Klebsiella pneumoniae*. Following environmental sampling, the source of the outbreak was determined to be a contaminated sink drain. In this present study, the washbasin was also swabbed adjacent to the drain. In this study, sampling of the drain outlet was investigated to determine if the bacteria contamination in wastewater pipe network was being pushed upwards by the dosing of the solutions. These observed reduction in biofilm formation in washbasin drains and washbasin indicates the effect ECA-solution effect on washbasins as well as the washbasin U-bend.

This study did not definitively demonstrate whether the decontamination approach would be of benefit in helping to control an actual hospital outbreak associated with contaminated washbasin or sink U-bends and/or drains. However, several previous studies managed to control such hospital outbreaks by replacing washbasins/sinks and U-bends or by treating U-bends with chemicals (La Forgia *et al.,* 2010; Stjarne Aspelund *et al.,* 2016; De Geyter *et al.,* 2017; Mahida *et al.*, 2017). Such approaches offer short-term solutions to the problem of U-bend contamination as new sanitary ware and pipework rapidly becomes recolonised from the wastewater network pipework (Hota *et al.,* 2009; La Forgia *et al.*, 2010; Roux *et al.*, 2013; Vergara-López *et al.*, 2013; Leitner *et al.*, 2015; Wendel *et al.*, 2015; Stjarne Aspelund *et al.*, 2016). DDUH operates on an outpatient basis only between 08.00 h and 17.00 h daily, which enabled significant out-of-hours access to the A&E Department in which we installed and tested the large-scale automated system. Such access would not be as readily available for system optimisation and sampling in a busy acute hospital setting.

In conclusion, the novel decontamination approach described in this chapter, provides an effective multi-facetted automated approach for minimising infection risks from drain outlets. This approach effectively reduced biofilm formation in washbasin U-bends, which comprise a ubiquitous contamination reservoir in healthcare facilities. This novel approach

demonstrated effective and consistent decontamination of multiple washbasin U-bends over a period of 62 cycles or 5 months. The use of automated U-bend decontamination would be of most benefit in ICUs and in hospital wards with vulnerable patient groups such as immunocompromised patients and cystic fibrosis patients. The system is capable of decontaminating multiple washbasin U-bends simultaneously and can be tailored to manage as many or as few washbasins and sinks as desired for a particular healthcare setting.

Chapter 4

Investigating the efficacy of automated decontamination of multiple washbasin U-bends with electrochemically-activated solutions over a period of 52 weeks and identifying the bacterial communities at multiple DDUH wastewater network locations using Illumina high-throughput 16S rRNA amplicon sequencing

4.1 Introduction

Hand washbasins, U-bends, associated fixtures and wastewater pipes have been increasingly identified as reservoirs of microorganisms responsible for nosocomial infections, including infections caused by antibiotic resistant organisms. The successful development of a large-scale programmable automated system for the simultaneous decontamination of multiple washbasin U-bends in the A&E Department of DDUH using sequential treatment with catholyte followed by anolyte solutions was previously described in Chapter 3. This system was shown to effectively and consistently reduce the bacterial bioburden within the 10 washbasin U-bends and drains over a period of five months (Deasy *et al.,* 2018). However, two main factors of a good decontamination protocol are the long-term feasibility-of-use of the decontamination approach and the long-term efficacy of the decontamination approach. Continuous chemical disinfection has previously been shown to lead to selective environmental pressures for the emergence of AMR organisms, alongside adverse effects on wastewater network pipes and components (Cooper *et al.*, 2010; Mao *et al.*, 2018; Jin *et al.*, 2020).

As previously discussed, a plethora of approaches have been described for the decontamination of washbasin wastewater drains, U-bends and associated fixtures. While today, chemical disinfectants and novel decontamination approaches continue to be developed, long-term studies are necessary for investigating the efficacy of decontamination within aging wastewater networks. One recent review compared the interventions used against CRO-associated outbreaks transmitted from wastewater drain biofilms. The review identified 23 wastewater drain-associated CRO outbreak studies between 1990 – 2018 (Carling, 2018). Comparison of routine disinfection approaches determined disinfection had limited, if any, impact on CRO drain colonisation, and generally necessitated the replacement of wastewater fittings to terminate outbreaks (Carling, 2018).

Since the discovery of microorganisms, scientists have aimed to identify potential pathogens associated with diseases. However, this process has not always been straightforward. A 2003 study determined that 50% of all sepsis cases documented between 1979 and 2000 within representative hospitals throughout the United States could not identify the causative pathogen by microbiological culture-based testing in a clinical setting (Martin *et al.*, 2003). Likewise, it has been estimated that > 99% of microorganisms in nature cannot be cultivated using standard laboratory techniques (Hugenholtz *et al.,* 1998). The advent of NGS

technologies brought about rapid, culture-independent and relatively cost-efficient methods to profile diverse microbial communities. NGS facilitates the sequencing of thousands of organisms in parallel compared to other techniques, such as PCR-based approaches and capillary sequencing, which were reduced in parallel sequencing capacities.

Wastewater networks are complex ecosystems that are exposed to differing levels of use. While parts of the wastewater network can be continuously utilised and subjected to continuous incorporation of microorganisms, organic materials and/or chemicals, other parts may remain dormant for considerable periods. Yet, all parts of healthcare facility wastewater networks are connected to and lead to sewer discharge points. The sewers also receive effluent from domestic, clinical and commercial discharge pipes and sometimes groundwater (Guo *et al*., 2019; McLellan and Roguet, 2019). While these sewers lead to wastewater treatment plants for processing of the effluent, sewers are ideal locations for microbial propagation and can potentially retro-contaminate healthcare facility discharge pipes. Wastewater network in healthcare facilities, especially hospitals, are generally exposed to high levels of antibiotics and disinfectants, which increase the risk of genetic transfer of genes conferring antimicrobial resistance to non-resistant organisms and also exert selective pressures on antibiotic resistant organisms (Perry *et al.*, 2019).

The field of microbial community analysis has rapidly expanded in recent years, with hundreds of articles published recently. One area of confusion in this field concerns the utilisation of certain terms, oftentimes used interchangeably or incorrectly. In dealing with this subject matter, a number of commonly used terms will be defined in Table 4.1 for the sake of clarity, including microbiota, microbiome, metagenome, and metagenomics. (Handelsman *et al.*, 1998). Metagenomics can analyse single genes within a population, such as 16S rRNA sequencing, but can also analyse multiple genes and genomes within a population, such as whole genome shotgun sequencing (Chen and Pachter, 2005). Characterisation of both the microbiome and microbiota can involve the application of metagenomic analysis, alongside other applications such as metabonomic, metatranscriptomic, and metaproteomic analyses (Marchesi and Ravel, 2015). Utilisation of these analyses can determine the bacterial community classification by taxonomic rank defined under the taxonomic hierarchy. Microorganisms are defined under the following taxa: domain, kingdom, phylum, class, order, family, genus and species.

Table 4.1 Terms used to describe microbial community analyses

Term	Definition
Microbiota	The collection of microorganisms within a defined environment.
Microbiome	The collection of microorganisms defined by their genomes and genes within a defined environment.
Metagenome	The total collection of genomes and genes of a collection of microorganisms.
Metagenomics	The term metagenomics is the study of genetic material recovered directly from environmental samples.

The two main NGS approaches for characterising microbial communities and metagenomic analyses include, amplicon sequencing and whole-genome shotgun sequencing (Rausch *et al*., 2019). Amplicon sequencing relies on PCR amplification and sequencing of conserved regions of bacterial genomes that contain phylogenetically informative polymorphisms (de Muinck *et al*., 2017). A number of phylogenetic molecular markers have been investigated, with the 16S ribosomal RNA (16S rRNA) gene being the most commonly adopted. This is due to a number of factors previously explored in Chapter 1. Other less adopted markers include the methyl coenzyme-M reductase (*mcrA*) gene and the *rpoB* gene that encodes the beta-subunit of bacterial RNA polymerase (Luton *et al*., 2002; Vos *et al*., 2012). Amplicon sequencing provides information on bacterial diversity, taxonomic composition and structure, and is utilised for monitoring bacterial communities. There are two major limitations of 16S rRNA sequencing: amplification bias and abundance bias. Amplification bias may occur when choosing the PCR primers and also, when template concentrations are not standardised in the PCR protocol (Větrovský and Baldrian, 2013). Abundance bias may occur based on the variability of 16S rRNA copy number in bacteria, where the average copy number per genome is four but can reach a maximum of 15 (Větrovský and Baldrian, 2013; Ibarbalz *et al*., 2014). For these reasons, the relative bacterial abundance in total populations identified utilising 16S rRNA sequencing are considered estimates. To date, Illumina 16S rRNA amplicon sequencing analysis of the variable V3 and V4 regions of the 16S rRNA genes have been utilised for experiments ranging from investigating bacterial communities within the gut microbiota of mice with and without acute colitis, to the bacterial communities within wastewater influent and effluent from wastewater treatment plants (Jones-Hall *et al.*, 2015; Limayem *et al.*, 2019).

The second approach, whole-genome shotgun sequencing, randomly shears the total extracted sample DNA, and parallel sequences all of the DNA fragments, which can be assembled computationally into larger contigs or contiguous DNA fragments (sets of overlapping DNA segments that together form a consensus region of DNA) (Quince *et al.*, 2017). Whole-genome shotgun sequencing enables the detection of genes, the determination of the gene functions following annotation and the determination of microorganism population structures, providing functional characterisation of the whole communities. This approach can be utilised to discover microorganisms acting as reservoirs of antibiotic resistance genes, study the epidemiology of transmission events in a microbial community, to detect novel genes, and to detect genes within viral communities that lack the 16S rRNA gene (Chen and Pachter, 2005; Li *et al*., 2015). However, currently amplicon sequencing is

more widely adopted due to the high costs associated with whole-genome shotgun sequencing (de Muinck *et al*., 2017).

Finally, a key limitation in comparing results and conclusions of environmental sampling studies is the high levels of variability between sampling tools and individual sampling techniques, that result in low reproducibility. Standardisation of environmental sampling of surfaces, like washbasin U-bends, and recovery rates of microorganisms are limited by a number of factors. These factors include the variability of surface material sampled, the density and diversity of microorganisms on the sampled surface, the surface area sampled and the type of sampling device (Jones *et al*., 2020). To date, swab sampling is the most common method for monitoring and detection of microorganisms on hard surfaces (Keeratipibul *et al*., 2017). While standardisation of environmental sampling techniques may not be feasible, the reporting of individual studies of determined individual rates of recovery of microorganisms would lead to more transparent and informative environmental sampling analyses.

4.2 Objectives

The main aims of this chapter were to determine the efficacy of long-term ECA decontamination of washbasin U-bends in a functioning out-patient clinic in DDUH, to investigate the bacterial communities within the DDUH wastewater network, and finally to determine the bacterial recovery rates using the swab sampling technique adopted in these studies.

Specifically this chapter aims:

- To determine the efficacy of automated decontamination of washbasin U-bends using ECA solutions over a relatively long period of time, 52 weeks. Bacterial counts from 10 untreated U-bends were recorded once weekly, and three times weekly from 10 identical ECA decontaminated U-bends. The three times points included sampling: immediately following decontamination, 24 h post decontamination and 48 h post decontamination. The additional sampling time point of 48 h post decontamination was included to determine longer term residual effects of ECA decontamination on washbasin U-bends.

- To investigate the bacterial communities present in multiple wastewater network locations in DDUH. The representative culturable bacterial communities were determined in one ECA-treated washbasin U-bend in A&E over a five week period and in all 10 untreated washbasin U-bends in Clinic 2 over two time points. Secondly, the bacterial communities were investigated in 16 wastewater network locations throughout DDUH utilising Illumina high throughput 16S rRNA amplicon sequencing.

- To determine the bioburden within washbasin U-bends and drain outlets seven months after the cessation of decontamination in the A&E Department and to investigate the spread of bacteria from HP and DP washbasins in DDUH.

- To determine the efficacy of the swab sampling technique used in these experiments using different *P. aeruginosa* isolates and to determine the relative bacterial recovery rates of three different types of swabs.

4.3 Materials and Methods

4.3.1 Test and control washbasins

The development of the large-scale automated ECA system for decontaminating washbasin U-bends, drains and proximal wastewater pipes in A&E was described in Chapter 3. In this Chapter, the same U-bends of the 10 A&E HP hand washbasins were subjected to an additional 52 weeks of routine ECA decontamination (as described in Chapter 3, Section 3.3.1). The 52 week test period was spaced over a period of greater than one year (Winter 2017 – Spring 2019) owing to the closure of DDUH due to public holidays. All ten washbasins were each connected by a vertical pipe 1-m in length that discharged into a common horizontal wastewater collection pipe, that was located directly beneath the A&E Department in the basement level of DDUH (Chapter 3, Figure 3.1).

In this study, Clinic 2 was chosen as the location for sampling control washbasin U-bends not subjected to routine ECA decontamination. Clinic 2 was refurbished exactly one year after the A&E Department, in the Summer of 2017. All washbasins, U-bends, wastewater pipes and associated fittings were identical to those in the A&E Department. Likewise, all control washbasins sampled were used for hand washing only and were similar use clinics. Tork Extra Mild Liquid Soap was used for hand washing at all A&E and Clinic 2 washbasins. The water supply to Clinic 2 washbasins was identical to that in A&E as described in Chapter 3, Section 3.2.1. Ten washbasins in Clinic 2 were chosen as controls to determine the bacterial bioburden within washbasin U-bends not subjected to routine ECA decontamination. The ten washbasins were sampled over the same 52 week test period as the ECA-treated washbasin U-bends. Clinic 2 is equipped with 15 washbasins located in five clinical bays, with three washbasins per bay (Figure 4.1). Two washbasin U-bends from each bay were selected for sampling in this study. The U-bend of each Clinic 2 washbasin is connected via a 1-m vertical pipe to one of a series of five horizontal wastewater pipes, each of which serviced three washbasins. Each horizontal pipe discharges wastewater into an individual vertical pipe, which passes through the building into the basement. Two vertical pipes (labelled A and B in Figure 4.1) connected to separate, larger common horizontal wastewater pipes that discharged wastewater to the municipal sewer at separate locations. A sampling port was incorporated into vertical pipe B to enable swab sampling (Figure 4.1). The other three vertical pipes (labelled C – E in Figure 4.1) all connected to a larger common horizontal wastewater pipe in the basement of DDUH that also services all the washbasins

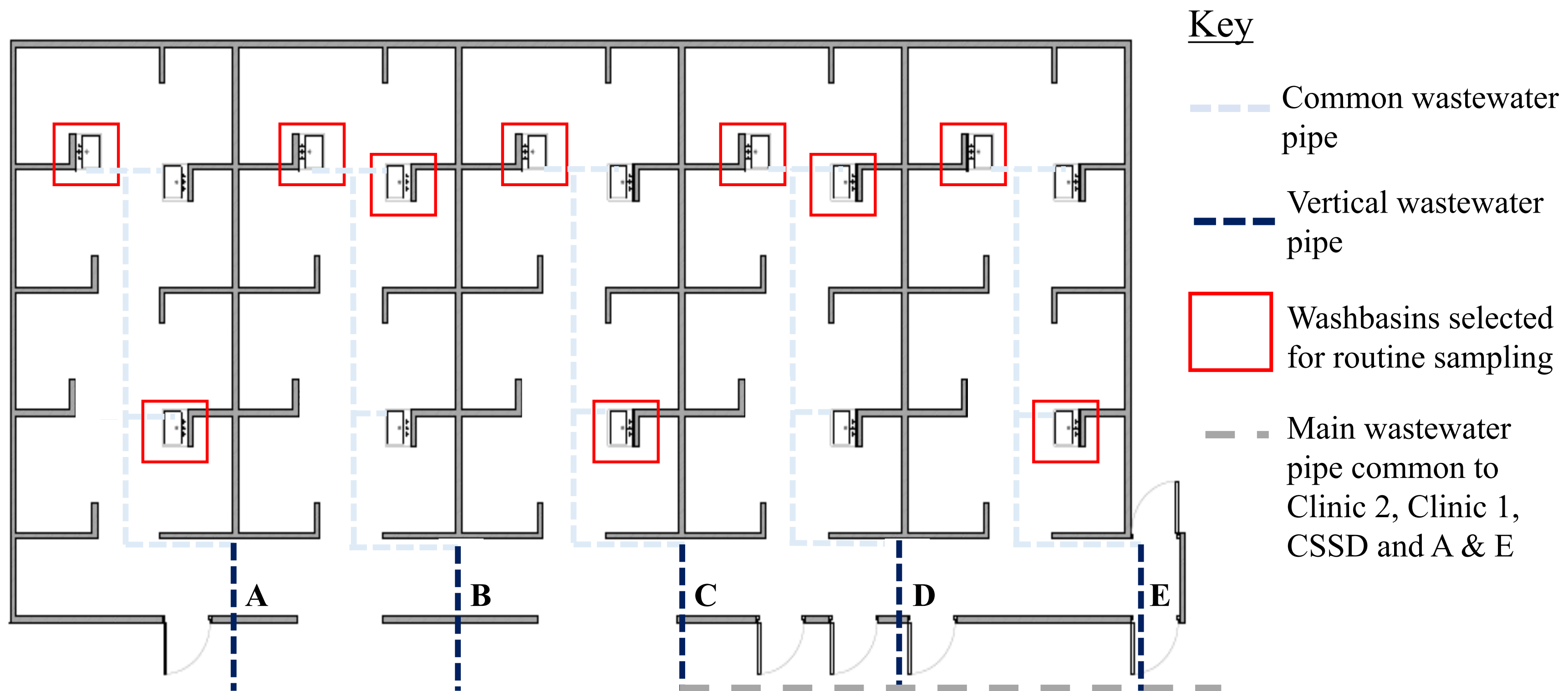

A
B
C
D
E
Key
Common wastewater pipe
Vertical wastewater pipe
Washbasins selected for routine sampling
Main wastewater pipe common to Clinic 2, Clinic 1, CSSD and A & E

Figure 4.1 Schematic diagram showing the layout of Clinic 2 in DDUH. Clinic 2 was equipped with 15 HP washbasins divided between five bays. For the purpose of this study, two washbasins per bay were samples as indicated in the schematic by a red box. The U-bend of each Clinic 2 washbasin was connected via a 1-m vertical pipe to one of a series of five horizontal wastewater pipes, each of which serviced three washbasins. Each of these horizontal pipes discharged water into an individual vertical pipe, which passed through the building into the basement. The five vertical pipes are labelled A – E. Vertical pipes A and B discharge wastewater to the municipal sewer at separate outlets. The three remaining vertical pipes (pipes labelled C – E in Figure 4.2) all connect to a larger common horizontal wastewater pipe in the basement of DDUH that discharges to the municipal sewer at the building perimeter. The U-bends of each A&E washbasin also discharge wastewater to the same larger common horizontal wastewater pipe servicing vertical pipes C – E from Clinic 2.

from A&E and discharges wastewater to the municipal sewer at the building perimeter (as labelled in Figure 4.1). Similarly to vertical pipe B, a sampling port was incorporated into the larger common horizontal wastewater pipe in the basement of DDUH at the point-of-discharge to enable swab sampling (Figure 4.1).

4.3.2 Electrochemically activated solutions

The anolyte and catholyte solutions used in this study are described in Chapter 2, Section 2.4.

4.3.3 Design of the automated ECA decontamination system for U-bends

The design of the ECA decontamination system is described in Chapter 3, Section 3.3.3.

4.3.4 Automated ECA decontamination cycles

Automated ECA decontamination cycle are described in Chapter 3, Section 3.3.4.

4.3.5 Microbiological culture

ECA decontamination efficacy within A&E U-bends was determined by quantitative microbiological culture of 10 U-bend sampled over a period of 52 weeks. A total of 156 ECA decontamination cycles of A&E U-bends occurred within the 52 week test period. Swab samples were taken from each U-bend at three time points once weekly over the test period as follows: immediately following disinfection (n = 520), 24 h post-treatment (n = 520) and 48 h post-treatment (n = 520). After completion of each decontamination cycle, A&E U-bends were flushed with tap water prior to sampling to void any retained residual anolyte. Water drainage in washbasins were monitored after every decontamination cycle to investigate if any blockages were occurring further down in the pipework.

Contemporaneous samples were also taken from 10 control Clinic 2 U-bends (n = 520 swabs) once weekly over the period of 52 weeks. Clinic 2 U-bends did not undergo ECA decontamination. In each case, the washbasin U-bends were swab sampled and processed following the protocol described in Chapter 2, Section 2.6.3.

4.3.6 Recolonisation of A&E U-bends seven months post cessation of ECA treatment

At one-time point seven months post cessation of ECA decontamination of the A&E U-bends, all 10 A&E washbasin U-bends and washbasin drain outlets were swab sampled to determine the average bacterial density in the U-bends. The tip of each swab was processed

as described in Chapter 2, Section 2.6.3. Bacterial colonies were counted following incubation on CBA, PSCN and R2A plates, as described in Chapter 2, Sections 2.2.1.

4.3.7 Use frequency of washbasins in A&E

The frequency of washbasin usage, the activities utilising the washbasins, and the inspection of incorrect disposals of chemicals and solutions in the A&E Department were recorded over a period of one week by a dental nurse.

4.3.8 Air sampling adjacent to washbasins with tap water flowing

Air sampling was carried out using an EM0100A model air sampler (Oxoid/Thermo Scientific, Fannin Healthcare, Dublin, Ireland) fitted with a 90 mm-diameter aluminium head with a 219-hole impactor (Thermo Fisher Scientific Oxoid, United Kingdom). The aluminium head was disinfected between use using Sani-Cloth 70% Alcohol Wipes (PDI, Flintshire, UK). Active air sampling was conducted by placing either a PSCN or a CBA agar plate into the air sampler head and the vacuum turned on. The test washbasin taps were turned on for the duration of the air sampling and the air sampler was tilted to a 45° angle from the flowing tap and held 50-cm away. Air samples were taken in duplicate from HP washbasins in Clinic 2 and A&E, and from one DP washbasin in West Clinic. The PSCN and CBA plates were incubated as previously described in Chapter 2, Section 2.2.1.

Following incubation, bacterial colonies were counted using a Stuart™ Scientific colony counter. The number of colonies counted on the surface of the agar plate needed to be corrected for the statistical possibility of multiple particles passing through the same impactor hole. The correction calculation was carried out by multiplying the most probable number (MPN; as determined in Table 4.2) by 1000. This number was divided by the volume of air sampled resulting in a number in colony forming units per 1000 L of air or CFU/m^3 (1000 L = 1 m^3).

4.3.9 The relative abundance of bacterial species identified from ECA-decontaminated and control U-bends

Similar to the identification of the range of bacterial species from the control and ECA-treated U-bends described in Chapter 3, the approximate relative abundance of culturable bacterial species were determined within washbasin U-bends in A&E and Clinic 2. The processed samples from one washbasin U-bend in A&E were investigated over a period of five weeks (winter 2018). The colonies were observed following the routine processing of

Table 4.2 Correction table of colony counts recovered from a 219-hole impactor using 90 mm petri plates. Adapted from the SAS SUPER 100/180, DUO SAS SUPER 360, SAS ISOLATOR Instruction Manual, 2006

Colonies (r)	MPN (Pr)	Colonies (r)	MPN (Pr)	Colonies (r)	MPN (Pr)	Colonies (r)	MPN (Pr)
1	1	**56**	64	**111**	154	**166**	309
2	2	**57**	66	**112**	156	**167**	313
3	3	**58**	67	**113**	158	**168**	317
4	4	**59**	69	**114**	160	**169**	322
5	5	**60**	70	**115**	162	**170**	326
6	6	**61**	71	**116**	165	**171**	331
7	7	**62**	73	**117**	167	**172**	335
8	8	**63**	74	**118**	169	**173**	340
9	9	**64**	76	**119**	171	**174**	344
10	10	**65**	77	**120**	173	**175**	349
11	11	**66**	78	**121**	175	**176**	354
12	12	**67**	80	**122**	178	**177**	359
13	13	**68**	81	**123**	180	**178**	365
14	14	**69**	83	**124**	182	**179**	370
15	15	**70**	84	**125**	185	**180**	375
16	17	**71**	86	**126**	187	**181**	381
17	18	**72**	87	**127**	189	**182**	387
18	19	**73**	88	**128**	192	**183**	393
19	20	**74**	90	**129**	194	**184**	399
20	21	**75**	92	**130**	196	**185**	405
21	22	**76**	93	**131**	199	**186**	412
22	23	**77**	95	**132**	201	**187**	418
23	24	**78**	96	**133**	204	**188**	425
24	25	**79**	98	**134**	206	**189**	432
25	26	**80**	99	**135**	209	**190**	439
26	28	**81**	101	**136**	212	**191**	447
27	29	**82**	102	**137**	214	**192**	455
28	30	**83**	104	**138**	217	**193**	463
29	31	**84**	106	**139**	220	**194**	471
30	32	**85**	107	**140**	222	**195**	480
31	33	**86**	109	**141**	225	**196**	489
32	34	**87**	110	**142**	228	**197**	499
33	36	**88**	112	**143**	231	**198**	508
34	37	**89**	114	**144**	234	**199**	519
35	38	**90**	116	**145**	237	**200**	530
36	39	**91**	117	**146**	240	**201**	542
37	40	**92**	119	**147**	243	**202**	554
38	42	**93**	121	**148**	246	**203**	567
39	43	**94**	122	**149**	249	**204**	580
40	44	**95**	124	**150**	252	**205**	595
41	45	**96**	126	**151**	255	**206**	611
42	46	**97**	128	**152**	258	**207**	627
43	48	**98**	130	**153**	261	**208**	646
44	49	**99**	131	**154**	265	**209**	666
45	50	**100**	133	**155**	268	**210**	687
46	51	**101**	135	**156**	271	**211**	712
47	53	**102**	137	**157**	275	**212**	739
48	54	**103**	139	**158**	278	**213**	770
49	55	**104**	141	**159**	282	**214**	807
50	57	**105**	142	**160**	286	**215**	851
51	58	**106**	144	**161**	289	**216**	905
52	59	**107**	146	**162**	293	**217**	978
53	60	**108**	148	**163**	297	**218**	1088
54	62	**109**	150	**164**	301	**219**	1307
55	63	**110**	152	**165**	305		

Abbreviations: r, colony forming units counted; MPN, most probable number; Pr, probable count.

swab samples from A&E U-bends, as described in Chapter 2, Section 2.6.3. The colonies were counted following incubation of CBA agar plates, as described in Chapter 2, Section 2.2.1. Due to the low bacterial counts observed from A&E washbasin U-bends following ECA decontamination, undiluted aliquots from processed swabs samples were spread in duplicate onto CBA plates. Following incubation, bacterial colony counts were recorded, along with the relative abundance of colonies with distinct morphologies. A representative of each distinct colony morphology type was stored as described previously in Chapter 2, Section 2.1.5.

Likewise, the relative abundance of culturable bacterial species was determined for control washbasins in Clinic 2. Unlike A&E, all 10 washbasins were investigated at two time points (winter 2018). The high bacterial counts recovered by swab sampling of washbasin U-bends in Clinic 2 required 10-fold dilutions of the bacterial solutions recovered from the processed swabs samples, and aliquots of the dilutions were spread in duplicate onto CBA plates. The relative abundance of each colony morphology type observed was recorded for each sample. A representative of each morphologically distinct colony was stored as described previously in Chapter 2, Section 2.1.5.

Stored bacterial isolates from A&E and Clinic 2 U-bends were identified using MALDI-TOF-MS, as described in Chapter 2, Section 2.2.4.

4.3.10 Illumina 16S rRNA amplicon sequencing

Following the determination of the relative abundance of culturable bacterial species identified from ECA-decontaminated and control U-bends, Illumina 16S rRNA amplicon sequencing was used to identify the range of bacterial species present in multiple wastewater network locations in DDUH. Sixteen distinct location within the wastewater pipe network in DDUH were sampled including: six washbasin U-bends from the individual staff bathrooms in DDUH (sample sites 1 – 6), five washbasin U-bends in Clinic 2 (sample sites 7 – 11), one washbasin U-bend in CSSD (sample site 12), the internal pipe surface of a vertical pipe connecting Clinic 1 and Clinic 2 (sample site 13), one washbasin U-bend from the A&E Department seven months post cessation of ECA decontamination (sample site 14), one washbasin U-bend in West Clinic (sample site 15), and the point-of-discharge sampling site of the main common wastewater pipe connecting Clinic 1, Clinic 2, CSSD and A&E (sample site 16) (Figure 4.2). The Illumina 16S rRNA amplicon sequencing protocol

Washbasin U-bend
Common wastewater pipe from individual clinics
Vertical common wastewater pipe

Figure 4.2 Schematic showing the locations of the 16 wastewater network sampling sites selected for Illumina 16S rRNA amplicon sequencing of the wastewater network in DDUH. Samples 1 – 12 and 14 – 15, were recovered by swab sampling of washbasin U-bends. Sample 13 was recovered following the removal of segments of wastewater pipe containing biofilm. Sample 16 was recovered by swab sampling of the internal surface of the point-of-discharge wastewater pipe.

was divided into four main stages, which included DNA isolation, library preparation, sequencing, and data interpretation.

4.3.10.1 DNA extraction for 16S rRNA amplicon sequencing

The bacterial populations of all selected wastewater network locations were investigated utilising the Illumina 16S rRNA amplicon sequencing analysis protocol. The wastewater pipe fixtures were sampled by one of two methods: swab sampling using sterile viscose transport swabs (Sarstedt, Nümbrecht, Germany), or the removal of 1-cm^2 of biofilm from the surface of removed segments of the pipework using a sterile scalpel. Ribosomal DNA was extracted from biofilm samples using the Qiagen DNeasy PowerSoil Kit (Qiagen). DNA was extracted from the 15 swab samples (viscose transport swabs; Sarstedt) by cutting off the swab tip and placing it into individual microcentrifuge tube (labelled Tube A) containing 1 ml of sterile 1X PBS. The tube was vortexed for 1 min, and the swab tip was removed and placed into a fresh microcentrifuge tube (labelled Tube B) containing 500 µl PBS and vortexed for 1 min. Tube A was centrifuged at 15,000 × *g* for 5 min, the supernatant discarded and the 500 µl PBS from Tube B transferred into Tube A and the pellet was resuspended. The entire volume of Tube A was then transferred into a fresh PowerBead tube (provided with the Qiagen DNeasy PowerSoil Kit) and vortexed for 10 s. DNA was extracted from biofilm removed directly from the surface of the selected pipework, by mechanically removing a standardised surface area of 1-cm^2 of biofilm using sterile forceps. The removed biofilm was transferred into a fresh PowerBead tube and vortexed for 10 s. Figure 4.3 shows examples of excised segments of a vertical wastewater pipe covered in biofilm; this biofilm was subsequently removed and DNA was extracted from the samples.

Once in the PowerBead tubes both initial extraction processes followed the same protocol. To each PowerBead tube, a volume of 60 µl of Solution C1 (Qiagen) was added and the tube were vortexed for 30 s. Each PowerBead tube was placed in a Fastprep FP120 Cell Dismembranator (Thermo-Scientific) bead beater set at 3,000 oscillations per min for 30 s. The process was repeated two times, with a 5 min ice incubation step in between. The tubes were centrifuged at 10,000 × *g* for 30 s. The supernatant of each tube was transferred to a fresh microcentrifuge tube and a volume of 250 µl Solution C2 (Qiagen) was added and vortexed for 30 s. The tubes were incubated at 2 – 8°C for 5 min. Following incubation, the tubes were centrifuged at 10,000 × *g* for 1 min and the supernatant was transferred to a fresh microcentrifuge tube and a volume of 200 µl Solution C3 (Qiagen) was added and vortexed

(a)

(b)

Figure 4.3 Photographs showing the build-up of sediment and biofilm on excised horizontal segments of a vertical wastewater pipe in DDUH. These segments were removed from a vertical wastewater pipe connecting Clinic 1 and Clinic 2, which passed through the building into the basement (Figure 4.2).

for 30 s. The tubes were incubated at 2 – 8°C for 5 min. Following the incubation, the tubes were centrifuged at 10,000 × *g* for 1 min and 750 µl of the supernatant was transferred to a fresh microcentrifuge tube and a volume of 1,200 µl Solution C4 (Qiagen) was added vortexed for 30 s.

For each sample, 650 µl of the Solution C4-supernatant solution was added to a fresh MB Spin Column (Qiagen). The column was centrifuged at 10,000 × *g* for 1 min, and the flow through was discarded. This step was repeated a total of three times, until all the Solution C4-supernatant solution had passed through the column. The column was placed into a fresh collection tube and 500 µl of Solution C5 (Qiagen) was added and centrifuged at 10,000 × *g* for 30 s. The collection tube and supernatant was discarded and the column was centrifuged for a further 30 s at 10,000 × *g*. The column was placed into a fresh microcentrifuge tube, and 50 µl of Solution C6 (Qiagen) was added directly onto the filter membrane of the column. The column was centrifuged at 10,000 × *g* for 30 s. The eluted DNA was stored in 1.5 ml microcentrifuge tubes were stored at 4°C for 4 – 6 weeks, or at -20°C for long term storage. The DNA was quantified and the quality was checked using the nanodrop 2000c spectrophotometer and Qubit Fluorometer 3.0, as described in Chapter 2, Section 2.3.

4.3.10.2 Library preparation and loading

This Illumina 16S rRNA amplicon sequencing protocol prepares DNA samples for sequencing of the variable V3 and V4 region of the 16S rRNA gene (approximately 460 bp) (Klindworth *et al.,* 2013). The 16S library preparation was broken into six steps: (i) the first stage amplicon PCR where primers target the V3 and V4 regions of the 16S rRNA gene in the DNA samples, (ii) a post amplicon PCR clean-up step, (iii) the second stage index PCR that adds Illumina sequencing adapters and dual-index barcodes to the amplicon target, (iv) a post index PCR clean-up step, (v) the library quantification and normalisation step, and finally (vi) the denaturation and MiSeq sampling loading.

4.3.10.2.1 First stage amplicon PCR

The first stage amplicon PCR was prepared for each sample. A reaction mixture was prepared in individual 0.2 ml PCR tubes containing 2.5 µl of 5 ng/µl DNA, 5 µl of 1 µM of amplicon PCR forward primer (Table 4.3), 5 µl of 1 µM of amplicon PCR reverse primer (Table 4.3), and 12.5 µl of 2X KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Roche, MA, USA). The total 25 µl reaction mixture was aspirated using a laboratory pipette (Gilson)

Table 4.3 Primers used for amplification of the V3 and V4 region of the 16S rRNA gene

Primer	**5' – sequence – 3'**	**Expected product length (bp)**	**Reference**
V3V4-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG	550	(Klindworth *et al.,* 2013)
V3V4-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC		

and placed in either a Kyratec Thermocycler model SC200 (Kyratec) or G-storm GSI Thermocycler (G-Storm). The cycle conditions consisted of an initial denaturation step at 95°C for 3 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and ending with an elongation step at 72°C for 5 min.

4.3.10.2.2 Post amplicon PCR clean-up step

The post amplicon PCR clean-up step began following completion of the PCR cycles. Each PCR tube was centrifuged at 1,000 × *g* for 1 min. Each PCR tube was transferred to a PCR tube rack where 20 µl of AMPure XP (Beckman Coulter Life Sciences, CA, USA) beads were added to each reaction tube. The mixture was aspirated using a laboratory pipette, vortexed for 10 s and incubated at room temperature for 5 min. Following incubation, the PCR tubes were placed on a magnetic stand (Thermo Fisher Scientific, Dublin, Ireland) for 2 min or until the supernatant cleared. Once cleared, the supernatant was discarded and each tube was washed twice with 200 µl freshly prepared 80% (v/v) ethanol. All liquid was removed from the PCR tubes and allowed to air dry for 2 min. The PCR tubes were then removed from the magnet and the pellet was resuspended in 52.5 µl 10 mM Tris pH 8.5 or Resuspension Buffer (RSB; Illumina) and incubated at room temperature for 2 min. The tubes were once again placed on the magnetic stand for 2 min, and 50 µl of the supernatants were transferred to fresh PCR tubes.

4.3.10.2.3 Second stage index PCR

The second stage index PCR was prepared for each sample. A reaction mixture was prepared in individual 0.2 ml PCR tubes containing 5 µl DNA from the post amplicon PCR clean-up step, 5 µl of 1 µM Nextera XT Index Primer 1 (Illumina), 5 µl of 1 µM Nextera XT Index Primer 2 (Illumina), 25 µl of 2X KAPA HiFi HotStart ReadyMix and PCR grade water. Each 50 µl reaction mixture was then aspirated using a laboratory pipette (Gilson) and placed in either a Kyratec Thermocycler model SC200 (Kyratec) or a G-storm GSI Thermocycler (G-Storm). The cycle conditions consisted of an initial denaturation step at 95°C for 3 min, followed by 8 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and ending with an elongation step at 72°C for 5 min.

4.3.10.2.4 Post index PCR clean-up step

The post index PCR clean-up step began following completion of the PCR cycles. Each PCR tube was transferred to a PCR tube rack and 56 µl of AMPure XP beads were added to each. The solution was mixed by aspiration using a pipette, vortexed for 10 s and incubated at

room temperature for 5 min. Following incubation, the PCR tubes were placed on a magnetic stand (Thermo Fisher) for 2 min or until the supernatant cleared. Once cleared, the supernatant was discarded and each tube was washed twice with 200 µl freshly prepared 80% (v/v) ethanol. All liquid was removed from the PCR tubes and allowed to air dry for 2 min. The PCR tubes were then removed from the magnet and the pellet was resuspended in 27.5 µl 10 mM Tris pH 8.5 or Resuspension Buffer (RSB; Illumina) and incubated at room temperature for 2 min. The tubes were then placed once again on the magnetic stand for 2 min, after which 25 µl of the supernatants were transferred to fresh PCR tubes.

4.3.10.2.5 Library quantification and normalization

The DNA library was quantified using the Qubit Fluorometer 3.0 (Thermo Fisher), as described in Chapter 2, Section 2.3. The average library fragment size was determined on an Agilent Technologies 211 Bioanalyzer trace (Agilent Technologies, CA, USA) prepared using Agilent High Sensitivity DNA kit (Agilent Technologies, CA, USA). The DNA dye concentrate, DNA gel matrix column, DNA chip, DNA marker were all produced by Agilent Technologies. The Gel-dye mix was prepared by bringing the DNA dye concentrate and DNA gel matrix column to room temperature for 30 min. A total volume of 15 µl of the DNA dye concentrate was added to the DNA gel matrix column, vortexed and centrifuged for 10 min at room temperature at 3800 × *g*. A total of 9 µl gel-dye mix was pipetted onto the four separate control wells on the DNA chip. A combination of 1 µl DNA was added to 5 µl DNA marker for up to 11 sample wells on the DNA chip. A total of 1 µl ladder was added to 5 µl DNA marker on the DNA chip. The DNA chip was then vortexed for 1 min at 610 × *g* and inserted into the Agilent 2100 Bioanalyzer for analysis. The expected average library fragment size was 630 bp.

Once the average library fragment size was determined, each sample was diluted to a concentration of 4 nM in nuclease free water to normalise the multiplex library. The DNA molarity was determined using the following formula, with 660 g/mol referring to the average weight of a single DNA base pair:

$$\frac{(\text{concentration in ng/ µl})}{(660 \text{ g/ mol x average library size})} \times 10^6 = \text{concentration in nM}$$

Once normalised, 5 µl of each sample were pooled into a fresh 1.5 ml microcentrifuge tube mixing into a single pooled library with unique indices. A quality control step was

incorporated into the protocol, where the pooled library was quantified using the Qubit Fluorometer.

4.3.10.2.6 Denaturation and MiSeq sampling loading

Once the library concentration was confirmed, 5 µl of the pooled library and 5 µl of freshly prepared 0.2 N NaOH were mixed in a microcentrifuge tube. The tube was incubated at room temperature for 5 min to denature the DNA into single strands, the 4nM library was diluted 1 in 100 into 990 µl Hybridization buffer (HT1; Illumina), resulting in 1 ml of 20 pM denatured library in 1 mM NaOH. Prior to loading onto the MiSeq sequencer, 20 pM of the PhiX sequencing control library was combined with the denatured 20 pM amplicon library. The 20 pM was diluted from the stock solution of 10 nM PhiX (Illumina). The 10 nM PhiX was diluted 2:5 in RSB resulting in 4 nM, and further diluted 1:1 4 nM PhiX and 0.2 N NaOH. The resulting 2 nM PhiX control was briefly vortexed and incubated at room temperature. The 2 nM PhiX library was further diluted 1 in 100 in pre-chilled HT1 reagent resulting in a 20 pM PhiX control.

The 16S rRNA pooled library was diluted to the loading concentration of 12 pM with a PhiX control representing 20% of the sample. The 12 pM loading solution contained 240 µl 20 pM denatured library, 240 µl HT1 reagent and 120 µl 20 pM PhiX. The combined 600 µl was kept on ice until immediate loading on a MiSeq Reagent kit v3 reagent cartridge (Illumina). On a heating block, the tube of 12 pM loading solution was set on a heating block for 2 min at 96°C. Immediately after the incubation the tube of 12 pM loading solution was inverted and mixed placed in ice-water bath for 5 min.

4.3.10.3 Cluster amplification and sequencing

All libraries were sequenced on the Illumina MiSeq platform (Illumina) using the MiSeq Reagent kit v3 (600 cycles) (Illumina) generating paired-end reads using the MiSeq sequencing platform (Illumina).

4.3.10.4 Data interpretation and analysis

Alignment and data interpretation of the raw reads from the Illumina MiSeq sequencer was sent on-line to the BaseSpace Sequence Hub (Illumina, Eindhoven, the Netherlands). The Illumina FASTQ files were uploaded through BaseSpace to the 16S Metagenomics Illumina application. This application performs rapid taxonomic classification of 16S rRNA target amplicon reads using a taxonomic database and utilises an algorithm adopted from the

Ribosomal Database Project (RDP) (Wang *et al.,* 2007). The output read data was classified into several taxonomic levels: kingdom, phylum, class, order, genus and species. This application clusters sequences into operational taxonomic units (OTU) at a cut-off of 97% similarity and the approximate relative abundance was calculated for each sample (Limayem *et al*., 2019).

4.3.10.5 Quality control

The quality metrics for each isolate was set at an average Phred quality score >30 (Q30). The Phred quality score is a prediction of the probability of errors occurring in base calling (Illumina Technical Note 2014, Pub. No. 770-2012-058). A higher quality score correlates to a lower probability of error. A base call with a quality score of Q30 predicts one base call in 1,000 is incorrect or an average base call accuracy greater than 99.9%. A number of control metrics were investigated, including determining the percentage of reads classified into genera, number of identified species, and use of the Shannon species diversity or Shannon-Wiener index of diversity, to measure the diversity of the investigated sample (Table 4.4).

4.3.11 Investigating the efficacy of swab sampling as a method of bacterial recovery from washbasin U-bends

4.3.11.1Comparison of different types of swabs for bacterial recovery

In this study, three different types of swabs were compared to determine the efficacy of bacterial recovery. This included the comparison of three swab materials: 20-mm cotton tip and 15-cm wooden stick dry swabs (Megro, Wesel, Germany), sterile viscose transport swabs (Sarstedt, Nümbrecht, Germany), and FLOQSwabs® Nylon® fibre swabs (Copan Diagnostics Inc., CA, USA) (Figure 4.4). The viscose transport swabs were transported in Remel Amies Transport Medium without charcoal. This medium contains potassium, calcium and magnesium salts, all of which maintain an osmotic equilibrium by regulating the permeability of bacterial cells. The *P. aeruginosa* reference strain ATCC 15442 was selected as the organism to test bacterial recovery. The isolate was reactivated by removing a single bead from a preserver vial containing plastic cryogenic beads (Microbank cryovials, Prolab Diagnostics, Cheshire, UK) using a sterile forceps. The bead was streaked onto a CBA plate using a sterile wire loop and incubating overnight in a static incubator (Gallenkamp, Leceister, UK) at 37°C. Following incubation, a single colony was transferred into a 13 ml tube containing 5 ml Trypticase Soy Broth (TSB; Oxoid, Basingstoke, UK) and held in a shaking incubator overnight at 37°C. Following incubation, 1 ml of the *P.*

Table 4.4 Quality control for Illumina 16S rRNA amplicon sequencing data

Sample Name	Number of reads PF[a]	Percentage of reads classified to genus (%)	Number of genera identified	Shannon Species Diversity[b]
1	861,464	95.82	421	1.302
2	264,567	90.85	469	2.352
3	1,307,372	90.55	549	1.862
4	1,641,396	91.15	552	2.099
5	2,962,473	85.07	573	1.869
6	1,660,892	84.32	573	2.263
7	1,493,612	94.67	323	2.172
8	1,310,902	98.75	291	1.789
9	1,986,171	96.04	382	2.292
10	1,784,524	93.26	450	2.2339
11	1,656,804	97.48	334	2.035
12	1,210,590	88.78	389	1.984
13	885,222	97.46	381	2.857
14	1,776,288	97.46	367	2.418
15	729,072	95.18	433	2.11
16	764,068	97.8	511	2.658

[a] Passing filter (PF) stands for the sequence reads which have passed Illumina chastity filters.

[b] Shannon Species Diversity, also known as Shannon-Wiener index of diversity, is a measurement of the diversity of a ‘ecosystem’. <1.5 represents low diversity. >1.5<x<2.5 represents medium diversity and >2.5 represents high diversity.

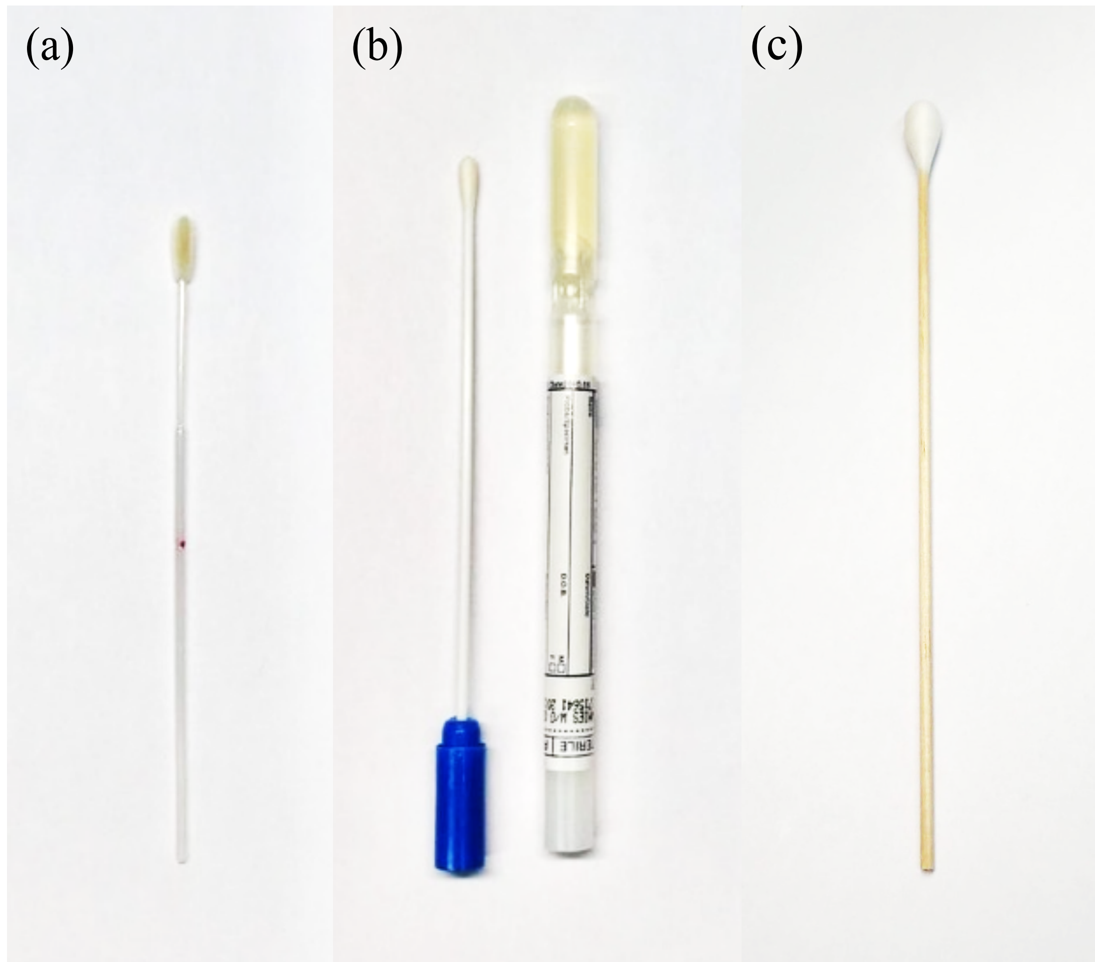


Figure 4.4 Photographs of the three types of swab used to test the efficacy of bacterial recovery from standardised inocula containing *P. aeruginosa* reference strain ATCC 15442. The three types of swabs were: **(a)** FLOQSwabs® Nylon® fibre swab, **(b)** sterile transport swab and transport tube containing Remel Amies Transport Medium, and **(c)** sterile cotton wool dry swab.

aeruginosa-TSB suspension was transferred into a 1.5 ml microcentrifuge tube and centrifuged at 2,260 × *g* for 5 min. The supernatant was discarded and the pellet was resuspended in 1000 µl 1X PBS. Standardised inocula were prepared for each experiment. The inocula were diluted ten-fold in 1 ml PBS in duplicate 1.5 ml microcentrifuge tubes (Tube A and Tube B). Tube A facilitated standardisation of the *P. aeruginosa* inocula per experiment. Aliquots of 100 µl were spread in duplicate on CBA and incubated at 37°C for 48 h. Following incubation, the colonies were counted using a Stuart™ Scientific colony counter and the average bacterial density of the suspension was determined. Tube B facilitated investigating the relative recovery of bacteria from the three different swab samples that were processed following the same protocol adopted in the ECA efficiency experiments. The tip of each tested swab was cut and incubated in a microcentrifuge tube containing 1 ml of the diluted *P. aeruginosa*-TSB suspension for 30 min.

Following incubation, individual swab tips were removed and placed in a fresh microcentrifuge tube and processed following the same approach used in previous experiments (Chapter 2, Section 2.6.3). Diluted aliquots of 100 µl were spread in duplicate on CBA and incubated at 37°C for 48 h. Following incubation, the colonies were counted using a Stuart™ Scientific colony counter and the average bacterial density of the suspension was determined following incubation and recovery from each sampling materials. The relative bacterial recovery rates using the three different swabs were determined by comparing bacterial colony counts from standardised inoculum plates and test plates.

4.3.11.2 Comparison of the bacterial recovery of morphologically distinct isolates of P. aeruginosa *by swab sampling*

The bacterial recovery from standardised inocula of an array of *P. aeruginosa* isolates was determined using the same sampling technique used in Chapter 3 and Chapter 4. A total of five isolates were selected from different U-bend locations in DDUH and their identities were confirmed by MALDI-TOF-MS. These included isolates from A&E (AE24Aug), a staff bathroom (LP3F2), and three isolates from Clinic 2 (19218_2232, B2D3dMay, and B5D4aJul). One refence strain (ATCC 15442) was included in this analysis. The isolates were chosen based on different colony morphology types including colony size after growth after 48 h incubation (large and small), colony colour and mucoid and non-mucoid colonies.

The bacterial densities of the suspensions were determined for each experiment and the relative recovery rate of each *P. aeruginosa* isolate followed the same protocol used for sterile viscose transport swabs (Sarstedt) as described in Section 4.3.7.3.

4.4 Results

4.4.1 Long term efficacy of ECA decontamination of washbasin U-bends

The automated decontamination system installed at the A&E Department of DDUH was used to investigate the automated decontamination of the drain outlets, U-bends and associated wastewater pipes of 10 identical hand washbasins by sequential treatment with the ECA solutions catholyte followed by anolyte over a period of five months. This chapter investigated the efficacy of ECA decontamination over a study period of 52 weeks in the DDUH A&E Department. The U-bends were exposed to three weekly decontamination treatment cycles (Monday, Wednesday and Friday), resulting in a total of 156 decontamination cycles throughout the study period. The 10 washbasin U-bends were sampled immediately following decontamination, 24 h post decontamination, and 48 h post decontamination for one decontamination cycle weekly. Ten non-decontaminated washbasin U-bends from a similar-use clinic, Clinic 2, were selected as controls and sampled once weekly over the same 52 week period. Swab samples were taken from the internal surfaces of the U-bends and quantitative bacterial counts recovered on CBA, R2A and PSCN agar media were determined. The average bacterial density recovered from the 10 untreated control U-bends in Clinic 2 during the study period on CBA, R2A and PSCN was 1.86×10^6 ($\pm 6.78 \times 10^5$), 1.55×10^6 ($\pm 8.08 \times 10^5$) and 1.13×10^6 ($\pm 8.40 \times 10^5$) CFU/swab, respectively, (Table 4.5). Swab samples taken immediately following ECA decontamination from the 10 A&E U-bends once weekly over the 52 weeks yielded average bacterial densities on CBA, R2A and PSCN of 28.6 (± 57.13), 52.64 (± 111.7) and 13.54 (± 77.63) CFU/swab, respectively (Table 4.5). The average bacterial densities from ECA-treated U-bends immediately following decontamination compared to control U-bends showed a >4.4 log reduction.

Similar to the five month study described in Chapter 3, swab samples were taken from the 10 ECA-treated U-bends 24 h post decontamination. The average bacterial densities recovered from the 10 ECA-treated U-bends on CBA, R2A and PSCN were 84.52 (± 147.7), 122.9 (± 198.5) and 41.06 (± 97.15) CFU/swab, respectively, (Table 4.5). The average reduction in bacterial counts from ECA-treated U-bends 24 h post decontamination relative to the corresponding bacterial counts from the 10 untreated control U-bends showed a >4.1 log reduction. Finally, an additional sampling time-point of 48 h post decontamination was taken for all 10 ECA-treated A&E U-bends. The average bacterial densities recovered from

Table 4.5 The average quantitative bacterial counts from 10 washbasin U-bends in A&E subjected to automated treatment with ECA solutions over a period of 52 weeks and the corresponding counts from ten untreated U-bends in Clinic 2

Agar medium	U-bend	Average bacterial counts in CFU/swab immediately following ECA-treatment (*n* = 52 cycles, 520 swabs) and control (*n* = 520 swabs) U-bends	SD	Range of bacterial counts in CFU/swab	*P* value
CBA	Treated	28.6	57.13	0 – 282.2	
	Untreated[1]	1.86×10^6	6.78×10^5	$1.96 \times 10^5 – 3.6 \times 10^6$	< 0.0001
R2A	Treated	52.64	111.7	0 – 606.1	
	Untreated[1]	1.55×10^6	8.08×10^5	$3.95 \times 10^5 – 4.27 \times 10^6$	< 0.0001
PSCN	Treated	13.54	77.63	0 – 596.9	
	Untreated[1]	1.13×10^6	8.40×10^5	$1.73 \times 10^5 – 3.26 \times 10^6$	< 0.0001
		Average bacterial counts in CFU/swab 24 h after ECA treatment (*n* = 52 cycles, 520 swabs) and control (*n* = 520 swabs) U-bends			
CBA	Treated	84.52	147.7	0 – 844.4	
	Untreated[1]	1.86×10^6	6.78×10^5	$1.96 \times 10^5 – 3.6 \times 10^6$	< 0.0001
R2A	Treated	122.9	198.5	$0 – 1.29 \times 10^3$	
	Untreated[1]	1.55×10^6	8.08×10^5	$3.95 \times 10^5 – 4.27 \times 10^6$	< 0.0001
PSCN	Treated	41.06	97.15	0 – 428.9	
	Untreated[1]	1.13×10^6	8.40×10^5	$1.73 \times 10^5 – 3.26 \times 10^6$	< 0.0001
		Average bacterial counts in CFU/swab 48 h after ECA treatment (*n* = 52 cycles, 520 swabs) and control (*n* = 520 swabs) U-bends			
CBA	Treated	306.7	439.6	$0 – 2.12 \times 10^3$	
	Untreated[1]	1.86×10^6	6.78×10^5	$1.96 \times 10^5 – 3.6 \times 10^6$	< 0.0001
R2A	Treated	502	593.3	$0 – 2.25 \times 10^3$	
	Untreated[1]	1.55×10^6	8.08×10^5	$3.95 \times 10^5 – 4.27 \times 10^6$	< 0.0001
PSCN	Treated	481.5	594.9	$0 – 2.74 \times 10^3$	
	Untreated[1]	1.13×10^6	8.40×10^5	$1.73 \times 10^5 – 3.26 \times 10^6$	< 0.0001

Abbreviations: ECA, electrochemically activated; CBA, Columbia blood agar; R2A, Reasoner's 2A agar; PSCN, *P. aeruginosa* selective agar; SD, standard deviation.

[1] Untreated U-bend bacterial counts were recovered from samples taken once weekly over the 52 week period as opposed to treated U-bends that were sampled three times weekly:

immediately following decontamination, 24 h post decontamination and 48 h post decontamination. For this reason, the same bacterial counts from untreated U-bends were compared to the bacterial counts from treated U-bends on each media for each sampled time point.

the ten-treated U-bends on CBA, R2A and PSCN were 306.7 (± 439.6), 502 (± 593.3) and 481.5 (± 594.9) CFU/swab, respectively, (Table 4.5). The average reduction in bacterial counts from ECA-treated U-bends 48 h post decontamination relative to the corresponding bacterial counts from the 10 untreated control U-bends showed a >3.3 log reduction.

At all-time points, reductions in average bacterial counts from treated U-bends on all media relative to the corresponding counts from untreated U-bends were highly significant ($P < 0.0001$), (Table 4.5).

4.4.2 Recolonisation of A&E washbasin U-bends and drains seven months post cessation of ECA treatment

Following the completion of the 52 week study, routine ECA decontamination of A&E U-bends ceased. Swab samples were taken from each of the 10 A&E washbasin U-bends seven months post cessation of ECA treatment in order to determine the average bacterial densities within U-bends following cessation of routine decontamination. As detailed in Section 4.4.1 above, throughout the 52 week test period, the average bacterial densities from the 10 ECA-treated U-bends immediately following decontamination on CBA, R2A and PSCN were 28.6 (± 57.13), 52.64 (± 111.7) and 13.54 (± 77.63) CFU/swab, respectively (Table 4.5). In contrast, the average bacterial densities from 10 U-bends seven months post cessation of routine ECA decontamination on CBA, R2A and PSCN were 1.59×10^5 ($\pm 1.34 \times 10^5$), 3.19×10^5 ($\pm 2.77 \times 10^5$) and 8.89×10^4 ($\pm 9.09 \times 10^4$) CFU/swab, respectively. The average bacterial densities in U-bends during the 52 week period of ECA-treatment compared to the same U-bends seven months following cessation of ECA decontamination exhibited a >3.75 log increase in bacterial counts on all media.

The washbasin drain outlets from the 10 washbasins in A&E were also sampled seven months post cessation of ECA decontamination. The average bacterial densities recovered on CBA, R2A and PSCN were 2.97×10^3 ($\pm 2.65 \times 10^3$), 6.1×10^3 ($\pm 4.1 \times 10^3$) and 1.16×10^4 ($\pm 1.6 \times 10^4$) CFU/swab, respectively. These drain outlet counts were compared to the bacterial counts recovered from six of A&E washbasin outlets determined during the initial five month ECA-decontamination test period described in Chapter 3, Section 3.4.4. In the five-month study, the average bacterial density recovered on CBA agar was 1 (± 5) CFU/swab, whereas no colonies were recovered on PSCN agar. The average bacterial densities in drain outlets during the five-month study of ECA-treatment compared to the

same drain outlets seven months following cessation of ECA decontamination displayed a >3.4 log increase in bacterial counts on all media.

4.4.3 Use frequency of washbasins in A&E

Over a period of one week, washbasin usage in the A&E Department was monitored by a dental nurse. The average daily use frequency was recorded as 12 times per day per washbasin. The average volume of water discharged from A&E washbasin taps during hand washing (n = 5) was 650 ml.

4.4.4 The spread of bacteria from HP and DP washbasins in DDUH

Air samples were taken from three washbasins (one DP in West Clinic; one HP in A&E; one HP in Clinic 2) throughout DDUH while the tap water was flowing. All three washbasins were not routinely decontaminated with ECAs. The A&E sample was taken seven months after the cessation of routine decontamination in the A&E. Air samples were taken to investigate if bacteria, and in particular *P. aeruginosa*, was aerosolised from drain outlets/washbasin surfaces while the tap water was flowing at these sites. Prior to sampling, the tap was turned on at each site, the air sampler was held at a distance of 50-cm away from the drain outlet and tilted to face the drain outlet. No colonies were recorded on PSCN agar plates sampling at all three sites. All sites were also sampled using CBA agar plates, yielding 132 CFU/m^3 from the DP washbasin in West Clinic, 92 CFU/m^3 from the HP washbasin tap in Clinic 2, and 214 CFU/m^3 from the HP washbasin tap in A&E.

4.4.5 Relative abundance of potentially pathogenic culturable bacteria in an ECA treated U-bend in A&E and untreated U-bend in Clinic 2

The approximate relative abundance of culturable bacterial species in ECA-treated washbasin U-bends in A&E and untreated control washbasin U-bends in Clinic 2, during the 52 week study period was investigated. The approximate relative abundance of potentially pathogenic culturable bacteria in one A&E U-bend was assessed over a five week period. A total of 1120 bacterial colonies were observed on CBA plates from swab samples within the U-bend at the three sampling time points per week over the five week period (n = 15). A representative selection of distinct colony morphology types (n = 23) were selected for identification, based on the colony morphology and colour, and identified using MALDI-TOF-MS.

MALDI-TOF-MS analysis successfully identified 91% of colonies (21/23). Gram-negative bacterial species represented 85% (18/21) of the identifiable colonies including: *P. aeruginosa* (13/21), and *Pseudomonas putida* (5/21). Gram-positive bacterial species represented 15% (3/21) of the identifiable colonies including: *Micrococcus luteus* (2/21) and *Dermacoccus nishinomiyaensis* (1/21).

The relative abundance of culturable bacterial species was determined for 10 control washbasins in Clinic 2. Unlike A&E, all 10 washbasins were investigated at two time points. The high bacterial counts recovered by swab sampling of washbasin U-bends in Clinic 2 required 10-fold dilutions of the bacterial solutions recovered from the processed swabs samples, and aliquots of the dilutions were spread in duplicate onto CBA plates. The relative abundance of each colony morphology type observed was recorded for each sample. A total of 33 representative colonies were selected for identification using MALDI-TOF-MS.

MALDI-TOF-MS analysis successfully identified all 33 colonies selected for identification resulting in the identification of nine bacterial species. Eight Gram-negative species were identified including: *P. aeruginosa* (10/33), *Stenotrophomonas maltophilia* (5/33), *Delftia acidovorans* (3/33), *Achromobacter denitrificans* (2/33), *Achromobacter xylosoxidans* (2/33), *Burkholderia cenocepacia* (2/33), *Enterobacter cloacae* (2/33), and *Elizabethkingia meningoseptica* (2/33). One Gram-positive species was identified, *Micrococcus luteus* (5/33).

4.4.6 Characterisation of bacterial communities in sixteen DDUH wastewater network locations by Illumina high-throughput 16S rRNA amplicon sequencing

The targeted variable V3 and V4 regions of 16S rRNA genes present within bacteria recovered from wastewater network samples were sequenced to determine the bacterial population structure across 16 sampled wastewater network locations in DDUH. The 16S rRNA sequencing was undertaken to provide an estimate of the total bacterial abundance in the DDUH wastewater system. The 16 sampled locations included: fourteen U-bend samples (five U-bends in Clinic 2, one U-bend in A&E, one U-bend in CSSD, six U-bends in six individual staff bathrooms, and one U-bend in West Clinic) and two wastewater pipe samples (one from a vertical wastewater pipe common to Clinic 2 and Clinic 1, and one site at the point of discharge of a wastewater pipe to the municipal sewer). All site locations are shown in Figure 4.3. Taxonomic annotation of the sequence read data revealed that the

bacterial domain predominated (average 99.997%), with the Archaea domain and viruses representing the remaining 0.003%.

4.4.6.1 The bacterial community in DDUH wastewater network

A total of 22,295,417 quality assured sequence reads were utilised in this study, with an average of 1,393,464 reads (range 729,072 – 2,962,473) obtained per sampled location (Table 4.4). An average of 93% of the total bacterial sequence reads (range 84.3% – 98.8%) could be classified at the genus level taxonomic ranking. The bacterial communities among the 16 samples were dominated by the following four phyla: Proteobacteria (median 86.6%, range 48.3% – 99.2%), Bacteroidetes (median 5.9%, range 0.29% – 17.8%), Actinobacteria (median 1.45%, range 0.01% – 39.8%) and Cyanobacteria (median 0.5%, range 0.01% – 3.4%).

The data were organised based on the relative abundance of the five most abundant bacterial families identified in each of the 16 sampled locations (n = 28), as shown in Figure 4.5. The median number of bacterial families observed among the population was 186.5 (range 126 – 232). The top five most abundant bacterial families identified in each of the 16 sampled locations represent an average 76.1% (range 39.9% – 98.9%) of the total abundance. The following families represented ≥10% of the relative total abundance in one or more of the 16 samples: *Alcaligenaceae*, *Burkholderiaceae*, *Caulobacteraceae*, *Comamonadaceae*, *Enterobacteriaceae*, *Flavobacteriaceae*, *Hyphomicrobiaceae*, *Methylobacteriaceae*, *Mycobacteriaceae*, *Oxalobacteraceae*, *Pseudanabaenaceae*, *Pseudomonadaceae*, *Rhodocyclaceae*, *Rhodospirillaceae*, and *Sphingomonadaceae*. Families *Comamonadaceae*, *Rhodocyclaceae, Pseudomonadaceae, Oxalobacteraceae* and *Rhodospirillaceae* were identified as one of the most abundant families in at least half of the samples.

Analysis of the data based on bacterial genera, a median number of 421 genera (range 291 – 573) were observed among the population. The top five most abundant bacterial genera identified in each of the 16 sampled locations represent an average 69.8% (range 32.2% – 96.1%) of the total abundance. The following genera represented ≥10% of the relative total abundance in one or more of the 16 samples: *Achromobacter*, *Acidovorax*, *Azospira*, *Burkholderia*, *Caulobacter*, *Cupriavidus*, *Delftia*, *Elizabethkingia*, *Enterobacter*, *Flavobacterium*, *Janthinobacterium*, *Magnetospirillum*, *Methylobacterium*, *Mycobacterium*, *Paracoccus*, *Pseudomonas*, *Rhodoplanes*, *Sphingomonas*, *Trabulsiella* and *Zoogloea*.

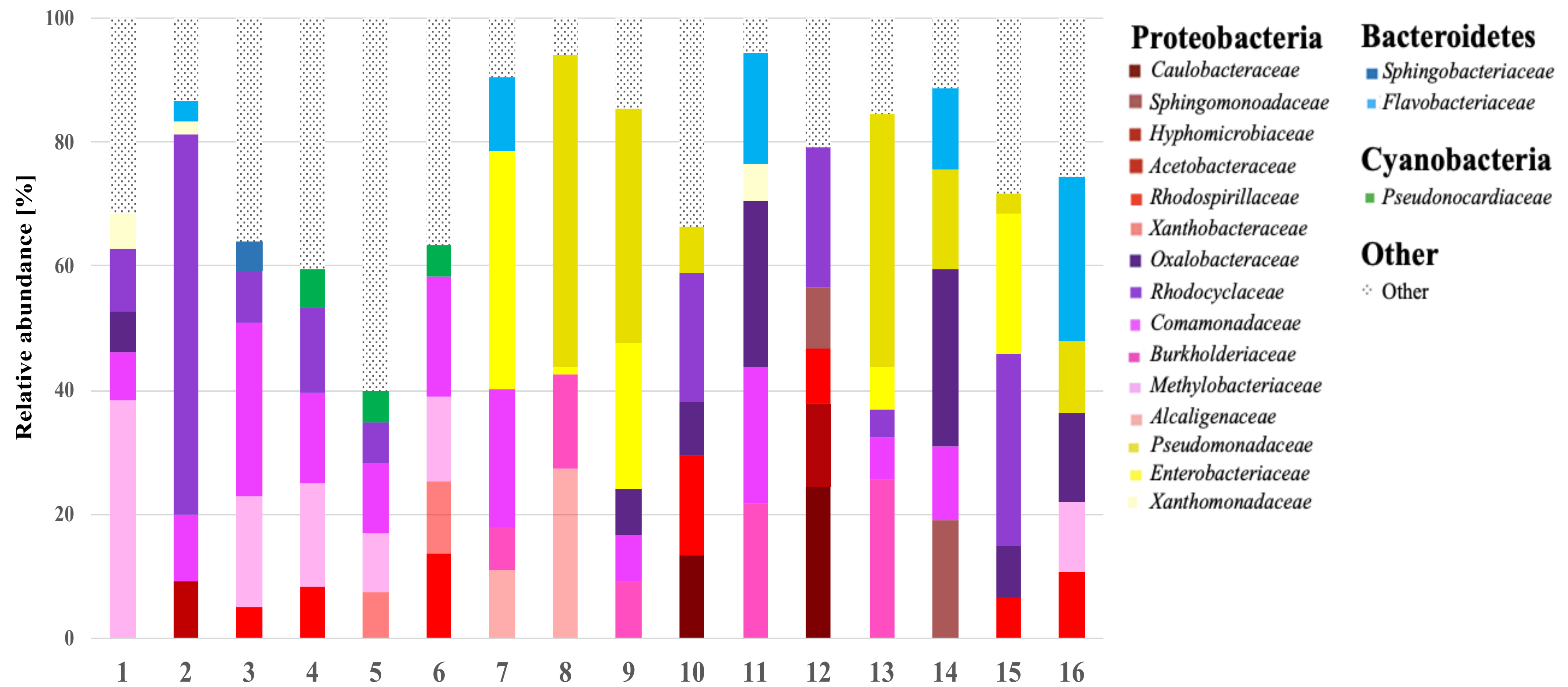
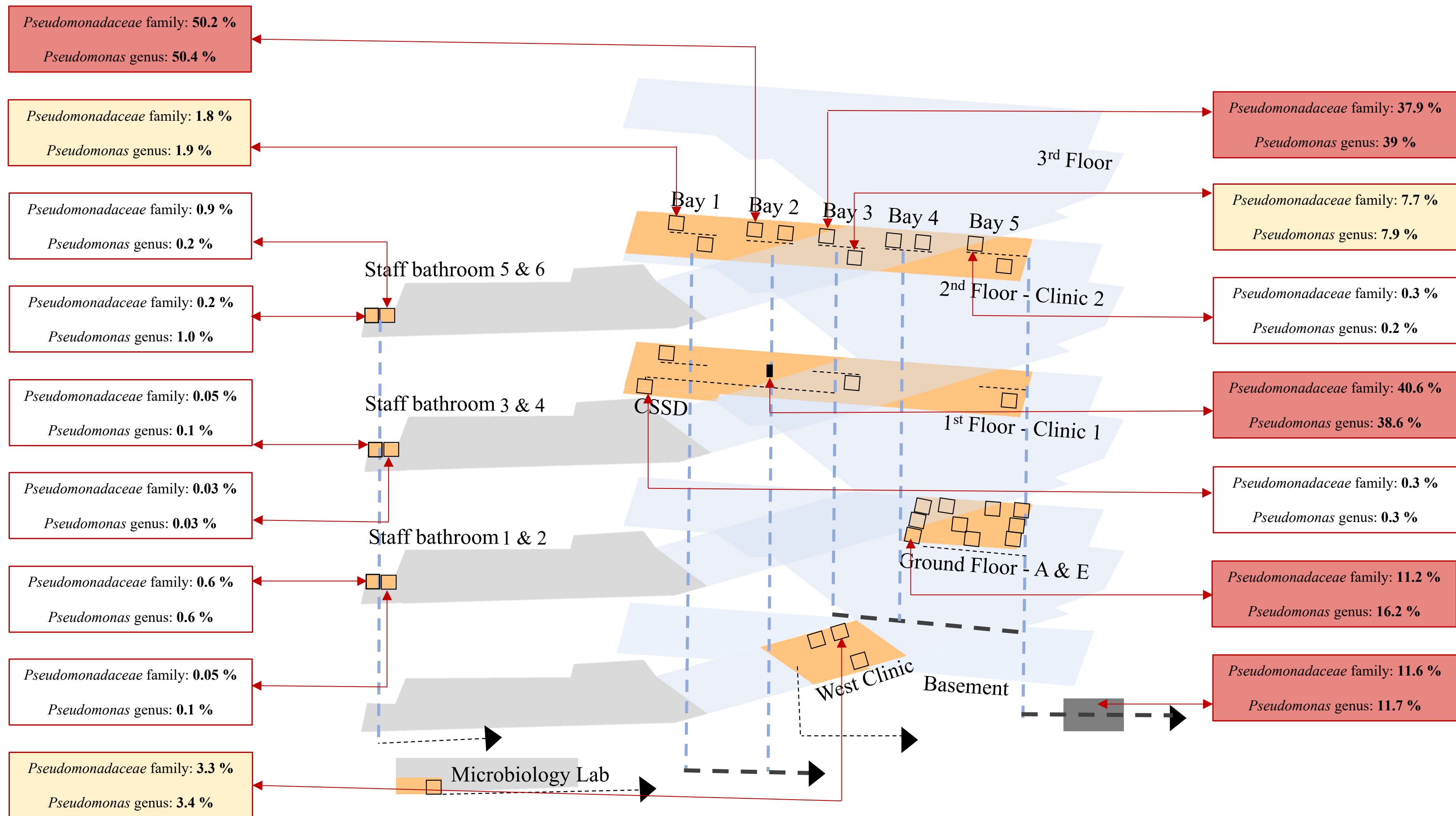

Relative abundance [%]
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Proteobacteria
Caulobacteraceae
Sphingomonoadaceae
Hyphomicrobiaceae
Acetobacteraceae
Rhodospirillaceae
Xanthobacteraceae
Oxalobacteraceae
Rhodocyclaceae
Comamonadaceae
Burkholderiaceae
Methylobacteriaceae
Alcaligenaceae
Pseudomonadaceae
Enterobacteriaceae
Xanthomonadaceae
Bacteroidetes
Sphingobacteriaceae
Flavobacteriaceae
Cyanobacteria
Pseudonocardiaceae
Other
Other

Figure 4.5 Stacked bar charts representing the relative abundance of the top five taxonomic rank families of each of the 16 samples from the DDUH wastewater network. The 16 distinct locations within the wastewater pipe network included: six washbasin U-bends from the individual staff bathrooms in DDUH (sample sites 1 – 6), five washbasin U-bends in Clinic 2 (sample sites 7 – 11), one washbasin U-bend in CSSD (sample site 12), the internal pipe surface of a vertical pipe connecting Clinic 1 and Clinic 2 (sample site 13), one washbasin U-bend from the A&E Department seven months post cessation of ECA decontamination (sample site 14), one washbasin U-bend in West Clinic (sample site 15), and the point-of-discharge sampling site of the main common wastewater pipe connecting Clinic 1, Clinic 2, CSSD and A&E (sample site 16). Segments of the stacked bar charts shown in red, purple or yellow represent families belonging to the Proteobacteria phylum: red represents class α-proteobacteria, purple represents class β-proteobacteria, and yellow represents class γ-proteobacteria. Segments of the stacked bar charts shown in blue represent families belonging to the Bacteroidetes phylum, green represents a family belonging to the Cyanobacteria phylum. All families not comprising the top five taxonomic rank families for each location were collectively grouped under Other.

The following bacterial species were identified as the most abundance organisms in one or more of the 16 samples: *Achromobacter insolitus*, *Acidovorax temperans*, *Azospira oryzae*, *Burkholderia vietnamiensis*, *Caulobacter crescentus*, *Chryseobacterium soli*, *Cupriavidus metallidurans*, *Cupriavidus pauculus*, *Delftia lacustris*, *Elizabethkingia meningoseptica*, *Flavobacterium succinicans*, *Janthinobacterium lividum*, *Leptolyngbya laminose*, *Mycobacterium abscessus*, *Mycobacterium ulcerans*, *Sphingopyxis taejonensis*, *Paracoccus aminovorans*, *Pseudomonas aeruginosa*, *Pseudomonas azotoformans*, and *Pseudoxanthomonas indica.*

4.4.6.2 The relative abundance of the family Pseudomonadaceae *and genus* Pseudomonas *at the 16 sampled locations*

At both the family level *Pseudomonadaceae* and genus level *Pseudomonas,* only 5/16 samples from the DDUH wastewater network locations contained *Pseudomonadaceae* and *Pseudomonas* that represented >10% of the relative population of that sample. However, *Pseudomonadaceae* and *Pseudomonas* was detected at all 16 locations of the wastewater pipe. The average relative abundance of *Pseudomonadaceae* per sample was 10.4%, with a median of 1.35%. Figure 4.6 shows the relative abundance of the genus *Pseudomonas* of the total bacterial population at each location sampled. Within Clinic 2, the genus *Pseudomonas* represents 50.4% of the relative abundance of bacterial genera from U-bend sample 8, and 39% of the relative abundance of bacterial genera from U-bend sample 9 (Figure 4.2). The genus *Pseudomonas* represents 38.6% of the relative abundance of bacterial genera from the internal pipe surface of a vertical pipe connecting Clinic 1 and Clinic 2 (sample site 13), 16.2% of the relative abundance of bacterial genera from U-bend sampled in the A&E Department seven months post cessation of ECA decontamination (sample site 14), and 11.7% of the relative abundance of bacterial genera from the point-of-discharge sampling site of the main common wastewater pipe connecting Clinic 1, Clinic 2, CSSD and A&E (sample site 16). Within two of the remaining samples, the genus *Pseudomonas* comprised >1% – 10% of the relative abundance of bacterial genera (7.9% of the relative bacterial genera from Clinic 2 U-bend sample 10 and 3.4% of the relative bacterial genera from West Clinic U-bend sample 15). The remaining nine samples consisted of the genus *Pseudomonas* comprising 0% – 1% of the relative bacterial genera. At a species, *P. aeruginosa* was detected in level 15/16 sampled locations.



Pseudomonadaceae family: **50.2 %**
Pseudomonas genus: **50.4 %**
Pseudomonadaceae family: **1.8 %**
Pseudomonas genus: **1.9 %**
Pseudomonadaceae family: **0.9 %**
Pseudomonas genus: **0.2 %**
Pseudomonadaceae family: **0.2 %**
Pseudomonas genus: **1.0 %**
Pseudomonadaceae family: **0.05 %**
Pseudomonas genus: **0.1 %**
Pseudomonadaceae family: **0.03 %**
Pseudomonas genus: **0.03 %**
Pseudomonadaceae family: **0.6 %**
Pseudomonas genus: **0.6 %**
Pseudomonadaceae family: **0.05 %**
Pseudomonas genus: **0.1 %**
Pseudomonadaceae family: **3.3 %**
Pseudomonas genus: **3.4 %**
Pseudomonadaceae family: **37.9 %**
Pseudomonas genus: **39 %**
Pseudomonadaceae family: **7.7 %**
Pseudomonas genus: **7.9 %**
Pseudomonadaceae family: **0.3 %**
Pseudomonas genus: **0.2 %**
Pseudomonadaceae family: **40.6 %**
Pseudomonas genus: **38.6 %**
Pseudomonadaceae family: **0.3 %**
Pseudomonas genus: **0.3 %**
Pseudomonadaceae family: **11.2 %**
Pseudomonas genus: **16.2 %**
Pseudomonadaceae family: **11.6 %**
Pseudomonas genus: **11.7 %**
3rd Floor
Bay 1
Bay 2
Bay 3
Bay 4
Bay 5
2nd Floor - Clinic 2
Staff bathroom 5 & 6
Staff bathroom 3 & 4
CSSD
1st Floor - Clinic 1
Staff bathroom 1 & 2
Ground Floor - A & E
West Clinic
Basement
Microbiology Lab

Figure 4.6 Schematic showing the relative abundance of the *Pseudomonadaceae* family and *Pseudomonas* genus recorded at the 16 separate DDUH wastewater network locations investigated. Fourteen of the 16 locations were U-bends, and two were sampling ports in wastewater pipes (the vertical wastewater pipe connecting Clinic 1 and Clinic 2 and the point-of-discharge large common wastewater pipe).White boxes display the percentage of *Pseudomonas* genus representing 0 – 1% of the relative population. Yellow boxes display the percentage of *Pseudomonas* genus representing >1% – 10% of the relative population. Red boxes display the percentage of *Pseudomonas* genus representing >10% of the relative population.

4.4.7 Efficacy of swab sampling as a method of bacterial recovery

4.4.7.1 Comparison of different swab types for bacterial recovery

Three different types of swabs were compared to determine the efficacy of bacterial recovery. The relative bacterial recovery rates of *P. aeruginosa* ATCC 15442 strain using three different kinds of sampling swabs (n = 7) were determined by comparing the relative bacterial colony counts on CBA plates recovered from standardised inocula. The average bacterial densities recovered from three swab types were determined as a percentage of the total bacterial density of standardised inocula. The average relative recovery rates were as follows: 19.4% bacterial recovery (± 7.60) using sterile viscose transport swabs, 40.7% bacterial recovery (± 15.14) using FLOQSwabs® Nylon® fibre swabs, and 45.5% bacterial recovery (± 17.96) using 20-mm cotton tip and 15-cm wooden stick dry swabs.

4.4.7.2 Bacterial recovery of morphologically distinct isolates of P. aeruginosa *by swab sampling*

The bacterial recovery of five confirmed morphology distinct *P. aeruginosa* isolates and one references *P. aeruginosa* ATCC 15442 strain was compared to determine whether colony morphology affected bacterial recovery. The relative bacterial recovery rates using the five morphologically distinct isolates and the reference strain (n = 6) were determined by comparing bacterial colony counts recovered from standardised inocula and following recovery from swabs. The average recovery rate of the six *P. aeruginosa* isolates from swab sampling using a sterile viscose transport swabs was 21.4%, which is in accordance with the experiment discribed in section 4.4.7.1.

The average relative recovery rates were as follows: a bacterial recovery rate of 24.76% (± 6.28%) was observed using the reference strain ATCC 15442, a bacterial recovery rate of 23.52% (± 17.15%) was observed using the mucoid displaying A&E originating isolate AE24Aug which produced small colonies, a bacterial recovery rate of 16.82% (± 7.36%) was observed using the non-mucoid small staff bathroom originating isolate LP3F2 which produced small colonies, a bacterial recovery rate of 14.88% (± 4.13%) was observed using the non-mucoid Clinic 2 originating isolate 19218_2232 which produced a rich pyoverdine pigment in large colonies, a bacterial recovery rate of 27.295% (± 14.48%) was observed using the non-mucoid Clinic 2 originating isolate B2D3dMay which produced a rich pyocyanin pigment in small colonies, and a bacterial recovery rate of 23.88% (± 28.76%) was observed using the Clinic 2 originating isolate B5D4aJul producing pyocyanin pigment large colonies.

4.5 Discussion

The escalating number of reports identifying washbasin U-bends as reservoirs of AMR bacteria highlights the necessity of developing and maintaining effective decontamination protocols. The present study examined the long-term efficacy of ECA-decontamination system described in Chapter 3 (Deasy *et al.,* 2018). This chapter monitored the effects of ECA decontamination on washbasin U-bend bioburden over a period of 52 weeks. Routine ECA decontamination of the A&E washbasins proved to be a consistently effective approach in reducing the bacterial bioburden within the wastewater environment. This chapter also accurately identified the bacterial communities within the wastewater network in DDUH. The accurate identification of the bacterial communities within these environments may lead to effective assessment of washbasin decontamination and mitigation strategies.

In this study, three main modifications were made to the large-scale U-bend decontamination system described in Chapter 3 and the experimental monitoring techniques adopted. Firstly, the untreated control washbasin U-bends were changed. Exactly one year after the refurbishment of the A&E Department, Clinic 2 on the second floor of DDUH was equipped with identical washbasins, U-bends and wastewater pipes (Figure 4.6). Clinic 2 and the A&E Department share a common water supply and have similar usage. This modification enabled the comparison of bacterial bioburden in washbasin U-bends in nearly identical test sites and conditions. Secondly, the weekly sampling frequency and the sampling period were altered. The five-month study described in Chapter 3 encompassed a total of 62 decontamination cycles with three monitored decontamination cycles per week (Monday, Wednesday, Friday). In the present study, the ECA-treated washbasin U-bends were subjected to a total of 156 decontamination cycles three times weekly over a period of 52 weeks. However the washbasin basin U-bends were sampled immediately following only one decontamination cycle per week (n = 52) (Table 4.5). Extension of the sampling period enabled adequate monitoring of the decontamination efficacy once weekly. A >4.4 log reduction was observed on all media immediately following decontamination over the test period between the 10 ECA-treated washbasin U-bends and the 10 untreated U-bends in Clinic 2 (Table 4.5). Furthermore, a >4.1 log reduction was observed between the ECA-treated U-bends and the 10 untreated U-bends in Clinic 2 on all media 24 h post decontamination over the 52 week test period (Table 4.5). A third modification implemented in this chapter was the addition of a third sampling time point: 48 h post ECA-decontamination. This sampling time point facilitated the investigation of the recovery of bioburden within the washbasin U-bends after

48 h treatment with ECA solutions. The bacterial recovery data 48 h after decontamination on all media tested showed a >3.3 reduction (Table 4.5). Results from these additional timepoints may reflect the residual effects of ECA decontamination on the biofilm within the pipework or may reflect slow recovery of the bacterial bioburden following ECA-treatment. Following completion of the study, the washbasin drains and U-bends were sampled seven months post cessation of ECA decontamination. The bacterial density in all washbasin U-bends had recovered and exhibited a >3.4 log increase in bacterial counts on all media relative to the average bacterial counts throughout the 52 week ECA-decontamination study.

The installation of washbasin U-bends with two access ports in the study described in Chapter 3 and in this chapter permitted the sampling of six selected sites in rotation to reduce the mechanical removal of biofilm by routine swab sampling. In Chapter 3, washbasin U-bends in the A&E Department were sampled following each decontamination cycle and 24 h after decontamination, resulted in six sampling events per week. In this chapter, the number of weekly U-bends sampling events was reduced to three time points per week. The availability of the average bacterial bioburdens within A&E washbasins from both studies enabled the comparison of these data and the investigation of the role mechanical removal may play on both these data. Both systems were subjected to identical decontamination processes but varied on the frequency of sampling and the total duration of the test periods. Interestingly, the bacterial recovery data immediately following ECA-treatment showed a slight reduction in the average counts on all media from the five-month study to the current study: 73.4 (± 258.2) compared to 28.6 (± 57.13) CFU/swab on CBA, 122.5 (± 371.3) compared to 52.64 (± 111.7) CFU/swab on R2A, and 15.3 (± 184.5) compared to 13.54 (± 77.63) CFU/swab on PSCN, respectively (Table 3.1 and Table 4.5). Likewise, both studies observed a >3.3 log reduction in bacterial counts in U-bends on all media relative to untreated controls (P <0.0001), (Table 3.1 and Table 4.5). Increased frequency of sampling in Chapter 3 did not appear to reduce the bacterial bioburden in washbasin U-bends as the average bacterial counts were larger compared to the corresponding data from this chapter.

Increased attention is now focusing on the misuse of hand washbasins in healthcare facilities. Hand washbasins are intended for hand washing only. However, not all healthcare facilities implement proper use protocols. In the present study, the frequency of use of A&E washbasins was monitored over a course of one week by a dental nurse based in the A&E Department. The washbasins were recorded as being utilised for hand washing events only,

and the observed frequency of use was determined of an average of 12 times per day. Furthermore, in this study, Tork Extra Mild Liquid Soap was the only hand washing preparation provided at hand washbasins. Increasingly, studies are investigating the improper usage of washbasins in healthcare facilities, as such events may lead to potential bacterial seeding or transmission of organisms within the wastewater network. Grabowski *et al.* (2018) investigated the range of activities being carried out using handwashing sinks in two patient rooms and two patient bathrooms in an ICU over 60 days. Sink use was observed by motion sensitive cameras and periodic in-person observations (Grabowski *et al.*, 2018). Unexpectedly, hand washing accounted for only 4% of the total observed activities. The remaining 96% of activities including: medical patient care activities such as short-term holding of medical items and emptying/filling syringes etc., and non-medical patient care activities, such as wetting of patient cleaning cloths, patient nutrition, and environmental cleaning (Grabowski *et al.,* 2018). The results of this study emphasised how misuse of washbasins can lead to contamination events of the washbasins and washbasins U-bends.

The effects of drainage and availablitity of nutrients have recently been identified as key factors in bacterial proliferation and spread in wastewater networks. The general drainage conditions were observed routinely following completion of the one decontamination cycle per week. Following each decontamination cycle, all washbasins were flushed with water. No blockages were observed or reported within the A&E U-bends throughout the study. The importance of maintaining good cleaning practises in washbasins and their assoicated drainage was highlighted in a paper by Aranega-Bou *et al.* (2018). This paper investigated how drainage rates effected the dispersal of a *Citrobacter freundii* strain, *K. pneumoniae* carbapenemase (KPC)-producing Enterobacteriaceae and other CRE strains from the drain outlets of HP and DP washbasins. Slow drainage in HP sinks resulted in 30-fold less dispersal of CREs from washbasin drain outlets than DP sinks, and when the drainage was fast, HP sinks released fewer CRE from sink drain oulets than DP sinks measured by settle plates placed around the perimeter of the sink (Aranega-Bou *et al.,* 2019). Secondly, the effects nutrients had on the growth of CRE in a model sink U-bend system has recently been studied (Kotay *et al.,* 2020). The study demonstrated that the four selected bacteria strains (*Citrobacter freundii*, *Enterobacter hormaechei*, *Serratia marcescens*, and *Klebsiella quasipneumoniae*) persisted in biofilms under nutrient deficit conditions within a sink U-bend. The bacteria rapidly proliferated when exposed to nutrient rich conditions and spread within the wastewater towards the drain outlets and further downstream (Kotay *et al.,* 2020).

While hand washing is vital for proper hygienic patient care, misuse or mismanagement of washbasins, the lack of efficient decontamination or cleaning practise, and the action of hand washing itself may lead to the increased risk of transmission of potential pathogens. The primary dispersion mechanism of potential pathogens from washbasin drains and U-bends was initially established to be caused by the generation of aerosols as airborne particles originating from washbasins (Hota *et al.,* 2009; Schneider *et al.,* 2012; Fusch *et al.*, 2015; De Geyter *et al.,* 2017). Aerosols are defined as airborne particles measuring ≤5 µm in diameter, as opposed to droplets that measure >5 µm in diameter (Siegel *et al.,* 2007; World Health Organization, 2014b). Following the cessation of ECA-decontamination, the air around the three washbasins (two HP and one DP) where the flow of water had been activated in DDUH was sampled. The counts were recorded on CBA and PSCN agar. No bacterial colonies were detected on PSCN agar, and similar bacterial counts were recovered from all three washbasins. On CBA agar, similar bacterial counts were observed from all three washbasins, irrespective of the washbasin design. Parameters known to affect air sampling include: quantity of air contaminants, the airflow in the environment, and the activity levels in the environment being sampled that may have resulted in similar bacterial counts in our study (Chia *et al.,* 2020). The previous assumption that aerosols were the primary dispersion mechanism of bacteria from washbasins was recently challenged. Kotay *et. al* (2019) demonstrated in a model system that droplets were the primarly method of bacterial transmission from contamined handwashing domestic pattern sink U-bends. Likewise, a Aranega-Bou *et al.* (2018) installed artifically contaminated and naturally contamined wastewater U-bends into a model laboratory-based sink system. Active and passive air sampling demonstrated the dispersal of pathogens from sinks was mainly droplet based and multidirectional (Aranega-Bou *et al.,* 2019). In either case, the generation of both types of airborne particles facilitate the dissemination of bacteria from washbasins.

The second main aim of this chapter was to identify the environmental bacterial communities within the wastewater network in DDUH. The two strategies adopted were: a culture-based approach utilising MALDI-TOS-MS, and a culture-independent approach utilising Illumina high throughput 16S rRNA amplicon sequencing. Over the past three decades, washbasin drain- and U-bend-associated outbreaks have primarily been linked with bacteria species (Carling, 2018). Only recently other microorganims, such as the fungal *Fusarium spp.* residing in the wastewater network, have been associated with hospital based outbreaks (Hino *et al.,* 2020). In that study, the clinical epidemiology of invasive fusariosis was

monitored in patients with haematological disease in Japan. Drain outlets were detected as a reservoir for *Fusarium spp.* and the high similarity of drain and clinical samples indicate a key role that washbasins colonisation may have in transmission of infection (Hino *et al.,* 2020). For this reason, the majority of reports have focused on bacterial communities and population structures in wastewater networks (Guo *et al.*, 2019; Limayem *et al.*, 2019; Numberger *et al.*, 2019; Constantinides *et al.*, 2020). In this chapter, MALDI-TOF-MS was utilised to determine the approximate media-specific culturable bacterial communities within one washbasin ECA-treated U-bends and within the 10 untreated Clinic 2 U-bends. Gram-negative bacterial species accounting for 85% of the identifiable selected colonies in the ECA-treated U-bends with *P. aeruginosa* the most predominant species (62%). *Pseudomonas aeruginosa* was also identified as the predominant organism (30%) identified among samples selected from 10 washbasin U-bends in Clinic 2. A wider range of microorganism were identified in the non-ECA treated Clinic 2 U-bends (nine bacterial species, eight Gram-negative and one Gram-positive) compared to the one tested A&E U-bend (four bacterial species, two Gram-negative and two Gram-positive). The utilisation of MALDI-TOF-MS provided a rough indication of the culturable bacterial species present in the selected washbasin U-bends.

Culture-based approaches are time consuming and limited in their ability to identify fastidious microorganisms and viable but non-culturable bacteria. For this reason, culture-independent methods have increasingly been adopted for more accurate characterisation of bacterial communities within wastewater networks (Guo *et al.*, 2019; Limayem *et al.*, 2019; Numberger *et al.*, 2019; Constantinides *et al.*, 2020). The bacterial communities within the wastewater networks reflect the environments they inhabit. These environments include the pipe materials, the nutrients and oxygen availabilty available to microorganisms, the ability of microorganisms to effectively compete with the large densities of viable bacteria and form biofilms, the ability of microorganisms to adapt to rapidly changing environmental conditions, and the selective pressures placed on the bacterial communities by antibiotic use and decontamination processes (McLellan and Roguet, 2019). Illumina high throughput sequencing of the V3-V4 regions of the 16S rRNA gene was used to determine the microbial composition across 16 sampled wastewater network locations in DDUH. This methodology was utilised because the V3-V4 regions have been shown to provide taxonomic accuracy, is one of the most commonly adopted regions for microbiota analyses and the MiSeq Illumina technology provides high-quality reads for futher analyses (Onywera and Meiring, 2020). The phlyum Proteobacteria represented a median 86.6% of all the sequence reads, followed

by Bacteroidetes (median 5.9%), Actinobacteria (median 1.45%) and Cyanobacteria (median 0.5%). The phylum Proteobacteria contains *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter spp.*, pathogens that have been identified in hospital wastewater and wastewater treatment effluent. These organisms have been listed as priority status pathogens for the development of new antibiotics due to the increased incidence of multi-drug resistance (World Health Organization, 2018; De Oliveira *et al.*, 2020). Proteobacteria have been identified as the dominant phylum in multiple metagenomic studies of wastewater samples as well as being a dominant phylum in biofilms formed on PVC, and stainless steel surfaces (Jiang *et al*., 2008; Li *et al*., 2014; Nascimento *et al.*, 2018; Ibekwe and Murinda, 2019).

At the bacterial family level, 15 familes were identified in the top five most abundant families. The families *Comamonadaceae*, *Rhodocyclaceae, Pseudomonadaceae, Oxalobacteraceae* and *Rhodospirillaceae* were identified in the top five families in at least half of the samples investigated (Figure 4.5). Classification to genus level is thought of as reliable using short-read sequencing technologies. Classification of short reads sequences to species level may lead to high levels of misclassification, however here the species identified are listed as an idicator of species within theses samples (Winand *et al.,* 2020). At a species level, of the 20 most abundant species eight have previously been identified as opportunistic pathogens for human diseases including: *P. aeruginosa*, *Cupriavidus pauculus, Delftia lacustris, Mycobacterium ulcerans, Achromobacter insolitus, Burkholderia vietnamiensis, Elizabethkingia meningoseptica,* and *Janthinobacterium lividum* (Sizaire *et al.*, 2006; Jassem *et al.,* 2011; Shin *et al.,* 2012; Almasy *et al.*, 2016; Li *et al.,* 2017). Interestingly, two species of note are *Elizabethkingia meningoseptica,* which is intrinsically resistant to many commonly utilised hospital antibiotics, and *Janthinobacterium lividum,* which produces violacein that has antibacterial, antiviral, and antifungal properties (Balm *et al.,* 2013; Valdes *et al.*, 2015). Also, the environmental species *Cupriavidus metallidurans* was listed as one of the most abundant species and is a Gram-negative bacterium previously shown to adapt for survival in heavy metal stresses such as copper, a prominent wastewater pipe material (Lal *et al.,* 2013). The bacterial diversity observed utilising high-throughput 16S rRNA amplicon sequencing demonstrates the limitations of culture-based approaches.

As previously discussed, *P. aeruginosa* is a microorganism of significant importance to human pathology, and is ubiquitous within wastewater networks (Hota *et al.,* 2009; Breathnach *et al.,* 2012; Leitner *et al.,* 2015; Stjarne Aspelund *et al.,* 2016). Carbapenem-

resistant *P. aeruginosa* is listed at critical level for research and development into new antibiotics (World Health Organization, 2018; De Oliveira *et al.*, 2020). The family *Pseudomonadaceae* was identified in all sampled locations, representing an average 10.4% (median 1.35%) of the relative abundance of families identified across the samples. A 2019 review by McLellan and Roguet reported that the family *Pseudomonadaceae* represented a relative abundance 2.38% of families examined in the metagenomic papers analysed focusing on sewerage bacterial composition. Likewise, the family *Pseudomonadaceae* represented a relative abundance 0.32% of families examined in the metagenomic papers analysed focusing on biofilm in sewer pipes bacterial composition. On the genus level, *Pseudomonas* was identified in all of the 16 DDUH wastewater network samples and identified in varying abundances (0.03% – 50.4%) (Figure 4.6).

Metagenomic studies have helped to establish how bacterial bioburden levels and composition in wastewater networks may lead to risks to patients in healthcare facilities (Numberger *et al.*, 2019; Constantinides *et al.,* 2020). The accurate identification of microorganims helps researchers understand community dynamics, indentify potential antimicrobial reservoirs and investigate the adverse effects of decontamination and/or antibiotic use on the microbial communities. All 16 samples from the wastewater network in DDUH were taken in the absence of an observed outbreak and from wastewater sites not subjected to routine deconatmination by ECAs. While no sample was taken for Illumina 16S rRNA amplicon sequencing from the A&E washbasins throughout the 52 week ECA-decontamination phase of the study, many studies have recently identified the role disinfection can have on bacterial community structures. A 2020 study by Jin *et al.* was the first of its kind to show that chlorine disinfection increased the frequency of natural transformation in the four tested bacterial species (*Escherichia coli, P. aeruginosa, Salmonella aberdeen* and *Enterococcus faecalis*) and therefore promoted horizontal transfer of antimicrobial resistant genes across bacterial genera (Jin *et al.*, 2020). This study used culturable chlorine-injured bacteria and showed an enrichment of antimicrobial resistant genes in the four bacterial strains after exposure to sodium hypochlorite (NaClO)(Jin *et al.*, 2020). A recent study by Dai *et al.* (2020) investigated the effects disinfectant residuals had on the drinking water microbiome. The drinking water microbiome was structurally and functionally less diverse in water treated with disinfectant residuals compared to systems not treated with disinfectant residuals (Dai *et al.,* 2020). The study also reported that the bacterial domain was the most abundant domain across residual treated and untreated systems, with archaea more abundant in residual untreated systems and eukaryota in residual treated

systems (Dai *et al.,* 2020). Likewise, a study by Bautista-de los Santos *et al.* (2016) carried out meta-analyses of 16S rRNA amplicon data from the published literature. The work investigated the microbiome population structure of drinking water distribution systems subjected to various levels of residuals disinfectants. The report showed that disinfectant residuals affected the microbial community structure and the disinfected systems were less diverse than their untreated counterparts (Bautista-de los Santos *et al.,* 2016).

The bacterial communities structures within wastewater systems are not fixed. A study by Guo *et al.* (2019) investigated the change in the microbial community structure and functional traits of the associated genes over a short time period. In the study, the authors investigated the microbial communities, using 16S rRNA gene amplicon sequencing, at 4 h intervals over 2 days (Guo *et al.,* 2019). The data showed large variation in the microbial community structure at phylum and genus levels. For example, the phylum Proteobacteria ranged from 44% to 63% of the total relative abundance. Likewise, a study by Perry *et al.* (2019) investigated the metagenomes of bacterial communities and AMR genes abundances from hospital effluent using whole-genome shotgun sequencing. The study compared the bacterial community structures and abundances of AMR genes from seven discharge points representing clinical specialities and therefore representing clinical activities and washbasin use in one hospital. The wastewater samples were taken over a period of 24 h from seven wastewater locations from a hospital and one community sewerage works (Perry *et al.*, 2019). The microbiota and AMR genes varied between all sites, however AMR genes were recovered in higher abundances in the hospital samples than the community samples and in samples associated with prolonged patient stays. Within the seven hospital wastewater sample sites and one community sample site, the most predominant genera were *Pseudomonas* and *Acinetobacter*. At a species level, *Pseudomonas fluorsecens* and *Acinetobacter johnsonii* were most abundant indicating high levels of environmental species associated with hospital pipe biofilm (Perry *et al.*, 2019).

Finally, the swab sampling technique utilised throughout the experiments in this thesis was investigated and the relative recovery rates were determined. As stated previously, the major factors affecting standardisation of environmental sampling include the type of sampling device, the variability of surface material sampled, the density and diversity of microorganisms on the sampled surface, and the surface area sampled (Jones *et al.,* 2020). All sampled U-bend surfaces were made from PVC materials while a standardised surface area was established as discussed in Chapter 3. For this reason, a variety of types of sampling

devices were investigated to determine the efficacy of the sampling device utilised. As environmental sampling is an integral part of many sectors including healthcare, manufacturing, food processing, the variety of swab types and materials are ever increasing. Swab sampling is currently the most widely adopted sampling device for microbiological testing of hard surfaces, however other surface sampling devices are increasingly becoming adopted including sponges, cloths, wipes and contact plates (Jones *et al.,* 2020).

Washbasin network sampling has primarily relied on swab sampling due to the reduced accessibility and restricted nature of wastewater pipes and U-bends. The large cotton tipped swab was most efficient at recovery bacteria owing in part to the size of the swab tip (45.5%), followed by the flocculated nylon fibre swab (40.7%), and then the sterile viscose transport swabs utilised throughout these studies (19.4%). Keeratipibul *et al.* (2017) determined the following recovery effieciency of different swabs on wet surfaces: polyurethane foam swabs (94.5%), cellulose sponge swabs (94.4%), gauze swabs (90.3%), and cotton swabs (84%). Lower recovery effieciency was found for all of the tested swabs on dry surfaces (Keeratipibul *et al.,* 2017). However, as environmental sampling is not standardised, U-bend sampling results presented in this study give an accurate indication of the relative recovery of the bacterial bioburden in DDUH washbasins.

The diversity and density of the microorganisms on the sampled wastewater network surfaces could not be standardised. However, as the previous chapter and studies conducted in DDUH showed, *P. aeruginosa* was the most prevalent culturable organism in DDUH washbasin U-bends (Swan *et al.,* 2016; Deasy *et al.,* 2018). *Pseudomonas aeruginosa* isolates recovered by the standardised swab sampling techniques was similar irrespective of the colony morphologically. Recently, attention has focused on rapid non-culturable testing techniques such as testing of adenosine triphosphate (ATP) levels to determine cleanliness levels of surfaces in hospitals. However, one limitation of this technique is that this should be used as an indication of bacterial contamination rather than a quantitative measurement (Whiteley *et al.*, 2016).

In conclusion, the automated ECA decontaminated system developed in Chapter 3 has proved to be an efficient and consistent approach in reducing the bacterial bioburden in washbasin U-bends. Regular decontamination was proven to be necessary for reducing the bacterial bioburden in washbasins and their associated drains, and the cessation of decontamination lead to increased levels of bacterial bioburden in the washbasin U-bends

and drains. As the literature states, increased bioburden increases the risk of transmission from washbasin U-bends and drains. The use of culture-based approaches only as a method to identify bacteria is limited and may portray misleading ranges of bacteria in washbasin U-bends. Finally, while the type of swab may affect the density of microorganisms recovered, the use of a consistent approach while defining the recovery rates lead to a more transparent approach for other researchers to interpret data.

Chapter 5

Whole-genome sequencing identifies highly related *Pseudomonas aeruginosa* in multiple washbasin U-bends at several locations in the Dublin Dental University Hospital: evidence for trafficking of potential pathogens via wastewater pipes

5.1 Introduction

Hand washing is vital for reducing infections in the healthcare environment. As outlined in Chapter 1, healthcare establishments facilitate hand hygiene in order to reduce opportunities for the transmission of microorganisms and thus reduce the spread of infection. While, ironically, the presence of hand washbasins in healthcare premises promotes more frequent hand washing, it also results in increased associated infection risks (Hota *et al*., 2009; Coleman *et al*., 2020). This is due to the fact that biofilm coated wastewater pipes servicing washbasin and sink drain outlets are open to the healthcare environment throughout hospital buildings in areas occupied by vulnerable patients.

Hospital washbasin and sink drains have been implicated in numerous episodes of nosocomial infection as described in Chapter 1. However, the mechanisms of dispersal of bacteria within the wastewater network and to patients have not been fully determined. Previous studies have demonstrated that bacteria present in washbasin and sink drains can be aerosolised by the impact of tap water flow or splash back and can contaminate the washbasin, taps and local environmental surfaces (Hota *et al*., 2009; Breathnach *et al*., 2012; De Geyter *et al*., 2017; Kotay *et al*., 2017; Aranega-Bou *et al*., 2019). A study by Hota *et al*. (2009) demonstrated the dispersal of water droplets up to 1 m from a contaminated sink drain using fluorescent markers when the sink was used for handwashing. Likewise, a 2017 study used a monoculture of a laboratory strain of *Escherichia coli* expressing green fluorescent protein to show that the biofilm in a sink U-bend model system grows upwards towards the sink drain outlet (Kotay *et al.,* 2017). Bacterial movement through the pipe network towards the sink drain was recorded at a rate of about 2.5-cm per day and colonisation of the sink drain outlet led to water splatter contaminating the bowl and surrounding area (Kotay *et al*., 2017). The trafficking of *E. coli* to adjacent sinks by means of the common wastewater pipes study, while very informative, did have a number of limitations (Kotay *et al*., 2017). *Escherichia coli* is an organism not largely associated with washbasin biofilms, and was introduced into the model system as a laboratory-grown monoculture and artificially supplemented with nutrients. Fundamentally, this study was an artificial model system that demonstrated how microorganisms can spread within the wastewater pipe network. The model system does not necessarily reflect the behaviour of complex populations of competing microorganisms present in U-bends servicing washbasins.

Today, next generation sequencing (NGS) platforms, such as those marketed by Illumina, are the 'gold standard' for genotyping bacterial isolates (van Dijk *et al.*, 2014). As described in Chapter 1, whole-genome sequencing (WGS) is the process of determining the complete DNA sequence of an organism's genome. Recently, studies have used WGS and comparative genomic data analyses to type *P. aeruginosa* isolates recovered from cystic fibrosis patients, from patients and environmental samples, and to determine and control outbreaks of specific strains in hospital wards (Blanc *et al.,* 2016; Decraene *et al.,* 2018; Wee *et al.,* 2018; Magalhães *et al.,* 2020). This high-throughput and high resolution method of typing bacterial isolates was adopted in the present study to investigate potential trafficking of *P. aeruginosa* in washbasin U-bends and the associated wastewater network in DDUH. While WGS and the accompanying comparative data approaches are utilised today, traditional methods for typing *P. aeruginosa* isolates for epidemiological and surveillance purposes have evolved significantly since the 1930's (Figure 5.1).

Typing of *P. aeruginosa* isolates began in the early twentieth century, based on phenotypic typing methods. The four main phenotypic methods for typing *P. aeruginosa* have been serotyping, pyocin typing, bacteriophage typing and biotyping (Pitt, 1988). Phenotyping is based on the observation of an organism's physical traits or characteristics. The most commonly used phenotypic approach adopted in the 1930s was serotyping. Two types of serotyping have been described for *P. aeruginosa*: O-antigen serotyping and H-antigen serotyping. The O-antigen is a repeat polysaccharide portion of outer membrane lipopolysaccharides (LPS) of Gram-negative bacteria (Lu *et al*., 2014). LPS contributes greatly to the structural integrity of the outer membrane of Gram-negative bacteria and also functions as bacterial endotoxin. Serotyping of *P. aeruginosa* is based on the significant structural diversity of the O-antigen, with the International Antigenic Scheme consisting of 20 standard O serotypes (Lu *et al*., 2014). The H-antigens are located on the single polar flagellum of *P. aeruginosa*. H-antigen serotyping was not largely adopted due to the difficulty in preparation of the specific anti-flagellar sera (Pitt, 1988). The major weakness of serotyping as an approach to type isolates of *P. aeruginosa* is the relatively poor discriminatory power of the technique, as isolates within a serotype can exhibit large genotypic variances.

While still used today, phenotype-based typing methods have largely been replaced by molecular genotyping methods. These include, but are not limited to, PCR-based random amplification of polymorphic DNA (RAPD) (also known as arbitrary primer PCR), pulsed

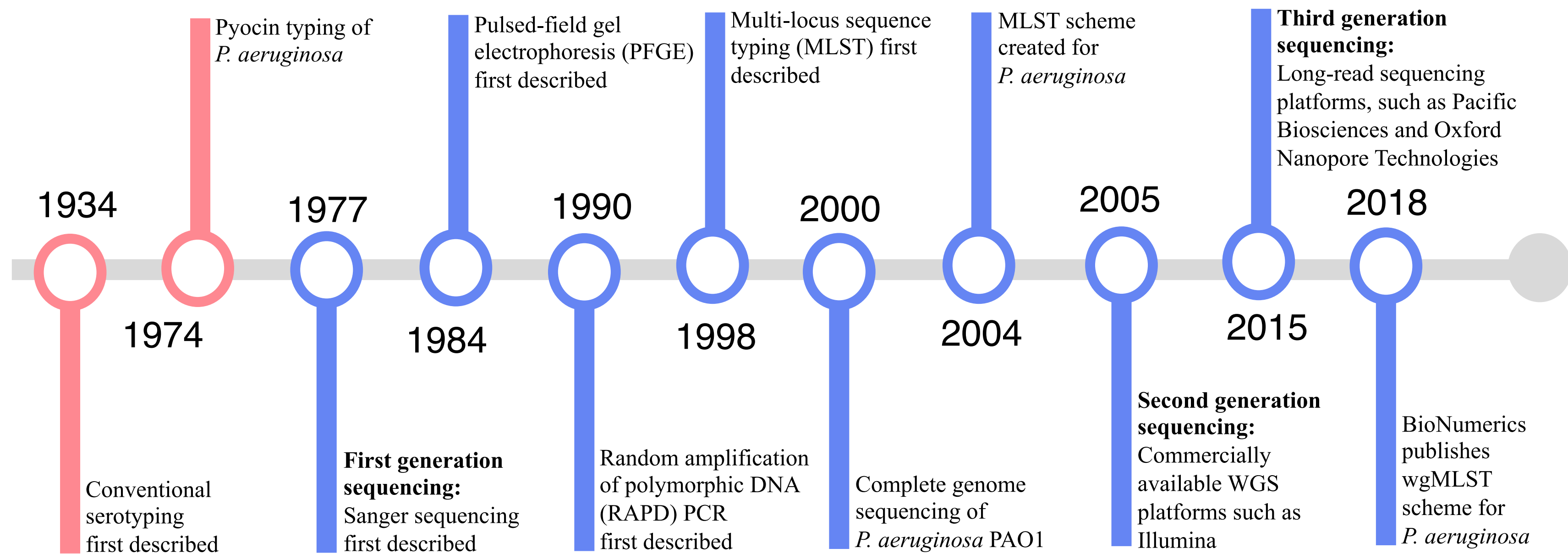


Figure 5.1 A schematic timeline of the development of sequencing technologies and selected phenotypic and molecular typing methods for *P. aeruginosa*. The selected phenotypic typing methods are indicated in pink and molecular typing methods are shown in blue. The timeline depicts the trend away from phenotypic to molecular methods for epidemiological and surveillance purposes overtime.

field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), and NGS or second generation sequencing technologies (Lopez-Canovas *et al*., 2019). Molecular genotyping methods were originally developed to provide enhanced isolate discrimination based on DNA or RNA analysis, relative to phenotypic typing methods based on chemotaxonomic approaches that provide information on the constituents of the microorganism (Donelli *et al*., 2013). RAPD PCR utilises variable short length oligonucleotide primers to bind at low stringency throughout the genome and yields a variety of amplimers that provide an isolate profile or pattern following separation by conventional agarose gel electrophoresis (Tazumi *et al*., 2009). This approach can rapidly differentiate *P. aeruginosa* isolates, however it is limited by problems associated with poor reproducibility.

PFGE became established as the 'gold-standard' for genotypic typing of a range of microorganisms in the 1980s, as it encompassed the four characteristics of a good typing method: typeability, reproducibility, improved discriminatory power, ease of realisation and interpretation (Lopez-Canovas *et al*., 2019). PFGE can separate DNA molecules up to 5 Mb in size and can readily resolve complex patterns of genomic DNA fragments generated following digestion of genomic DNA by restriction endonucleases that cleave DNA infrequently. These banding patterns provide a DNA profile or fingerprint for each isolate analysed. However, the discriminatory power of PFGE is somewhat limited. The limitations are based on the methods itself, and the experience of the scientist. These include the generation of DNA restriction fragment patterns that can vary slightly between technicians, the necessity of a defined nomenclature for banding pattern difference interpretation for a given organism, changes in a single restriction endonuclease cleavage site may lead to one or more band changes, only nucleotide changes in restriction endonuclease recognition sites can be detected and some strains are not typable by PFGE. These limitations indicate that PFGE should be used only as a guide for isolate relatedness (Lüth *et al*., 2018). Nonetheless, PFGE has been a key tool for comparing variation among *P. aeruginosa* isolates recovered from the wastewater environment. Hota *et al*. (2009) used PFGE for investigating the genotypic relatedness of multidrug-resistant *P. aeruginosa* isolates recovered from 36 patients and the patient environment. The source of the outbreak was traced to biofilm located in the drain outlet of a handwashing sink. Likewise, the relatedness of multidrug resistant hospital outbreak isolates of *Klebsiella oxytoca* and contemporary isolates from hospital sinks was analysed using PFGE by Lowe *et al*. (2012). Using this approach the outbreak associated clones were also identified in sink drains in the ICU, in the majority of patient rooms and from staff washrooms (Lowe *et al.*, 2012).

In 1998, MLST was first described as an approach to provide enhanced molecular typing of microorganisms. The first MLST typing scheme for *P. aeruginosa* was described in 2004, which permitted the genetic relatedness of isolates to be determined based on sequence variation within seven predetermined housekeeping genes or loci (Maiden *et al.*, 1998; Curran *et al*., 2004). MLST schemes assign sequence types (STs) to isolate collections that are useful for population genetic studies. However, due to the conservative number of housekeeping genes used, conventional MLST lacks the discriminatory power to differentiate bacterial isolates in outbreak investigations.

Prior to the advent of WGS, phenotypic and genotypic typing methods were limited in their ability to discriminate between isolates of *P. aeruginosa*. In this study, two comparative genomic approaches were used to determine the relatedness of isolates investigated from the WGS data: (i) single nucleotide variant (SNV), and (ii) gene-by-gene analysis, such as whole-genome MLST (wgMLST) analysis. SNVs are variations within the DNA sequence occurring when a single nucleotide in the genome differs to the comparison or reference genome(s). The terms SNVs and single nucleotide polymorphisms (SNP) are sometimes used interchangeably, however there is a difference between these two terms. While SNVs refer to the change of a single nucleotide in the genome with no reference to the frequency of this observed change in the population, SNPs are single nucleotide changes that have become established amongst a population that are observed in at least 1% of population (He *et al.,* 2014). Standard SNV analysis involves the use of a selected reference genome to identify SNVs throughout the query genomes. The sequence reads of the query genomes are aligned to the reference using mapping algorithms and variations can be identified between the sequences (Li and Durbin, 2010). SNV filters are essential to identify true point mutations in contrast to assembly errors or recombination events. SNVs are useful biological markers to examine evolutionary change within populations. A study published in 2018 utilised Illumina NGS technology and SNV analysis to compare *Sphingomonas koreensis* isolates recovered from an infected patient with environmental isolates from sink faucets in the patient environment (Johnson *et al*., 2018). A single transmission event was identified in which a patient and a faucet isolate recovered 15 days later were found to be very closely related, differing by only 5 SNVs. Three recent studies further utilised Illumina NGS technology and SNV analysis to investigate the relatedness of microorganisms from the washbasin environment and patients. A study by Feng *et al.* (2019) demonstrated that three carbapenem-resistant *Klebsiella pneumonia* isolates recovered from two separate patient

samples, and a washbasin differed from each other by 4 - 12 SNVs. This data revealed that a common washbasin was the source of colonisation and infection in both patients. Another study by Buhl *et al.* (2019) evaluated the molecular evolution of extensively-drug resistant *P. aeruginosa* isolates from patient samples and the hospital environment at two adjacent hospitals during a prolonged outbreak. SNV analysis revealed adaptation within two genes associated with a multi-drug efflux pump and a mercury detoxification operon, which may result in resistance to antimicrobials in *P. aeruginosa* isolates (Buhl *et al.,* 2019). Finally, a study by Hopman *et al.* (2019) confirmed the transmission of carbapenemase-producing *P. aeruginosa* strain between a patient, the air and the hospital environment. This study demonstrated that *P. aeruginosa* isolates from a patient, the shower drains in eight patient rooms, one sink sample, and air samples all differed by range of 1–12 SNVs. The study highlighted the potential spread of *P. aeruginosa* via the wastewater network connecting the shower drain in eight separate patient rooms, and the data suggested airborne transmission was the most likely source of transmission to patients. One limitation of this study however was the low read coverage (or depth) (cut-off of 20x) of isolate sequences used for downstream SNP analysis. While Illumina WGS accurately detects individual nucleotides, sequencing errors can occur, and a coverage of 50x (meaning every read has an average of 50 copies) ensures higher confidence in the detection of true point mutations.

A whole genome (wg)MLST scheme for comparison of *P. aeruginosa* isolates was first described in 2018 (https://www.applied-maths.com/sites/default/files/extra/Release-Note-Pseudomonas-aeruginosa-schema.pdf). wgMLST analysis provides a gene-by-gene approach to the analysis of the entire bacterial genome. While wgMLST provides lower resolution than SNV analysis, wgMLST analysis records various types of nucleotide differences (SNVs, variable number tandem repeat, and insertion-deletion mutations or INDELs) for every open reading frame of an organism and provides standardisation of a comparative genomic approach (Kingry *et al.*, 2016). The wgMLST scheme provides high discriminatory power for the comparison of multiple isolates, utilising the 15,136 validated loci with the addition of the traditional 7 housekeeping gene MLST scheme for *P. aeruginosa*. A recent study utilised wgMLST analysis to determine the relatedness of isolates of *Legionella anisa* recovered from dental chair unit output water from separate outlets in a hospital dental clinic (Fleres *et al.*, 2018). The results of wgMLST analysis revealed that all isolates investigated belonged to the same wgMLST cluster and exhibited between two and four allelic differences. These findings indicated a common contamination source for the *Legionella anisa*.

5.2 Objectives

The objective of this study was to investigate if bacteria are trafficked between washbasin U-bends and the associated wastewater pipe network in a hospital setting. The distribution of highly related isolates may provide evidence for isolate trafficking throughout via the wastewater network. The specific aims of the study were:

- To use *P. aeruginosa* isolates recovered from washbasin U-bends and other wastewater pipe network sites in DDUH as marker organisms to investigate trafficking in U-bends and associated wastewater pipes in a hospital setting. As one of the most frequently encountered bacteria from washbasin U-bends, the genetic relatedness of *P. aeruginosa* isolates recovered from multiple washbasin U-bends located at adjacent and distant sites in DDUH were investigated in detail using WGS analysis.

- To investigate the impact of routine decontamination of washbasin U-bends with ECA solutions on the *P. aeruginosa* population.

5.3 Materials and Methods

5.3.1 Hand washbasins

Thirty-one ceramic hand washbasins in the DDUH were included in the study. Twenty-seven were hospital-pattern (HP) washbasins, without overflow outlets and with offset drain outlets (Swan *et al*., 2016; Deasy *et al*., 2018). Four were domestic pattern (DP) washbasins with the drain located directly below the tap water flow. Each HP washbasin faucet was equipped with a thermostatic mixing valve set to provide water at 38°C. The DP washbasins each had a manual mixer tap. All washbasins were in frequent daily use on weekdays and were fitted with identical polypropylene U-bends with two sampling ports for bacterial isolate recovery, as described in Chapter 2, Section 2.6.

Washbasins were selected to represent the diversity of large clinics and other areas in different DDUH locations. Figure 5.2 shows the relative locations in DDUH of all washbasins included in the study. Ten HP washbasins were located on the ground floor A&E (equipped with 11 HP washbasins in total), and 10 in Clinic 2 (equipped with 15 HP washbasins in total) on the second floor. Clinic 2 is divided into five separate treatment bays, each equipped with three washbasins and one sink. Clinic 2 and A&E were refurbished in August 2017 and 2016, respectively, and equipped with identical new washbasins, faucets, U-bends and wastewater pipes (Deasy *et al*., 2018). Cold water to washbasin faucets was provided from a 15,000-L water storage tank supplied with mains water, which also supplied a calorifier providing faucet hot water. Hot and cold water supplied to DDUH washbasins is treated with residual anolyte (2.5 ppm), an electrochemically activated disinfectant solution composed predominately of hypochlorous acid (Boyle *et al*., 2012). A&E operates seven days a week with lower use-frequency at weekends, while Clinic 2 operates Monday-Friday. Both clinics are out-patient facilities. Additional HP washbasins from different locations in DDUH were included: one on the first floor in CSSD, three on the first floor Clinic 1 and three in West Clinic on the ground floor. The four DP washbasins were located distant from clinics in the following areas: (i) a third-floor staff bathroom, (ii) a second-floor staff bathroom, (iii) a first floor staff bathroom, and (iv) the DDUH microbiology laboratory.

The U-bend of each Clinic 2 washbasin was connected via a 1-m vertical pipe to one of

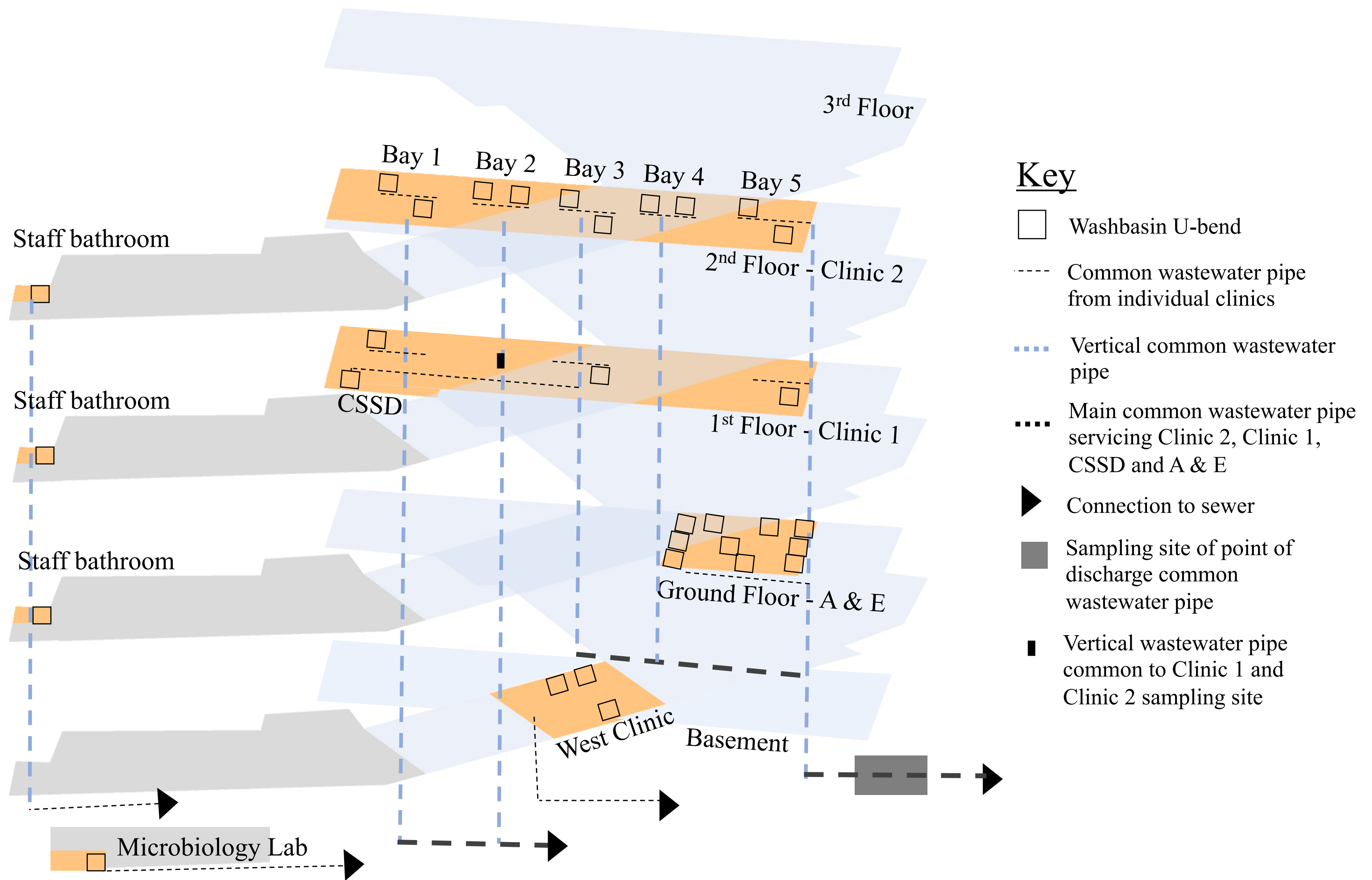

3rd Floor
Bay 1
Bay 2
Bay 3
Bay 4
Bay 5
Staff bathroom
2nd Floor - Clinic 2
Staff bathroom
CSSD
1st Floor - Clinic 1
Staff bathroom
Ground Floor - A & E
West Clinic
Basement
Microbiology Lab
Key
Washbasin U-bend
Common wastewater pipe from individual clinics
Vertical common wastewater pipe
Main common wastewater pipe servicing Clinic 2, Clinic 1, CSSD and A & E
Connection to sewer
Sampling site of point of discharge common wastewater pipe
Vertical wastewater pipe common to Clinic 1 and Clinic 2 sampling site

Figure 5.2 Schematic showing the relative locations of the washbasins/U-bends and associated wastewater pipes in the DDUH investigated in the study. Clinic 1 and Clinic 2 located on the first and second floors of DDUH, respectively, have an identical layout. Both clinics are divided into five treatment bays, with each bay containing three hospital pattern (HP) washbasins; two of the three washbasins per bay in Clinic 2 were sampled recurrently during this study. While a total of three washbasins were sampled from Clinic 1. The 10 sampled HP washbasins in Accident & Emergency Department (A&E) are all connected by one common wastewater pipe. All washbasins in the grey section of the schematic shown on the left were domestic pattern fixtures, whereas all washbasins indicated in the blue section of the schematic (Clinics 1 & 2, A&E and CSSD) were HP fixtures. Abbreviations: A&E, Accident and Emergency Department; CSSD, Central Sterile Services Department.

a series of five horizontal wastewater pipes, each of which serviced three washbasins (two washbasins from each bay were included in the study) (Figure 5.2). Each pipe discharged water into an individual larger vertical pipe which passed through the building into the basement (Figure 5.2). Three of the five vertical pipes connected to a larger common horizontal wastewater pipe connected to the municipal sewer at the building perimeter. The other two vertical pipes connected to a separate common horizontal wastewater pipe that discharged wastewater to the municipal sewer at a separate outlet. The U-bends of each A&E washbasin were connected via 1-m vertical pipes to a common horizontal wastewater pipe that discharged water to the same point of discharge to the municipal sewer as the wastewater collection pipe servicing the three vertical wastewater collection pipes from Clinic 2 (Figure 5.2). The layout of Clinic 1 washbasins and wastewater pipes was identical to Clinic 2, with both clinics discharging wastewater through the same common vertical wastewater pipes (Figure 5.2). The CSSD washbasin discharged water into one of the vertical wastewater collection pipes common to Clinic 1 and Clinic 2. The washbasin U-bends in West Clinic, the single DP washbasin in the microbiological laboratory and the three DP washbasins in staff bathrooms discharge wastewater to the sewer system at different outlets to Clinic 2 and A&E (Figure 5.2).

Since their installation in August 2016 until March 2019, A&E washbasin U-bends underwent automated decontamination three times weekly, involving sequential treatments with two electrochemically-activated (ECA) solutions generated from brine; catholyte (80 ppm NaOH) with detergent properties and 800 ppm anolyte with disinfectant properties (Deasy *et al*., 2018). The decontaminations cycles are described in both Chapter 3, Section 3.2.4 and Chapter 4, Section 4.3.4. All other DDUH washbasin U-bends were not decontaminated during the study.

5.3.2 Recovery of *P. aeruginosa* from U-bends

Pseudomonas aeruginosa isolates were recovered from washbasin U-bends by swab sampling and the swabs were processed as described in Chapter 2, Section 2.6.3.

5.3.3 Testing water and washbasin faucets for *P. aeruginosa*

Seventy-two 1-L water samples, eight from the washbasin cold water supply, eight from the DDUH mains supply, and 56 from washbasin faucets (including 16 each from A&E and Clinic 2) were tested for *P. aeruginosa*. Samples were taken in sterile glass bottles, neutralised with 0.5% sodium thiosulfate and vacuum filtered through 0.45 μm filters

(Sartorius, Göttingen, Germany), followed by incubation on PSCN agar plates. Swab samples of 20 representative DDUH washbasin faucets (including 5 each from A&E and Clinic 2) were sampled four times at six-month intervals (i.e. 80 samples in total) with swabs dipped in sodium thiosulphate (0.5%) and cultured on PSCN as described in Chapter 2, Section 2.2.1.

5.3.4 Study design and isolate selection

Clinic 2 was selected as a model clinic to investigate the population of *P. aeruginosa* in non-decontaminated washbasins U-bends (n = 10) by WGS. *Pseudomonas aeruginosa* isolates recovered from other washbasin U-bend locations in DDUH (A&E, West Clinic, CSSD, Clinic 1, three staff bathrooms and the microbiology laboratory; Figure 5.2), the wastewater pipe network in DDUH (the main point of discharge wastewater collection pipe common to Clinic 2 and A&E, and one larger vertical wastewater collection pipe common to Clinic 1 and Clinic 2; Figure 5.2), as well as external comparators and reference strains were incorporated into this study.

A six-month time frame was established for the selection of *P. aeruginosa* isolates from Clinic 2 to be sequenced (February – July 2018) to reduce WGS costs. Overall 55 isolates were sequenced (Table 5.1). These included five *P. aeruginosa* isolates from at least three independent U-bends recovered monthly for the six-month period (n = 30). An additional 17 *P. aeruginosa* from one individual U-bend (B2D3) consisted of isolates recovered at intervals of at least a week over the six-months. The remaining eight isolates consisted of separate *P. aeruginosa* isolates recovered in February 2019 from B2D3, sampled at one time point following completion of the sampling period.

Twenty-six *P. aeruginosa* isolates from ECA-treated A&E U-bends were investigated (Table 5.1). Isolates were recovered over a longer sampling period (January 2017 – November 2019) for WGS due to reduced number of *P. aeruginosa* isolates recovered from ECA-treated U-bends (Deasy *et al*., 2018). Isolates from four time points were investigated: immediately after ECA treatment (n = 7), 24-h post-ECA treatment (n = 7), 48-h post-ECA treatment (n = 7), and seven months after the cessation of regular three times weekly ECA decontamination (n = 5) of the washbasin U-bends.

Additional isolates from other locations in DDUH were included in this study: three from separate West Clinic washbasin U-bends (June and July 2017), two from a CSSD washbasin

Table 5.1 Recovery timeline and sequence types of the 118 *P. aeruginosa* environmental isolates and two reference strains investigated by Illumina whole-genome sequencing

Sequence Type	Number of isolates	Isolate name	Date of isolation	Location[1]
ST179	34	AE24Jan	January 2017	A&E – washbasin 5 (24 h post-decontamination)
		LP3F1	May 2017	DP washbasin in third-floor staff bathroom
		AE24Aug	August 2017	A&E – washbasin 9 (24 h post-decontamination)
		LP3F2	August 2017	DP washbasin in third-floor staff bathroom
		B2D3bFeb	February 2018	Clinic 2 – washbasin B2D3
		B2D3cMar	March 2018	Clinic 2 – washbasin B2D3
		B3D4Apr	April 2018	Clinic 2 – washbasin B3D4
		B2D3aMay	May 2018	Clinic 2 – washbasin B2D3
		B2D3bMay	May 2018	Clinic 2 – washbasin B2D3
		B2D3cMay	May 2018	Clinic 2 – washbasin B2D3
		B3D2May	May 2018	Clinic 2 – washbasin B3D2
		B2D3dMay	May 2018	Clinic 2 – washbasin B2D3
		B3D4May	May 2018	Clinic 2 – washbasin B3D4
		B1D2Jun	June 2018	Clinic 2 – washbasin B1D2
		B2D3aJun	June 2018	Clinic 2 – washbasin B2D3
		B3D2Jun	June 2018	Clinic 2 – washbasin B3D2
		B3D4Jun	June 2018	Clinic 2 – washbasin B3D4
		B4D3Jun	June 2018	Clinic 2 – washbasin B4D3
		B2D3cJun	June 2018	Clinic 2 – washbasin B2D3
		B1D4bJul	July 2018	Clinic 2 – washbasin B1D4
		B1D4cJul	July 2018	Clinic 2 – washbasin B1D4
		B2D3aJul	July 2018	Clinic 2 – washbasin B2D3
		B2D3bJul	July 2018	Clinic 2 – washbasin B2D3
		B2D3SNAP1	February 2019	Clinic 2 – washbasin B2D3
		B2D3SNAP2	February 2019	Clinic 2 – washbasin B2D3
		B2D3SNAP3	February 2019	Clinic 2 – washbasin B2D3
		B2D3SNAP4	February 2019	Clinic 2 – washbasin B2D3
		B2D3SNAP5	February 2019	Clinic 2 – washbasin B2D3
		B2D3SNAP6	February 2019	Clinic 2 - washbasin B2D3
		B2D3SNAP7	February 2019	Clinic 2 - washbasin B2D3
		B2D3SNAP8	February 2019	Clinic 2 – washbasin B2D3
		C1B1D2a	November 2019	Clinic 1 – washbasin B1D2

Table 5.1 continued overleaf

Table 5.1 (continued)

Sequence Type	Number of isolates	Isolate name	Date of isolation	Location[1]
		C1B1D2b	November 2019	Clinic 1 – washbasin B1D2
		C1B5D4	November 2019	Clinic 1 – washbasin B5D4
ST560	27	AE24aMay	May 2017	A&E – washbasin 4 (24 h post-decontamination)
		AE24cMay	May 2017	A&E – washbasin 3 (24 h post-decontamination)
		CSSD1	May 2017	Central Sterile Services Department
		CSSD2	June 2017	Central Sterile Services Department
		AEDaJul	July 2017	A&E - washbasin 4 (immediately after decontamination)
		B1D4Feb	February 2018	Clinic 2 – washbasin B1D4
		B2D3aFeb	February 2018	Clinic 2 – washbasin B2D3
		B2D3cFeb	February 2018	Clinic 2 – washbasin B2D3
		B3D4Feb	February 2018	Clinic 2 – washbasin B3D4
		B1D4Mar	March 2018	Clinic 2 – washbasin B1D4
		B2D2Mar	March 2018	Clinic 2 – washbasin B2D2
		B2D3aMar	March 2018	Clinic 2 – washbasin B2D3
		B2D3bMar	March 2018	Clinic 2 – washbasin B2D3
		B3D4Mar	March 2018	Clinic 2 – washbasin B3D4
		B4D3Mar	March 2018	Clinic 2 – washbasin B4D3
		B2D3aApr	April 2018	Clinic 2 – washbasin B2D3
		B2D3bApr	April 2018	Clinic 2 – washbasin B2D3
		B2D3cApr	April 2018	Clinic 2 – washbasin B2D3
		B3D2Apr	April 2018	Clinic 2 – washbasin B3D2
		B2D3bJun	June 2018	Clinic 2 – washbasin B2D3
		B1D4aJul	July 2018	Clinic 2 – washbasin B1D4
		B2D3cJul	July 2018	Clinic 2 – washbasin B2D3
		B5D4Jul	July 2018	Clinic 2 – washbasin B5D4
		B5D4aJul	July 2018	Clinic 2 – washbasin B5D4
		AEDaOct	October 2018	A&E – washbasin 3 (immediately after decontamination)
		CWP3	May 2019	Common point of discharge wastewater pipe[2]
		CWP6	May 2019	Common point of discharge wastewater pipe[2]
ST298	9	B5D2Feb	February 2018	Clinic 2 – washbasin B5D2
		B5D2Apr	April 2018	Clinic 2 – washbasin B5D2
		B5D4Apr	April 2018	Clinic 2 – washbasin B5D4
		B5D2Jun	June 2018	Clinic 2 – washbasin B5D2
		B5D4bJul	July 2018	Clinic 2 – washbasin B5D4

Table 5.1 continued overleaf

Table 5.1 (continued)

Sequence Type	Number of isolates	Isolate name	Date of isolation	Location[1]
		DH11	November 2018	Hospital 2[3]
		LP2R	March 2019	DP washbasin in second-floor staff bathroom
		AER6	November 2019	A&E - washbasin 6 (seven months post ECA decontamination cessation)
		LP1R	November 2019	DP washbasin in first-floor staff bathroom
ST308	7	AEDbJul	July 2017	A&E – washbasin 7 (immediately after decontamination)
		AEDApr	April 2018	A&E – washbasin 1 (immediately after decontamination)
		AE48aJun	June 2018	A&E – washbasin 1 (48 h post-decontamination)
		AE48bJun	June 2018	A&E – washbasin 7 (48 h post-decontamination)
		AE24Feb	February 2019	A&E – washbasin 1 (24 h post-decontamination)
		AE24Mar	March 2019	A&E – washbasin 1 (24 h post-decontamination)
		AE48Mar	March 2019	A&E – washbasin 1 (48 h post-decontamination)
ST27	6	AE24bMay	May 2017	A&E – washbasin 2 (24 h post-decontamination)
		West1	June 2017	West Clinic
		West2	June 2017	West Clinic
		West3	July 2017	West Clinic
		AER1	November 2019	A&E – washbasin 1 (seven months post ECA decontamination cessation)
		AER10	November 2019	A&E – washbasin 10 (seven months post ECA decontamination cessation)
ST252	6	B5D4Feb	February 2018	Clinic 2 – washbasin B4D3
		B5D4Mar	March 2018	Clinic 2 – washbasin B4D3
		B1D2aMay	May 2018	Clinic 2 – washbasin B1D2
		B1D2bMay	May 2018	Clinic 2 – washbasin B1D2
		ATCC15442[4]	N/A	American Type Culture Collection 15442
		RdRm	November 2019	Basement level microbiology lab washbasin
ST773	5	AE48Aug	August 2018	A&E – washbasin 5 (48 h post decontamination)
		AEDJan	January 2019	A&E – washbasin 7 (immediately after decontamination)
		AE48aJan	January 2019	A&E – washbasin 5 (48 h post-decontamination)
		AE48bJan	January 2019	A&E – washbasin 1 (48 h post-decontamination)
		AER5	November 2019	A&E – washbasin 5 (seven months post ECA decontamination cessation)
ST1320	5	DenS1	2005[e]	Recovered from dental suction systems
		DenS2	2005[e]	Recovered from dental suction systems
		DenS3	2005[e]	Recovered from dental suction systems
		DenS5	2005[e]	Recovered from dental suction systems
		DenS6	2005[e]	Recovered from dental suction systems
ST296	4	AEDJun	June 2018	A&E – washbasin 7 (immediately after decontamination)

Table 5.1 continued overleaf

Table 5.1 (continued)

Sequence Type	Number of isolates	Isolate name	Date of isolation	Location[1]
		AE48Sep	September 2018	A&E – washbasin 2 (48 h post-decontamination)
		AEDbOct	October 2018	A&E – washbasin 7 (immediately after decontamination)
		AER8	November 2019	A&E – washbasin 8 (seven months post ECA decontamination cessation)
ST253	3	DH6	November 2018	Hospital 2[3]
		CWP2	May 2019	Common point of discharge wastewater pipe[2]
		DenS4	2005[e]	Recovered from dental suction systems
ST17	2	LH1	March 2019	Hospital 1[3]
		LH4	March 2019	Hospital 1[3]
ST313	2	LPDP1	February 2019	Water sample from dental chair unit water reservoir outside DDUH
		LPDP2	February 2019	Water sample from dental chair unit water reservoir outside DDUH
ST395	2	DH13	November 2018	Hospital 2[3]
		LH3	March 2019	Hospital 1[3]
ST2685	2	DenS9	2005[5]	Recovered from dental suction systems
		DSS	2005[5]	Recovered from dental suction systems
ST309	1	DPC1C	November 2019	Vertical wastewater collection pipe common to Clinic 1 and Clinic 2
ST282	1	DH1	November 2018	Hospital 2[3]
ST348	1	DH10	November 2018	Hospital 2[3]
ST390	1	LH2	March 2019	Hospital 1[3]
ST549	1	**PAO1[4]**	N/A	Genbank (AE004091.2) (Stover *et al*., 2000)
ST606	1	C1B3D4	November 2019	Clinic 1 – washbasin B3D4

Abbreviations: DDUH, Dublin Dental University Hospital; A&E, Accident and Emergency Department; DP, domestic pattern washbasin; CSSD, Central Sterile Services Department. [1]All isolates were recovered from swab sampled U-bends unless otherwise stated. [2] Common wastewater pipe collecting wastewater from Clinic 2, CSSD and A&E. [3] Hospitals 1 and 2 are located 121 km and 8 km from DDUH, respectively. [4] The two *P. aeruginosa* strains PAO1 and ATCC15442 were included in the investigation as reference strains and are shown in bold typeface. [5] (O'Donnell *et al*., 2005).

U-bend (May and June 2017), four from three Clinic 1 washbasin U-bends (November 2019) four from the three DP staff bathroom washbasin U-bends (August 2017, March 2019 and October 2019) and one from a DP washbasin in the microbiology laboratory (November 2019). Three additional isolates recovered in May 2019 from the main point of discharge wastewater collection pipe common to Clinic 2 and A&E at the point of discharge into the municipal sewer were also investigated (Figure 5.2). One isolate was included from one of the larger vertical wastewater collection pipe common to Clinic 1 and Clinic 2 (Figure 5.2).

A total of 99 isolates recovered from DDUH U-bends and other wastewater pipes were sequenced in this study. These included 95 U-bend isolates, one isolate recovered from the vertical wastewater pipe common to Clinic 1 and Clinic 2 and three additional isolates recovered from the point of discharge wastewater collection pipe, were selected for sequencing from DDUH (Table 5.1).

A selection of *P. aeruginosa* comparator isolates from separate washbasin U-bends from two other Irish hospitals (n = 9), from a dental chair water reservoir from a clinic outside of DDUH (n = 2) and isolates previously recovered from dental suction systems (n = 8) (O'Donnell *et al*., 2005) were investigated as comparator isolates. The low abundance of comparator isolates incorporated into this study was due to the financial costs associated with sequencing a large bacterial genome. The *P. aeruginosa* reference strains PAO1 (Stover *et al*., 2000) and ATCC 15442 (Wang *et al*., 2014) were also included.

In total, 118 environmental *P. aeruginosa* isolates and the reference ATCC 15442 strain were sequenced (Table 5.1). The genome sequence of the PAO1 reference strain data was imported from GenBank (Genbank accession number AE004091.2) into BioNumerics (Applied Maths) for analysis.

5.3.5 Whole-Genome Sequencing

The Illumina WGS protocol is divided into three main stages: library preparation, cluster amplification and sequencing, and alignment and data interpretation as described in Chapter 1, Section 1.7.2. A simplified overview of the protocol is shown in Figure 5.3.

5.3.5.1 Library preparation

All *P. aeruginosa* isolates sequenced underwent genomic DNA extraction by enzymatic lysis using the Qiagen DNeasy Blood and Tissue kit (Qiagen, West Sussex, UK) as

Step 1: Library preparation

Tagmentation:
- Fragmentation of DNA and tagging of adapter sequence

Amplification:
- PCR amplification of DNA libraries
- Primer ligation to fragmented ends

Fragment size selection, quantification and pooling of pooled library

Denaturing and dilution:
- DS DNA denatured for MiSeq loading

Step 2: Cluster amplification and sequencing

Paired-end Illumina NGS MiSeq sequencing

Step 3: Alignment and data interpretation

Demultiplexing and primer trimming

Export FASTQ files:
- Export data to BaseSpace and download to BioNumerics

Data interpretation depending on desired outcome:
- Assignment of ST:
  - BioNumerics PubMLST plug-in
- wgMLST analysis
  - Assembly-free allele calling
  - Assembly-based allele calling
- SNV analysis
- Resistance gene finder
  - ResFinder
- Visualization of data:
  - Minimum Spanning Trees

Figure 5.3 A simplified workflow diagram of the three stages of whole genome-sequencing and analyses of *P. aeruginosa* isolates. Abbreviations: DS, double stranded; NGS, next generation sequencing; ST, Sequence type.

described in Chapter 2, Section 3. The sequencing library was prepared using the Nextera Flex DNA Library Preparation kit (Illumina, Eindhoven, The Netherlands) and the Nextera Flex Index kits (96 well index plate) (Illumina). A maximum of 20 DNA samples were prepared per sequencing run based on the minimum desired 50x average read coverage.

5.3.5.1.1 Tagmentation and post tagmentation clean-up

A reaction mixture for each *P. aeruginosa* sample was prepared in individual 0.2 ml PCR tubes containing 15 µl gDNA (100 – 500 ng/µl) (extracted as described in Chapter 2, Section 2.3), 5 µl Bead Linked Transposomes (BLT; provided in the Nextera Flex DNA Library Preparation kit) and 5 µl Tagmentation Buffer (TB1; Illumina). The total reaction mixture was aspirated using a laboratory pipette (Gilson) and incubated at 55°C for 10 min using the Kyratec Thermocycler model SC200 (Kyratec) or G-storm GSI Thermocycler (G-Storm). Immediately following incubation, 5 µl Tagmentation Stop Buffer (TSB; Illumina) was added to each tube, aspirated using a pipette and incubated for a further 15 min at 37°C. Once complete, the contents of each PCR tube were transferred into a 96 well plate (Corning Inc. Co-star, NY, USA) and placed on a 96 well magnetic stand (Invitogen, Thermo Scientific, MA, USA) until the solution went clear (~ 3 min) and the supernatant was discarded. The 96 well plate was then removed from the magnetic stand and 50 µl Tagmentation Wash Buffer (TWB; Illumina) was added to each sample and the pellet was resuspended. The PCR plate was placed back on the magnetic stand until the solution went clear (~ 3 min) and the supernatant was discarded. This TWB wash step was repeated a total of three times.

5.3.5.1.2 Amplification of the DNA libraries

Each washed tagmented DNA sample was resuspended with 10 µl Enhanced PCR Mix (EPM; Illumina), 10 µl nuclease free water and 5 µl index adapters (A1 – H12) from the 96-well index primer plate (Illumina) and transferred to individual 0.2 ml PCR tubes. Each unique adapter was selected to allow for multiple DNA samples (multiplexing) to be sequenced on a single run. The entire volume of each sample was aspirated using a pipette a minimum of 10 times. Each mixture underwent amplification using a Kyratec Thermocycler model SC200 (Kyratec) under the following conditions: 68°C for 3 min, 98°C for 3 min, six cycles of 98°C for 45 s, 62°C for 30 s and 68°C for 2 min, 68°C for 1 min and hold at 10°C.

5.3.5.1.3 PCR clean-up

Following amplification, the contents of each PCR tube was transferred to a 96-well plate (Corning) and placed on a Magnetic stand – 96 (Invitogen, Thermo Fischer Scientific, USA) until the solution went clear (~ 5 min). Once clear, 22.5 µl of each PCR supernatant was transferred to a fresh Abgene™ 96 Well 0.8 mL Polypropylene Deepwell Storage Plate (ThermoScientific, MA, USA). The PCR clean-up step was performed using Sample Purification Beads (SPB; Illumina). To each sample, 22.5 µl of room temperature SPB and 20 µl nuclease free water were added. The entire volume of each sample was aspirated using a pipette a minimum of 10 times. This step is crucial for the proper size distribution of the libraries. The samples were then incubated at room temperature for 5 min and placed on the magnetic stand until the solution went clear (~ 5 min).

The supernatant (62.5 µl) was then transferred to a fresh Abgene™ 96-well plate (ThermoScientific) and 7.5 µl SPB were added to each sample. The entire volume of each sample was then aspirated using a pipette a minimum of 10 times. The samples were incubated at room temperature for 5 min and placed on the magnetic stand until the solution went clear (~ 5 min). The supernatant was then discarded and with the plate still on the magnetic stand, 100 µl freshly prepared 80% (v/v) ethanol was washed over each pellet for 30 s and subsequently discarded. The ethanol wash was repeated twice. The beads were left to air dry for a maximum of 5 min, after which the plate was removed from the magnet and 17 µl of Resuspension Buffer (RSB; Illumina) were added to each sample beads and aspirated using a pipette. The samples were incubated for a total 2 min at room temperature and then placed on the magnetic stand for a further 2 min, after which a final volume of 15 µl of each sample was transferred to a fresh microcentrifuge tube.

5.3.5.1.4 Determining DNA fragment size

The size range of the DNA libraries were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) as described in Chapter 4, Section 4.3.10.2.5.

5.3.5.1.5 Pooling, denaturation and diluting

Following library clean up, the DNA concentration in each sample was determined using the Quibit Fluorometer 3.0 (Thermo Fisher). The Qubit dsDNA High Sensitivity Assay Kit (Thermo Fisher) was used to quantify each DNA sample. The Quibit Working solution was prepared by diluting the Qubit dsDNA HS Reagent (Thermo Fisher) 1:200 in Qubit dsDNA HS Buffer (Thermo Fisher). A final volume of 198 µl Quibit Working solution and 2 µl

DNA sample were prepared and incubated at room temperature for 2 min before samples were read in ng/µl.

Each sampled was diluted to a concentration of 4 nM in nuclease free water to normalise the multiplex library. The DNA molarity was determined using the following formula, with 660 g/mol referring to the average weight of a single DNA base pair:

$$\frac{\text{(concentration of the pooled library in ng/µl)}}{\text{(660 g/mol x the average library size in bp)}} \times 10^6 = \text{concentration in nM}$$

Once normalised, 5 µl of each sample were pooled into a fresh 1.5 ml microcentrifuge tube. A quality control step was incorporated into the protocol, where the pooled library was quantified using the Quibit Fluorometer. Once the library concentration was confirmed, 5 µl of the pooled library and 5 µl of freshly prepared 0.2 N NaOH were mixed in a microcentrifuge tube. The tube was incubated at room temperature for 5 min, and 990 µl Hybridization buffer (HT1; Illumina) was added resulting in 1 ml of 20 pM denatured library. For loading onto the MiSeq sequencer, the library was further diluted to 12 pM in a total volume of 600 µl, by added 360 µl of the 20 pM library to 240 µl of the HT1 buffer. Each run was spiked with PhiX DNA (Illumina) as an internal sequencing control. The control PhiX library is included into the prepared sequencing library as an internal quality control for cluster generation, alignment and to balance the nucleotide diversity in low-diversity input samples. From the 600 µl loading volume, 6 µl was discarded using a pipette and 6 µl of 12 pM PhiX (Illumina) was added. A control library generated by the PhiX virus is included in each library preparation.

5.3.5.2 Cluster amplification and sequencing

All libraries were sequenced on the Illumina MiSeq platform (Illumina) using the MiSeq Reagent kit v2 (500 cycles) (Illumina) generating paired-end reads using the MiSeq sequencing platform (Illumina).

5.3.5.3 Alignment and data interpretation

All forward and reverse FASTQ files generated per isolate sequenced were uploaded from the Illumina MiSeq sequence platform into BaseSpace Sequence Hub (Illumina, Eindhoven, the Netherlands). The BioNumerics v7.6 (Applied Maths, Sint-Martens-Latem, Belgium) suite of software applications were used to analyse WGS data from Illumina FASTQ files.

The default quality control settings were applied for the import of the sequence reads files into BioNumerics: all base calls below an average quality of 10% were removed, all read lengths below 10% of the average read length were removed, and all read ends below 25% of the average read end were removed. Raw reads were *de novo* assembled and contigs generated utilising SPAdes v6.4 (Bankevich *et al*., 2012). SPAdes is an example of a De Bruijn graph method *de novo* assembler which is designed to assemble bacterial genomes (Bankevich *et al.,* 2012). The parameters of each assembly were determined automatically by the SPAdes software.

5.3.5.3.1 MLST analysis

The MLST profile of all 118 environmental *P. aeruginosa* isolates sequenced and the two reference strains was determined using PubMLST plug-in using BioNumeric (https://pubmlst.org).

5.3.5.3.2 wgMLST analysis

Whole genome MLST analysis was performed using the BioNumerics v7.6 (Applied Maths) *P. aeruginosa* wgMLST scheme. This wgMLST scheme consists of 15,136 loci and 7 public MLST loci (http://www.applied-maths.com/sites/default/files/extra/Release-Note-Pseudomonas-aeruginosa-schema.pdf) and includes core and accessory genome (i.e. pan-genome) loci identified from 400 annotated *P. aeruginosa* genomes. These loci have been further validated using 2,286 additional annotated publicly available genomes. Two different algorithms were utilised to generate a consensus wgMLST profile for each isolate: assembly-free allele calling and assembly-based allele calling. The assembly free approach uses a k-mer based algorithm to determine which loci are present and the allelic identity from the wgMLST scheme. The approach identifies all sub-sequences (of length k) in every raw sequence read. The parameters set for retention of alleles into the consensus wgMLST profile for this approach include a default k-mer length of 35 and the retention of loci with a minimum total coverage of 5x (with 1x coverage in both direction). Assembly-based allele calling uses an approach to detect alleles in contigs using BLAST (Altschul *et al*., 1990). The parameters set for retention of alleles into the consensus wgMLST profile for this approach include applying a minimum sequence similarity of 75% (single base threshold 75%, double base threshold 85%, triple base threshold 95% and gap threshold of 50%), and exclusion of loci with more than one allelic variant.

5.3.5.3.3 SNV analysis

Pairwise SNV analysis was performed using the BioNumerics v7.6 (Applied Maths). Each experiment required selection of a reference isolate. The generated contigs of the reference isolate were used to map other query sequence read sets against (Li and Durbin, 2010). The tool for mapping query sequence read sets against the reference, was the Burrows-Wheeler Aligner (incorporated into BioNumerics v7.6) (Li and Durbin, 2010). The parameters set for base correction during read mapping were set by BioNumerics. These parameters include a single base threshold of 0.75, a double base threshold of 0.85 and a triple base threshold of 0.95. The single base threshold requires that at a given position, the most frequently called base must be present in at least 75% of all base calls at that position to be included into the consensus sequence. The double base threshold requires that at a given position, the two most frequently called bases must be present in at least 85% of all base calls at that position to be included into the consensus sequence. Double base threshold was utilised for bases that did not fulfil the criterion for the single base calling threshold. The triple base threshold requires that at a given position, the three most frequently called bases must be present in at least 95% of all base calls at that position to be included into the consensus sequence. Triple base threshold was utilised for bases that did not fulfil the criterion for the single or double base calling thresholds. SNV filter exclusion parameters were set to remove potential indel-related SNVs (SNVs occurring within 12 bp), positions with ambiguous base calls caused by mapping artifacts and contamination, gaps or non-covered regions, and SNVs in repeat regions. SNVs with at least 5x coverage (including 1x in both direction), were included in this analysis.

5.3.5.3.4 Minimum Spanning Trees

Minimal spanning trees (MSTs) were generated using BioNumerics (Applied Maths), for wgMLST and SNV data. The minimum spanning tree was generated based on Kruskal's algorithm, connecting all nodes at the shortest distance (Kruskal, 1956; Kwapien *et al*., 2017). Six MSTs were constructed based on wgMLST and SNV analysis in this chapter.

5.3.5.4.5 Quality control

The quality metrics for each isolate was set at an average Phred quality score >30 (Q30) meaning the base call accuracy was greater than 99.9%, and all had an average read coverage of ≥50x (Table 5.2). Other quality control parameters include the average N50, that is defined as the minimum contig length required to cover 50 % of the genome, the average number of contigs, the average number of confirmed loci in the wgMLST scheme, the average number of assembly-based allele calls present and the average number of assembly-

Table 5.2 Whole-genome sequencing quality assurance data

Quality parameter	Average result
Trimmed read quality	33.8
Trimmed assembly coverage	86.9
N50 [1]	270,047.3
No. of contigs per isolate	105
No. assembly-based allele calls present	6189.9
No. assembly-free allele calls present	6157.2
No. of confirmed loci [2]	5202

[1] The N50 is the minimum contig length needed to cover 50% of the genome.
[2] In a scheme of 15,136 loci and 7 housekeeping genes.

free allele calls present (Schatz *et al.,* 2010). Read qualities were checked using Galaxy software tools (Afgan *et al*., 2016), which utilises FastQC software and where necessary, the reads with a Phred score of <30 were trimmed using Trimmomatic software using default settings (Afgan *et al.,* 2016).

5.3.5.3.6 Identification of resistance genes in P. aeruginosa *isolates*

All *P. aeruginosa* isolates sequenced in this study were analysed using the ResFinder version 3.2, to identify antibiotic resistance genes (Camacho *et al*., 2009). The resistance genes are associated with the following antimicrobial agents only: aminoglycosides, beta-lactams, colistin, fluoroquinolone, fosfomycin, fusidic acid, glycopeptides, MLS (macrolide, lincosamide,and streptogramin B), nitroimidazole, oxazolidinone, phenicol, rifampicin, sulphonamide, tetracycline, and trimethoprim. The default parameters were set at 90% sequence identity and 60% sequence coverage.

5.3.6 Antimicrobial susceptibility testing of *P. aeruginosa*

Forty-nine *P. aeruginosa* isolates were selected for antimicrobial agent susceptibility testing. These were selected to represent the diversity of the 120 *P. aeruginosa* investigated based on the STs identified throughout DDUH and the comparator isolates. Antimicrobial agent susceptibility testing was undertaken using the VITEK 2 system (bioMérieux, Marcy-l'Étoile, France) with additional manual E-test testing (bioMérieux) for meropenem-vaborbactam and netilmicin. All tests were carried out at the NMRSARL by the laboratory staff. The following 15 antimicrobial agents were tested: piperacillin-tazobactam (TZP), ticarcillin-clavulanic acid (TIM), cefepime (FEP), ceftazidime (CAZ), imipenem (IPM), meropenem (MEM), meropenem-vaborbactam (MVB), aztreonam (ATM), ciprofloxacin (CIP), levofloxacin (LVX), amikacin (AMK), gentamicin (GEN), netilmicin (NET), tobramycin (TOB), and colistin (CST) (Table 5.3).

Antimicrobial susceptibility testing on the Vitek 2 system was carried out by initially suspending *P. aeruginosa* culture into Muller-Hinton broth and incubated at 35ºC for 18 h in a shaking incubator. The desired inoculum is 5×10^5 CFU/ml adjusted in 0.45% sodium chloride. Suspensions of isolates for testing were loaded onto AST N352 cards (bioMérieux) and inserted into the Vitek 2 system. AST N352 cards include three to eight concentrations of each tested antibiotic: TZP, 2/4 – 48/8 µg/ml ; TIM, 8 – 64 µg/ml; FEP, 0.25 – 32 µg/ml; CAZ, 0.25 – 32 µg/ml; IPM, 1 – 12 µg/ml; MEM, 0.5 – 12 µg/ml; ATM, 2 – 32 µg/ml; CIP, 0.5 – 4 µg/ml; LVX, 0.25 – 8 µg/ml; AMK, 8 – 64 µg/ml; GEM, 4 – 32 µg/ml; TOB, 8 – 64

Table 5.3 Antimicrobial classes and agents

Antimicrobial class	Antibiotic
Penicillins	Piperacillin-Tazobactam
	Ticarcillin-Clavulanic acid
Cephalosporins	Cefepime
	Ceftazidime
Carbapenems	Imipenem
	Meropenem
	Meropenem-Vaborbactam
Monobactams	Aztreonam
Fluoroquinolones	Ciprofloxacin
	Levofloxacin
Aminoglycosides	Amikacin
	Gentamicin
	Netilmicin
	Tobramycin
Miscellaneous	Colistin

µg/ml; CST, 4 – 32 µg/ml. The system automatically analyses the turbidity of the suspension every 15 min for 18 h for each antimicrobial agent-containing test well.

Antibiotic susceptibility testing with E-test strips was carried out in parallel to tests undertaken with the Vitek 2 system to determine the MIC of meropenem-vaborbactam (bioMérieux) and netilmicin (bioMérieux). *Pseudomonas aeruginosa* isolates were grown at 37°C for 24 h in a shaking incubator, adjusted to a turbidity of 0.5 MacFarland standard, lawn plated onto a Muller-Hinton agar plate and incubated at 37°C for 24 h. The minimum inhibitory concentrations (MIC) were read directly from the scale on the E-test strip placed on the agar plate. The MIC breakpoints of netilmicin are susceptible ≤ 4 mg/l and R > 4 mg/l and of meropenem-vaborbactam are susceptible ≤ 8 mg/l and R > 8 mg/l. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) MIC breakpoints were used to determine whether isolates were recorded as susceptible (S), intermediate (I) or resistant (R) (http://www.eucast.org/clinical_breakpoints/).

5.4 Results

5.4.1 *P. aeruginosa* from Clinic 2 and A&E U-bends

5.4.1.1 P. aeruginosa *STs in DDUH U-bends*

Whole-genome sequencing analysis of 47 selected *P. aeruginosa* isolates recovered from Clinic 2 U-bends over a period of six months (February – July 2018) and 8 *P. aeruginosa* selected isolates recovered from one U-bend on completion of the study (February 2019) yielded four STs from the 55 isolates sequenced (ST179, ST252, ST298, ST560). Clinic 2 U-bends were not subjected to decontamination during the study period. ST179 and ST560 accounted for 49% (27/55) and 35% (19/55) of isolates, respectively (Table 5.1). The recovery locations of the ST179 and ST560 isolates are shown in Figure 5.4. Clinic 2 U-bend B2D3 was sampled weekly during the same six-month period (February – July 2018) and 17 isolates from separate samples belonged to ST179 (9 isolates, average allelic difference of 1 [median 0, range 0 – 2]) and ST560 (8 isolates, average allelic difference of 1 [median 1, range 0 – 2]). Following the completion of the initial study period (February – July 2018), eight isolates from one washbasin (B2D3) sample (February 2019) were sequenced to provide a snapshot of the total *P. aeruginosa* population in one washbasin. These eight isolates recovered from one B2D3 sample belonged to ST179 (average allelic difference of 2 [median 0, range 0 – 7]). Analysis of all Clinic 2 ST560 (n = 19) and ST179 isolates (n = 27) showed that isolates within each ST were very closely related (average allelic difference of 1 [median 1, range 0 – 4] and 2 [median 1, range 0 – 14]), respectively. ST298 and ST252 accounted for 9% (5/55) and 7% (4/55) of isolates, respectively (Table 5.1). Analysis of the ST298 and ST252 isolates showed that isolates within each ST were very closely related (average allelic difference of 1 [median 0, range 0 – 4] and 1 [median 1, range 0 – 3]), respectively.

Concurrently, *P. aeruginosa* isolates recovered from washbasin U-bends in the DDUH A&E Department, which were decontaminated three-times weekly with ECA solutions, were sequenced. Twenty-one *P. aeruginosa* isolates were selected from seven A&E U-bends over a period of 24 months (January 2017 – January 2019). The longer recovery period relative to Clinic 2 was chosen because of the low abundance of *P. aeruginosa* in decontaminated A&E U-bends, as described in Chapter 3, Section 3.2.4 and Chapter 4, Section 4.2.4.

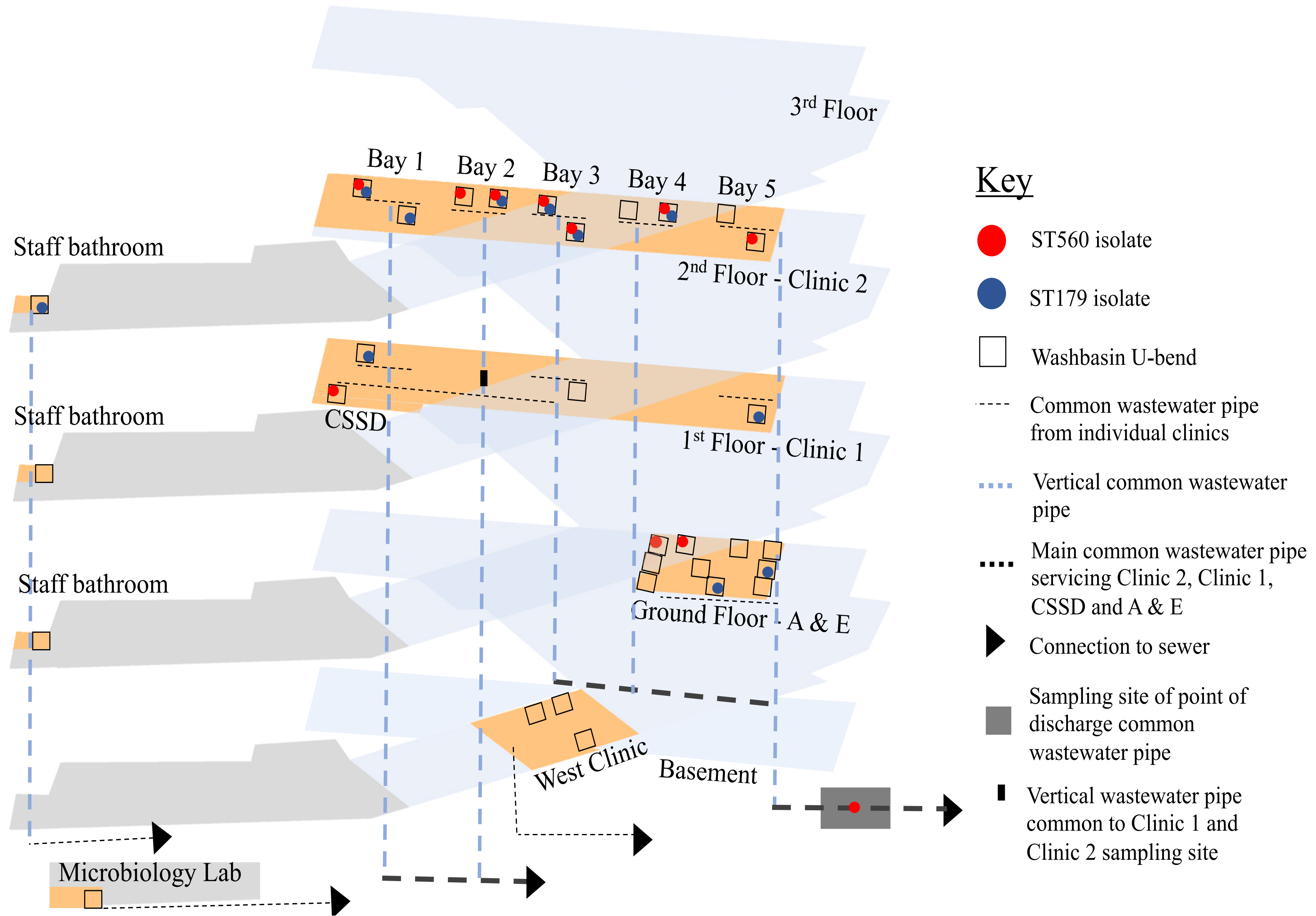

3rd Floor
Bay 1
Bay 2
Bay 3
Bay 4
Bay 5
Staff bathroom
2nd Floor - Clinic 2
Staff bathroom
CSSD
1st Floor - Clinic 1
Staff bathroom
Ground Floor - A & E
West Clinic
Basement
Microbiology Lab
Key
ST560 isolate
ST179 isolate
Washbasin U-bend
Common wastewater pipe from individual clinics
Vertical common wastewater pipe
Main common wastewater pipe servicing Clinic 2, Clinic 1, CSSD and A & E
Connection to sewer
Sampling site of point of discharge common wastewater pipe
Vertical wastewater pipe common to Clinic 1 and Clinic 2 sampling site

Figure 5.4 Schematic showing the relative locations of DDUH washbasins investigated showing the distribution of the two predominant *P. aeruginosa* sequence types (STs) identified (ST560 and ST179) in U-bends. The pale blue (clinical) and grey (administrative) sections represent different areas of DDUH. The 15 washbasins in each of Clinic 1 and Clinic 2 are located in five bays, each with three washbasins, on both clinics. Wastewater from each bay is discharged into a separate vertical wastewater pipe that passes down through the building into the basement. The vertical pipes servicing bays 3 – 5 from each clinic connect to a large common horizontal wastewater pipe in the DDUH basement where it discharges to the municipal sewer at the building perimeter. This common wastewater pipe also receives washbasin wastewater from the Accident and Emergency Department (A&E) and the Central Sterile Supply Department (CSSD). Wastewater from clinical bays 1 and 2 connect to a separate horizontal wastewater pipe in the basement and discharge wastewater to the municipal sewer at a different exit point. Washbasin wastewater from bathrooms in the administration area and West Clinic discharge wastewater to the municipal sewer at separate connections. Ten washbasins each from Clinic 2 and A&E, three from Clinic 1, one from CSSD, three from West Clinic, one from three separate staff bathrooms and one from an independent lab were included in the study. Clinic 2 and A&E are equipped with 15 and 11 washbasins, respectively.

ST560 was recovered from 11 washbasin U-bends in Clinic 2, A&E, CSSD, and the common wastewater collection pipe servicing clinical bays 3 – 5 just prior to discharge into the municipal sewer. ST179 was recovered from 11 washbasin U-bends in Clinic 1, Clinic 2, A&E, and the staff bathroom. SNV analysis of the 27 ST560 isolates grouped the isolates into two clusters. In the present study the allelic and SNV thresholds of relatedness for *P. aeruginosa* isolates were set at ≤14 allelic differences and ≤37 SNVs. Isolates from Cluster I ($n = 25$) exhibit an average SNV difference of 2 (median 1, range 0 – 8) and were recovered in Clinic 2, A&E, CSSD and the common wastewater pipe. Cluster II exhibits five SNV differences between two isolates recovered in A&E.

SNV analysis of the 34 ST179 isolates exhibit an average pairwise SNV difference of 10 SNVs (median 5, range 0 – 38). However, interestingly, there was no SNV differences between isolates recovered from the A&E and the staff bathroom.

Five additional isolates recovered from five A&E U-bends recovered seven months after the cessation of regular ECA decontamination in November 2019 were also sequenced. Sequencing of the 26 *P. aeruginosa* isolates from seven A&E U-bends yielded seven STs, including ST308 (*n* = 7), ST773 (*n* = 5), ST560 (*n* = 4), ST296 (*n* = 4), ST27 (*n* = 3), ST179 (*n* = 2), and ST298 (*n* = 1). The four ST560 and two ST179 isolates exhibited an average allelic difference of 14 (median 0.5, range 0 – 35) and 0 – 7 allelic differences, respectively. Overall, the two most abundant STs recovered from Clinic 2 and A&E combined were ST179 and ST560 and exhibited an average allelic difference of 3 (median 1, range 0 – 17) and 10 (median 1, range 0 – 64), respectively.

Fourteen *P. aeruginosa* isolates were sequenced from other DDUH washbasin U-bend locations including, one U-bend in CSSD (*n* = 2), three U-bends in West Clinic (*n* = 3), three U-bends in Clinic 1 (*n* = 4), a single DP washbasin U-bend from the microbiology laboratory (*n* = 1), four staff bathroom washbasin U-bends (*n* = 4) and one isolate from one wastewater pipe sampling site in a vertical wastewater collection pipe common to Clinic 1 and Clinic 2 (Figure 5.2). These sixteen isolates yielded seven STs (ST27, ST179, ST252, ST253, ST309, ST560 and ST606) (Table 5.1). Three additional isolates belonging to ST253 and ST560 were recovered from the main common wastewater pipe receiving wastewater from A&E, Clinic 1, Clinic 2 and CSSD at the point of discharge to the municipal sewer (Figure 5.2, Table 5.1).

5.4.1.2 P. aeruginosa STs among comparator isolates

The 11 *P. aeruginosa* isolates investigated from three other healthcare facilities (including 9 isolates from washbasin U-bends in two acute hospitals) yielded eight STs (ST17, ST253, ST282, ST298, ST313, ST348, ST390 and ST395) (Table 5.1). Only two of these (ST253 and ST298) were identified in DDUH. The ST298 isolate from Hospital 2 exhibited at least 134 allelic differences to the ST298 isolates (*n* = 8) from Clinic 2, A&E and the first floor and second-floor staff bathroom isolates LP1R and LP2R. Furthermore, the three ST253 isolates identified in Hospital 2, dental suction systems and the point of discharge wastewater pipe common to Clinic 2 and A&E, exhibited an average of 26 (median 24.5, range 0 – 58) allelic differences from each other. *Pseudomonas aeruginosa* isolates recovered from dental suction systems (*n* = 8) yielded three STs including ST1320 (*n* = 5; average of allelic differences 4 [median 4, range 0–11]), ST253 (*n* = 1) and ST2865 (*n* = 2).

5.4.1.3 Population structure of DDUH and comparator P. aeruginosa

A MST based on wgMLST profiles was generated showing the STs of all isolates investigated (Figure 5.5). Overall eleven STs were identified among 99 DDUH isolates. These included 95 isolates from DDUH U-bends, one isolate from a vertical collection pipe common to Clinic 1 and Clinic 2, and the three isolates recovered from the point of discharge wastewater pipe common to Clinic 2, Clinic 1 and A&E (ST27, ST179, ST252, ST253, ST296, ST298, ST308, ST309, ST560, ST606, and ST773). One of the predominant DDUH STs, ST179 (n = 34), exhibited an average allelic difference of 3 (median 2, range 0 – 17), indicating these isolates were very closely related (Figure 5.5). Isolates of the second predominant ST, ST560 (n = 27), exhibited an average allelic difference of 7 (median 1, range 0 – 64), suggesting these isolates were more diverse. However, two isolate clusters were evident within ST560; Cluster I (n = 25; average allelic difference of 3 [median 1, range 0 – 21]) and Cluster II (n = 2; no allelic differences) (Figure 5.5).

ST560 and ST179 isolates were also investigated by pairwise SNV analysis; these isolates exhibited an average of 9 SNVs (median 1, range 0 – 66) and 10 SNVs (median 5, range 0 – 38), respectively (Figure 5.6 (a) and (b)). ST560 Cluster I isolates exhibited an average of 2 SNVs (median 1, range 0 – 8) (including isolates from Clinic 2, A&E, CSSD and the common wastewater pipe at the point of discharge to the municipal sewer), whereas the two ST560 Cluster II isolates (from A&E) exhibited five SNVs. Cluster II was differentiated from Cluster I by 59 SNVs (Figure 5.6 (a)). ST179 isolates (including isolates from Clinic 2, Clinic 1, A&E, and the isolate LP3F1 from the third-floor staff bathroom) exhibited an average of 10 SNVs (median 5, range 0 – 38) (Figure 5.6 (b)). Two isolates from U-bends in A&E and the staff bathroom exhibited no SNVs (Figure 5.6 (b)). The relative locations in which all ST179 and ST560 isolates sequenced in this study were originally recovered are shown in Figure 5.5.

5.4.2 Effects of A&E U-bend decontamination on the *P. aeruginosa* population structure

Of the 26 isolates sequenced from A&E, four STs were identified immediately after U-bend decontamination (ST296, ST308, ST560 and ST773), four 24 h-post decontamination (ST27, ST179, ST308 and ST560), three 48 h-post decontamination the (ST296, ST308, ST773), and five seven-months after cessation of routine decontamination (ST27, ST296, ST298, ST773). Isolates from STs ST27, ST308, ST296, ST773, and ST560 were identified between two or more of the sampling time points (Figure 5.7). Of all the *P. aeruginosa* STs identified in DDUH, ST308 was found exclusively in the washbasin U-bends in A&E.

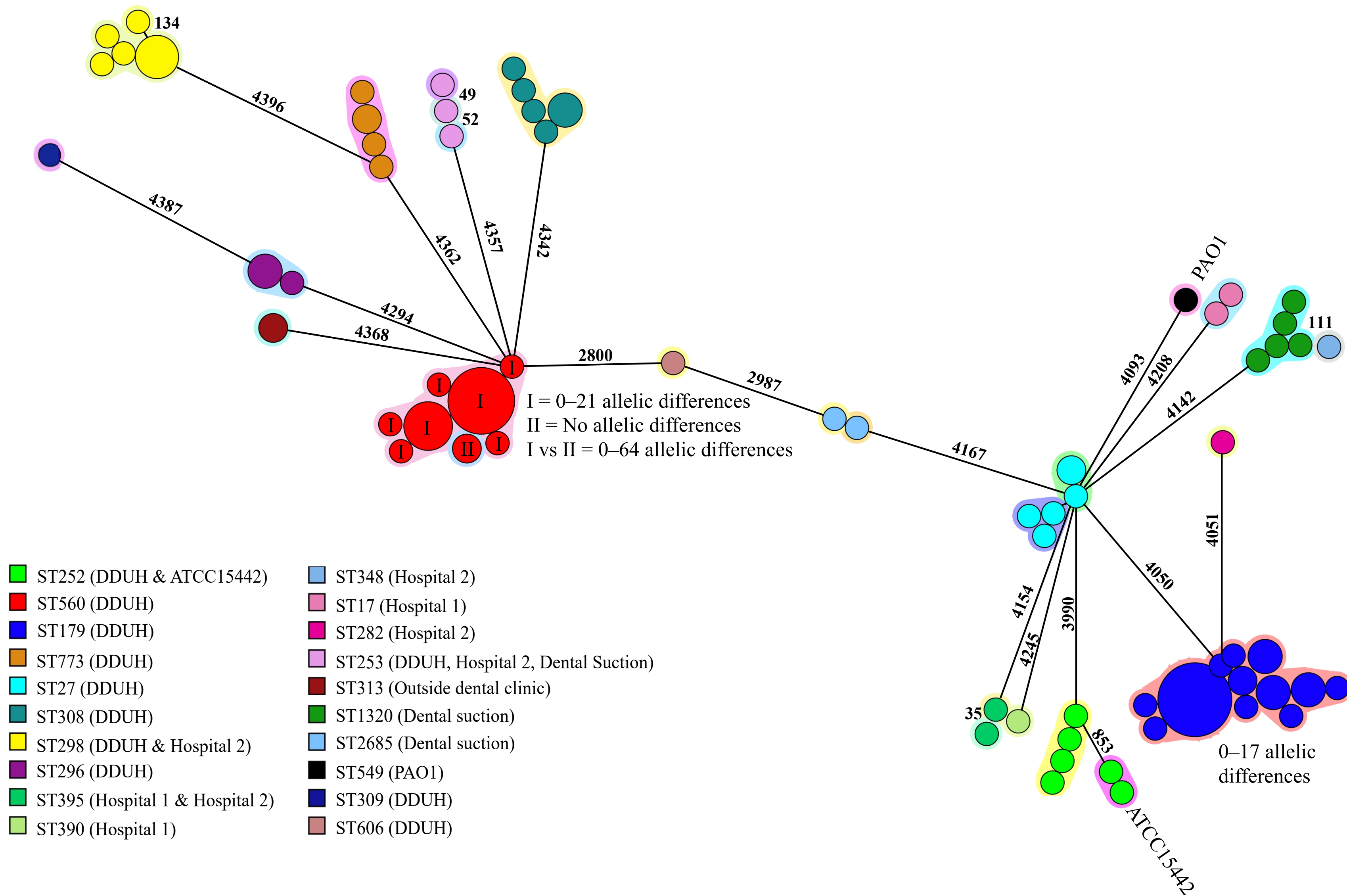

134
4396
49
52
4387
4362
4357
4342
4294
4368
2800
2987
I = 0–21 allelic differences
II = No allelic differences
I vs II = 0–64 allelic differences
4167
PAO1
4093
4208
111
4142
4051
4050
4154
4245
3990
35
853
ATCC15442
0–17 allelic differences
ST252 (DDUH & ATCC15442)
ST560 (DDUH)
ST179 (DDUH)
ST773 (DDUH)
ST27 (DDUH)
ST308 (DDUH)
ST298 (DDUH & Hospital 2)
ST296 (DDUH)
ST395 (Hospital 1 & Hospital 2)
ST390 (Hospital 1)
ST348 (Hospital 2)
ST17 (Hospital 1)
ST282 (Hospital 2)
ST253 (DDUH, Hospital 2, Dental Suction)
ST313 (Outside dental clinic)
ST1320 (Dental suction)
ST2685 (Dental suction)
ST549 (PAO1)
ST309 (DDUH)
ST606 (DDUH)

Figure 5.5 Minimum spanning tree (MST) based on whole-genome multi-locus sequence typing (wgMLST) of *P. aeruginosa* isolates. The MST is based on wgMLST data of all 120 *P. aeruginosa* (118 environmental isolates and reference strains PAO1 and ATCC 15442) investigated showing the relationships between the sequence types (STs). The colour-coded key identifies STs and the origin of isolates within each ST. Ninety-five isolates were recovered from DDUH U-bends (ST27, ST179, ST252, ST296, ST298, ST308, ST560, ST606, ST773), one (ST309) from a vertical wastewater pipe common to Clinic 1 and Clinic 2 (see Figure 5.2), and three (ST253 and ST560) from the point of discharge wastewater collection pipe sampling port common to washbasins in Clinic 2, Clinic 1, and A&E.

Each node indicates at least one isolate and the colour of the node represents the ST. The allelic threshold of relatedness for *P. aeruginosa* isolates has been set at ≤14 allelic differences (Mellmann *et al.,* 2016; Schürch *et al.,* 2018). A uniform colour surrounding the node indicates that all the nodes are within the threshold of relatedness or form a cluster.

ST179 and ST560 accounted for 34% (34/99) and 27% (27/99) of the total DDUH isolates sequenced. ST560 isolates had two distinct clusters; Cluster I consisted of 25 isolates, whereas Cluster II consisted of two isolates. ST179 and ST560 were not identified among comparator isolates investigated.

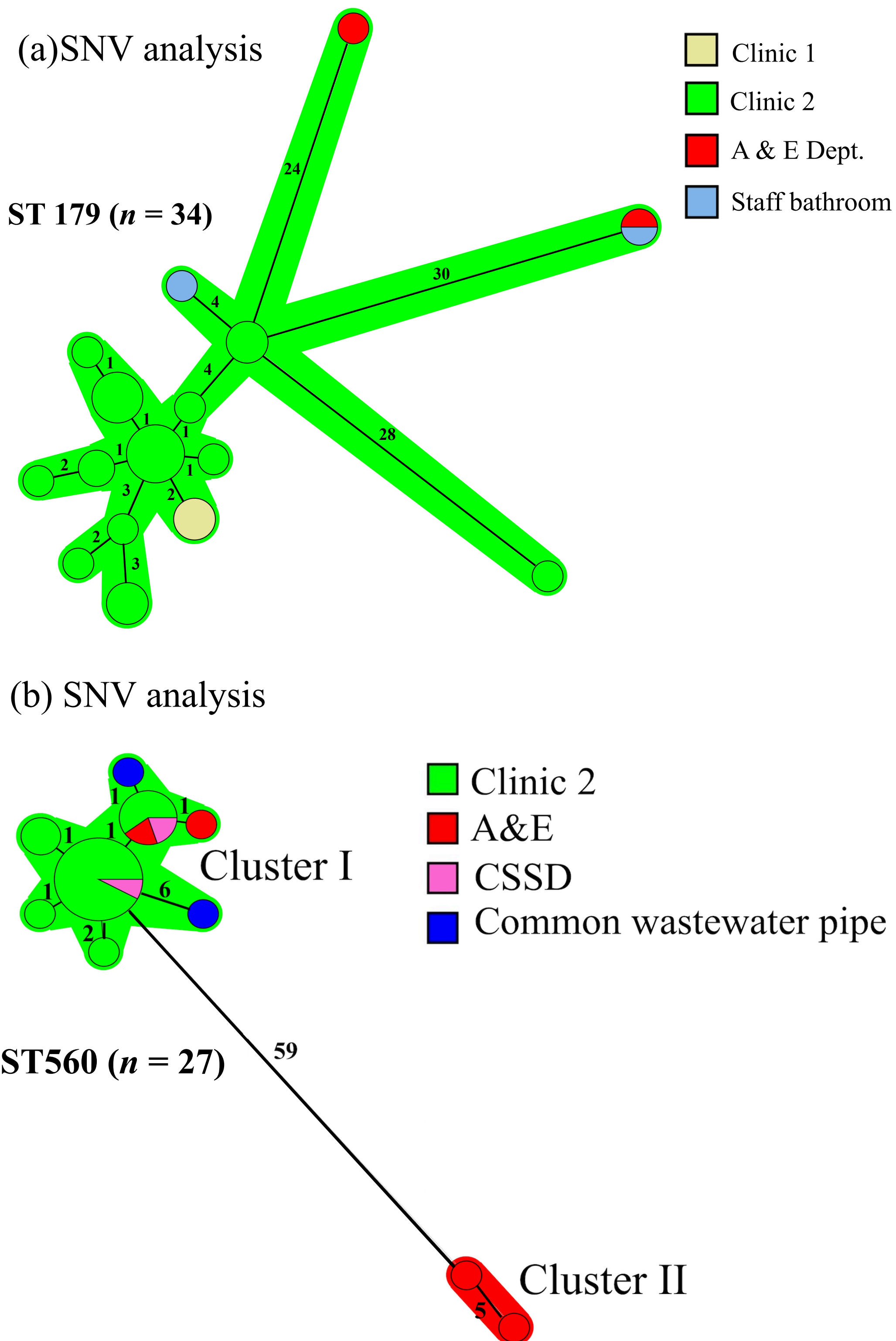


Figure 5.6 Minimum spanning trees (MSTs) based on single nucleotide variation (SNV) data of *P. aeruginosa* isolates. The threshold of isolate relatedness was set as ≤37 SNV differences. Panel **(a)** An MST based on the SNV profiles of the 34 ST179 DDUH isolates recovered from Clinic 1, Clinic 2, A&E and the third-floor staff bathroom. Isolates differed by an average of 10 SNVs (median 5, range 0 – 38). Panel **(b)** An MST based on SNV

analysis of the 27 ST560 DDUH isolates recovered from Clinic 2, A&E, CSSD and the point of discharge wastewater pipe common to A&E, Clinic 1 and Clinic 2. The isolates formed two distinct groups, Clusters I and II, differentiated by 59 SNVs. The average SNVs within the 25 isolates of Clusters I was 2 (median 1, range 0 – 8), whereas the two Cluster II isolates differed by five SNVs. Isolates within each of the two clusters revealed by SNV analysis corresponded to the same isolates identified within the two clusters identified within ST560 by wgMLST analysis. These findings confirmed that isolates within each cluster were very closely related.

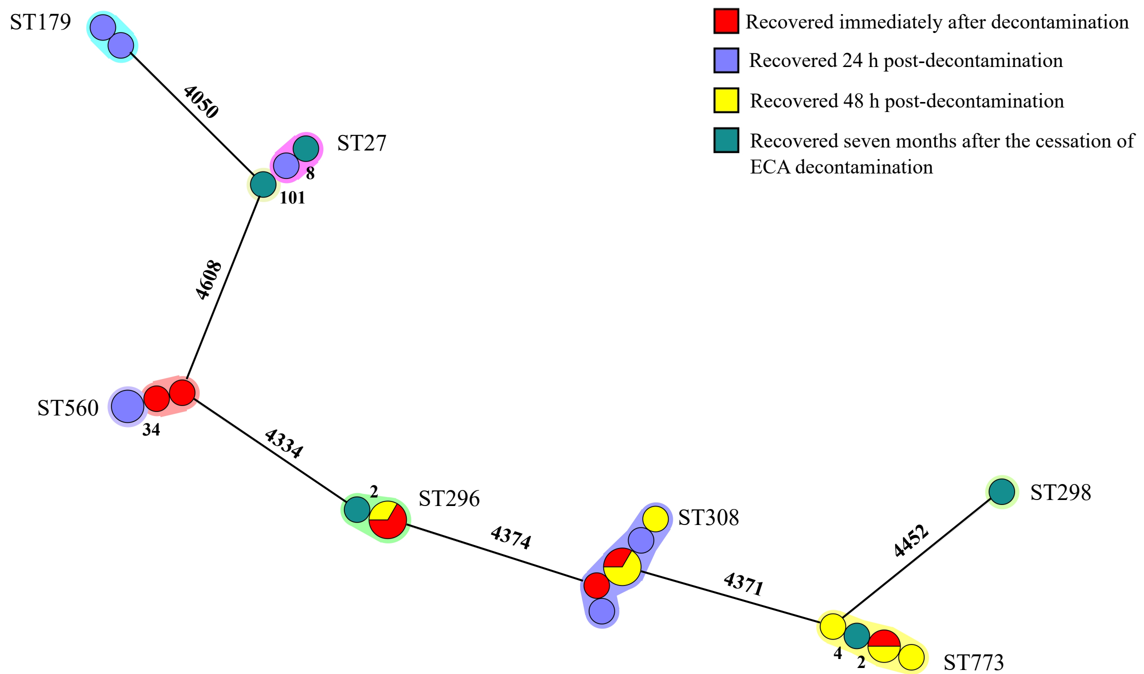


Figure 5.7 Minimum spanning tree (MST) based on whole-genome multi-locus sequence typing (wgMLST) of the 26 *P. aeruginosa* isolates recovered from A&E. The key on the right-hand side represents the four times points over which the isolates were recovered: immediately after decontamination, 24 h post- decontamination, 48 h post- decontamination, or seven months after the cessation of ECA decontamination. Five of the seven STs recovered (ST27, ST560, ST296, ST308, ST773) contain isolates from at least two different sampling time-points.

Focusing on the ST296 cluster, the isolate recovered from the washbasin U-bend seven months after the cessation of ECA decontamination exhibited two allelic differences from the isolates recovered immediately after decontamination and 48 h post- decontamination. This close relatedness is also reflected in isolates within ST773 and ST27 indicating conserved wgMLST profiles in the wastewater pipe network in DDUH. ST308, ST179 and ST560 were not recovered from washbasin U-bends seven months after the cessation of ECA decontamination. ST179 and ST560 isolates were recovered from multiple location in DDUH throughout the course of the study, ST308 was recovered only from the A&E department suggesting possible adaptation of the ST to routine decontamination.

5.4.3 Testing DDUH water outlets for *P. aeruginosa*

Testing of 72 1-L samples of DDUH water during the study failed to detect *P. aeruginosa* including the potable mains water supply (*n* = 8), the anolyte-treated water storage tank supplying washbasin faucets (*n* = 8), and washbasin faucet output water (*n* = 56). Previous studies over 54 weeks at DDUH showed average aerobic heterotrophic bacterial densities in hot and cold tap washbasin water of 1 (± 4) and 2 (± 4) CFU/ml, respectively (Boyle *et al.*, 2012). Swab sampling of 20 DDUH washbasin faucets including five each from Clinic 2 and A&E on four occasions each (total of 80 samples) during the study also failed to yield *P. aeruginosa*.

5.4.4 Detection of antimicrobial resistance genes in *P. aeruginosa*

All *P. aeruginosa* isolates sequenced in this study were investigated for resistance genes using the ResFinder plugin. ResFinder is a database that captures antimicrobial resistance genes from whole-genome data sets. All 120 *P. aeruginosa* harboured a chromosomal aminoglycoside phosphotransferase gene (*aph(3')-IIb*), an oxacillinase gene (*blaOXA-50*), a beta-lactamase resistance gene (*blaPAO*), a fosfomycin resistance glutathione transferase gene (*fosA*) and all, apart from three isolates (AE24May, West3, AER1; Table 5.1) harboured a chloramphenicol acetyltransferase gene (*catB7*). Isolate CWP2 harboured two additional resistance genes including a sulphonamide resistance gene (*sul1*) and an aminoglycoside adenyltransferase gene (*aadA7*). Isolate DH6 harboured an additional two streptomycin resistance genes (*strA* and *strB*), a sulphonamide resistance gene (*sul1*), two streptomycin resistance genes (*aadB* and *aadA11*), and an additional oxacillinase gene (*blaOXA-10*).

5.4.5 Phenotypic resistance to antibiotics by *P. aeruginosa*

Of the 120 *P. aeruginosa* sequenced in this study, 49 isolates were selected for phenotypic susceptibility testing to a range of antibiotics based on the diversity of STs identified recovered from DDUH and comparator locations. The 15 antimicrobials chosen for susceptibility testing were based on compounds for which EUCAST breakpoint information was available (Table 5.4). The 49 isolates were grouped into four observed phenotypic profiles: (i) susceptible to all 15 antimicrobials tested, (ii) resistance to one class of antimicrobials, (iii) resistance to two classes of antimicrobials, and (iv) resistance to three or more classes of antimicrobials or multi-drug resistant (MDR).

Table 5.4 Phenotypic antibiotic resistance profiles of a selection of 49 *P. aeruginosa* isolates

		No. of isolates containing specific resistance genes[2]														
		Penicillins		Cephalosporins		Carbapenems			Monobactams	Fluoroquinolones		Aminoglycosides				Miscellaneous
Isolates	AR profile[1]	TZP	TIM	FEP	CAZ	IPM	MEM	MVB	ATM	CIP	LVX	AMK	GEN	NET	TOB	CST
Sequence Type 27																
AE24bMay	Non-MDR	S	S	S	S	S	S	S	S	**R**	**R**	S	S	S	S	S
West1	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Sequence Type 179																
B1D2Jun	Non-MDR	S	**R**	S	S	S	S	S	S	S	S	S	S	S	S	S
B1D4cJul	Non-MDR	S	S	S	S	**R**	**I**	S	S	S	S	S	S	S	S	S
B2D3cMar	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
B2D3dMay	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
B2D3cJun	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
B2D3bJul	MDR	**R**	**R**	**R**	S	**R**	S	S	S	**R**	**R**	**R**	**R**	**R**	**R**	**R**
B2D3SNAP1	Non-MDR	S	S	S	S	**R**	**I**	S	S	S	S	S	S	S	S	S
B3D2Jun	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
B3D4Apr	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	**R**	S	S
B3D4Jun	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
B4D3Jun	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
AE24Aug	Non-MDR	S	S	**R**	**R**	S	S	S	S	S	**R**	S	S	S	S	S
LP3F1	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Sequence Type 252																
B1D2May	MDR	S	**R**	**R**	S	S	S	S	**R**	S	**R**	S	S	S	S	S
B5D4Feb	MDR	S	**R**	**R**	S	S	S	S	**R**	S	**R**	S	S	**R**	S	S

Table 5.4 continued overleaf

Table 5.4 (continued)

Isolates	AR profile[1]	TZP	TIM	FEP	CAZ	IPM	MEM	MVB	ATM	CIP	LVX	AMK	GEN	NET	TOB	CST
Sequence Type 253																
CWP2	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	**R**	S	S
DH6	Non-MDR	S	S	S	S	S	S	S	S	S	**R**	S	S	**R**	S	S
Sequence Type 282																
DH1	Non-MDR	S	**R**	S	S	S	S	S	S	S	S	S	S	**R**	S	S
Sequence Type 296																
AEDbOct	MDR	S	**R**	S	**R**	S	**I**	S	S	**R**	S	S	**R**	S	S	S
Sequence Type 298																
B5D2Feb	Non-MDR	S	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S
B5D2Jun	Non-MDR	S	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S
B5D4Apr	Non-MDR	S	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S
B5D4bJul	Non-MDR	S	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S
DH11	Non-MDR	ND	S	ND	ND	S	ND	S	ND	**R**	**R**	S	S	**R**	S	S
Sequence Type 308																
AE24Mar	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
AE48bJun	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	**R**	S	S
Sequence Type 348																
DH10	MDR	S	**R**	S	S	S	**I**	S	S	**R**	**R**	S	S	**R**	S	S
Sequence Type 560																
B1D4Feb	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
B1D4aJul	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
B2D2Mar	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
B2D3aFeb	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Table 5.4 continued overleaf

Table 5.4 (continued)

Isolates	AR profile[1]	TZP	TIM	FEP	CAZ	IPM	MEM	MVB	ATM	CIP	LVX	AMK	GEN	NET	TOB	CST
B2D3cFeb	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
B2D3aMar	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
B2D3cApr	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
B2D3bJun	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
B2D3cJul	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
B3D2Apr	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
B3D4Feb	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
B4D3Mar	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
AEDaOCT	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
AE24cMay	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
CSSD1	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
CWP3	Non-MDR	S	**R**	S	S	S	S	S	S	S	S	S	S	S	S	S
Sequence Type 773																
AEDJan	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
AE48Aug	Non-MDR	S	**R**	S	S	S	S	S	S	S	S	S	S	**R**	S	S
Sequence Type 1320																
DenS1	Non-MDR	S	S	S	S	S	S	S	S	**R**	**R**	S	S	S	S	S
Sequence Type 2685																
DenS7	Non-MDR	S	S	S	S	S	S	S	S	S	S	S	S	**R**	S	S

[1]All disc-diffusion susceptibility testing was carried out following the European Committee on Antimicrobial Susceptibility Testing Clinical breakpoints (EUCAST, 2019). The two antibiotic resistance (AR) profiles documented are Multi-Drug Resistant (MDR) and Non-MDR.

[2] The breakpoint results are given in S (susceptibility), I (Intermediate) or R (Resistant), as quantified in Table 5.3. Not determined (ND) was given for results not obtained. Abbreviations: TZP, piperacillin-tazobactam; TIM, ticarcillin-clavulanic acid; FEP, cefepime; CAZ, ceftazidime; IPM, imipenem;

MEM, meropenem; MVB, meropenem-vaborbactam; ATM, aztreonam; CIP, ciprofloxacin; LVX, levofloxacin; AMK, amikacin; GEN, gentamicin; NET, netilmicin; TOB, tobramycin; CST, colistin.

A total of 23 of the *P. aeruginosa* isolates tested (23/49; 47%) were resistant to at least one antimicrobial agent and five (5/49; 10.2%) were MDR. Four of the five MDR isolates were recovered from DDUH (Clinic 2, $n = 3$; A&E, $n = 1$). The STs of the individual MDR isolates are as follows: B2D3bJul (ST179), B1D2May (ST252), B5D4Feb (ST252), AEDbOct (ST296) and DH10 (ST348) (Table 5.1).

As the phenotypically most diverse ST based on antimicrobial susceptibility, ST179 was chosen to contrast observed phenotypic differences with genomic SNV and wgMLST data (Figure 5.8). Of the 34 ST179 isolates investigated throughout this study, 13 isolates were included in antimicrobial susceptibility testing. All four observed antimicrobial susceptibility phenotypes were observed among the 13 ST179 isolates, which exhibited no allelic differences (Figure 5.8(a)). wgMLST analysis detected no allelic differences between isolates that exhibited phenotypic resistance to one, two, and three antimicrobial classes isolates compared with isolates that were susceptible to all antimicrobial classes tested (Figure 5.8(a)). Similarly, using SNV analysis, a cluster of seven isolates demonstrated that no SNVs were detected among isolates that were susceptible to all antimicrobial classes tested compared with isolates expressing resistance to just one antibiotic class (Figure 5.8(b)).

(a) wgMLST analysis

ST 179 (*n* = 34)

- Resistance to one antibiotic class
- Resistance to two antibiotic classes
- Resistance to three or more antibiotic classes
- No observed resistance
- Not phenotypically tested

(b) SNV analysis

ST 179 (*n* = 34)

- Resistance to one antibiotic class
- Resistance to two antibiotic classes
- Resistance to three or more antibiotic classes
- No observed resistance
- Not phenotypically tested

Figure 5.8 Minimum spanning trees (MSTs) based on the **(a)** wgMLST data and **(b)** SNV data, of the 34 ST179 *P. aeruginosa* isolates recovered from DDUH indicating the resistance phenotypes. All breakpoints were selected from EUCAST clinical minimum inhibitory concentration (MIC) breakpoint Tables 2019 (EUCAST, 2019).

As the phenotypically most diverse ST based on antimicrobial susceptibility, ST179 was chosen to contrast observed phenotypic differences with genomic SNV and wgMLST data. Isolates that had not been investigated for their phenotypic resistance profiles based on the 15 antibiotics tested, are indicated in white. Of the 34 ST179 isolates investigated throughout

this study, 13 isolates were included in antimicrobial susceptibility testing. The three phenotypes displayed are: no observed resistance (yellow), phenotypic resistance to one antibiotic class tested (green), resistance shown to two antibiotics tested (red), and resistance to three or more antibiotic classes (blue). **(a)** All four observed antimicrobial susceptibility phenotypes were observed among the 13 ST179 isolates, which exhibited no allelic differences. wgMLST analysis detected no allelic differences between isolates that exhibited phenotypic resistance to one, two, and three antimicrobial classes isolates compared with isolates that were susceptible to all antimicrobial classes tested.

(b) Similarly, using SNV analysis, the central cluster of seven isolates demonstrated that no SNVs were detected among isolates that were susceptible to all antimicrobial classes tested compared with isolates expressing resistance to just one antibiotic class.

5.5 Discussion

Washbasin U-bends and drains have increasingly been identified as reservoirs for nosocomial infections (Lowe *et al.,* 2012; Kotay *et al.,* 2017; Feng *et al.,* 2019; Hopman *et al.,* 2019; Snitkin, 2019; Coleman *et al*., 2020; Jung *et al.,* 2020). However, little is known about trafficking of bacteria in wastewater pipe networks in the hospital environment. The present study is the first to report trafficking of bacteria in hospital wastewater pipes using WGS and comparative data analysis using SNV and wgMLST. *Pseudomonas aeruginosa* was used as a marker organism for washbasin U-bend contamination, where it is virtually ubiquitous, and many reports have linked nosocomial transmission of *P. aeruginosa* to contaminated U-bends (Decker and Palmore, 2013; Ferranti *et al*., 2014; Dekker and Frank, 2016; Kotay *et al*., 2017).

This study focused on investigating the distribution of *P. aeruginosa* STs in washbasin U-bends in DDUH in two separate clinics located on different floors, each with a common water supply, similar usage and equipped with identical washbasins, use of same Tork Extra Mild Liquid Soap (SCA Hygiene Products Ltd), U-bends and wastewater pipes. The vertical wastewater pipes from each clinic discharged into three individual point of discharge wastewater outflow pipes connected to the municipal sewer (Figure 5.2). Three of the five wastewater pipes connect to a common point of discharge pipe, which also services A&E (Figure 5.2). To investigate the relatedness of the *P. aeruginosa* isolates recovered from Clinic 2 and A&E U-bends, isolates were investigated by SNV, MLST and wgMLST analyses. MLST analysis of *P. aeruginosa* isolates from Clinic 2 U-bends yielded only four STs among 55 isolates sequenced, of which ST179 and ST560 predominated (83.6%; 46/55). Seven STs were identified among 26 *P. aeruginosa* isolates from A&E U-bends, with ST179 and ST560 accounting for 23% (6/26) of the isolates sequenced. ST560 and ST179 have both been identified as intercontinental STs associated with human outbreaks and sometimes exhibit MDR phenotypes (Waine *et al*., 2009; Cholley *et al*., 2014; Duong *et al*., 2015; Haenni *et al*., 2015; Kidd *et al*., 2015; Ruiz-Roldán *et al*., 2018). All other STs identified were previously recovered from environmental sources, and all except ST296, have been associated with clinical infections (Domitrovic *et al*., 2016; Hilliam *et al*., 2017; Ruiz-Roldán *et al*., 2018; Subedi *et al*., 2018; Kocsis *et al*., 2019). The relatively narrow range of STs observed in U-bends contrasts with the results of a previous study that investigated the *P. aeruginosa* population diversity at two wastewater sampling sites in a French hospital using MLST, which identified 15 different STs from 30 samples (Slekovec *et al*., 2012).

The two predominant STs identified, ST179 and ST560, were further analysed using wgMLST and SNV analyses. The allelic and SNV thresholds of relatedness for *P. aeruginosa* isolates have previously been set at ≤14 allelic differences using core genome MLST (cgMLST) analysis and ≤37 SNVs (Snyder *et al.,* 2013; Dekker and Frank, 2016; Mellmann *et al.*, 2016; Schürch *et al.*, 2018). Isolates within ST179 and ST560, respectively, were very closely related based on wgMLST and SNV analyses regardless of the location of recovery (Figures 5.4 – 5.5). ST560 represented 27% of all isolates recovered from DDUH and was only recovered from DDUH samples (Clinic 2, A&E and CSSD U-bends, and the point of discharge wastewater pipe common to Clinic 2, Clinic 1 and A&E; Figure 5.4). The average allelic differences and SNVs within ST560 isolates was 7 (median 1, range 0 – 64), and 9 (median 1, range 0 – 66), respectively (Figure 5.5 and 5.6(b)). However, on closer inspection the 27 ST560 isolates group into two clusters, Cluster I (n = 25) and Cluster II (n = 2) (Figure 5.6(b)). Cluster I isolates exhibited average allelic and SNV differences of 3 (median 1, range 0 – 21) and 2 (median 1, range 0 – 8), respectively, whereas Cluster II isolates exhibited no allelic differences and five SNVs (Figure 5.5 and 5.6(b)). While the allelic threshold for isolate relatedness of Cluster I isolates is outside the previously suggested threshold of ≤14 allelic differences (Mellmann *et al.*, 2016), the average allelic differences between the 25 isolates was 3 with a median of 1. In this regard, it is important to highlight that the threshold of ≤14 allelic differences suggested by Mellmann *et al* was based on cgMLST, whereas the present study used wgMLST (Mellmann *et al.,* 2016). All of these findings indicate that the ST560 isolates recovered from DDUH were closely related.

Similarly, ST179 accounted for 34% of all isolates investigated and were only recovered from DDUH U-bends (Clinic 2, Clinic 1, A&E and the third-floor staff bathroom; Figure 5.4). The average allelic differences and SNVs within ST179 isolates was 3 (median 2, range 0 – 17) and 10 (median 5, range 0 – 38), respectively, indicating that the ST179 isolates were closely related (Figure 5.5 and 5.6(a)). The previously suggested allelic and SNV thresholds of relatedness for *P. aeruginosa* isolates were set at ≤14 allelic differences and ≤37 SNVs (Snyder *et al.,* 2013; Dekker and Frank, 2016; Mellmann *et al.*, 2016; Schürch *et al.*, 2018). Interestingly, an ST179 isolate (AE24Aug) from an A&E U-bend and an ST179 isolate (LP3F2) from the staff bathroom U-bend were indistinguishable (i.e. exhibited zero SNVs) (Figure 5.6(a)). These U-bends are located at opposite ends of DDUH, separated by a distance of approximately 132 m. Overall, these results revealed the presence of very closely

related *P. aeruginosa* isolates in washbasin U-bends in several different areas of DDUH (i.e. Clinic 2, Clinic 1, A&E, CSSD, third-floor staff bathroom) and in the point of discharge main common wastewater pipe sampling site. A study by Magalhães *et al.* 2020 utilised WGS to investigate the epidemiology of *P. aeruginosa* isolates in one hospital. Isolates were collected from patients, tap samples, sink trap samples and environmental swabs from five ICUs. Interestingly, isolates recovered 10 years apart from sink U-bends in adjacent units, were highly similar (<14 SNP differences) (Magalhães *et al.,* 2020). The authors suggest the slow evolution may be indicative the absence of or low-level selective pressures.

Clinic 2 washbasin U-bend B2D3 was selected to investigate the diversity of isolates in an individual U-bend over time. Seventeen isolates from this U-bend recovered within the six-month study period at intervals of at least a week belonged to ST179 (n = 9) and ST560 (n = 8) and isolates within each ST were very closely related (both with an average allelic difference of 1 [median 1, range 0 – 2]). These findings reveal the persistence and stability of isolates in an individual U-bend; at least during the six-month period isolates were sequenced. At the end of the study, eight isolates from one sample from B2D3 belonged to ST179 and exhibited an average allelic difference of 2 (median 0, range 0 – 7). These samples were included to demonstrate a representation of the total STs identified in one washbasin U-bend at one time-point.

Isolates from A&E U-bends were also investigated to address the second aim of this work: to investigate the impact of routine decontamination of washbasin U-bends with ECA solutions on the *P. aeruginosa* population. The abundance and prevalence of *P. aeruginosa* in A&E U-bends was significantly lower than non-decontaminated U-bends elsewhere in DDUH and the bacterial densities recovered from Clinic 2 versus A&E exhibit >3.3 log reduction (see Chapter 4, Section 4.4.1). The range of STs recovered was slightly higher with seven STs, compared to the four recovered in Clinic 2. STs ST27, ST296, ST773 and ST298 were recovered in A&E seven months after the cessation of U-bend decontamination with ECA solutions and all, apart from ST298, were highly related to isolates within these STs recovered at least seven months previously (e.g. ST773 isolates were recovered from August 2018 – November 2019, and ST296 were recovered from June 2018 – November 2019) (Figure 5.7). ST298 was only recovered in A&E seven months after the cessation of U-bend decontamination with ECA solutions.

Overall eleven *P. aeruginosa* STs, identified according to traditional MLST analysis, were identified among the 99 isolates sequenced that were recovered from DDUH U-bends and wastewater pipes (ST27, ST179, ST252, ST253, ST296, ST298, ST308, ST309, ST560, ST606, ST773). The *P. aeruginosa* population structure has been revisited many times since the organism was first described in 1882 (Pirnay *et al.*, 2009). Recently, the existence of dominant epidemic high-risk (EHR) clones of *P. aeruginosa* have been described (Mulet *et al.*, 2013; Oliver *et al.*, 2015; Abdouchakour *et al.*, 2018; Slekovec *et al.*, 2019). Of the eleven STs recovered in the present study from DDUH U-bends and wastewater pipes, two EHR STs were identified (ST253 and ST308) and two EHR STs were identified among the comparator strains (ST253 and ST395) investigated (Slekovec *et al.*, 2019). The underlying reasons why EHR clones of *P. aeruginosa* strains are strongly associated with hospital outbreaks internationally are probably associated with the ability of these strains to acquire resistance genes in areas of high antibiotic selective pressure and/or the ability of these strains to survive in the hospital environment (Petitjean *et al.*, 2017). Evidence for the significant survival of ST395 isolates in copper solution was previously confirmed phenotypically and may account for their recovery from the plumbing systems of hospital where outbreaks occurred (Petitjean *et al.*, 2017; Slekovec *et al.*, 2019). Likewise, two studies from Germany and France demonstrated how ST308 was isolated from siphon water leading to the colonisation of handwash basins in Germany, and in France, ST308 was also isolated from tap water (Willmann *et al.*, 2014; Abdouchakour *et al.*, 2018). In these studies, the hospital water environment acted as a reservoir for patient exposure and resulted in prolonged outbreaks.

Seventy-two water samples were taken from the potable water supply, the residual anolyte-treated water storage tanks and from washbasin faucet output water. Alongside this, 20 washbasin faucets from Clinic 2 and A&E were swab sampled (*n* = 80). None of the water or swab samples tested yielded *P. aeruginosa*. Nonetheless, *P. aeruginosa* was recovered from every washbasin U-bends investigated at one point during the study. Failure to detect *P. aeruginosa* in washbasin faucet supply water, washbasin faucet water or the faucets themselves was not surprising as water supplying DDUH washbasins has been routinely treated with residual anolyte (2.5 ppm) since 2012 (Boyle *et al.*, 2012). The results of this study and the previous study by Boyle *et al.* (2012) reveal that anolyte treatment of washbasin supply water is effective at maintaining washbasin supply and output water and the faucets themselves free of detectable *P. aeruginosa*. These findings indicated that the *P.*

aeruginosa present in washbasin U-bends very likely did not originate from faucets or faucet supply water.

The detection of highly related *P. aeruginosa* isolates in U-bends in multiple locations (Clinic 2, Clinic 1, A&E, CSSD, and staff bathroom) throughout DDUH, some separated by considerable distances, indicates that the wastewater pipe network contributes to U-bend contamination (Figure 5.4). There are three possible routes of entry for *P. aeruginosa* into washbasin U-bends in DDUH; upstream contamination from the water supply, contamination at the point-of-use of the washbasins and downstream contamination from wastewater pipes (Figure 5.9). Upstream contamination may be caused by *P. aeruginosa* entering through the water supply and/or contaminated water supply pipes and/or water storage tanks. However, as discussed above, all water samples tested throughout this study failed to yield *P. aeruginosa*, most likely due to residual treatment of supply water with anolyte. Entry of *P. aeruginosa* into washbasin U-bends at the point of use may be attributed to handwashing and/or the contamination of the washbasin faucet, as previously described (Walker *et al.*, 2014; Bédard *et al.*, 2016; Hutchins *et al.*, 2017). However, all 80 swab samples of faucets tested throughout this study also failed to yield *P. aeruginosa*, again probably due to residual treatment of supply water with anolyte. A previous year-long study from DDUH also failed to detect *P. aeruginosa* in anolyte-treated washbasin faucet water or faucets from the same sites DDUH (Boyle *et al*., 2012). While *P. aeruginosa* can be carried transiently on the hands, if hand washing were a frequent contributor of *P. aeruginosa* to U-bends, a far wider range of STs would be anticipated (Widmer *et al.*, 1993). Throughout DDUH, highly related isolates of *P. aeruginosa,* as demonstrated by the clustering of ST179 and ST560 isolates, were located in geographically distinct locations of DDUH (Figure 5.4). Interestingly, ST560 and ST179 isolates were recovered from U-bends in adjacent clinical bays in Clinic 2. Washbasins in individual bays in Clinic 2 do not share common proximal wastewater pipes, common pipework occurs more distally in the network, suggesting trafficking of isolates occurs from more downstream regions (Figure 5.4). All of these results strongly suggest that the wastewater network is the most probable source of *P. aeruginosa* entry into the DDUH washbasin U-bends.

Trafficking of bacteria in wastewater pipes could occur primarily by the carriage of microorganisms within the wastewater flow, bacterial motility and air currents. Water discharged down washbasin drains can traffic bacteria in U-bends and pipes to distal sites in the wastewater network. The model U-bend system described by Kotay *et al*. (2017)

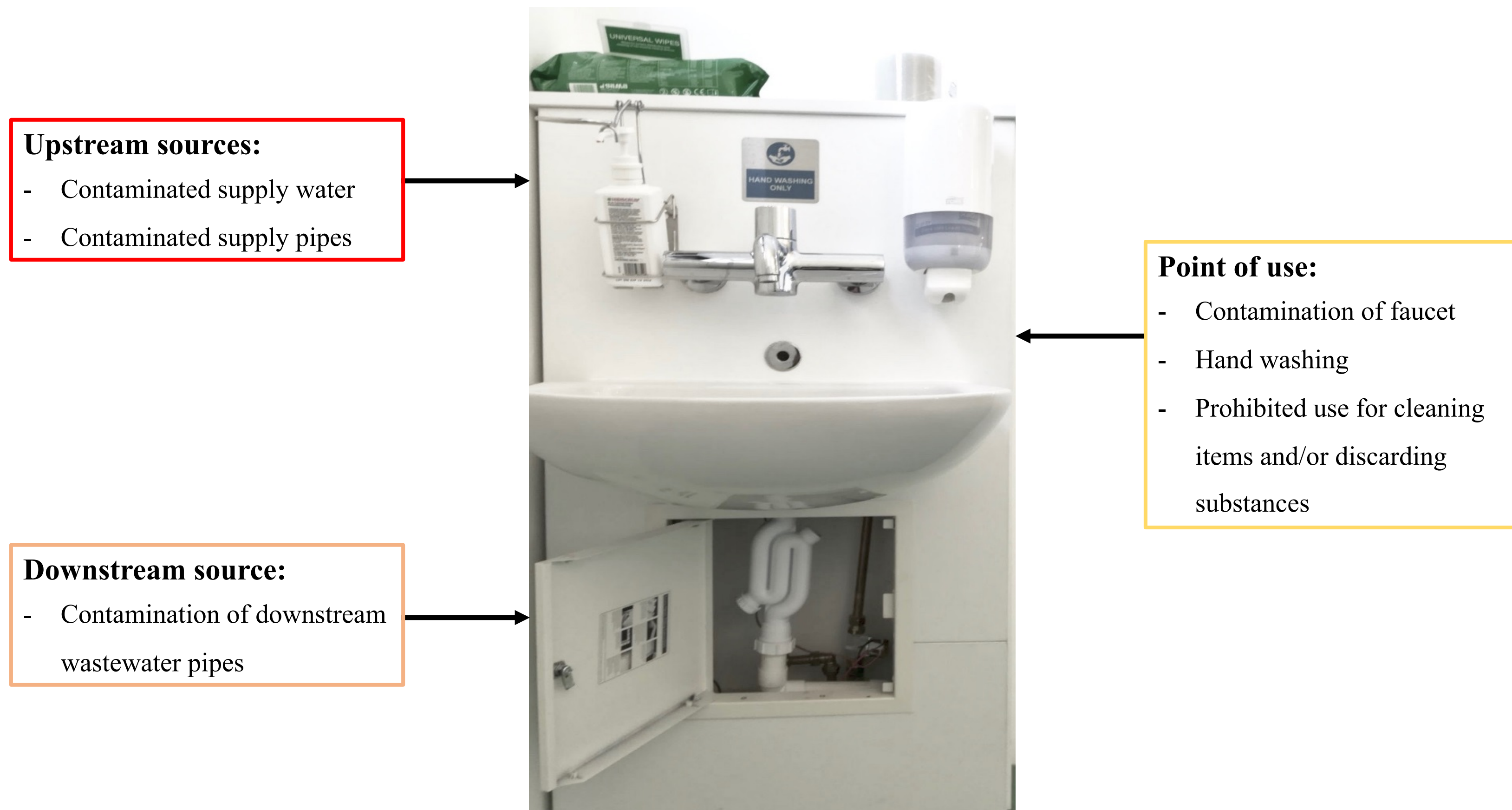


Figure 5.9 The possible routes of entry of *P. aeruginosa* in the washbasin U-bends and wastewater pipes in DDUH. The three main routes are by contamination upstream, at the point of use, or downstream. While washbasins in healthcare facilities are strictly for hand washing use only, washbasins have been used for inappropriate disposal of waste (Akici *et al.,* 2018). An example of this was described in a 2020 paper, confirming the frequent disposal of human waste, such as dialysis fluid, into handwash sinks (Jung *et al.,* 2020).

demonstrated that an *E. coli* strain expressing GFP exhibited an average growth of 2.5 cm per day along the pipework. Flagellar motility has been shown to be an essential element in the ability of *P. aeruginosa* to form biofilms on surfaces and tissue (Vater *et al.*, 2014). Likewise, air currents carry potential pathogens in wastewater pipes both by airflow down into the sewer, and inversely, within wastewater networks (Gormley *et al.*, 2017). The flow of water in pipes results in a partial vacuum that draws air behind the flow of water. A previous study by Gormley *et al.* (2017) built a test plumbing system representing two floors of a building that simulated toilet flushes on the bottom floor. Test water including *Pseudomonas putida* was flushed into the pipework on the bottom floor that expelled the liquid into a collection tank, to represent single or multiple toilet flushes (Gormley *et al.,* 2017). While on the top floor, a test chamber was constructed to mimic a bathroom. The bathroom contained a toilet, with an emptied U-bend, and an extractor fan, which an air sampler could be adapted for sampling. The flushing of the *P. putida* contaminated test water created turbulence to aerosolise *P. putida* in the wastewater pipes. The airflows within the pipes were capable of carrying the aerosolised particles from the bottom floor, into the top floor, and post flush, all parts of the system were found to be contaminated (Gormley *et al.*, 2017). *Pseudomonas putida* was observed to enter the bathroom environment from the wastewater system due to the empty U-bend. One final method that may lead to trafficking of microorganisms in wastewater systems is wastewater pipe blockages. Blockages can occur frequently in hospitals due to poor structural design of the wastewater pipe networks, such as the presence of T-junctions (Breathnach *et al.,* 2012). These blockages facilitate the backflow of water from downstream in the wastewater pipe upwards towards the hospital environment, facilitating the spread of microorganisms. Over a five-year study, Breathnach *et al.* (2012) determined tha a protracted outbreak of MDR *P. aeruginosa* resulted from slow drainage and sewage backflows in a hospital that recorded an average of 391 notification of blocked washbasins, toilets or sluices per year. Wastewater pipe blockages have not been recorded in DDUH and therefore cannot be a method for trafficking of microorganisms. Wastewater flow, bacterial motility and air currents are all methods likely to contribute to the distribution of microorganisms throughout a wastewater network and are likely to have facilitated the dissemination of highly related, and possibly adapted, STs of *P. aeruginosa* within the DDUH washbasin wastewater network.

Washbasin U-bends are areas of microbial proliferation and persistence. The ability of *P. aeruginosa* to thrive and cause hospital outbreaks is due in part to the ability of these organisms to acquire resistance genes in biofilms. Washbasins in particular are recognised

as hotspots of resistance gene transfer (Weingarten *et al.*, 2018). The tolerance of microbial biofilms to antimicrobial agents is multifaceted and includes physical, physiological and genetic factors. Individual bacteria in biofilms can develop or acquire antibiotic resistance by mutations in the genome, alongside the acquisition of antibiotic resistance genes on mobile genetic elements by horizontal gene transfer (Ciofu and Tolker-Nielsen, 2019). The genotypic and phenotypic profiles of *P. aeruginosa* isolates from U-bends and wastewater pipes was investigated in this study.

The genotypic resistance profiles of all isolates sequenced in this study were determined by the identification of antimicrobial resistance genes utilising ResFinder. ResFinder captures antimicrobial resistance genes from whole-genome data sets based on known genes associated with resistance to antimicrobial agents. The genotypic profiles of 97% (116/118) of the environmental isolates investigated harboured two beta-lactam, one aminoglycoside, one fosfomycin and one chloramphenicol resistance genes. The remaining three isolates lacked the chloramphenicol resistance gene. These resistance genes have been previously observed in the genotypic resistance profiles of *P. aeruginosa* (Murugan *et al.*, 2016; Subedi *et al.,* 2018). However, the use of ResFinder is limited in the number of antimicrobial genes it captures, and does not investigate chromosomal mutations.

The phenotypic resistance profiles of a select number of isolates were determined based on 15 antimicrobials, for which EUCAST breakpoint information is available. A total of 49 isolates were selected to give a representation of the diversity of STs recovered during this study. These include isolates from DDUH washbasin U-bends (n = 43), dental suction systems in DDUH (n = 2), and an external hospital located 8 km from DDUH (n = 4). The phenotypic resistance profiles of the 43 DDUH washbasin U-bends demonstrated 42% (18/43) of isolates exhibited resistance to at least one class of antimicrobial. The 2018 European Antimicrobial Resistance Surveillance Network (EARS-NET) report reported 32.1 % of all reported invasive *P. aeruginosa* isolates were resistant to one or more antibiotics (European Centre for Disease Prevention and Control, 2018). The antibiotics included in the surveillance report were piperacillin ± tazobactam, fluoroquinolones, ceftazidime, aminoglycosides and carbapenems. Overall, a low level of resistance would be expected among isolates in the wastewater network in DDUH due to the low level of antibiotics administered in dental hospitals relative to acute hospitals, resulting in reduced selective pressure for resistance in the wastewater network. However, with almost half of the investigated isolates in the present study resistant to at least one group of antimicrobials,

these data may be indicative of the growing problem of antimicrobials in the wastewater environment and sewerage systems. Such selective pressures can result in in the acquisition of antimicrobial resistance genes by HGT (Jendrzejewska and Karwowska, 2018).

Interestingly, three of the 13 STs phenotypically tested (ST252, ST253 and ST298) showed resistance between all isolates for at least one of the antimicrobials tested (Table 5.4). However, not one of the 13 STs phenotypically tested exhibited identical resistance profiles among all tested isolates. The 13 ST179 isolates exhibited the most diverse phenotypic differences (i.e. resistance/susceptibility to antimicrobials) of all STs tested. Figure 5.8 displays the diversity of phenotypic resistance profiles of ST179 based on MST generated of SNV and wgMLST analyses. MSTs are undirected graphs used to connect all points, in this case *P. aeruginosa* isolates, creating the minimum possible total edge weight (Kruskal, 1956). In other words, MSTs are created by continuously adding entries to the tree where the SNV or wgMLST sequencing data of each added *P. aeruginosa* isolates, is compared to the existing SNV or wgMLST sequencing data, creating the minimal distance between those two isolates and between all isolates within the complete tree. Generation of an MST based on a wgMLST scheme is based on the formation of a consensus wgMLST profile. The wgMLST scheme is based on the pan-genome, where each isolate contains varying numbers of loci, loci missing between entries are ignored and the MST is based on the number of different alleles in the shared loci present between isolates.

In this study, the wgMLST analysis data was compared to the phenotypic resistance profiles of ST179 isolates. While the *P. aeruginosa* isolates are very closely related using wgMLST data, no detected differences between isolates expressing different phenotypic resistance/susceptibility profiles were observed using the wgMLST data (Figure 5.7(a)). Previously a study by Schaumburg *et al.* demonstrated the use of an *ad hoc P. aeruginosa* cgMLST scheme to display the diversity of phenotypically-observed ceftolozane-tazobactam resistance among a range of *P. aeruginosa* isolates. In the study, the ceftolozane-tazobactam resistance was significantly associated with one ST and all isolates displayed at least an allelic difference between resistant and susceptible isolates (Schaumburg *et al.,* 2017). A number of factors contributed to the disparity between the observed phenotypic profiles and genotypic profiles by wgMLST analysis in this present study: (i) expression of phenotypic resistance through physiological changes rather than genotypic, (ii) the resistance genes were not included in the consensus wgMLST profile due to limitations of using only short-read sequencing, where issues may arise in the assemblage of large

plasmids encoding resistance genes and/or (iii) the plasticity of *P. aeruginosa* for acquiring resistance genes. wgMLST analysis is a tool that can record various types of nucleotide differences (SNVs, variable number tandem repeat, and insertion-deletion mutations or INDELs) for every open reading frame of an organism (Kingry *et al.*, 2016). Further resolution of the plasmid encoded genes can be achieved by the creation of hybrid assemblies of short reads generated by Illumina sequencing with long reads generated by platforms such as MinION sequencing using ONT (Egan *et al.,* 2020). Likewise, the SNV analysis data was compared to the phenotypic susceptibility/resistance profiles of ST179 isolates and no detected differences were observed between isolates expressing different phenotypic resistance/susceptibility profiles (Figure 5.8(b)). This is a result of the use of strict SNV filters in this study. All potential indel-related SNVs that include SNVs occurring within 12 bp of each other, SNV positions with ambiguous base calls, and SNVs in repeat regions identified in repeat regions were excluded. Therefore, all plasmid encoded acquired resistance genes were not included in this analysis.

In conclusion, the evidence presented in this chapter demonstrates *P. aeruginosa* entry into DDUH washbasin U-bends is most likely from the wastewater pipe network. This evidence is supported by all water and swab samples of faucets and stored supply water testing negative for *P. aeruginosa* throughout the course of this study. The present study is the first of its kind to utilise the high-resolution WGS molecular typing method to demonstrate the trafficking of a microorganism *in situ* in a healthcare facilities wastewater network. This study utilises comparative genomic wgMLST and SNV analyses to demonstrate the distribution of highly related isolates of *P. aeruginosa* in multiple washbasin U-bends in different locations, some separated by significant distances, in a hospital setting.

Chapter 6

General Discussion

6.1 Developing an automated decontamination system

The automated decontamination system described in Chapters 3 and 4 of this thesis is the first of its kind to demonstrate the long term effective decontamination of washbasin drains, U-bends, and associated pipework in an active healthcare facility. The success of this approach relied on four strategies to maximise decontamination efficiency: (1) sequential treatment of U-bends and associated wastewater pipework with the ECA solutions catholyte and anolyte, harnessing the detergent properties of the former and the disinfectant properties of the latter, as a decontamination strategy, (2) the incorporation of a valve downstream of the U-bend that permitted complete filling and retention of ECA solutions in the pipework to increase contact time and to facilitate retro-filling of the solutions upstream of the valve into each washbasin, (3) the automated control of the downstream ball valve and the automated generation and dosing of ECA solutions into the proximal wastewater system (washbasins, U-bends and associated pipework; see Figure 3.2), and (4) the inclusion of multi-disciplinary personnel and expertise in the development and maintenance of the system. Utilising a multi-faceted decontamination approach has proved to be an effective long-term, cost efficient and environmentally friendly alternative to previous U-bend decontamination strategies.

Previous approaches that used chemical solutions to decontaminate washbasin drains and U-bends relied on the utilisation of either a detergent or disinfectant solution as the means to minimise microbial biofilm (Clarivet *et al.*, 2016; Stjarne Aspelund *et al.,* 2016; Kossow *et al.*, 2017; Parkes and Hota, 2018; Buchan *et al.*, 2019). However, as reported by the CDC in 2008, the combined process of removing organic and inorganic materials with detergents products prior to disinfection is necessary to achieve high-level disinfection. The presence of organic and inorganic materials can interfere with disinfectants reducing their efficacy (Centers of Disease Control and Prevention, 2008). The U-bend decontamination system developed in DDUH uses both catholyte and anolyte solutions generated onsite (Swan *et al.*, 2016; Swan, 2017). In DDUH, the ECA solution anolyte is used routinely to decontaminate dental unit waterlines and associated water networks, water provided to washbasins and washbasin taps. The present study extended the use of ECA solutions (catholyte and anolyte) for decontamination of selected washbasin U-bends and associated wastewater pipework in DDUH. The utilisation of ECA solutions for a range of automated decontamination processes in one healthcare facility helps to maximise the benefits of purchasing an ECA

generator, reduces staff involvement and reduces the risks associated with long term storage of chemicals.

The incorporation of valves downstream of washbasin U-bends in the wastewater network has been adopted as part of a decontamination strategy in a number of studies, including the present study (Vergara-López *et al.,* 2013; Swan *et al.,* 2016; Cadnum *et al.*, 2019; Deasy *et al.*, 2019). In all of these studies, valves were used to close off wastewater pipework downstream of U-bends to enable retention of detergents/disinfectant solutions in the pipework and thus increase the contact time between the solutions and the interior pipework. Under normal circumstances, disinfectants poured down washbasin drains are rapidly carried downstream by wastewater pipes and the pipework itself is never completely filled with disinfectant. The work undertaken in Swan *et al*. (2016) and in the present study was the first to retro-fill decontamination solutions into the U-bend and adjacent pipework from below, minimising the introduction of air bubbles into the pipework (Swan *et al.,* 2016). Expanding on this intervention, the automated decontamination system utilised in this study could be configured to decontaminate entire wastewater networks, from drain outlets in washbasins, showers and sluices, to the point of discharge into the municipal sewer.

In Chapter 5, *P. aeruginosa* was used as a model organism to investigate the spread of potentially pathogenic microorganisms throughout a wastewater network (Figure 5.4). Highly related isolates were identified throughout the wastewater network in DDUH, which likely originated from the wastewater pipe network, as all washbasin output water, faucets and supply water samples tested consistently failed to yield *P. aeruginosa* throughout the course of this study. Incorporation of a ball valve just upstream of where the wastewater network discharges into the municipal sewer would facilitate the decontamination of a significant proportion of the wastewater network pipework and may further limit the spread of potentially pathogenic bacteria.

Throughout this study, emphasis was placed on the detection of adverse effects resulting from use of the automated decontamination system. While no adverse outcomes were detected, previous studies have shown that engineering measures employed to mitigate the spread and risk of infection can ultimately give rise to new reservoirs of contamination and infection or cause other issues (Walker *et al*., 2014; Weinbren *et al.,* 2017). Flow straighteners were initially incorporated into washbasin taps to regulate and narrow the flow of water from the tap outlet into the washbasin. However, non-maintained flow straighteners

have been shown to be ideal locations for biofilm formation. A study conducted by Walker *et al.* (2014) found that similar *P. aeruginosa* isolates were found in both biofilms in flow straighteners tested from neonatal unit taps and samples from four neonates that died from *P. aeruginosa* bacteraemia in Northern Ireland. Likewise, a study by Weinbren *et al.* (2017) conducted in two clinical rooms investigating elbow-operated taps use, determined hands were used to operate the taps in 97% of the observed instances. Incorrect use of these taps may lead to HAIs resulting from colonisation of the taps by microorganisms present on contaminated hands. This study concluded the diversity of taps used throughout the healthcare facility, lack of instructions and the oftentimes incorrectly installed units led to the misuse (Weinbren *et al.,* 2017). These studies highlight that despite the universally acknowledged importance of handwashing in safe and clean environments, large gaps can still exist in the functionality, design and in training of the correct use of hand wash stations.

The automation of the decontamination approach used in this study provided three main advantages: mitigation of the risk of adverse effects to cleaning staff handling disinfectants and detergents solutions, increased cleaning in parts of the network not accessible or difficult to reach by cleaning staff, and the negation of errors commonly introduced while manually cleaning/decontaminating systems. In general, occupations involving frequent use of disinfectants and detergents, like janitorial staff and cleaning staff, have long been associated with poor respiratory health (Folletti *et al.*, 2014; Cummings and Virji, 2018). A systematic review conducted for the period 1976 – 2012 by Folletti *et al.* (2014) explored the epidemiological links between work activities involving cleaning and the risk of asthma and rhinitis. The review determined that specific cases of asthma and rhinitis could be linked to cleaning staff's exposure to specific chemicals used for cleaning (e.g. bleach) and the mixing of cleaning products (Folletti *et al.,* 2014). While ECAs are considered non-toxic, the improper handling of catholyte solutions can result in burns due to the alkaline nature of the solution (pH 12). Automation of decontamination practices would significantly reduce work related illnesses due to handling of cleaning solutions.

The automated ECA decontamination system developed in the present study could be successfully adapted to a number of domestic and clinical applications. This system would be of most benefit in areas utilised by the most medically vulnerable patient groups in domestic and healthcare settings. A metagenomic study by Perry e*t al.* (2019) demonstrated that the abundance of AMR genes from hopsital wastewater in multiple departments in one hospital was influenced by the length of stay of the patients and hospital antimicrobial usage.

Adapting the automated ECA solution system to decontaminate the wastewater network in ICUs, neonatal ICUs and in hospital wards catering to immunocompromised patients, burn patients and cystic fibrosis patients may provide the most benefit. The decontamination approach described in this study has been shown to be scalable and can decontaminate one or multiple washbasin U-bends simultaneously (Swan *et al.,* 2016; Swan, 2017; Deasy *et al.,* 2018). One additional area of interest is the adaptation of the decontamination approach to showers drain outlets and shower hoses and shower heads. The inhalation of potentially pathogenic bacteria during showering has been associated with respiratory infections caused by *Legionella* species, nontuberculous *Mycobacteria* (NTM), and *P. aeruginosa* (Halstrom *et al.,* 2015; Proctor *et al.,* 2018; Cates and Torkzadeh, 2020). Adaptation of this decontamination approach to decontaminate shower hoses and outlets may result in reduced rates of aerosolisation of potentially pathogenic bacteria and therefore reduce the risk of infection associated with showering (Cates and Torkzadeh, 2020). Adopting this approach in the homes of cystic patients vulnerable to *P. aeruginosa* infections could be used to decontaminate multiple high-risk areas, like bathroom washbasins, kitchen sinks, shower hoses and drains and bath drains.

Finally, the research group in DDUH harnessed the knowledge of multi-disciplinary personnel in developing the automated ECA decontamination system. A fundamental requirement of any effective IPC strategy is a competent and educated workforce (Health Information and Quality Authority, 2017). However too often the burden of management of wastewater networks is placed on staff that lack the expertise to accurately assess and comprehend the risk of infection from these environments. This lack of knowledge from facility managers oftentimes results in the selection of decontamination approaches based on cost. Healthcare facilities should encourage multi-disciplinary communication and encourage personnel in upgrading healthcare facilities using evidence- and experience-based information (O'Connell and Humphreys, 2000). The development of any efficient safe decontamination approach relies on the use of interdisciplinary, evidence-based approaches communicated clearly. In DDUH, the decontamination approach was developed, monitored, and maintained utilising the knowledge and expertise of microbiologists, IPC personnel, engineers, cleaning staff and facility staff.

6.2 Rethinking the wastewater network designs in healthcare facilities

The implementation of functionally useful wastewater network designs and regular auditing of wastewater networks are essential for providing safe healthcare. Healthcare built environments oftentimes receive little attention by governing bodies and policy makers following the initial construction of the facilities. This is due to the inflexible or inaccessible nature of these systems, built to last as operational facilities for decades (Mills *et al.,* 2015). Wastewater networks in healthcare facilities are often developed from existing designs that can be outdated or were not designed employing scientific evidence. Three obvious examples of poor wastewater structural designs are the use of DP hand washbasins in hospitals, hand operated taps in toilet facilities and the location of shower drains directly below the shower head flow of water, for reasons previously described (Weinbren, 2020). Two innovative approaches may provide a large-scale overhaul of the wastewater network in hospitals: 'water-free' patient care and zoning of the wastewater network.

'Water-free' patient care was first introduced as a means to mitigate the risk of infection from wastewater networks in healthcare facilities (Hopman *et al.,* 2017). The study demonstrated that the removal of sinks in wards catering to inpatients and the introduction of 'water-free' patient care resulted in significant reduction in ICU patients becoming colonised by GNB and among patients with long stays in the ICU (Hopman *et al.,* 2017). While the removal of all washbasins from the clinical environment would be counterproductive in terms of ensuring the maintenance of good hand hygiene, increasing the distance between patient beds and contaminated washbasins while maintaining other hand hygiene strategies has been shown to reduce the incidence of HAIs (Hopman *et al.,* 2017). Secondly, the implementation of zoning and infrastructural controls in the wastewater network would mitigate the risk of infection from these networks. Currently, water supplies across healthcare facilities are zoned. Zoning reduces the spread of pathogens, such as *Legionella,* throughout the entire facility and also reduces the likelihood of an overall failure should one section be compromised. Chapter 5 demonstrated how *P. aeruginosa* can spread throughout DDUH within the wastewater pipe network by the carriage of microorganisms within the wastewater flow, bacterial motility and air currents. Zoning of the wastewater network would impede the spread of microorganisms rapidly throughout the entire wastewater network. It has been shown that bacterial movement through a model pipe network towards the sink drain was recorded at a rate of about 2.5-cm per day (Kotay *et al*., 2017), so regular decontamination of the entire wastewater network may limit the rapid recolonisation of proximal wastewater pipework. However, such an undertaking would require vast quantities of decontamination solutions as the wastewater network in most

hospitals is extensive and likely to be many kilometres in length. In a hospital facility it may be more practical to specifically zone wastewater pipework in areas housing particularly vulnerable patients, such as ICUs, limiting the volume of disinfectant solution required for wastewater pipe decontamination and would maximise benefit to patients.

6.3 Highlighting the role wastewater networks may play in the spread of the SARS-Co-V2 virus

In the context of the current COVID-19 pandemic, strong evidence suggests the wastewater network, including U-bends, may facilitate the spread of aerosolised microorganisms like the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus. Following the 2003 SARS epidemic, an investigation by the World Health Organization determined one 'superspreading event' occurred in a housing complex in Hong Kong due to dry U-bends in bathroom floor drains (World Health Organization, 2003). These empty U-bends acted as open sources for contaminated droplets from the sewerage system to enter households, and over-sized extractor fans facilitated the spread of the virus particles up to 200 metres (Nghiem *et al.,* 2020). Current studies identify washbasins as potential reservoirs for the spread of the SARS-CoV-2 virus (Döhla *et al.*, 2020; Gormley *et al.*, 2020; Ong *et al.*, 2020).

Work carried out by the Gormley group demonstrated that microorganisms dispersed by flushing of a test toilet can spread throughout a multi-story wastewater plumbing network in a test system (Gormley *et al.*, 2017; Gormley *et al.*, 2020). As discussed above, zoning of the wastewater network in healthcare facilities could reduce the potential for spread of microorganisms in this manner. Likewise, the use of HP washbasins would also reduce the spread of microorganisms that can be aerosolised by the impact of tap water flow on the drain outlet. A study conducted by Döhla *et al.* (2020) investigated the prevalence of SARS-CoV-2 from sampled washbasin wastewater in 21 domestic German households under quarantine conditions. The study shows that 19.23% (5/26) of wastewater samples were positive for SARS-CoV-2 (Döhla *et al.*, 2020). The increase in numbers of people self-isolating and/or quarantining may lead to higher use of the wastewater networks in domestic settings potentially increasing the risk of SARS-Co-V2 transmission.

6.4 Antimicrobial agents and the environment

Antibiotic resistance is one of the major public health concerns of the twenty-first century. Owing to increasing population growth and longer life expectancies, the use of antimicrobial agents has increased worldwide. In the past few decades, Ireland has increased its antibiotic use in humans and animals to approximately 100 tonnes per year (Morris *et al.,* 2016). This increased usage has led to the increased presence of AMR in the environment (Morris *et al.,* 2016). A study by Lepuschitz *et al.* (2019) investigated the prevalance of AMR genes in the surface water in the vicinity of the following Austrian cities: Vienna, Linz, Innsbruck, and Klagenfurt. All five water samples taken from a river upstream of both the city and its wastewater treatment plant in each case were negative for ESBL and carbapenemase-producing *K. pneumoniae*, while all five samples taken 3 km downstream yielded resistant *K. pneumoniae* isolates (Lepuschitz *et al.*, 2019). In total, three ESBL-producing isolates and two carbapenemase-producing isolates were recovered downstream of the following cities: three ESBL-producing isolates from Linz and Klagenfurt, and two carbapenemase-producing isolates from Vienna and Innsbruck (Lepuschitz *et al.*, 2019). This study demonstrates the anthropogenic effect on multi-drug resistance in the environment and the limitations of wastewater treatment strategies.

While water is the most abundant resource on Earth, in the developed world this resource is oftentimes undervalued. In Ireland, high water quality standards are taken for granted, from water processed for drinking and supply to water found in rivers and seas. Wastewater contamination of surface water is not a new phenomenon. A recent report stated that urban wastewater contamination accounted for more than half the reported pollution events to Irish coastal waters (Environmental Protection Agency Ireland, 2020). Increasingly, reports highlight the many routes wastewater effluent can pollute the environment, including wastewater leakages from aged pipes, inadequate decontamination protocols and the use of wastewater treatment plants running at overcapacity leading to failures in sewer overflows (Olds *et al.*, 2018; Kauppinen *et al.*, 2019; Rizzo *et al.*, 2013). With increasing media attention on pollution events stemming from wastewater effluent, government backed funding and incentives may reduce the impact these pollution channels have on the environment.

6.5 The future of bacterial typing

Over the past 20 years, the advent of NGS and third generation sequencing technologies have revolutionised every field of life sciences. These technologies have resulted in a greater

understanding of bacterial communities in ecosystems ranging from the human gut to washbasin U-bends. Likewise, these technologies can provide information about naturally occurring AMR reservoirs in wastewater networks, enable the investigation of virulence and fitness strategies in highly diverse and competitive environments, and accurately monitor interventions (Olds *et al*., 2018; Kauppinen *et al.*, 2019). Throughout this study, bacteria were identified using both culture-based and culture-independent methods. Culture-dependent approaches are widely used due to the establishment of standardised analyses and the low costs associated with these technologies. However, the ever reducing turnaround-times and costs of genomic technologies will lead to their use in routine diagnoses and in the establishment of treatments (Fournier *et al*., 2014). The establishment of quality assurance parameters and data interpretation criteria, and the standardisation of computation analyses such cgMLST and SNV analyses must be determined to facilitate widespread adoption of WGS-based typing in Europe (European Centre for Disease Prevention and Control, 2016).

One current limitation of NGS platforms like the Illumina MiSeq platform are the short-read sequences generated. The assembly process of short read sequencing technologies is complicated in genome repetitive regions. Repetitive regions are located across the genome sequence but are algorithmically fragmented into only a few sequences during assembly, which is due to their similarity (Baptista *et al.,* 2018). The current limitation of long read sequences produced by third generation sequencing platforms like ONT are the associated high error rates in the production of read sequences. The combination of long read and short read sequences can be used to generate hybrid assemblies that take advantage of the strengths of each technology individually; i.e. the accuracy of short read sequencing and the extended length of long read sequences (Baptista *et al.*, 2018). These hybrid assemblies yield reconstructions with uniform coverage of the bacterial genomes and increase structural accuracy of the downstream assembly (Lee *et al*., 2016; Giani *et al.*, 2020). The ever-increasing development in sequencing technologies will lead to increased resolution in bacterial identification and typing, enabling better surveillance, diagnostic and therapeutic strategies.

Finally, metagenomic analyses holds the potential of becoming a routine surveillance strategy for identifying complex bacterial communities. Metagenomic shotgun sequencing data provides more diversity in community structure than amplicon sequencing approaches, like the one adopted in this study. Likewise, metagenomic shotgun sequencing data provides

information on metabolic processes possible in the community (Guo *et al.*, 2016). However, the high costs associated with shotgun metagenomic sequencing currently limit its widespread adoption. To date, metagenomic analysis of human infections have not identified multitudes of novel microorganisms. However, metagenomic studies have been increasingly used to detect novel genomes in the environment and as commensal carriage organisms in animals (Driscoll *et al.,* 2017; Taylor-Brown *et al.,* 2017). The lack of publications highlighting novel potential pathogens may be due to the lack of novel microorganisms contributing to clinical infections. However it would seem more probable that investigator biases limit these analyses. Metagenomic analysis produces vast quantities of data that may lead researchers to focus on organisms previously associated with infection.

6.6 Future perspectives

Continued research into the role washbasin U-bends play in microbial colonisation and transmission is necessary to provide safer healthcare. In relation to the work described in this thesis, three main avenues present themselves as the next logical steps for investigation. Firstly, this study demonstrated the scalability of the decontamination approach from the previous work described by Swan *et al.* (2016). Determining the limits of this decontamination system would provide insight into the most efficient running of this particular system and may guide future endeavours in adapting this approach. The limits of ECAs decontamination can be conducted by testing the efficacy of each concentration of solution separately and in combination in the active healthcare clinic.

Secondly, high-throughput Illumina 16S rRNA amplicon sequencing is an accurate and effective tool for undertaking snapshots of bacterial communities. This analysis was utilised in Chapter 4 to determine the bacterial communities throughout the wastewater network in DDUH. During the study, no samples were taken for 16S rRNA amplicon sequencing from A&E washbasins while the washbasins were subjected to routine decontamination with ECAs. Investigating the bacterial communities in washbasin U-bends while subjected to routine ECA decontamination may provide an understanding of what organisms can persist in environments of high hypochlorous acid-based stress. Understanding the microbial populations continually treated with ECA solutions may highlight potential selection of resistant bacterial populations in the wastewater network, and guide future environmental decontamination policies.

Finally, the spread of *P. aeruginosa* within the wastewater network of DDUH was investigated in Chapter 5 utilising three main comparative genomic tools: MLST, wgMLST and SNV analyses. During the study, wgMLST was utilised as the genome wide allele-based approach to compare the relatedness of *P. aeruginosa* isolates. However, a limitation of wgMLST analyses is in the creation of the consensus profile between all tested isolates. Only alleles present in all isolates are included in the consensus profile, thus reducing the degree of comparison between more distantly related isolates. In contrast, cgMLST schemes contain defined numbers of loci, selected to represent specific species, and are standardised in both the genotypic nomenclature and analyses of isolates of that species. Prior to May 2020, only two previous groups utilised *ad hoc* cgMLST *P. aeruginosa* schemes, neither of which has been used outside their own research groups and neither were externally validated (Mellmann *et al.,* 2016; Royer *et al.,* 2020). As of May 2020, a proposed scheme was published by de Sales *et al.* (2020) aiming to standardise the comparison of *P. aeruginosa* isolates. Analysing the data using cgMLST analysis may provide more accurate determinations of the relatedness of populations of *P. aeruginosa* isolates.

6.7 Concluding remarks

Washbasins U-bends are reservoirs of potential pathogens that are increasingly identified as contributing to human infection. It is not feasible to maintain sterile wastewater systems, however poor building design in healthcare facilities wastewater networks and/or suboptimal decontamination approaches may increase the risk of infection. This study is the first of its kind to develop an effective multi-facetted automated approach for minimising infection risks from drain outlets in an active healthcare department with minimum, to no disruption to the clinic. The regular sequential use of catholyte and anolyte solutions resulted in reduced bioburden in the washbasin U-bends and drains, thus reducing the risk of transmission. This study also investigated the bacterial communities within the wastewater network in DDUH using culture-dependent and culture-independent approaches. The comparison of MALDI-TOF-MS analysis and Illumina high throughput 16S rRNA amplicon sequencing data highlighted the disparity among the diversity of bacteria observed within the washbasins and associated pipework in DDUH.

Finally, the present study is the first of its kind to utilise the high-resolution WGS to demonstrate the trafficking of a microorganism *in situ* in a healthcare facility's wastewater network. This work demonstrated that the entry of *P. aeruginosa* into DDUH washbasin U-

bends is most likely from the wastewater pipe network, as all water and swab samples of faucets and stored supply water tested negative for *P. aeruginosa* throughout the course of this study. This study utilised comparative genomic wgMLST and SNV analyses to demonstrate the distribution of highly related isolates of *P. aeruginosa* in multiple washbasin U-bends in different locations, some separated by significant distances, in a hospital setting. These findings indicate that a hospital wastewater network can act as a highway for trafficking potentially pathogenic microorganisms throughout the facility.

The aim of this research has been to highlight the roles washbasin U-bends play in the spread of potential pathogens and to emphasise the need for adequate, safe and effective decontamination protocols. This will only be achieved by widespread education highlighting the risk these reservoirs pose, incentivising preventative long-term effective decontamination approaches aided by regular monitoring, and the implementation of effective policies and guidelines by regulatory authorities.

Bibliography

Abdouchakour, F., Aujoulat, F., Licznar-Fajardo, P., Marchandin, H., Toubiana, M., Parer, S., Lotthé, A. and Jumas-Bilak, E. (2018). Intraclonal variations of resistance and phenotype in *Pseudomonas aeruginosa* epidemic high-risk clone ST308: a key to success within a hospital? *Int. J. Med. Microbiol.* 308, 279–289. Available at: https://doi.org/10.1016/j.ijmm.2017.11.008

Abraham, T., Schooling, S. R., Beveridge, T. J., and Katsaras, J. (2008). Monolayer film behavior of lipopolysaccharide from *Pseudomonas aeruginosa* at the air-water interface. *Biomacromolecules* 9, 2799–2804. Available at: https://doi.org/10.1021/bm800562r

Afgan, E., Baker, D., Van den Beek, M., Blankenberg, D., Bouvier, D., Čech, M., Chilton, J., Clements, D., Coraor, N. and Eberhard, C. (2016). The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. *Nucleic Acids Res.* 44, 3–10. Available at: https://doi.org/10.1093/nar/gkw343

Akici, A., Aydin, V., and Kiroglu, A. (2018). Assessment of the association between drug disposal practices and drug use and storage behaviors. *Saudi Pharm. J.* 26, 7–13. Available at: https://doi.org/10.1016/j.jsps.2017.11.006

Almasy, E., Szederjesi, J., Rad, P., and Georgescu, A. (2016). A fatal case of community acquired *Cupriavidus pauculus* pneumonia. *J. Crit. Care Med.* 2, 201–204. Available at: https://doi.org/0.1515/jccm-2016-0027

Amoureux, L., Riedweg, K., Chapuis, A., Bador, J., Siebor, E., Péchinot, A., Chrétien, M-L., de Curraize, C., and Neuwirth, C. (2017). Nosocomial infections with IMP-19−producing *Pseudomonas aeruginosa* linked to contaminated sinks, France. *Emerg. Infect. Dis.* 23, 304. Available at: https://doi.org/10.3201/eid2301.160649

Anaissie, E. J., Penzak, S. R., and Dignani, M. C. (2002). The hospital water supply as a source of nosocomial infections: a plea for action. *Arch. Intern. Med.* 162, 1483–1492. Available at: http://doi.org/10.1001/archinte.162.13.1483

Anwar, M. A., and Choi, S. (2014). Gram-negative marine bacteria: structural features of lipopolysaccharides and their relevance for economically important diseases. *Mar. Drugs* 12, 2485–2514. Available at: http://doi.org/10.3390/md12052485

Aranega-Bou, P., George, R. P., Verlander, N. Q., Paton, S., Bennett, A., Moore, G., Aiken, Z., Akinremi, O., Ali, A., Cawthorne, J., Cleary, P., Crook, D. W., Decraene, V., Dodgson, A., Doumith, M., Ellington, M., Eyre, D. W., George, R. P., Grimshaw, J., Guiver, M., Hill, R., Hopkins, K., Jones, R., Lenney, C., Mathers, A. J., McEwan, A., Moore, G., Neilson, M., Neilson, S., Peto, T. E. A.Phan, H. T. T.,Regan, M., Seale, A. C., Stoesser, N., Turner-Gardner, J., Watts, V., Walker, J., Sarah Walker,

A., Wyllie, D., Welfare, W., and Woodford, N. (2019). Carbapenem-resistant *Enterobacteriaceae* dispersal from sinks is linked to drain position and drainage rates in a laboratory model system. *J. Hosp. Infect.* 102, 63–69. Available at: https://doi.org/10.1016/J.JHIN.2018.12.007

Armstrong, D. G., Bohn, G., Glat, P., Kavros, S. J., Kirsner, R., Snyder, R., and Tettelbach, W. (2015). Expert recommendations for the use of hypochlorous solution: science and clinical application. *Ostomy Wound Manage.* 61, 2–19. Available at: http://online.fliphtml5.com/zxoes/ebfp/#p=2

Bakhir, V. M. (1992). Electrochemical activation. *M. VNIIIMT*. 2–657

Bakhir, V.M.; Zadorozhny, Y. G. (1996). A flow-through electrochemical modular cell of FEM-3 type. *Electrochem. Cell. US Pat. No 5,635,040, filed11.03.1996, pbl. 3.06.1997*

Balm, M. N. D., Salmon, S., Jureen, R., Teo, C., Mahdi, R., Seetoh, T., Teo, J., Lin, R., and Fisher, D. A. (2013). Bad design, bad practices, bad bugs: frustrations in controlling an outbreak of *Elizabethkingia meningoseptica* in intensive care units. *J. Hosp. Infect.* 85, 134–140. Available at: https://doi.org/10.1016/j.jhin.2013.05.012

Balvočiute, M., and Huson, D. H. (2017). SILVA, RDP, Greengenes, NCBI and OTT - how do these taxonomies compare? *BMC Genomics* 18, 1–8. Available at: https://doi.org/10.1186/s12864-017-3501-4

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Lesin, V. M., Nikolenko, S. I., Pham, S., Prjibelski, A. D. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. Available at: https://doi.org/10.1089/cmb.2012.0021

Baptista, R. P., Reis-Cunha, J. L., DeBarry, J. D., Chiari, E., Kissinger, J. C., Bartholomeu, D. C., and Macedo, A. M. (2018). Assembly of highly repetitive genomes using short reads: the genome of discrete typing unit III *Trypanosoma cruzi* strain 231. *Microb. Genomics* 4, e000156. Available at: https://doi.org/10.1099/mgen.0.000156

Bautista-de los Santos, Q. M., Schroeder, J. L., Blakemore, O., Moses, J., Haffey, M., Sloan, W., and Pinto, A. J. (2016). The impact of sampling, PCR, and sequencing replication on discerning changes in drinking water bacterial community over diurnal time-scales. *Water Res.* 90, 216–224. Available at: https://doi.org/10.1016/j.watres.2015.12.010

Bédard, E., Prévost, M., and Déziel, E. (2016). *Pseudomonas aeruginosa* in premise plumbing of large buildings. *Microbiologyopen* 5, 937–956. Available at: https://doi.org/10.1002/mbo3.391

Bertrand, X., Thouverez, M., Talon, D., Boillot, A., Capellier, G., Floriot, C., and Hélias, J. (2001). Endemicity, molecular diversity and colonisation routes of *Pseudomonas aeruginosa* in intensive care units. *Intensive Care Med.* 27, 1263–1268. Available at: https://doi.org/10.1007/s001340100979

Blanc, D. S., Gomes Magalhaes, B., Abdelbary, M., Prod'hom, G., Greub, G., Wasserfallen, J. B., Genoud, P., Zanetti, G., and Senn, L. (2016). Hand soap contamination by *Pseudomonas aeruginosa* in a tertiary care hospital: no evidence of impact on patients. *J. Hosp. Infect.* 93, 63–67. Available at: https://doi.org/10.1016/j.jhin.2016.02.010

Blom, K. (2015). Drainage systems, an occluded source of sanitation related outbreaks. *Arch. Public Heal.* 73, 8. Available at: https://doi.org/10.1186/s13690-014-0056-6

Bloomfield, S., Exner, M., Flemming, H.-C., Goroncy-Bermes, P., Hartemann, P., Heeg, P., Ilschner, C., Krämer, I., Merkens, W., and Oltmanns, P. (2015). Lesser-known or hidden reservoirs of infection and implications for adequate prevention strategies: where to look and what to look for. *GMS Hyg. Infect. Control* 10, 04.

Boesl, U. (2017). Time of flight mass spectrometry: introduction to the basics. *Mass Spectrom. Rev.* 36, 86–109. Available at: https://doi.org/10.1002/mas.21520

Boyle, M. A., O'Donnell, M. J., Miller, A., Russell, R. J., and Coleman, D. C. (2012). Control of bacterial contamination of washbasin taps and output water using Ecasol: a one-year study. *J. Hosp. Infect.* 80, 288–292. Available at: https://doi.org/10.1016/j.jhin.2012.01.011

Boyle, M. A., O'Donnell, M. J., Russell, R. J., and Coleman, D. C. (2010). Lack of cytotoxicity by Trustwater Ecasol™ used to maintain good quality dental unit waterline output water in keratinocyte monolayer and reconstituted human oral epithelial tissue models. *J. Dent.* 38, 930–940. Available at: https://doi.org/10.1016/j.jdent.2010.08.00

Breathnach, A. S., Cubbon, M. D., Karunaharan, R. N., Pope, C. F., and Planche, T. D. (2012). Multidrug-resistant *Pseudomonas aeruginosa* outbreaks in two hospitals: association with contaminated hospital waste-water systems. *J. Hosp. Infect.* 82, 19–24. Available at: https://doi.org/10.1016/j.jhin.2012.06.007

Buchan, B. W., Arvan, J. A., Graham, M. B., Tarima, S., Faron, M. L., Nanchal, R., and Munoz-Price, L. (2019). Effectiveness of a hydrogen peroxide foam against bleach for the disinfection of sink drains. *Infect. Control Hosp. Epidemiol.* 40, 724–726. Available at: https://doi.org/10.1017/ice.2019.72

Buhl, M., Kästle, C., Geyer, A., Autenrieth, I. B., and Peter, S. (2019). Molecular

evolution of extensively drug-resistant (XDR) *Pseudomonas aeruginosa* strains from patients and hospital environment in a prolonged outbreak. *Frontiers in Microbiol.* 10, 1742. Available at: https://doi.org/10.3389/fmicb.2019.01742

Bush, K. (2018). Past and present perspectives on β-Lactamases. *Antimicrob. Agents Chemother.* 62, e01076-18. Available at: https://doi.org/10.1128/AAC.01076-18

Cadnum, J. L., Livingston, S. H., Gestrich, S. A., Jencson, A. L., Wilson, B. M., and Donskey, C. J. (2019). Use of a stop valve to enhance disinfectant exposure may improve sink drain disinfection. *Infect. Control Hosp. Epidemiol.* 40, 254–256. Available at: https://doi.org/10.1017/ice.2018.318

Cai, Z. (2005). Characterisation of electrochemically activated solutions for use in environmental remediation. *A thesis submitted to University of the West of England, Bristol.* Available at: https://ethos.bl.uk/OrderDetails.do?uin=uk.bl.ethos.418216

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., and Madden, T. (2009). BLAST+: architecture and applications. *BMC Bioinformatics* 10, 421. Available at: https://doi.org/10.1186/1471-2105-10-421

Carling, P. C. (2018). Wastewater drains: epidemiology and interventions in 23 carbapenem-resistant organism outbreaks. *Infect. Control Hosp. Epidemiol.* 39, 972–979. Available at: https://doi.org/10.1017/ice.2018.138

Cates, E. L., and Torkzadeh, H. (2020). Can incorporation of UVC LEDs into showerheads prevent opportunistic respiratory pathogens? – microbial behavior and device design considerations. *Water Res.* 168, 115–163. Available at: https://doi.org/10.1016/j.watres.2019.115163

Centers for Disease Control and Prevention (2003). Guidelines for Environmental Infection Control in Healthcare Facilities. Atlanta, United States. pp. 1–241. Available at: https://www.cdc.gov/infectioncontrol/pdf/guidelines/environmental-guidelines-P.pdf

Chakravorty, S., Helb, D., Burday, M., Connell, N., and Alland, D. (2007). A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *J. Microbiol. Methods* 69, 330–339. Available at: https://doi.org/10.1016/j.mimet.2007.02.005

Chapuis, A., Amoureux, L., Bador, J., Gavalas, A., Siebor, E., Chrétien, M.-L., Caillot, D., Janin, M., de Curraize, C., and Neuwirth, C. (2016). Outbreak of extended-spectrum beta-lactamase producing *Enterobacter cloacae* with high MICs of quaternary ammonium compounds in a hematology ward associated with contaminated sinks. *Front. Microbiol.* 7, 1070. Available at: https://doi.org/10.3389/fmicb.2016.01070

Chen, K., and Pachter, L. (2005). Bioinformatics for whole-genome shotgun sequencing of microbial communities. *PLOS Comput. Biol.* 1, e24. Available at: https://doi.org/10.1371/journal.pcbi.0010024

Chia, P. Y., Sengupta, S., Kukreja, A., S.l. Ponnampalavanar, S., Ng, O. T., and Marimuthu, K. (2020). The role of hospital environment in transmissions of multidrug-resistant gram-negative organisms. *Antimicrob. Resist. Infect. Control* 9, 1–11. Available at: https://doi.org/10.1186/s13756-020-0685-1

Cholley, P., Ka, R., Guyeux, C., Thouverez, M., Guessennd, N., Ghebremedhin, B., Frank, T., Bertrand, X., and Hocquet, D. (2014). Population structure of clinical *Pseudomonas aeruginosa* from west and central African countries. *PLoS One* 9, e107008. Available at: https://doi.org/10.1371/journal.pone.0107008

Cholley, P., Thouverez, M., Floret, N., Bertrand, X., and Talon, D. (2008). The role of water fittings in intensive care rooms as reservoirs for the colonization of patients with *Pseudomonas aeruginosa*. *Intensive Care Med.* 34, 1428. Available at: https://doi.org/10.1007/s00134-008-1110-z

Churko, J. M., Mantalas, G. L., Snyder, M. P., and Wu, J. C. (2013). Overview of high throughput sequencing technologies to elucidate molecular pathways in cardiovascular diseases. *Circ. Res.* 112, 1613–1623. Available at: https://doi.org/10.1161/CIRCRESAHA.113.300939

Ciofu, O., and Tolker-Nielsen, T. (2019). Tolerance and resistance of *Pseudomonas aeruginosa* biofilms to antimicrobial agents-how *Pseudomonas aeruginosa* can escape antibiotics. *Front. Microbiol.* 10, 913. Available at: https://doi.org/10.3389/fmicb.2019.00913

Clarivet, B., Grau, D., Jumas-Bilak, E., Jean-Pierre, H., Pantel, A., Parer, S., and Lotthé, A.(2016). Persisting transmission of carbapenemase-producing *Klebsiella pneumoniae* due to an environmental reservoir in a university hospital, France, 2012 to 2014. *Euro Surveill.* 21, 30213. Available at: https://doi.org/10.2807/1560-7917.es.2016.21.17.30213

Cochran, W. L., Suh, S. J., McFeters, G. A., and Stewart, P. S. (2000). Role of RpoS and AlgT in *Pseudomonas aeruginosa* biofilm resistance to hydrogen peroxide and monochloramine. *J. Appl. Microbiol.* 88, 546–553. Available at: https://doi.org/10.1046/j.1365-2672.2000.00995.x

Cole, K., and Talmadge, J. E. (2019). Mitigation of microbial contamination from waste water and aerosolization by sink design. *J. Hosp. Infect.* 103, 193–199. Available at: https://doi.org/10.1016/j.jhin.2019.05.011

Coleman, D.C., Deasy, E.C., Moloney, E.M., Swan, J.S., and O'Donnell, M.J. (2020). Decontamination of Hand Washbasins and Traps in Hospitals. *In:* Decontamination in Hospitals and Healthcare Facilities (Walker, J.T, Editor), Woodhead Publishing, Duxford, United Kingdom. pp. 135–162

Collier, S. A., Stockman, L. J., Hicks, L. A., Garrison, L. E., Zhou, F. J., and Beach, M. J. (2012). Direct healthcare costs of selected diseases primarily or partially transmitted by water. *Epidemiol. Infect.* 140, 2003–2013. Available at: https://doi.org/10.1017/S0950268811002858

Constantinides, B., Chau, K. K., Quan, T. P., Rodger, G., Andersson, M., Jeffery, K. J., Lipworth, S., Gweon, S., Peniket, A., Pike, G., Millo, J., Byukusenge, M., Holdaway, M., Gibbons, C., Mathers, A., Crook, D., Peto, T., Walker, A., and Stoesser, N. (2020). Multi-omic surveillance of *Escherichia coli* and *Klebsiella* spp. in hospital sink drains and patients. *bioRxiv*, 19, 952366. Available at: https://doi.org/10.1101/2020.02.19.952366

Cooper, I. R., and Hanlon, G. W. (2010). Resistance of *Legionella pneumophila* serotype 1 biofilms to chlorine-based disinfection. *J. Hosp. Infect.* 74, 152–159. Available at: https://doi.org/10.1016/j.jhin.2009.07.005

Corcoran, E. (2010). Sick water?: the central role of wastewater management in sustainable development: a rapid response assessment. UNEP/Earthprint. pp. 1–88. Available at: http://wedocs.unep.org/bitstream/handle/20.500.11822/9156/Sick%20Water.pdf?sequence=1&isAllowed=y

Cummings, K. J. and Virji, M. A. (2018). The long-term effects of cleaning on the lungs. *Am J Respir Crit Care Med.* 197, 1099–1101. Available at: https://doi.org/10.1164/rccm.201801-0138ED

Cundell, T. (2015). The limitations of the colony-forming unit in microbiology. *Eur. Pharm. Rev.* 20, 11–13.

Curran, B., Jonas, D., Grundmann, H., Pitt, T., and Dowson, C. G. (2004). Development of a multilocus sequence typing scheme for the opportunistic pathogen *Pseudomonas aeruginosa*. *J. Clin. Microbiol.* 42, 5644–5649. Available at: https://doi.org/10.1128/JCM.42.12.5644-5649.2004

Dai, Z., Sevillano-Rivera, M. C., Calus, S. T., Bautista-De Los Santos, Q. M., Eren, A. M., Van Der Wielen, P. W. J. J., Ijaz, U Z., and Pinto, A. J. (2020). Disinfection exhibits systematic impacts on the drinking water microbiome. *Microbiome* 8, 1–19. Available at: https://doi.org/10.1186/s40168-020-00813-0

Dancer, S. J. (2014). Controlling hospital-acquired infection: focus on the role of the environment and new technologies for decontamination. *Clin. Microbiol. Rev.* 27, 665–690. Available at: https://doi.org/10.1128/cmr.00020-14

Danylkovych, A. G., Lishchuk, V. I., and Romaniuk, O. O. (2016). Use of electrochemically activated aqueous solutions in the manufacture of fur materials. *Springerplus* 5, 1–11. Available at: https://doi.org/10.1186/s40064-016-1784-6

Davis, J. R., Stager, C. E., and Araj, G. F. (1983). 4-h Identification of *Pseudomonas aeruginosa* with 9-chloro-9-(4-diethylaminophenyl)-10-phenylacridan. *J. Clin. Microbiol.* 17, 1054–1056. Available at: https://10.1128/jcm.17.6.1054-1056.1983

De Geyter, D., Blommaert, L., Verbraeken, N., Sevenois, M., Huyghens, L., Martini, H., Covens, L., Pierard, D., and Wybo, I. (2017). The sink as a potential source of transmission of carbapenemase-producing *Enterobacteriaceae* in the intensive care unit. *Antimicrob. Resist. Infect. Control.* 6, 24. Available at: https://doi.org/10.1186/s13756-017-0182-3

de Jonge, E., de Boer, M. G. J., van Essen, E. H. R., Dogterom-Ballering, H. C. M., and Veldkamp, K. E. (2019). Effects of a disinfection device on colonization of sink drains and patients during a prolonged outbreak of multidrug-resistant *Pseudomonas aeruginosa* in an intensive care unit. *J. Hosp. Infect.* 102, 70–74. Available at: https://doi.org/10.1016/j.jhin.2019.01.003

de Muinck, E. J., Trosvik, P., Gilfillan, G. D., Hov, J. R., and Sundaram, A. Y. M. (2017). A novel ultra high-throughput 16S rRNA gene amplicon sequencing library preparation method for the Illumina HiSeq platform. *Microbiome.* 5, 68. Available at: https://doi.org/10.1186/s40168-017-0279-1

De Oliveira, D. M. P., Forde, B. M., Kidd, T. J., Harris, P. N. A., Schembri, M. A., Beatson, S. A., Paterson, D. L., and Walker, M. J. (2020). Antimicrobial resistance in ESKAPE pathogens. *Clin. Microbiol. Rev.* 33, 1–49. Available at: https://doi.org/10.1128/CMR.00181-19

De Vos, D., Lim, A. J., Pirnay, J. P., Struelens, M., Vandenvelde, C., Duinslaeger, L., Vanderkelen, A., and Cornelis, P. (1997). Direct detection and identification of *Pseudomonas aeruginosa* in clinical samples such as skin biopsy specimens and expectorations by multiplex PCR based on two outer membrane lipoprotein genes, *oprI* and *oprL*. *J. Clin. Microbiol.* 35, 1295–1299. Available at: https://doi.org/10.1128/jcm.35.6.1295-1299.1997

Deasy, E. C., Moloney, E. M., Boyle, M. A., Swan, J. S., Geoghegan, D. A., Brennan, G. I., Fleming, T. E., O'Donnell, M. J., and Coleman, D. C. (2018). Minimizing

microbial contamination risk simultaneously from multiple hospital washbasins by automated cleaning and disinfection of U-bends with electrochemically activated solutions. *J. Hosp. Infect.* 100, 98–104. Available at: https://doi.org/10.1016/j.jhin.2018.01.012

Decker, B. K., and Palmore, T. N. (2013). The role of water in healthcare-associated infections. *Curr. Opin. Infect. Dis.* 26, 345. Available at: https://doi.org/10.1097/qco.0b013e3283630adf

Decraene, V., Phan, H. T. T., George, R., Wyllie, D. H., Akinremi, O., Aiken, Z., Cleary, P., Dodgson, A., Pankhurst, L., Crook, D. W., Lenney, C., Walker, A. S., Woodford, N., Sebra, R., Fath-Ordoubadi, F., Mathers, A. J., Seale, A. C., Guiver, M., McEwan, A., Watts, V., Welfare, W., Stoesser, N., and Cawthorne, J. (2018). A large, refractory nosocomial outbreak of *Klebsiella pneumoniae* carbapenemase-producing *Escherichia coli* demonstrates carbapenemase gene outbreaks involving sink stes require novel approaches to infection control. *Antimicrob. Agents Chemother.* 62. Available at: https://doi.org/10.1128/AAC.01689-18

Dekker, J. P., and Frank, K. M. (2016). Commentary: next-generation epidemiology using real-time core genome multilocus sequence typing to support infection control policy. *J. Clin. Microbiol.* 54, 2850–2853. Available at: https://doi.org/10.1128/jcm.01714-16

Denton, M., Rajgopal, A., Mooney, L., Qureshi, A., Kerr, K. G., Keer, V., Pollard, K., Peckham, D. G., and Conway, S. P. (2003). *Stenotrophomonas maltophilia* contamination of nebulizers used to deliver aerosolized therapy to inpatients with cystic fibrosis. *J. Hosp. Infect.* 55, 180–183. Available at: https://doi.org/10.1016/s0195-6701(03)00299-8

Department of Health UK (2013a). Health Building Note 00-09: Infection Control in the Built Environment. London, United Kingdom. pp. 1–47. Available at: https://www.gov.uk/government/publications/guidance-for-infection-control-in-the-built-environment

Department of Health UK (2013b). Health Building Note 00-10 Part C: Sanitary Assemblies. London, United Kingdom. pp 1–32. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/148497/HBN_00-10_Part_C_Final.pdf

Dettenkofer, M., and Spencer, R. C. (2007). Importance of environmental decontamination - a critical view. *J. Hosp. Infect.* 65, 55–57. Available at: https://doi.org/10.1016/S0195-6701(07)60016-4

Döhla, M., Wilbring, G., Schluye, B., Kummerer, B.K., Diegmann, C., Sib, E., Richter, E.,

Haag, A., Engelhart, S., Eis-Hubinger, A.M., Exner, M., Streeck, H., and Schmithausen, R. M. (2020). SARS-CoV-2 in environmental samples of quarantined households. *MedRxiv*. 49, 1–19. Available at: https://doi.org/10.1101/2020.05.28.20114041

Domitrovic, T. N., Hujer, A. M., Perez, F., Marshall, S. H., Hujer, K. M., Woc-Colburn, L. E., Parta, M., and Bonomo, R. A. (2016). Multidrug resistant *Pseudomonas aeruginosa* causing prosthetic valve endocarditis: a genetic-based chronicle of evolving antibiotic resistance. *Open Forum Infect. Dis.* 3, ofw188. Available at: https://doi.org/10.1093/ofid/ofw188

Donelli, G., Vuotto, C., and Mastromarino, P. (2013). Phenotyping and genotyping are both essential to identify and classify a probiotic microorganism. *Microb. Ecol. Health Dis.* 24. Available at: https://doi.org/10.3402/mehd.v24i0.20105

Döring, G., Ulrich, M., Müller, W., Bitzer, J., Schmidt-Koenig, L., Münst, L., Grupp, H., Wolz, C., Stern, M., and Botzenhart, K. (1991). Generation of *Pseudomonas aeruginosa* aerosols during handwashing from contaminated sink drains, transmission to hands of hospital personnel, and its prevention by use of a new heating device. *Zentralblatt fur Hyg. und Umweltmedizin / Int. J. Hyg. Environ. Med.* 191, 494–505

Driscoll, C. B., Otten, T. G., Brown, N. M., and Dreher, T. W. (2017). Towards long-read metagenomics: complete assembly of three novel genomes from bacteria dependent on a diazotrophic cyanobacterium in a freshwater lake co-culture. *Stand. Genomic Sci.* 12, 9. Available at: https://doi.org/10.1186/s40793-017-0224-8

Duncan, M. W., Roder, H., and Hunsucker, S. W. (2008). Quantitative matrix-assisted laser desorption/ionization mass spectrometry. *Briefings Funct. Genomics* 7, 355–370. Available at: https://doi.org/10.1093/bfgp/eln041

Duong, J., Booth, S. C., McCartney, N. K., Rabin, H. R., Parkins, M. D., and Storey, D. G (2015). Phenotypic and genotypic comparison of epidemic and non-epidemic strains of *Pseudomonas aeruginosa* from individuals with cystic fibrosis. *PLoS One.* 10, e0143466. Available at: https://doi.org/10.1371/journal.pone.0143466

Eftekharizadeh, F., Dehnavieh, R., Hekmat, S. N., and Mehrolhassani, M. H. (2016). Health technology assessment on super oxidized water for treatment of chronic wounds. *Med. J. Islam. Repub. Iran* 30, 384.

Egan, S. A., Shore, A. C., O'Connell, B., Brennan, G. I., and Coleman, D. C. (2020). Linezolid resistance in *Enterococcus faecium* and *Enterococcus faecalis* from hospitalized patients in Ireland: high prevalence of the MDR genes *optrA* and *poxtA* in isolates with diverse genetic backgrounds. *J. Antimicrob. Chemother.* 75, 1704–

1711. Available at: https://doi.org/10.1093/jac/dkaa075

European Centre for Disease Prevention and Control (2018). Surveillance of Antimicrobial Resistance in Europe 2017. Stockholm, Swedan. pp. 1–108. Available at: https://www.ecdc.europa.eu/sites/default/files/documents/AMR%202017_Cover%2BInner-web_v3.pdf

European Centre for Disease Prevention and Control (2016). Expert Opinion on Whole Genome Sequencing for Public Health Surveillance: Strategy to Harness Whole Genome Sequencing to Strengthen EU Outbreak Investigations and Public Health Surveillance. Stockholm, Swedan. pp. 1–13. Available at: https://doi.org/10.2900/12442

European Union Drinking Water Regulations 2014. (2014) SI 2014/122. Available at: http://www.irishstatutebook.ie/eli/2014/si/122/made/en/pdf

Environmental Protection Agency Ireland (2020). Bathing Water Quality in Ireland - A Report for the Year 2019. Wexford, Ireland. pp. 1–49. Available at: https://www.epa.ie/pubs/reports/water/bathing/Bathing Water Quality in Ireland 2019.pdf

Environmental Protection Agency Ireland (2017). Urban Waste Water Treatment in 2017. Wexford, Ireland. pp. 1–49. Available at: https://www.epa.ie/pubs/reports/water/wastewater/Final%20report%20for%20website.pdf

Environmental Protection Agency Ireland (2014). European Union (Drinking Water) Regulations. Wexford, Ireland. pp. 1–34. Available at: http://www.irishstatutebook.ie/eli/2014/si/122/made/en/pdf

Enviornmental Protection Agency United States (2012). Saving Water in Hospitals. Washington, United States. pp. 0–1. Available at: https://www.epa.gov/sites/production/files/2017-01/documents/ws-commercial-factsheet-hospitals.pdf

Feng, Y., Wei, L., Zhu, S., Qiao, F., Zhang, X., Kang, Y., Cai, L., Kang, M., McNally, A., and Zong, Z. (2019). Handwashing sinks as the source of transmission of ST16 carbapenem-resistant *Klebsiella pneumoniae*, an international high-risk clone, in an intensive care unit. *J. Hosp. Infect.* 104, 492–496. Available at: https://doi.org/10.1016/j.jhin.2019.10.006

Ferranti, G., Marchesi, I., Favale, M., Borella, P., and Bargellini, A. (2014). Aetiology, source and prevention of waterborne healthcare-associated infections: a review. *J. Med. Microbiol.* 63, 1247–1259. Available at: https://doi.org/10.1099/jmm.0.075713-

0

Ferroni, A., Nguyen, L., Pron, B., Quesne, G., Brusset, M. C., and Berche, P. (1998). Outbreak of nosocomial urinary tract infections due to *Pseudomonas aeruginosa* in a paediatric surgical unit associated with tap-water contamination. *J. Hosp. Infect.* 39, 301–307. Available at: https://doi.org/0.1016/s0195-6701(98)90295-x

Fleres, G., Couto, N., Lokate, M., van der Sluis, L. W. M., Ginevra, C., Jarraud, S., Deurenberg, R. H., Rossen, J. W., García-Cobos, S., and Friedrich, A. W. (2018). Detection of *Legionella anisa* in water from hospital dental chair units and molecular characterization by whole-genome sequencing. *Microorganisms* 6, 71. Available at: https://doi.org/10.3390/microorganisms6030071

Folletti, I., Zock, J.-P., Moscato, G., Siracusa, A. (2014). Asthma and rhinitis in cleaning workers: a systematic review of epidemiological studies. *Journal of Asthma.* 51, 18–28. Available at: https://doi.org/10.3109/02770903.2013.833217

Fournier, P.-E., Dubourg, G., and Raoult, D. (2014). Clinical detection and characterization of bacterial pathogens in the genomics era. *Genome Med.* 6, 114. Available at: https://doi.org/10.1186/s13073-014-0114-2

Fox, G. E., Magrum, L. J., Balch, W. E., Wolfe, R. S., and Woese, C. R. (1977). Classification of methanogenic bacteria by 16S ribosomal RNA characterization. *Proc. Natl. Acad. Sci. U. S. A.* 74, 4537–4541. Available at: https://doi.org/10.1073/pnas.74.10.4537

Franco, L. C., Tanner, W., Ganim, C., Davy, T., Edwards, J., and Donlan, R. (2020). A microbiological survey of handwashing sinks in the hospital built environment reveals differences in patient room and healthcare personnel sinks. *Sci. Rep.* 10, 8234. Available at: https://doi.org/10.1038/s41598-020-65052-7

Fuentefria, D. B., Ferreira, A. E., and Corcao, G. (2011). Antibiotic-resistant *Pseudomonas aeruginosa* from hospital wastewater and superficial water are they genetically related? *J. Environ. Manage.* 92, 250–255. Available at: https://doi.org/10.1016/j.jenvman.2010.09.001

Fuks, G., Elgart, M., Amir, A., Zeisel, A., Turnbaugh, P. J., Soen, Y., and Shental, N. (2018). Combining 16S rRNA gene variable regions enables high-resolution microbial community profiling. *Microbiome.* 6, 1–13. Available at: https://doi.org/10.1186/s40168-017-0396-x

Fusch, C., Pogorzelski, D., Main, C., Meyer, C., el Helou, S., and Mertz, D. (2015). Self-disinfecting sink drains reduce the *Pseudomonas aeruginosa* bioburden in a neonatal intensive care unit. *Acta Paediatr.* 104, 344–349. Available at:

https://doi.org/10.1111/apa.13005

Gbaguidi-Haore, H., Varin, A., Cholley, P., Thouverez, M., Hocquet, D., and Bertrand, X. (2018). A bundle of measures to control an outbreak of *Pseudomonas aeruginosa* associated with P-trap contamination. *Infect. Control Hosp. Epidemiol.* 39, 164–169. Available at: https://doi.org/10.1017/ice.2017.304

Gélinas, P., and Goulet, J. (1983). Neutralization of the activity of eight disinfectants by organic matter. *J. Appl. Bacteriol.* 54, 243–247. Available at: https://doi.org/10.1111/j.1365-2672.1983.tb02613.x

Giani, A. M., Gallo, G. R., Gianfranceschi, L., and Formenti, G. (2020). Long walk to genomics: history and current approaches to genome sequencing and assembly. *Comput. Struct. Biotechnol. J.* 18, 9–19. Available at: https://doi.org/10.1016/j.csbj.2019.11.002

Gionco, B., Tavares, E. R., de Oliveira, A. G., Yamada-Ogatta, S. F., do Carmo, A. O., Pereira, U. de P., Chideroli, R. T., Simionato, A. S., Navarro, M. O. P., and Chryssafidis, A. L. (2017). New insights about antibiotic production by *Pseudomonas aeruginosa*: a gene expression analysis. *Front. Chem.* 5, 66. Available at: https://doi.org/10.3389/fchem.2017.00066

Goodman, K. E., Simner, P. J., Klein, E. Y., Kazmi, A. Q., Gadala, A., Toerper, M. F., Levin, S., Tamma, P. D., Rock, C., Cosgrove, S. E., Maragakis, L. L., and Milstone, A. M. (2019). Predicting probability of perirectal colonization with carbapenem-resistant *Enterobacteriaceae* (CRE) and other carbapenem-resistant organisms (CROs) at hospital unit admission. *Infect. Control Hosp. Epidemiol.* 40, 541–550. Available at: https://doi.org/10.1017/ice.2019.42

Gormley, M., Aspray, T. J. and Kelly, D. A. (2020). COVID-19: mitigating transmission via wastewater plumbing systems. *Lancet Global Health.* 20, 30112. Available at: https://doi.org/10.1016/S2214-109X(20)30112-1

Gormley, M., Aspray, T. J., Kelly, D. A., and Rodriguez-Gil, C. (2017). Pathogen cross-transmission via building sanitary plumbing systems in a full scale pilot test-rig. *PLoS One.* 12, e0171556. Available at: https://doi.org/10.1371/journal.pone.0171556

Grabowski, M., Lobo, J. M., Gunnell, B., Enfield, K., Carpenter, R., Barnes, L., and Mathers, A. J. (2018). Characterizations of handwashing sink activities in a single hospital medical intensive care unit. *J. Hosp. Infect.* 100, 115–122. Available at: https://doi.org/10.1016/j.jhin.2018.04.025

GreenHealthcare (2016). Water use benchmarks for Irish hospitals. Available at: https://www.greenhealthcare.ie/wp-content/uploads/2017/12/Water-Benchmark-Fact-

Sheet.pdf

Gross, J. H. (2006). Mass spectrometry: a textbook. *In*: *Springer Sci. & Bus. Media.* (Smith Z and Smith Y, Editors), Oxford University Press, Oxford, United Kingdom, pp 1–507

Guleri, A., Palmer, R., Jackson, P., Przybylo, M., Sharma, R., Peel, A., Bird, M., Moorhouse, L., Clitheroe, S., and King, S. (2012). Outbreak report of multidrug-resistant *Pseudomonas aeruginosa* (MDR-PA) in a cardiac intensive care unit at a Lancashire cardiac centre O122. *Clin. Microbiol. Infect.* 18, 13. Available at: http://dx.doi.org/10.1111/j.1469-0691.2012.03801.x

Guo, B., Liu, C., Gibson, C., and Frigon, D. (2019). Wastewater microbial community structure and functional traits change over short timescales. *Sci. Total Environ.* 662, 779–785. Available at: https://doi.org/10.1016/j.scitotenv.2019.01.207

Guo, J., Cole, J. R., Zhang, Q., Brown, C. T., and Tiedje, J. M. (2016). Microbial community analysis with ribosomal gene fragments from shotgun metagenomes. *Appl. Environ. Microbiol.* 82, 157–166. Available at: https://doi.org/10.1128/aem.02772-15

Hadi, S. F., Khaliq, T., Bilal, N., Sikandar, I., Saaiq, M., Zubair, M., and Aurangzeb, S (2007). Treating infected diabetic wounds with superoxidized water as anti-septic agent: a preliminary experience. *J Coll Physicians Surg Pak.* 17, 740–743

Haenni, M., Hocquet, D., Ponsin, C., Cholley, P., Guyeux, C., Madec, J.-Y., and Bertrand, X. (2015). Population structure and antimicrobial susceptibility of *Pseudomonas aeruginosa* from animal infections in France. *BMC Vet. Res.* 11, 9. Available at: https://doi.org/10.1186/s12917-015-0324-x

Halstrom, S., Price, P., and Thomson, R. (2015). Environmental mycobacteria as a cause of human infection. *Int. J. Mycobacteriology* 4, 81–91. Available at: https://doi.org/10.1016/j.ijmyco.2015.03.002

Hammerum, A. M., Hansen, F., Nielsen, H. L., Jakobsen, L., Stegger, M., Andersen, P. S., Jensen, P., Nielsen, T. K., Hansen, L. H., and Hasman, H. (2016). Use of WGS data for investigation of a long-term NDM-1-producing *Citrobacter freundii* outbreak and secondary in vivo spread of bla NDM-1 to *Escherichia coli, Klebsiella pneumoniae* and *Klebsiella oxytoca*. *J. Antimicrob. Chemother.* 71, 3117–3124. Available at: https://doi.org/10.1093/jac/dkw289

Handelsman, J., Rondon, M. R., Brady, S. F., Clardy, J., and Goodman, R. M. (1998). Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. *Chem. Biol.* 5, 245-9. Available at:

https://doi.org/10.1016/s1074-5521(98)90108-9

Hanlin, J. H., and Myers, E. R. (2018). Healthcare plumbing systems are sources and vectors of infection for HAIs. *Infect. Control Today.* Available at: https://www.infectioncontroltoday.com/environmental-hygiene/healthcare-plumbing-systems-are-sources-and-vectors-infection-hais

Hata, G., Uemura, M., Weine, F. S., and Toda, T. (1996). Removal of smear layer in the root canal using oxidative potential water. *J. Endod.* 22, 643–645. Available at: https://doi.org/10.1016/s0099-2399(96)80056-2

Hawley, R. J., and Kozlovac, J. P. (2005). Decontamination. In: Biological Weapons Defense (Lindler, L.E., Lebeda,F.J., Korch, GW, Editors). Springer, Berlin and Germany. pp. 333–348. Available at: https://doi.org/10.1385/1-59259-764-5:333

He, Q., He, Q., Liu, X., Wei, Y., Shen, S., Hu, X., and Li, Q. (2014). Genome-wide prediction of cancer driver genes based on SNP and cancer SNV data. *Am J Cancer Res.* 4, 394–410

Health Information and Quality Authority. (2017). National Standards for the Prevention and Control of Healthcare-associated Infections in Acute Healthcare Services. Dublin, Ireland. pp. 1–120. Available at: https://www.hiqa.ie/sites/default/files/2017-05/2017-HIQA-National-Standards-Healthcare-Association-Infections.pdf

Health Protection Surveillance Centre. (2015). Guidelines for the Prevention and Control of Infection from Water Systems in Healthcare Facilities. Dublin, Ireland. pp. 1–98. Available at: http://www.hpsc.ie/AboutHPSC/ScientificCommittees/Sub-CommitteesofHPSCSAC/WaterGuidelinesSub-Committee/File,14451,en.pdf

Health Protection Surveillance Centre. (2009). Health Protection Surveillance Centre National Guidelines for the Control of Legionellosis in Ireland. Dublin, Ireland. pp. 1–127. Available at: https://www.hpsc.ie/abouthpsc/scientificcommittees/publications/File,3936,en.pdf

Hilliam, Y., Moore, M. P., Lamont, I. L., Bilton, D., Haworth, C. S., Foweraker, J., Walshaw, M. J., Williams, D., Fothergill, J. L., and De Soyza, A. (2017). *Pseudomonas aeruginosa* adaptation and diversification in the non-cystic fibrosis bronchiectasis lung. *Eur. Respir. J.* 49, 1602108. Available at: https://doi.org/10.1183/13993003.02108-2016

Hino, Y., Muraosa, Y., Oguchi, M., Yahiro, M., Yarita, K., Watanabe, A., Sakaida, E., Yokote, K., and Kamei, K. (2020). Drain outlets in patient rooms as sources for invasive fusariosis: an analysis of patients with haematological disorders. *J. Hosp. Infect.* 105, 518–526. Available at: https://doi.org/10.1016/j.jhin.2020.04.029

Hocquet, D., Muller, A., and Bertrand, X. (2016). What happens in hospitals does not stay in hospitals: antibiotic-resistant bacteria in hospital wastewater systems. *J. Hosp. Infect.* 93, 395–402. Available at: https://doi.org/10.1016/j.jhin.2016.01.010

Hopman, J., Meijer, C., Kenters, N., Coolen, J. P. M., Ghamati, M. R., Mehtar, S., van Crevel, R., Morshuis, W. J., Verhagen, A. F. T. M.,van den Heuvel, M. M., Voss, A., and Wertheim, H. F. L. (2019). Risk assessment after a severe hospital-acquired infection associated with carbapenemase-producing *Pseudomonas aeruginosa. JAMA Netw. Open.* 2, e187665. Available at: https://doi.org/10.1001/jamanetworkopen.2018.7665

Hopman, J., Tostmann, A., Wertheim, H., Bos, M., Kolwijck, E., Akkermans, R., Sturm, P., Voss, A., Pickkers, P., and Hoeven, H. (2017). Reduced rate of intensive care unit acquired gram-negative bacilli after removal of sinks and introduction of 'water-free' patient care. *Antimicrob. Resist. Infect. Control.* 6, 1–9. Available at: https://doi.org/10.1186/s13756-017-0213-0

Hota, S., Hirji, Z., Stockton, K., Lemieux, C., Dedier, H., Wolfaardt, G., and Gardam, M. A (2009). Outbreak of multidrug-resistant *Pseudomonas aeruginosa* colonization and infection secondary to imperfect intensive care unit room design. *Infect. Control Hosp. Epidemiol.* 30, 25–33. Available at: https://doi.org/10.1086/592700

Huang, Y. R., Hung, Y. C., Hsu, S. Y., Huang, Y. W., and Hwang, D. F. (2008). Application of electrolyzed water in the food industry. *Food Control.* 19, 329–345. Available at: https://doi.org/j.foodcont.2007.08.012

Hugenholtz, P., Goebel, B. M., and Pace, N. R. (1998). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* 180, 6793. Available at: https://doi.org/10.1128/JB.180.24.6793-6793.1998

Hutchins, C. F., Moore, G., Thompson, K.-A., Webb, J., and Walker, J. T. (2017). Contamination of hospital tap water: the survival and persistence of *Pseudomonas aeruginosa* on conventional and 'antimicrobial' outlet fittings. *J. Hosp. Infect.* 97, 156–161. Available at: https://doi.org/10.1016/j.jhin.2017.06.005

Ibarbalz, F. M., Pérez, M. V., Figuerola, E. L. M., and Erijman, L. (2014). The bias associated with amplicon sequencing does not affect the quantitative assessment of bacterial community dynamics. *PLoS One.* 99, e99722. Available at: https://doi.org/10.1371/journal.pone.0099722

Ibekwe, A. M., and Murinda, S. E. (2019). Linking microbial community composition in treated wastewater with water quality in distribution systems and subsequent health effects. *Microorganisms* 7, 660. Available at:

https://doi.org/10.3390/microorganisms7120660

International PBI Spa. (2006). SAS SUPER 100/180, DUO SAS SUPER 360, SAS ISOLATOR Instruction Manual. pp 1–48. Available at: https://archive-resources.coleparmer.com/Manual_pdfs/33687-02.pdf

Illumina (2017). An Introduction to Next-GenerationSequencing Technology. pp. 1–16. Available at: http://www.illumina.com/content/dam/illumina-marketing/documents/products/illumina_sequencing_introduction.pdf

Illumina Technical Note 2014, Understanding Illumina Quality Scores. San Diego, CA: Illumina, Inc., Pub. No. 770-2012-058. Available at: https://www.illumina.com/content/dam/illumina-marketing/documents/products/technotes/technote_understanding_quality_scores.pdf

International Human Genome Sequencing Consortium (2004). Finishing the euchromatic sequence of the human genome. *Nature* 431, 931–945. Available at: https://doi.org/10.1038/nature03001

Jami Al-Ahmadi, G., and Zahmatkesh Roodsari, R. (2016). Fast and specific detection of *Pseudomonas aeruginosa* from other *Pseudomonas* species by PCR. *Ann. Burns Fire Disasters* 29, 264–267.

Jassem, A. N., Zlosnik, J. E. A., Henry, D. A., Hancock, R. E. W., Ernst, R. K., and Speert, D. P. (2011). In vitro susceptibility of *Burkholderia vietnamiensis* to aminoglycosides. *Antimicrob. Agents Chemother.* 55, 2256–2264. Available at: https://doi.org/10.1128/AAC.01434-10

Jayaraman, P., Sakharkar, M. K., Lim, C. S., Tang, T. H., and Sakharkar, K. R. (2010). Activity and interactions of antibiotic and phytochemical combinations against *Pseudomonas aeruginosa in vitro*. *Int. J. Biol. Sci.* 6, 556–568. Available at: https://doi.org/10.7150/ijbs.6.556

Jendrzejewska, N., and Karwowska, E. (2018). The influence of antibiotics on wastewater treatment processes and the development of antibiotic-resistant bacteria. *Water Sci. Technol.* 77, 2320–2326. Available at: https://doi.org/10.2166/wst.2018.153

Jiang, X., Ma, M., Li, J., Lu, A., and Zhong, Z. (2008). Bacterial diversity of active sludge in wastewater treatment plant. *Earth Sci. Front.* 15, 163–168. Available at: https://doi.org/10.1016/S1872-5791(09)60015-4

Jin, M., Liu, L., Dong, D. W., Liu, Y. W., Yin, J., Wang, Z. Y. H., Shen, Z. Q. Z., Li, D. S. H., and Li, J. G. J. (2020). Chlorine disinfection promotes the exchange of antibiotic resistance genes across bacterial genera by natural transformation. *ISME J.* 14, 1847–1856. Available at: https://doi.org/10.1038/s41396-020-0656-9

Johnson, R. C., Deming, C., Conlan, S., Zellmer, C. J., Michelin, A. V, Lee-Lin, S., Thomas, P. J., Park, M., Weingarten, R. A., Less, J., Dekker, J. P., Frank, K. M., Musser, K. A., McQuiston, J. R., Henderson, D. K., Lau, A. F., Palmore, T. N. and Segre, J. A. (2018). Investigation of a cluster of *Sphingomonas koreensis* Infections. *N. Engl. J. Med.* 379, 2529–2539. Available at: https://doi.org/10.1056/NEJMoa1803238

Jones-Hall, Y. L., Kozik, A., and Nakatsu, C. (2015). Ablation of tumor necrosis factor is associated with decreased inflammation and alterations of the microbiota in a mouse model of inflammatory bowel disease. *PLoS One* 10, e0125309. Available at: https://doi.org/10.1371/journal.pone.0119441

Jones, L. D., Mana, T. S. C., Cadnum, J. L., Jencson, A. L., Silva, S. Y., Wilson, B. M., and Donskey, C. J. (2020). Effectiveness of foam disinfectants in reducing sink-drain gram-negative bacterial colonization. *Infect. Control Hosp. Epidemiol.* 41, 280–285. Available at: https://doi.org/10.1017/ice.2019.325

Jones, S. L., Ricke, S. C., Keith Roper, D., and Gibson, K. E. (2020). Swabbing the surface: critical factors in environmental monitoring and a path towards standardization and improvement. *Crit. Rev. Food Sci. Nutr.* 60, 225–243. Available at: https://doi.org/10.1080/10408398.2018.1521369

Jung, J., Choi, H. S., Lee, J. Y., Ryu, S. H., Kim, S. K., Hong, M. J., Kwak, S. H., Kim, H. J., Lee, M. S., Sung, H., Kim, M. N., and Kim, S. H. (2020). Outbreak of carbapenemase-producing *Enterobacteriaceae* associated with a contaminated water dispenser and sink drains in the cardiology units of a Korean hospital. *J. Hosp. Infect.* 104, 476–483. Available at: https://doi.org/10.1016/j.jhin.2019.11.015

Kamil, Z. H. (2014). *In vitro* studies comparing activities of antimicrobial photodynamic therapy and electrochemically activated solutions. *A thesis submitted to the Faculty of Health and Applied Science, University of West Englend.* Available at: https://uwe-repository.worktribe.com/output/806200/in-vitro-studies-comparing-activities-of-antimicrobial-photodynamic-therapy-and-electrochemically-activated-solutions

Kappstein, I., Grundmann, H., Hauer, T., and Niemeyer, C. (2000). Aerators as a reservoir of *Acinetobacter junii*: an outbreak of bacteraemia in paediatric oncology patients. *J. Hosp. Infect.* 44, 27–30. Available at: https://doi.org/10.1053/jhin.1999.0648

Kauppinen, A., Pitkänen, T., Al-Hello, H., Maunula, L., Hokajärvi, A. M. and Rimhanen-Finne, R. (2019). Two drinking water outbreaks caused by wastewater intrusion including sapovirus in Finland. *Int. J. of Env. Res. Pub. He.* 16, 1–14. Available at: https://doi.org/10.3390/ijerph16224376

Keeratipibul, S., Laovittayanurak, T., Pornruangsarp, O., Chaturongkasumrit, Y., Takahashi, H., and Techaruvichit, P. (2017). Effect of swabbing techniques on the efficiency of bacterial recovery from food contact surfaces. *Food Control.* 77, 139–144. Available at: https://doi.org/10.1016/j.foodcont.2017.02.013

Kidd, T. J., Ramsay, K. A., Vidmar, S., Carlin, J. B., Bell, S. C., Wainwright, C. E., Grimwood, K., Wainwright, C. E., Grimwood, K., Francis, P. W., Dakin, C., Cheney, J., George, N., Carlin, J. B., Robertson, C. F., Vidmar, S., Moodie, M.,Carzino, R., Carter, R., Armstrong, D. S., Cooper, P. J., McKay, K., Martin, A, Whitehead, B., Hunter, J., Byrnes, C. A., Tiddens, H. A., Graniel, K., Gerbrands, K., and Mott, L. (2015). *Pseudomonas aeruginosa* genotypes acquired by children with cystic fibrosis by age 5-years. *J. Cyst. Fibros.* 14, 361–369. Available at: https://doi.org/10.1016/j.jcf.2014.12.007

Kim, O.-S., Cho, Y.-J., Lee, K., Yoon, S.-H., Kim, M., Na, H., Park, S-C., Jeon, Y. S., Lee, J-H., and Yi, H. (2012). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int. J. Syst. Evol. Microbiol.* 62, 716–721. Available at: https://doi.org/10.1099/ijs.0.038075-0

Kingry, L. C., Rowe, L. A., Respicio-Kingry, L. B., Beard, C. B., Schriefer, M. E., and Petersen, J. M. (2016). Whole genome multilocus sequence typing as an epidemiologic tool for *Yersinia pestis*. *Diagn. Microbiol. Infect. Dis.* 84, 275–280. Available at: https://doi.org/10.1016/j.diagmicrobio.2015.12.003

Kizny-Gordon, A. E., Mathers, A. J., Cheong, E. Y. L., Gottlieb, T., Kotay, S., Walker, A. S., Peto, T. E. A., Crook, D. W., and Stoesser, N. (2017). The hospital water environment as a reservoir for carbapenem-resistant organisms causing hospital-acquired infections - a systematic review of the literature. *Clin. Infect. Dis.* 64, 1435–1444. Available at: https://doi.org/10.1093/cid/cix132

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., and Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41, 1. Available at: https://doi.org/10.1093/nar/gks808

Kocsis, B., Toth, A., Gulyas, D., Ligeti, B., Katona, K., Rokusz, L., and Szabo, D. (2019). Acquired qnrVC1 and blaNDM-1 resistance markers in an international high-risk *Pseudomonas aeruginosa* ST773 clone. *J. Med. Microbiol.* 68, 336–338. Available at: https://doi.org/10.1099/jmm.0.000927

Kossow, A., Kampmeier, S., Willems, S., Berdel, W. E., Groll, A. H., Burkhardt, B., Rossig, C., Groth, C., Idelevich, E. A., Kipp, F., Mellmann, A., and Stelljes, M.

(2017). Control of multidrug-resistant *Pseudomonas aeruginosa* in allogeneic hematopoietic stem cell transplant recipients by a novel bundle including remodeling of sanitary and water supply systems. *Clin. Infect. Dis.* 65, 935–942. Available at: https://doi.org/10.1093/cid/cix465

Kotay, S. M., Parikh, H. I., Barry, K., Gweon, H. S., Guilford, W., Carroll, J., and Mathers, A. J. (2020). Nutrients influence the dynamics of *Klebsiella pneumoniae* carbapenemase producing enterobacterales in transplanted hospital sinks. *Water Res.* 176, 115707. Available at: https://doi.org/10.1016/j.watres.2020.115707

Kotay, S. M., Donlan, R. M., Ganim, C., Barry, K., Christensen, B. E., and Mathers, A. J. (2019). Droplet- rather than aerosol-mediated dispersion is the primary mechanism of bacterial transmission from contaminated hand-washing sink traps. *Appl. Environ. Microbiol.* 85, 1–12. Available at: https://doi.org/10.1128/AEM.01997-18

Kotay, S., Chai, W., Guilford, W., Barry, K., and Mathers, A. J. (2017). Spread from the sink to the patient: *in situ* study using green fluorescent protein (GFP)-expressing *Escherichia coli* to model bacterial dispersion from hand-washing sink-trap reservoirs. *Appl. Environ. Microbiol.* 83, e03327-16. Available at: https://doi.org/10.1128/AEM.03327-16

Kramer, A., Schwebke, I., and Kampf, G. (2006). How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infect. Dis.* 6, 130. Available at: https://doi.org/10.1186/1471-2334-6-130

Kruskal, J. B. (1956). On the shortest spanning subtree of a graph and the traveling salesman problem. *Proc. Am. Math. Soc.* 7, 48–50. Available at: https://doi.org/10.1090/s0002-9939-1956-0078686-7

Kwapien, J., Oswiecimka, P., Forczek, M., and Drozdz, S. (2017). Minimum spanning tree filtering of correlations for varying time scales and size of fluctuations. *Phys. Rev.* 95, 52313. Available at: https://doi.org/10.1103/PhysRevE.95.052313

La Forgia, C., Franke, J., Hacek, D. M., Thomson Jr, R. B., Robicsek, A., and Peterson, L. R. (2010). Management of a multidrug-resistant *Acinetobacter baumannii* outbreak in an intensive care unit using novel environmental disinfection: a 38-month report. *Am. J. Infect. Control* 38, 259–263. Available at: https://doi.org/10.1016/j.ajic.2009.07.012

Lal, D., Nayyar, N., Kohli, P., and Lal, R. (2013). *Cupriavidus metallidurans*: A Modern Alchemist. *Indian J. Microbiol.* 53, 114–115. Available at: https://doi.org/10.1007/s12088-013-0355-6

Landelle, C., Legrand, P., Lesprit, P., Cizeau, F., Ducellier, D., Gouot, C., Bréhaut, P.,

Soing-Altrach, S., Girou, E., and Brun-Buisson, C. (2013). Protracted outbreak of multidrug-resistant *Acinetobacter baumannii* after intercontinental transfer of colonized patients. *Infect. Control Hosp. Epidemiol.* 34, 119–124. Available at: https://doi.org/10.1086/669093

Lata, S., Mohanty, S. K., Pradhan, P. K., Patri, G., Sinha, S. P., and Agrawal, P. (2016). Anti bacterial effectiveness of electro- chemically activated (ECA) water as a root canal irrigant - an i*n-vitro* comparative study. *J. Clin. Diagn. Res.* 10, 138–142. Available at: https://doi.org/10.7860/JCDR/2016/22148.8699

Lavigne, J.-P., Espinal, P., Dunyach-Remy, C., Messad, N., Pantel, A., and Sotto, A. (2013). Mass spectrometry: a revolution in clinical microbiology? *Clin. Chem. Lab. Med.* 51, 257–270. Available at: https://doi.org/10.1515/cclm-2012-0291

Lee, H. Gurtowski, J., Yoo, S., Nattestad, M., Marcus, S., Goodwin, S., McCombie, W. R., and Schatz, M. (2016). Third-generation sequencing and the future of genomics. *bioRxiv*, 048603. Available at: https://doi.org/10.1101/048603

Lehtola, M. J., Miettinen, I. T., Keinänen, M. M., Kekki, T. K., Laine, O., Hirvonen, A., Vartiainen, T., and Martikainen, P. J. (2004). Microbiology, chemistry and biofilm development in a pilot drinking water distribution system with copper and plastic pipes. *Water Res.* 38, 3769–3779. Available at: https://doi.org/10.1016/j.watres.2004.06.024

Leitner, E., Zarfel, G., Luxner, J., Herzog, K., Pekard-Amenitsch, S., Hoenigl, M., Valentin, T., Feierl, G., Grisold, A. J., and Högenauer, C. (2015). Contaminated handwashing sinks as the source of a clonal outbreak of KPC-2-producing *Klebsiella oxytoca* on a hematology ward. *Antimicrob. Agents Chemother.* 59, 714–716. Available at: https://doi.org/10.1128/aac.04306-14

Lepuschitz, S., Schill, S., Stoeger, A., Pekard-Amenitsch, S., Huhulescu, S., Inreiter, N., Hartl, R., Kerschner, H., Sorschag, S., Springer, B., Brisse, S., Allerberger, F., Mach, R. L., and Ruppitsch, W. (2019). Whole genome sequencing reveals resemblance between ESBL-producing and carbapenem resistant *Klebsiella pneumoniae* isolates from Austrian rivers and clinical isolates from hospitals. *Sci. Total Environ.,* 662, 227–235. Available at: https://doi.org/10.1016/j.scitotenv.2019.01.179

Li, B., Yang, Y., Ma, L., Ju, F., Guo, F., Tiedje, J. M., and Zhang, T. (2015). Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes. *ISME J.* 9, 2490–2502. Available at: https://doi.org/10.1038/ismej.2015.59

Li, G., Zhang, T., Yang, L., Cao, Y., Guo, X., Qin, J., Yang, Q., You, S., Yuan, G., Jiang,

K., Luo, J., Li, Z., Gao, L., Jiang, K., Wu, L., and Zheng, J. (2017). Complete genome sequence of *Achromobacter* insolitus type strain LMG 6003T, a pathogen isolated from leg wound. *Pathog. Dis.* 75, ftx037. Available at: https://doi.org/10.1093/femspd/ftx037

Li, L., Mendis, N., Trigui, H., Oliver, J. D., and Faucher, S. P. (2014). The importance of the viable but non-culturable state in human bacterial pathogens. *Front. Microbiol.* 5, 258. Available at: https://doi.org/10.3389/fmicb.2014.00258

Li, H., and Durbin, R. (2010). Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* 26, 589–595. Available at: https://doi.org/10.1093/bioinformatics/btp698

Liato, V., Hammami, R., and Aïder, M. (2017). Influence of electro-activated solutions of weak organic acid salts on microbial quality and overall appearance of blueberries during storage. *Food Microbiol.* 64, 56–64. Available at: https://doi.org/10.1016/j.fm.2016.12.010

Liato, V., Labrie, S., Viel, C., Benali, M., and Aïder, M. (2015). Study of the combined effect of electro-activated solutions and heat treatment on the destruction of spores of *Clostridium sporogenes* and *Geobacillus stearothermophilus* in model solution and vegetable puree. *Anaerobe* 35, 11–21. Available at: https://doi.org/10.1016/j.anaerobe.2015.06.004

Limayem, A., Wasson, S., Mehta, M., Pokhrel, A. R., Patil, S., Nguyen, M., Chen, J., and Nayak, B. (2019). High-throughput detection of bacterial community and its drug-resistance profiling from local reclaimed wastewater plants. *Front. Cell. Infect. Microbiol.* 9, 303. Available at: https://doi.org//10.3389/fcimb.2019.00303

Livingston, S. H., Cadnum, J. L., Gestrich, S., Jencson, A. L., and Donskey, C. J. (2018). A novel sink drain cover prevents dispersal of microorganisms from contaminated sink drains. *Infect. Control Hosp. Epidemiol.* 39, 1254–1256. Available at: https://doi.org/10.1017/ice.2018.192

Lomholt, J. A., Poulsen, K., and Kilian, M. (2001). Epidemic population structure of *Pseudomonas aeruginosa*: evidence for a clone that is pathogenic to the eye and that has a distinct combination of virulence factors. *Infect. Immun.* 69, 6284–6295. Available at: https://doi.org/10.1128/iai.69.10.6284-6295.2001

Lopez-Canovas, L., Martinez Benitez, M. B., Herrera Isidron, J. A., and Flores Soto, E. (2019). Pulsed Field Gel Electrophoresis: past, present, and future. *Anal. Biochem.* 573, 17–29. Available at: https://doi.org/10.1016/j.ab.2019.02.020

Lowe, C., Willey, B., O’Shaughnessy, A., Lee, W., Lum, M., Pike, K., Larocque, C.,

Dedier, H., Dales, L., and Moore, C. (2012). Outbreak of extended-spectrum β-lactamase–producing *Klebsiella oxytoca* infections associated with contaminated handwashing sinks. *Emerg. Infect. Dis.* 18, 1242. Available at: https://doi.org/10.3201/eid1808.111268

Lu, Q., Eggimann, P., Luyt, C. E., Wolff, M., Tamm, M., François, B., Mercier, E., Garbino, J., Laterre, P. F., Koch, H., Gafner, V., Rudolf, M. P., Mus, E., Perez, A., Lazar, H., Chastre, J., and Rouby, J. J. (2014). *Pseudomonas aeruginosa* serotypes in nosocomial pneumonia: prevalence and clinical outcomes. *Crit. Care* 18, 1–9. Available at: https://doi.org/10.1186/cc13697

Lüth, S., Kleta, S., and Al Dahouk, S. (2018). Whole genome sequencing as a typing tool for foodborne pathogens like *Listeria monocytogenes* – the way towards global harmonisation and data exchange. *Trends Food Sci. Technol.* 73, 67–75. Available at: https://doi.org/10.1016/j.tifs.2018.01.008

Luton, P. E., Wayne, J. M., Sharp, R. J., and Riley, P. W. (2002). The *mcrA* gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations in landfill. *Microbiology* 148, 3521–3530. Available at: https://doi.org/10.1099/00221287-148-11-3521

Magalhães, B., Valot, B., Abdelbary, M. M. H., Prod'hom, G., Greub, G., Senn, L., and Blanc, D. S. (2020). Combining standard molecular typing and whole genome sequencing to investigate *Pseudomonas aeruginosa* epidemiology in intensive care units. *Front. Public Heal.* 8, 3. Available at: https://doi.org/10.3389/fpubh.2020.00003

Mahida, N., Clarke, M., White, G., Vaughan, N., and Boswell, T. (2017). Outbreak of *Enterobacter cloacae* with New Delhi metallo-β-lactamase (NDM)-1: challenges in epidemiological investigation and environmental decontamination. *J. Hosp. Infect.* 97, 64–65. Available at: https://doi.org/10.1016/j.jhin.2017.05.016

Maiden, M. C., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D. A., Feavers, I. M., Achtman, M., and Spratt, B. G. (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc. Natl. Acad. Sci. U. S. A.* 95, 3140–3145. Available at: https://doi.org/10.1073/pnas.95.6.3140

Mao, G., Song, Y., Bartlam, M., and Wang, Y. (2018). Long-term effects of residual chlorine on *Pseudomonas aeruginosa* in simulated drinking water fed with low AOC medium. *Front. Microbiol.* 9, 879. Available at: https://doi.org/10.3389/fmicb.2018.00879

Marchesi, J. R., and Ravel, J. (2015). The vocabulary of microbiome research: a proposal. *Microbiome* 3, 1–3. Available at: https://doi.org/10.1186/s40168-015-0094-5

Mardis, E. R. (2008). Next-generation DNA sequencing methods. *Annu. Rev. Genomics Hum. Genet.* 9, 387–402. Available at: https://doi.org/10.1146/annurev.genom.9.081307.164359

Martin, G. S., Mannino, D. M., Eaton, S., and Moss, M. (2003). The epidemiology of sepsis in the United States from 1979 through 2000. *N. Engl. J. Med.* 348, 1546–1554. Available at: https://doi.org/10.1056/NEJMoa022139

Martínez-De Jesús, F. R., Ramos-De la Medina, A., Remes-Troche, J. M., Armstrong, D. G., Wu, S. C., Lázaro Martínez, J. L., and Beneit-Montesinos, J. V. (2007). Efficacy and safety of neutral pH superoxidised solution in severe diabetic foot infections. *Int. Wound J.* 4, 353–362. Available at: https://doi.org/10.1111/j.1742-481X.2007.00363.x

Mathers, A. J., Crook, D., Vaughan, A., Barry, K. E., Vegesana, K., Stoesser, N., Parikh, H. I., Sebra, R., Kotay, S., and Walker, A. S. (2019). *Klebsiella quasipneumoniae* provides a window into carbapenemase gene transfer, plasmid rearrangements, and patient interactions with the hospital environment. *Antimicrob. Agents Chemother.* 63, e02513-18. Available at: https://doi.org/10.1128/aac.02513-18

Mathers, A. J., Vegesana, K., German Mesner, I., Barry, K. E., Pannone, A., Baumann, J., Crook, D. W., Stoesser, N., Kotay, S., Carroll, J., and Sifri, C. D. (2018). Intensive care unit wastewater interventions to prevent transmission of multispecies *Klebsiella pneumoniae* carbapenemase–producing organisms. *Clin. Infect. Dis.* 67, 171–178. Available at: https://doi.org/10.1093/cid/ciy052

Maxam, A. M., and Gilbert, W. (1977). A new method for sequencing DNA. *Proc. Natl. Acad. Sci.* 74, 560–564. Available at: https://doi.org/10.1073/pnas.74.2.560

McLellan, S. L., and Roguet, A. (2019). The unexpected habitat in sewer pipes for the propagation of microbial communities and their imprint on urban waters. *Curr. Opin. Biotechnol.* 57, 34–41. Available at: https://doi.org/10.1016/j.copbio.2018.12.010

McReynolds, D. E. (2018). The novel use of electrochemically activated pH neutral hypochlorous acid solutions as biodecontaminants for removable PMMA dental prostheses. *A thesis submitted to Dublin Dental University Hospital, Trinity College Dublin.* Available at: http://www.tara.tcd.ie/bitstream/handle/2262/85288/Final%20Thesis%20-%20D%20McReynolds%20Thesis%206th%20Nov%202018.pdf?sequence=1&isAllowed=y

Meletis, G. (2016). Carbapenem resistance: overview of the problem and future perspectives. *Ther. Adv. Infect. Dis.* 3, 15–21. Available at: https://doi.org/10.1177/2049936115621709

Mellmann, A., Bletz, S., Böking, T., Kipp, F., Becker, K., Schultes, A., Prior, K., and Harmsen, D. (2016). Real-time genome sequencing of resistant bacteria provides precision infection control in an institutional setting. *J. Clin. Microbiol.* 54, 2874–2881. Available at: https://doi.org/10.1128/JCM.00790-16

Mills, G. R. W., Phiri, M., Erskine, J., and Price, A. D. F. (2015). Rethinking healthcare building design quality: an evidence-based strategy. *Build. Res. Inf.* 43, 499–515. Available at: https://doi.org/10.1080/09613218.2015.1033880

Mir-Tutusaus, J. A., Parlade, E., Llorca, M., Villagrasa, M., Barcelo, D., Rodriguez-Mozaz, S., Martinez-Alonso, M., Gaju, N., Caminal, G., and Sarra, M. (2017). Pharmaceuticals removal and microbial community assessment in a continuous fungal treatment of non-sterile real hospital wastewater after a coagulation-flocculation pretreatment. *Water Res.* 116, 65–75. Available at: https://doi.org/10.1016/j.watres.2017.03.005

Mombini, S., Rezatofighi, S. E., Kiyani, L., and Motamedi, H. (2019). Diversity and metallo-β-lactamase-producing genes in *Pseudomonas aeruginosa* strains isolated from filters of household water treatment systems. *J. Environ. Manage.* 231, 413–418. Available at: https://doi.org/10.1016/J.JENVMAN.2018.10.068

Montagna, M. T., Triggiano, F., Barbuti, G., Bartolomeo, N., De Giglio, O., Diella, G., Lopuzzo, M., Rutigliano, S., Serio, G., and Caggiano, G. (2019). Study on the *in vitro* activity of five disinfectants against nosocomial bacteria. *Int. J. Environ. Res. Public Health* 16, 1895. Available at: https://doi.org/10.3390/ijerph16111895

Moradali, M. F., Ghods, S., and Rehm, B. H. A. (2017). *Pseudomonas aeruginosa* lifestyle: a paradigm for adaptation, survival, and persistence. *Front. Cell. Infect. Microbiol.* 7, 39. Available at: https://doi.org/10.3389/fcimb.2017.00039

Morita, C., Sano, K., Morimatsu, S., Kiura, H., Goto, T., Kohno, T., Hong, W. U., Miyoshi, H., Iwasawa, A., Nakamura, Y., Tagawa, M., Yokosuka, O., Saisho, H., Maeda, T., and Katsuoka, Y. (2000). Disinfection potential of electrolyzed solutions containing sodium chloride at low concentrations. *J. Virol. Methods* 85, 163–174. Available at: https://doi.org/10.1016/s0166-0934(99)00165-2

Morris, D., Harris, S., Morris, C., Commins, E., and Cormican, M. (2016). Hospital Effluent: Impact on the Microbial Environment and Risk to Human Health. Available at:

http://www.epa.ie/researchandeducation/research/researchpublications/researchreports/EPA%20162%20final%20web.pdf

Mulet, X., Cabot, G., Ocampo-Sosa, A. A., Domínguez, M. A., Zamorano, L., Juan, C., Tubau, F., Rodríguez, C., Moyà, B., Peña, C., Martínez-Martínez, L., and Oliver, A. (2013). Biological markers of *Pseudomonas aeruginosa* epidemic high-risk clones. *Antimicrob. Agents Chemother.* 57, 5527–5535. Available at: https://doi.org/10.1128/AAC.01481-13

Murugan, N., Malathi, J., Umashankar, V., and Madhavan, H. N. (2016). Unraveling genomic and phenotypic nature of multidrug-resistant (MDR) *Pseudomonas aeruginosa* VRFPA04 isolated from keratitis patient. *Microbiol. Res.* 193, 140–149. Available at: https://doi.org/10.1016/j.micres.2016.10.002

Muzslay, M., Moore, G., Alhussaini, N., and Wilson, A. P. R. (2017). ESBL-producing Gram-negative organisms in the healthcare environment as a source of genetic material for resistance in human infections. *J. Hosp. Infect.* 95, 59–64. Available at: https://doi.org/10.1016/j.jhin.2016.09.009

Nascimento, A. L., Souza, A. J., Andrade, P. A. M., Andreote, F. D., Coscione, A. R., Oliveira, F. C., and Regitano, J. B. (2018). Sewage sludge microbial structures and relations to their sources, treatments, and chemical sttributes. *Front. Microbiol.* 9, 1462. Available at: https://doi.org/10.3389/fmicb.2018.01462

Nolan, L. M., Turnbull, L., Katrib, M., Osvath, S. R., Losa, D., and Whitchurch, C. B. (2019). *Pseudomonas aeruginosa* is capable of natural transformation in biofilms. *bioRxiv*, 859553. Available at: https://doi.org/10.1101/859553

Nghiem, L. D. Morgan, B., Donner, E., and Short, M.D. (2020). The COVID-19 pandemic: considerations for the waste and wastewater services sector, *Case Studies in Chemical and Environmental Engineering* 1, 100006. Available at: https://doi.org/10.1016/j.cscee.2020.100006

Numberger, D., Ganzert, L., Zoccarato, L., Mühldorfer, K., Sauer, S., Grossart, H. P., and Greenwood, A. (2019). Characterization of bacterial communities in wastewater with enhanced taxonomic resolution by full-length 16S rRNA sequencing. *Sci. Rep.* 9, 1–14. Available at: https://doi.org/10.1038/s41598-019-46015-z

O'Connell, N. H., and Humphreys, H. (2000). Intensive care unit design and environmental factors in the acquisition of infection. *J. Hosp. Infect.* 45, 255–262. Available at: https://doi.org/10.1053/jhin.2000.0768

O'Donnell, M. J., Boyle, M., Swan, J., Russell, R. J., and Coleman, D. C. (2009). A centralised, automated dental hospital water quality and biofilm management system

using neutral Ecasol™ maintains dental unit waterline output at better than potable quality: a 2-year longitudinal study. *J. Dent.* 37, 748–762. Available at: https://doi.org/10.1016/j.jdent.2009.06.001

O'Donnell, M. J., Shore, A. C., Russell, R. J., and Coleman, D. C. (2007). Optimisation of the long-term efficacy of dental chair waterline disinfection by the identification and rectification of factors associated with waterline disinfection failure. *J. Dent.* 35, 438–451. Available at: https://doi.org/10.1016/j.jdent.2007.01.001

O'Donnell, M. J., Shore, A. C., and Coleman, D. C. (2006). A novel automated waterline cleaning system that facilitates effective and consistent control of microbial biofilm contamination of dental chair unit waterlines: a one-year study. *J. Dent.* 34, 648–661. Available at: https://doi.org/10.1016/j.jdent.2005.12.006

O'Donnell, M. J., Tuttlebee, C. M., Falkiner, F. R., and Coleman, D. C. (2005). Bacterial contamination of dental chair units in a modern dental hospital caused by leakage from suction system hoses containing extensive biofilm. *J. Hosp. Infect.* 59, 348–360. Available at: https://doi.org/10.1016/j.jhin.2004.10.001

Olds, H. T., Corsi, R., Dila, K., Halmo, K., Bootsma, M., and McLellan, S. (2018). High levels of sewage contamination released from urban areas after storm events: a quantitative survey with sewage specific bacterial indicators, *PLoS Medicine*, 15, 1–23. Available at: https://doi.org/10.1371/journal.pmed.1002614

Oliver, A., Mulet, X., López-Causapé, C., and Juan, C. (2015). The increasing threat of *Pseudomonas aeruginosa* high-risk clones. *Drug Resist. Updat.* 21, 41–59. Available at: https://doi.org/10.1016/j.drup.2015.08.002

Ong, S. W., Tan, Y. K., and Chia, P.Y. (2020). Air, surface environmental, and personal protective equipment contamination by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) from a symptomatic patient, *JAMA*, 323, 1610–1612. Available at: https://doi.org/10.1001/jama.2020.3227

Onywera, H., and Meiring, T. L. (2020). Comparative analyses of Ion Torrent V4 and Illumina V3-V4 16S rRNA gene metabarcoding methods for characterization of cervical microbiota: taxonomic and functional profiling. *Sci. African* 7, e00278. Available at: https://doi.org/10.1016/j.sciaf.2020.e00278

Pachori, P., Gothalwal, R., and Gandhi, P. (2019). Emergence of antibiotic resistance *Pseudomonas aeruginosa* in intensive care unit; a critical review. *Genes Dis.* 6, 109–119. Available at: https://doi.org/10.1016/j.gendis.2019.04.001

Parkes, L. O., and Hota, S. S. (2018). Sink-related outbreaks and mitigation strategies in healthcare facilities. *Curr. Infect. Dis. Rep.* 20. 42. Available at:

https://doi.org/10.1007/s11908-018-0648-3

Petitjean, M., Martak, D., Silvant, A., Bertrand, X., Valot, B., and Hocquet, D. (2017). Genomic characterization of a local epidemic *Pseudomonas aeruginosa* reveals specific features of the widespread clone ST395. *Microb. Genomics* 3. 10. Available at: https://doi.org/10.1099/mgen.0.000129

Perry, M.R, van Bunnik, B, McNally, L, Munk. P, Warr, A, Moore, B, Kalima, P, Philip, C, de Roda Husman, A, Aerestrup, F, Woolhouse, M. (2019). Secrets of the hospital underbelly: abundance of antimicrobial resistance genes in hospital wastewater reflects hospital antimicrobial use and inpatient length of stay. *medRxiv* 19006858. Available at: https://doi.org/10.1101/19006858

Pirnay, J.-P., Bilocq, F., Pot, B., Cornelis, P., Zizi, M., Van Eldere, J., Deschaght, P., Vaneechoutte, M., Jennes, S., Pitt, T., and De Vos, D. (2009). *Pseudomonas aeruginosa* population structure revisited. *PLoS One* 4, e7740. Available at: https://doi.org/10.1371/journal.pone.0007740

Pitt, T. L. (1988). Epidemiological Typing of *Pseudomonas aeruginosa. Eur. J. Clin. Microbiol. Infect. Dis* 7, 238–247. Available at: https://doi.org/10.1007/bf01963095

Prestinaci, F., Pezzotti, P., and Pantosti, A. (2015). Antimicrobial resistance: a global multifaceted phenomenon. *Pathog. Glob. Health* 109, 309–318. Available at: https://doi.org/10.1179/2047773215Y.0000000030

Proctor, C. R., Reimann, M., Vriens, B., and Hammes, F. (2018). Biofilms in shower hoses. *Water Res.* 131, 274–286. Available at: https://doi.org/10.1016/j.watres.2017.12.027

Pullar, J. M., Vissers, M. C., and Winterbourn, C. C. (2000). Living with a killer: the effects of hypochlorous acid on mammalian cells. *IUBMB Life* 50, 259–266. Available at: https://doi.org/10.1080/713803731

Quainoo, S., Coolen, J. P. M., van Hijum, S. A. F. T., Huynen, M. A., Melchers, W. J. G., van Schaik, W., and Wertheim, H. F. L. (2017). Whole-genome sequencing of bacterial pathogens: the future of nosocomial outbreak analysis. *Clin. Microbiol. Rev.* 30, 1015–1063. Available at: https://doi.org/10.1128/CMR.00016-17

Quince, C., Walker, A. W., Simpson, J. T., Loman, N. J., and Segata, N. (2017). Shotgun metagenomics, from sampling to analysis. *Nat. Biotechnol.* 35, 833–844. Available at: https://doi.org/10.1038/nbt.3935

Ramos-Castaneda, J. A., Faron, M. L., Hyke, J., Bell-Key, D., Buchan, B. W., Nanchal, R., Pintar, P., Graham, M. B., Huerta, S., and Munoz-Price, L. S. (2020). How frequently should sink drains be disinfected? *Infect. Control Hosp. Epidemiol.* 41, 358–360.

Available at: https://doi.org/10.1017/ice.2019.316

Rausch, P., Rühlemann, M., Hermes, B. M., Doms, S., Dagan, T., Dierking, K., Domin, H., Fraune, S., Von Frieling, J., Hentschel, U., Heinsen, F. A., Höppner, M., Jahn, M. T., Jaspers, C., Kissoyan, K. A. B., Langfeldt, D., Rehman, A., Reusch, T. B.H., Roeder, T., Schmitz, R. A., Schulenburg, H., Soluch, R., Sommer, F., Stukenbrock, E., Weiland-Bräuer, N., Rosenstiel, P., Franke, A., Bosch, T., and Baines, J. F. (2019). Comparative analysis of amplicon and metagenomic sequencing methods reveals key features in the evolution of animal metaorganisms. *Microbiome* 7, 1–19. Available at: https://doi.org/10.1186/s40168-019-0743-1

Regev-Yochay, G., Smollan, G., Tal, I., Zade, N. P., Haviv, Y., Nudelman, V., Gal-Mor, O., Jaber, H., Zimlichman, E., and Keller, N. (2018). Sink traps as the source of transmission of OXA-48–producing *Serratia marcescens* in an intensive care unit. *Infect. Control Hosp. Epidemiol.* 39, 1307–1315. Available at: https://doi.org/10.1017/ice.2018.235

Regulation and Quality Improvement Authority (2012). Independent Review of Incidents of *Pseudomonas aeruginosa* Infection in Neonatal Units in Northern Ireland Final Report. Belfast, Northern Ireland. pp. 1–88. Available at: https://www.rqia.org.uk/RQIA/files/ee/ee76f222-a576-459f-900c-411ab857fc3f.pdf

Rizzo, L., Manaia, C., Merlinc, C., Schwartz, T., Dagote, C., Ploy, M.C., Michael, I., and Fatta-Kassinosg, D. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. *Sci. Total Environ.* 447, 345–360. Available at: https://doi.org/10.1016/j.scitotenv.2013.01.032

Robinson, G. M., Lee, S. W.-H., Greenman, J., Salisbury, V. C., and Reynolds, D. M. (2010). Evaluation of the efficacy of electrochemically activated solutions against nosocomial pathogens and bacterial endospores. *Lett. Appl. Microbiol.* 50, 289–294. Available at: https://doi.org/10.1111/j.1472-765X.2009.02790.x

Robinson, G., Thorn, R., and Reynolds, D. (2012). The effect of long-term storage on the physiochemical and bactericidal properties of electrochemically activated solutions. *Int. J. Mol. Sci.* 14, 457–469. Available at: https://doi.org/10.3390/ijms14010457

Roumpeka, D. D., Wallace, R. J., Escalettes, F., Fotheringham, I., and Watson, M. (2017). A review of bioinformatics tools for bio-prospecting from metagenomic sequence data. *Front. Genet.* 8, 1–10. Available at: https://doi.org/10.3389/fgene.2017.00023

Roux, D., Aubier, B., Cochard, H., Quentin, R., and Van Der Mee-Marquet, N. (2013). Contaminated sinks in intensive care units: an underestimated source of extended-

spectrum beta-lactamase-producing *Enterobacteriaceae* in the patient environment. *J. Hosp. Infect.* 85, 106–111. Available at: https://doi.org/10.1016/j.jhin.2013.07.006

Royer, G., Fourreau, F., Boulanger, B., Mercier-Darty, M., Ducellier, D., Cizeau, F., Potron, A., Podglajen, I., Mongardon, N., and Decousser, J-W (2020). Local outbreak of extended-spectrum β-lactamase SHV2a-producing *Pseudomonas aeruginosa* reveals the emergence of a new specific sub-lineage of the international ST235 high-risk clone, *J. Hosp. Infect*. Elsevier, 104, 33–39. Available at: https://doi.org/10.1016/j.jhin.2019.07.014

Ruiz-Roldán, L., Bellés, A., Bueno, J., Azcona-Gutiérrez, J. M., Rojo-Bezares, B., Torres, C., Castillo, F. J., Sáenz, Y., and Seral, C. (2018). *Pseudomonas aeruginosa* isolates from Spanish children: occurrence in faecal samples, antimicrobial resistance, virulence, and molecular typing. *Biomed Res. Int.* 8, 8060178. Available at: https://doi.org/10.1155/2018/8060178

Rutala, W., Weber, D., and The Healthcare Infection Control Practices Advisory Committee (2008). Guideline for Disinfection and Sterilization in Healthcare Facilities, 2008. North Carolina, United States. pp. 1–163. Available at: https://www.cdc.gov/infectioncontrol/pdf/guidelines/disinfection-guidelines-H.pdf

Salm, F., Deja, M., Gastmeier, P., Kola, A., Hansen, S., Behnke, M., Gruhl, D., and Leistner, R. (2016). Prolonged outbreak of clonal MDR *Pseudomonas aeruginosa* on an intensive care unit: contaminated sinks and contamination of ultra-filtrate bags as possible route of transmission? *Antimicrob. Resist. Infect. Control* 5, 53. Available at: https://doi.org/10.1186/s13756-016-0157-9

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* 74, 5463–5467. Available at: https://doi.org/10.1073/pnas.74.12.5463

Schatz, M. C., Delcher, A. L., and Salzberg, S. L. (2010). Assembly of large genomes using second-generation sequencing. *Genome Res.* 20, 1165–1173. Available at: https://doi.org/10.1101/gr.101360.109

Schaumburg, F., Bletz, S., Mellmann, A., Becker, K., and Idelevich, E. A. (2017). Susceptibility of MDR *Pseudomonas aeruginosa* to ceftolozane/tazobactam and comparison of different susceptibility testing methods. *J. Antimicrob. Chemother.* 72, 3079–3084. Available at: https://doi.org/10.1093/jac/dkx253

Schneider, H., Geginat, G., Hogardt, M., Kramer, A., Dürken, M., Schroten, H., and Tenenbaum, T. (2012). *Pseudomonas aeruginosa* outbreak in a pediatric oncology care unit caused by an errant water jet into contaminated siphons. *Pediatr. Infect. Dis.*

J. 31, 648–650. Available at: https://doi.org/10.1097/inf.0b013e31824d1a11

Schürch, A. C., Arredondo-Alonso, S., Willems, R. J. L., and Goering, R. V (2018). Whole genome sequencing options for bacterial strain typing and epidemiologic analysis based on single nucleotide polymorphism versus gene-by-gene–based approaches. *Clin. Microbiol. Infect.* 24, 350–354. Available at: https://doi.org/10.1016/j.cmi.2017.12.016

Schwartz, T., Hoffmann, S., and Obst, U. (1998). Formation and bacterial composition of young, natural biofilms obtained from public bank-filtered drinking water systems. *Water Res.* 32, 2787–2797. Available at: https://doi.org/10.1016/s0043-1354(98)00026-8

Selkon, J. B., Babb, J. R., and Morris, R. (1999). Evaluation of the antimicrobial activity of a new super-oxidized water, Sterilox, for the disinfection of endoscopes. *J. Hosp. Infect.* 41, 59–70. Available at: https://doi.org/10.1016/s0195-6701(99)90038-5

Shaw, E., Gavaldà, L., Càmara, J., Gasull, R., Gallego, S., Tubau, F., Granada, R. M., Ciercoles, P., Dominguez, M. A., Mañez, R., Carratalà, J., and Pujol, M. (2018). Control of endemic multidrug-resistant Gram-negative bacteria after removal of sinks and implementing a new water-safe policy in an intensive care unit. *J. Hosp. Infect.* 98, 275–281. Available at: https://doi.org/10.1016/j.jhin.2017.10.025

Shin, S. Y., Choi, J. Y., and Ko, K. S. (2012). Four cases of possible human infections with *Delftia lacustris*. *Springer Infect.* 40, 709–712. Available at: https://doi.org/10.1007/s15010-012-0339-1

Siegel, J. D., Rhinehart, E., Jackson, M., and Chiarello, L. (2007). Guideline for isolation precautions: preventing transmission of infectious agents in health care settings. *Am. J. Infect. Control* 35, 65. Available at: https://doi.org/10.1016/j.ajic.2007.10.007

Singh, M., Ganguli, S., and Ghosh, M. M. (2019). Comparative metagenomic dataset of hospital effluent microbiome from rural and urban hospitals in West Bengal. *Data Br.* 25, 104264. Available at: https://doi.org/10.1016/j.dib.2019.104264

Sizaire, V., Nackers, F., Comte, E., and Portaels, F. (2006). *Mycobacterium ulcerans* infection: control, diagnosis, and treatment. *Lancet Infect. Dis.* 6, 288–296. Available at: https://doi.org/10.1016/s1473-3099(06)70464-9

Slekovec, C., Plantin, J., Cholley, P., Thouverez, M., Talon, D., Bertrand, X., and Hocquet, D. (2012). Tracking down antibiotic-resistant *Pseudomonas aeruginosa* isolates in a wastewater network. *PLoS One* 7, e49300. Available at: https://doi.org/10.1371/journal.pone.0049300

Slekovec, C., Robert, J., Van Der Mee-Marquet, N., Berthelot, P., Rogues, A.-M., Derouin,

V., Cholley, P., Thouverez, M., Hocquet, D., and Bertrand, X. (2019). Molecular epidemiology of *Pseudomonas aeruginosa* isolated from infected ICU patients: a French multicenter 2012–2013 study. *Eur. J. Clin. Microbiol. Infect. Dis.* 38, 921–926. Available at: https://doi.org/10.1007/s10096-019-03519-w

Snitkin, E. S. (2019). Contamination of hospital plumbing: a source or a sink for antibiotic-resistant organisms? Contamination of hospital plumbing invited commentary. *JAMA Netw. Open* 2, e187660–187660. Available at: https://doi.org/10.1001/jamanetworkopen.2018.7660

Snyder, L. A., Loman, N. J., Faraj, L. J., Levi, K., Weinstock, J., Boswell, T. C., Pallen, M. J., and Ala'Aldeen, D. A. (2013). Epidemiological investigation of *Pseudomonas aeruginosa* isolates from a six-year-long hospital outbreak using high-throughput whole genome sequencing. *Eurosurveillance* 18, 42. Available at: https://doi.org/10.2807/1560-7917.es2013.18.42.20611

Solovyeva, A. M., and Dummer, P. M. (2000). Cleaning effectiveness of root canal irrigation with electrochemically activated anolyte and catholyte solutions: a pilot study. *Int. Endod. J.* 33, 494–504. Available at: https://doi.org/10.1046/j.1365-2591.2000.00342.x

Sorensen, S. J., Bailey, M., Hansen, L. H., Kroer, N., and Wuertz, S. (2005). Studying plasmid horizontal transfer in situ: a critical review. *Nat. Rev. Microbiol.* 3, 700–710. Available at: https://doi.org/10.1038/nrmicro1232

Srinivasan, R., Karaoz, U., Volegova, M., MacKichan, J., Kato-Maeda, M., Miller, S., Nadarajan, R., Brodie, E. L., and Lynch, S. V. (2015). Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. *PLoS One* 10, 1–22. Available at: https://doi.org/10.1371/journal.pone.0117617

Starlander, G., and Melhus, Å. (2012). Minor outbreak of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* in an intensive care unit due to a contaminated sink. *J. Hosp. Infect.* 82, 122–124. Available at: https://doi.org/10.1016/j.jhin.2012.07.004

Stewart, P. S., and Costerton, J. W. (2001). Antibiotic resistance of bacteria in biofilms. *Lancet* 358, 135–138. Available at: https://doi.org/10.1016/s0140-6736(01)05321-1

Stjarne Aspelund, A., Sjostrom, K., Olsson Liljequist, B., Morgelin, M., Melander, E., and Pahlman, L. I. (2016). Acetic acid as a decontamination method for sink drains in a nosocomial outbreak of metallo-beta-lactamase-producing *Pseudomonas aeruginosa*. *J. Hosp. Infect.* 94, 13–20. Available at: https://doi.org/10.1016/j.jhin.2016.05.009

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S. L., Hufnagle, W. O., Kowalik, D. J., and Lagrou, M. (2000).

Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406, 959. Available at: https://doi.org/10.1038/35023079

Su, Y.-C., Liu, C., and Hung, Y.-C. (2007). Electrolyzed water: principles and applications. *ACS Symposium Series* 309, 322. Available at: https://doi.org/10.1021/bk-2007-0967.ch014

Subedi, D., Vijay, A. K., Kohli, G. S., Rice, S. A., and Willcox, M. (2018). Comparative genomics of clinical strains of *Pseudomonas aeruginosa* strains isolated from different geographic sites. *Sci. Rep.* 8, 15668. Available at: https://doi.org/10.1038/s41598-018-34020-7

Swan, J. S. (2017). Minimisation of microbial reservoirs and infection risks from washbasin U-bend traps using a novel automated disinfection system with electrochemically activated solutions. *A thesis submitted to University of Dublin, Trinity College Dublin.* Available at: http://hdl.handle.net/2262/81904

Swan, J. S., Deasy, E. C., Boyle, M. A., Russell, R. J., O'Donnell, M. J., and Coleman, D. C. (2016). Elimination of biofilm and microbial contamination reservoirs in hospital washbasin U-bends by automated cleaning and disinfection with electrochemically activated solutions. *J. Hosp. Infect.* 94, 169–174. Available at: https://doi.org/10.1016/j.jhin.2016.07.007

Tagawa, M., Yamaguchi, T., Yokosuka, O., Matsutani, S., Maeda, T., and Saisho, H. (2000). Inactivation of a hepadnavirus by electrolysed acid water. *J. Antimicrob. Chemother.* 46, 363–368. Available at: https://doi.org/10.1093/jac/46.3.363

Tagini, F., and Greub, G. (2017). Bacterial genome sequencing in clinical microbiology: a pathogen-oriented review. *Eur. J. Clin. Microbiol. Infect. Dis.* 36, 2007–2020. Available at: https://doi.org/10.1007/s10096-017-3024-6

Taharaguchi, M., Takimoto, K., Zamoto-Niikura, A., and Yamada, Y.K. (2014). Effect of weak acid hypochlorous solution on selected viruses and bacteria of laboratory rodents. *Exp. Anim.* 63, 141–147. Available at: https://doi.org/10.1538/expanim.63.141

Taylor-Brown, A., Spang, L., Borel, N., and Polkinghorne, A. (2017). Culture-independent metagenomics supports discovery of uncultivable bacteria within the genus *Chlamydia*. *Sci. Rep.* 7, 10661. Available at: https://doi.org/10.1038/s41598-017-10757-5

Tazumi, A., Maeda, Y., Buckley, T., Millar, B. C., Goldsmith, C. E., Dooley, J. S. G., Elborn, J. S., Matsuda, M., and Moore, J. E. (2009). Moleculer epidemiology of clinical isolates of *Pseudomonas aeruginosa* isolated from horses in Ireland. *Ir. Vet. J.*

62, 456–459. Available at: https://doi.org/10.1186/2046-0481-62-7-456

Tenzin, S., Ogunniyi, A. D., Khazandi, M., Ferro, S., Bartsch, J., Crabb, S., Abraham, S., Deo, P., and Trott, D. J. (2019). Decontamination of aerosolised bacteria from a pig farm environment using a pH neutral electrochemically activated solution (Ecas4 anolyte). *PLoS One* 14, 1–15. Available at: https://doi.org/10.1371/journal.pone.0222765

Thorn, R. M. S., Lee, S. W. H., Robinson, G. M., Greenman, J., and Reynolds, D. M. (2012). Electrochemically activated solutions: evidence for antimicrobial efficacy and applications in healthcare environments. *Eur. J. Clin. Microbiol. Infect. Dis.* 31, 641–653. Available at: https://doi.org/10.1007/s10096-011-1369-9

Tracy, M., Ryan, L., Samarasekara, H., Leroi, M., Polkinghorne, A., and Branley, J. (2020). Removal of sinks and bathing changes to control multidrug-resistant Gram-negative bacteria in a neonatal intensive care unit: a retrospective investigation. *J. Hosp. Infect.*, 1–3. Available at: https://doi.org/10.1016/j.jhin.2020.01.014

Tulchinsky, T. H. (2018). John Snow, Cholera, the Broad street pump; waterborne diseases then and now. *Case Stud. Public Heal.*, 77–99. Available at: https://doi.org/10.1016/B978-0-12-804571-8.00017-2

Valdes, N., Soto, P., Cottet, L., Alarcon, P., Gonzalez, A., Castillo, A., Corsini, G., and Tello, M. (2015). Draft genome sequence of *Janthinobacterium lividum* strain MTR reveals its mechanism of capnophilic behavior. *Stand. Genomic Sci.* 10, 110. Available at: https://doi.org/10.1186/s40793-015-0104-z

Valentini, M., Gonzalez, D., Mavridou, D. A., and Filloux, A. (2018). Lifestyle transitions and adaptive pathogenesis of *Pseudomonas aeruginosa*. *Curr. Opin. Microbiol.* 41, 15–20. Available at: https://doi.org/10.1016/j.mib.2017.11.006

van Dijk, E. L., Auger, H., Jaszczyszyn, Y., and Thermes, C. (2014). Ten years of next-generation sequencing technology. *Trends Genet.* 30, 418–426. Available at: https://doi.org/10.1016/J.TIG.2014.07.001

Varin, A., Valot, B., Cholley, P., Morel, C., Thouverez, M., Hocquet, D., and Bertrand, X. (2017). High prevalence and moderate diversity of *Pseudomonas aeruginosa* in the U-bends of high-risk units in hospital. *Int. J. Hyg. Environ. Health* 220, 880–885. Available at: https://doi.org/10.1016/j.ijheh.2017.04.003

Vater, S. M., Weiße, S., Maleschlijski, S., Lotz, C., Koschitzki, F., Schwartz, T., Obst, U., and Rosenhahn, A. (2014). Swimming behavior of *Pseudomonas aeruginosa* studied by holographic 3D tracking. *PLoS One* 9, e87765–e87765. Available at: https://doi.org/10.1371/journal.pone.0087765

Vergara-López, S., Domínguez, M. C., Conejo, M. C., Pascual, Á., and Rodríguez-Baño, J. (2013). Wastewater drainage system as an occult reservoir in a protracted clonal outbreak due to metallo-β-lactamase-producing *Klebsiella oxytoca*. *Clin. Microbiol. Infect.* 19, 490–498. Available at: https://doi.org/10.1111/1469-0691.12288

Větrovský, T., and Baldrian, P. (2013). The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. *PLoS One* 8, e57923. Available at: https://doi.org/10.1371/journal.pone.0057923

Vo, T. N. N., Chu, D. T., Duong, D. L., Bui, V. N., Tong, M. S., Nguyen, T. T. P., Pham, Van H., and Chu-Dinh, T. (2019). Efficacy of electrochemically activated water solution in gingivitis treatment. *J. Pharm. Investig.* 49, 323–329. Available at: https://doi.org/10.1007/s40005-018-00419-7

Vos, M., Quince, C., Pijl, A. S., de Hollander, M., and Kowalchuk, G. A. (2012). A comparison of rpoB and 16S rRNA as markers in pyrosequencing studies of bacterial diversity. *PLoS One* 7, e30600. Available at: https://doi.org/10.1371/journal.pone.0030600

Waine, D. J., Honeybourne, D., Smith, E. G., Whitehouse, J. L., and Dowson, C. G. (2009). Cross-sectional and longitudinal multilocus sequence typing of *Pseudomonas aeruginosa* in cystic fibrosis sputum samples. *J. Clin. Microbiol.* 47, 3444 – 3448. Available at: https://doi.org/10.1128/JCM.00459-09

Waines, P. L., Moate, R., Moody, A. J., Allen, M., and Bradley, G. (2011). The effect of material choice on biofilm formation in a model warm water distribution system. *Biofouling* 27, 1161–1174. Available at: https://doi.org/10.1080/08927014.2011.636807

Walker, J. T., Jhutty, A., Parks, S., Willis, C., Copley, V., Turton, J. F., Hoffman, P. N., and Bennett, A. M. (2014). Investigation of healthcare-acquired infections associated with *Pseudomonas aeruginosa* biofilms in taps in neonatal units in Northern Ireland. *J. Hosp. Infect.* 86, 16–23. Available at: https://doi.org/10.1016/j.jhin.2013.10.003

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. Available at: https://doi.org/10.1128/AEM.00062-07

Wang, Y., Li, C., Gao, C., Ma, C., and Xu, P. (2014). Genome sequence of the nonpathogenic *Pseudomonas aeruginosa* strain ATCC 15442. *Genome Announc.* 2, e00421-14. Available at: https://doi.org/10.1128/genomea.00421-14

Wee, B. A., Tai, A. S., Sherrard, L. J., Ben Zakour, N. L., Hanks, K. R., Kidd, T. J.,

Ramsay, K., Lamont, I., Whiley, D., Bell, S., and Beatson, S. (2018). Whole genome sequencing reveals the emergence of a *Pseudomonas aeruginosa* shared strain sub-lineage among patients treated within a single cystic fibrosis centre. *BMC Genomics* 19, 1–11. Available at: https://doi.org/10.1186/s12864-018-5018-x

Weinbren, M., Bree, L., Sleigh, S., and Griffiths, M. (2017). Giving the tap the elbow? An observational study. *J. Hosp. Infect.* 96, 328–330. Available at: https://10.1016/j.jhin.2017.05.009

Weinbren, M. J. (2020). Dissemination of antibiotic resistance and other healthcare waterborne pathogens. The price of poor design, construction, usage and maintenance of modern water/sanitation services. *J. Hosp. Infect.* 105, 406–411. Available at: https://10.1016/j.jhin.2020.03.034

Weingarten, R. A., Johnson, R. C., Conlan, S., Ramsburg, A. M., Dekker, J. P., Lau, A. F., Khil, P., Odom, R. T., Deming, C., and Park, M. (2018). Genomic analysis of hospital plumbing reveals diverse reservoir of bacterial plasmids conferring carbapenem resistance. *MBio* 9, e02011-17. Available at: https://doi.org/10.1128/mbio.02011-17

Wendel, A. F., Kolbe-Busch, S., Ressina, S., Schulze-Röbbecke, R., Kindgen-Milles, D., Lorenz, C., Pfeffer, K., and MacKenzie, C. R. (2015). Detection and termination of an extended low-frequency hospital outbreak of GIM-1–producing *Pseudomonas aeruginosa* ST111 in Germany. *Am. J. Infect. Control* 43, 635–639. Available at: https://doi.org/10.1016/j.ajic.2015.02.024

Whiteley, G. S., Glasbey, T. O., and Fahey, P. P. (2016). A suggested sampling algorithm for use with ATP testing in cleanliness measurement. *Infect. Dis. Heal.* 21, 169–175. Available at: https://doi.org/10.1016/j.idh.2016.11.003

Widmer, A. F., Wenzel, R. P., Trilla, A., Bale, M. J., Jones, R. N., and Doebbeling, B. N. (1993). Outbreak of *Pseudomonas aeruginosa* infections in a surgical intensive care unit: probable transmission via hands of a health care worker. *Clin. Infect. Dis.* 16, 372–376. Available at: https://doi.org/10.1093/clind/16.3.372

Willmann, M., Bezdan, D., Zapata, L., Susak, H., Vogel, W., Schröppel, K., Liese, J., Weidenmaier, C., Autenrieth, I. B., Ossowski, S., and Peter, S. (2014). Analysis of a long-term outbreak of XDR *Pseudomonas aeruginosa*: a molecular epidemiological study. *J. Antimicrob. Chemother.* 70, 1322–1330. Available at: https://doi.org/10.1093/jac/dku546

Winand, R., Bogaerts, B., Hoffman, S., Lefevre, L., Delvoye, M., Van Braekel, J., Fu, Q., Roosens, N. H. C., De Keersmaecker, S. C. J., and Vanneste, K. (2020). Targeting the 16s rRNA gene for bacterial identification in complex mixed samples: comparative

evaluation of second (Illumina) and third (Oxford Nanopore Technologies) generation sequencing technologies. *Int. J. Mol. Sci.* 21, 1–22. Available at: https://doi.org/10.3390/ijms21010298

Wise, J. (2012). Three babies die in *Pseudomonas* outbreak at Belfast neonatal unit. *BMJ* 344. Available at: https://doi.org/10.1136/bmj.e592

World Health Organization (2018). WHO Report on Surveillance of Antibiotic Consumption. pp. 1–128. Available at: https://apps.who.int/iris/bitstream/handle/10665/277359/9789241514880-eng.pdf

World Health Organization (2014a). Antimicrobial Resistance: an Emerging Water, Sanitation and Hygiene Issue. pp. 1–16. Available at: https://apps.who.int/iris/handle/10665/204948

World Health Organization (2014b). Infection Prevention and Control of Epidemic-and Pandemic-prone Acute Respiratory Infections in Health Care. pp. 1–156. Available at: https://apps.who.int/iris/bitstream/handle/10665/112656/9789241507134_eng.pdf?sequence=1

World Health Organization (2013). How Much Water is Needed in Emergencies. pp. 1–4. Available at: https://www.who.int/water_sanitation_health/publications/2011/tn9_how_much_water_en.pdf%0Ahttp://www.who.int/water_sanitation_health/emergencies/WHO_TN_09_How_much_water_is_needed.pdf?ua=1

World Health Organization (2017). Guidelines for Drinking-water Quality. pp. 1–631. Available at: https://apps.who.int/iris/bitstream/handle/10665/254637/9789241549950-eng.pdf?sequence=1

World Health Organization (2003). Consensus Document on the Epidemiology of Severe Acute Respiratory Syndrome (SARS). pp. 1–46. Available at: https://apps.who.int/iris/handle/10665/70863

Yoon, S.-H., Ha, S.-M., Kwon, S., Lim, J., Kim, Y., Seo, H., and Chun, J. (2017). Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int. J. Syst. Evol. Microbiol.* 67, 1613–1617. Available at: https://doi.org/10.1099/ijsem.0.001755

Zeng, X., and Lin, J. (2013). Beta-lactamase induction and cell wall metabolism in Gram-negative bacteria. *Front. Microbiol.* 4, 128. Available at: https://doi.org/10.3389/fmicb.2013.00128

Zhou, Z. Y., Hu, B. J., Gao, X. D., Bao, R., Chen, M., and Li, H. Y. (2016). Sources of

sporadic *Pseudomonas aeruginosa* colonizations/infections in surgical ICUs: association with contaminated sink trap. *J. Infect. Chemother.* 22, 450–455. Available at: https://doi.org/10.1016/j.jiac.2016.03.016

Appendix

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Minimizing microbial contamination risk simultaneously from multiple hospital washbasins by automated cleaning and disinfection of U-bends with electrochemically activated solutions

E.C. Deasy [a,†], E.M. Moloney [a,†], M.A. Boyle [a], J.S. Swan [b], D.A. Geoghegan [b], G.I. Brennan [c], T.E. Fleming [c], M.J. O'Donnell [a], D.C. Coleman [a,*]

[a] *Microbiology Research Unit, Division of Oral Biosciences, Dublin Dental University Hospital, University of Dublin, Trinity College Dublin, Dublin, Ireland*
[b] *Facilities Department, Dublin Dental University Hospital, Dublin, Ireland*
[c] *National MRSA Reference Laboratory, St. James's Hospital, Dublin, Ireland*

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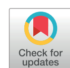

S U M M A R Y

Background: Outbreaks of infection associated with microbial biofilm in hospital hand washbasin U-bends are being reported increasingly. In a previous study, the efficacy of a prototype automated U-bend decontamination method was demonstrated for a single non-hospital pattern washbasin. It used two electrochemically activated solutions (ECA) generated from brine: catholyte with detergent properties and anolyte with disinfectant properties.
Aim: To develop and test a large-scale automated ECA treatment system to decontaminate 10 hospital pattern washbasin U-bends simultaneously in a busy hospital clinic.
Methods: A programmable system was developed whereby the washbasin drain outlets, U-bends and proximal wastewater pipework automatically underwent 10-min treatments with catholyte followed by anolyte, three times weekly, over five months. Six untreated washbasins served as controls. Quantitative bacterial counts from U-bends were determined on Columbia blood agar, Reasoner's 2A agar and *Pseudomonas aeruginosa* selective agar following treatment and 24 h later.
Findings: The average bacterial densities in colony-forming units/swab from treated U-bends showed a >3 log reduction compared with controls, and reductions were highly significant ($P<0.0001$) on all media. There was no significant increase in average bacterial counts from treated U-bends 24 h later on all media ($P>0.1$). *P. aeruginosa* was the most prevalent organism recovered throughout the study. Internal examination of untreated U-bends using electron microscopy showed dense biofilm extending to the washbasin drain outlet junction, whereas treated U-bends were free from biofilm.

* Corresponding author. Address: Microbiology Research Unit, Division of Oral Biosciences, Dublin Dental University Hospital, University of Dublin, Trinity College Dublin, Lincoln Place, Dublin 2, Ireland. Tel.: +353 1 6127276; fax: + 353 1 6127295.
E-mail address: david.coleman@dental.tcd.ie (D.C. Coleman).
† These two authors contributed equally to this article.

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Conclusion: Simultaneous automated treatment of multiple hospital washbasin U-bends with ECA consistently minimizes microbial contamination and thus the associated risk of infection.

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Introduction

Over the last two decades, many studies have reported hospital outbreaks, due particularly to Gram-negative bacteria, associated directly or indirectly with contaminated washbasin and sink drains [1–7]. U-bends are pieces of pipework fitted beneath washbasins that retain a volume of water, creating a seal preventing sewer gas from entering buildings from pipework downstream. This water may stagnate for considerable periods, encouraging the development of biofilms. These can spread as far as the washbasin drain, contaminating the washbasin and surrounding area [8,9].

U-bend biofilms are usually heterogenous communities consisting of a range of opportunistic bacterial pathogens, including *Pseudomonas* spp., *Acinetobacter* spp., *Klebsiella* spp. and *Enterobacter* spp., which can exhibit resistance to the major classes of antibiotics [2,4,6,10]. Furthermore, recent reports are increasingly highlighting the importance of wastewater pipework as a reservoir for the nosocomial transmission of carbapenemase-producing Enterobacteriaceae, an emerging global health threat [11].

A variety of approaches to U-bend decontamination have been investigated with varying success, most of which involve disruption to service and have financial implications, including the replacement of fixtures and/or associated pipework [2,6,10]. Replacement is ineffective in the long term as new washbasins and pipework rapidly become recolonized with micro-organisms. Disinfectants such as bleach may have diminished efficacy against dense biofilms, temporarily reducing bioburden but necessitating regular application [2,3,10]. Another approach involves thermal disinfection and vibrational cleaning of U-bends, but is not in widespread use [12].

Previously, the authors showed that long-term use of a pH-neutral electrochemically activated solution (ECA) (anolyte) as a disinfectant was effective to minimize microbial contamination of dental unit water and washbasin tap water [13,14]. ECA is produced by passing dilute brine through an electric field in an electrolytic cell, which generates two solutions of opposite charge [13,14]. The positively charged solution (anolyte) consists of a mixture of oxidants (predominantly hypochlorous acid), which is highly microbicidal [13]. The negatively charged antioxidant solution (catholyte) has detergent-like properties consisting predominantly of NaOH. Recently, the authors described the development of a programmable automated prototype system for minimizing microbial contamination of a domestic pattern washbasin U-bend by treating the system sequentially with catholyte to reduce organic material followed by disinfection with anolyte [8]. Average bacterial counts from the treated U-bend over 35 decontamination cycles on a variety of culture media showed a >4 log reduction relative to controls. This pilot study established proof of concept for automated U-bend decontamination using ECA.

The purpose of this study was to develop a large-scale automated ECA treatment system capable of decontaminating 10 hospital pattern washbasin U-bends and drains simultaneously, and to assess the efficacy of the system in a busy hospital clinical department.

Methods

Anolyte and catholyte

Anolyte and catholyte solutions were produced by electrochemical activation of an NaCl solution using a Qlean-Genie UL-75a ECA generator (Qlean Tech Enterprises, Mendota Heights, MI, USA) [8]. The generator was configured to produce anolyte measured at 800 parts per million (ppm) free available chlorine (FAC) at pH 7.0, having an oxidation-reduction potential (ORP) of +880 mV and consisting of approximately 632 ppm hypochlorous acid (79%) and 162 ppm OCl^- (20.2%). Catholyte is an amphoteric surfactant with a surface tension of 63 mN force and was produced at pH 12.5 with an ORP of approximately −1000 mV, consisting of approximately 400 ppm NaOH. Freshly generated anolyte was used undiluted. FAC levels in anolyte were measured using a Hach Pocket Colorimeter II (Hach, Ames, IA, USA) [8]. Freshly generated catholyte was diluted 1:5 with heated mains water with a temperature after dilution of approximately 33°C.

Test and control washbasins

Ten new ceramic hospital pattern washbasins with an offset drain outlet in the back wall of the basin (Armitage Shanks, Stoke on Trent, UK) were installed at the Accident & Emergency Department of the Dublin Dental University Hospital (DDUH) for ECA decontamination studies. Six identical washbasins located in different DDUH clinics were used as controls. Washbasins were used solely for handwashing. Tork Extra Mild Liquid Soap (SCA Hygiene Products Ltd, Dunstable, UK) was used for handwashing at all washbasins. Cold water supplied to test and control washbasin taps was provided from a 15,000-L tank supplied with potable quality mains water. This tank also supplied the calorifier, which provided hot water to all the washbasin taps. Automatic temperature recording was fitted on the out and return legs of the hot water network. Washbasin taps are fitted with a thermostatic mixing valve and provided output water at an average temperature of 38°C. Hot and cold water supplied to washbasins at DDUH has been treated with residual anolyte (2.5 ppm) for several years. Previous studies over 54 weeks showed average bacterial densities in hot and cold tap water of 1 [standard deviation (SD) 4] and 2 (SD 4) colony-forming units (cfu)/mL, respectively [14]. All washbasins were in frequent daily use from Monday to Friday. Three months prior to the study, washbasins were equipped with new

polypropylene U-bends (McAlpine Plumbing Products, Glasgow, UK) with two access ports (Figure 1).

Design of automated ECA treatment system for U-bends

A large-scale system was developed to decontaminate 10 washbasin U-bends, drains and proximal wastewater pipework simultaneously (Figure 2). A vertical wastewater pipe below each U-bend was connected to a horizontal common wastewater collection pipe. The pipes and fittings were made of polyvinylchloride (PVC) or acrylonitrile-butadiene-styrene (ABS), both compatible with long-term exposure to anolyte and catholyte. All pipe connections apart from U-bends were chemically welded to minimize the potential for leaks. ECA reservoirs were manufactured from ultraviolet-stabilized linear polyethylene designed for chemical storage. Each reservoir supplied a dosing pump (Grundfos, Bjerringbro, Denmark) connected by 25-mm ABS pipework to the common wastewater pipe (Figure 2).

A Praher unplasticized-PVC S4 ball valve (Schwertberg, Austria) was fitted to the common wastewater pipe downstream of the ECA pump connections to which an H-004 electric actuator (Actuated Solutions Ltd, Bognor Regis, UK) was fitted for automated valve operation. With the valve closed, the volume of ECA required to completely fill the wastewater pipework, U-bends and the washbasins to a level 5 cm above the drain outlets was determined (approximately 220 L). The timing, sequence of activation and duration of activation of the actuator-controlled valve, dosing pumps and ECA reservoir outlet valves was managed by a programmable electronic process controller (Open System Solutions Ltd, Southampton, UK) (Figure 2).

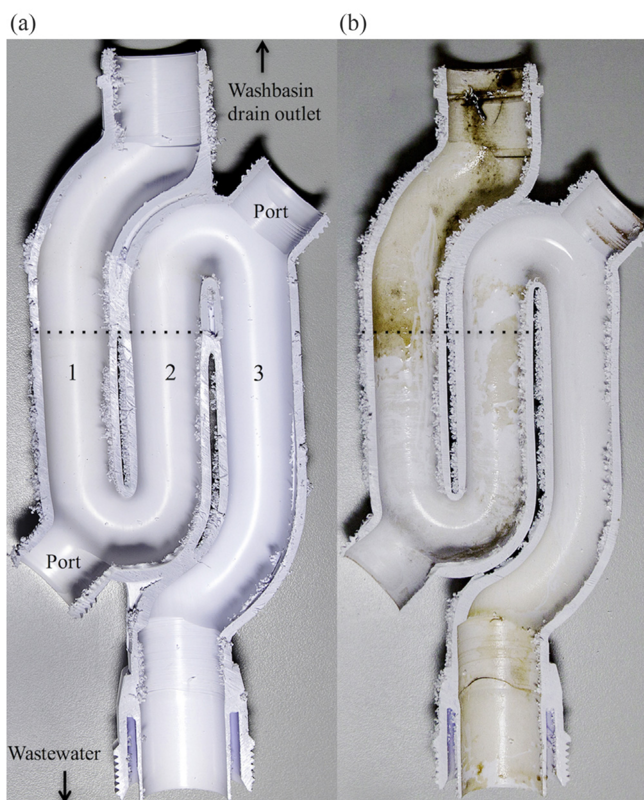

Figure 1. (a) A longitudinal section of a U-bend following 62 cycles of electrochemically activated solution (ECA) treatment over a five-month period. (b) A longitudinal section of a control U-bend at the end of the study. Both U-bends were installed at the same time. The dashed lines indicate the water level within the U-bends. Following each ECA treatment cycle, treated and control U-bends were swab sampled through the ports indicated. To avoid sampling the same part of each U-bend continually, six internal sampling sites were selected and sampled in rotation. Three of these (labelled 1–3) are shown in (a). The additional three sites were located on the other, mirror image half of the U-bend. The treated U-bend is noticeably free from visible biofilm, whereas the control U-bend contains slimy biofilm, especially above the waterline and at the junctions connecting to the washbasin drain outlet and wastewater discharge outlets.

Automated ECA decontamination cycles

Decontamination cycles began with the process controller activating the actuator and closing the valve on the common wastewater pipe. After a 30-s delay, the catholyte dosing pump was activated and dosed catholyte into the common wastewater pipe, and retro-filled this pipe, each washbasin's wastewater pipe, U-bend and washbasin drain outlet over a 3.5-min period. Catholyte was left *in situ* for 10 min and then voided to waste by automated opening of the valve on the common wastewater pipe. Following a further 30-s delay, the actuator closed the valve, and after 30 s, the anolyte pump activated and dosed anolyte into the system. Anolyte was left *in situ* for 10 min and then voided to waste, completing the cycle. Control washbasin drains and U-bends were flushed with mains water instead of ECA.

Microbiological culture

Decontamination efficacy was determined by semi-quantitative microbiological culture of U-bend samples ($N = 620$) immediately after each of 62 treatment cycles. Additional samples ($N = 420$) were taken 24 h after treatment for 42 cycles to assess microbial recovery. Samples were taken from control U-bends ($N = 372$) following each treated U-bend decontamination cycle. U-bends were flushed with tap water after each decontamination cycle to void residual anolyte. The interior surfaces of U-bends were sampled through the access ports using sterile cotton wool swabs (Venturi, Transystem, Copan, Italy) dipped in neutralizing solution (0.5% w/v sodium thiosulphate) [8]. Six internal sites were sampled in rotation to avoid sampling the same parts of the U-bends continually (Figure 1a). One site was sampled after each treatment cycle, and swabs were processed immediately. The tip of each swab was cut off and vortexed for 1 min in 1 mL of sterile phosphate-buffered saline, serially diluted and plated in duplicate on to Columbia blood agar (CBA) (Lip Diagnostic Services, Galway, Ireland), Reasoner's 2A (R2A) agar (Lip) and *Pseudomonas aeruginosa* selective agar (PAS) (Oxoid Ltd, Basingstoke, UK). PAS, CBA and R2A plates were incubated at 30°C for 48 h, 37°C for 48 h and 20°C for 10 days, respectively. Colony counts were recorded as cfu/swab [8]. The characteristics of different colony types and their abundance were recorded, and selected colonies of each were stored [8].

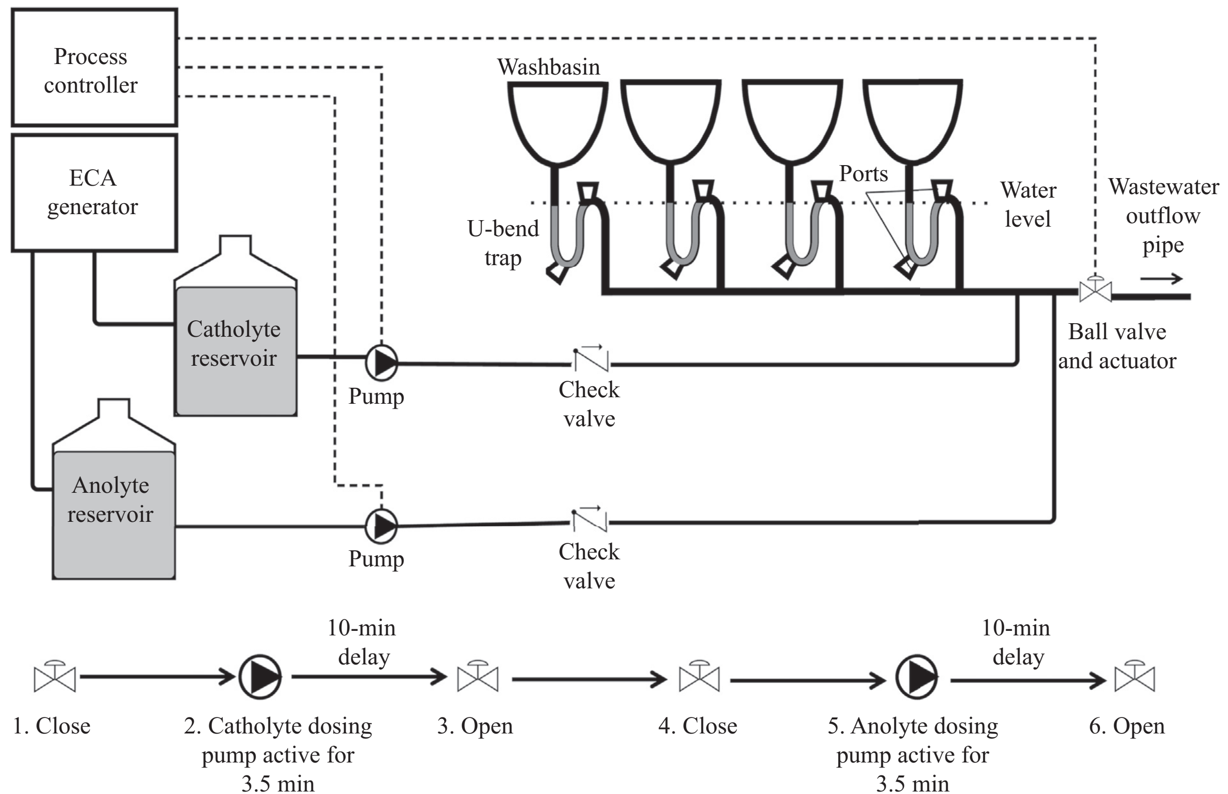

Figure 2. A schematic of the automated system for the simultaneous decontamination of 10 washbasin U-bends, drain outlets and wastewater pipes by sequential treatment with catholyte followed by anolyte used in the present study. Only four washbasins are shown for clarity. Each U-bend had two ports to facilitate sampling. The lower part of the figure shows a process control schematic for automated decontamination. The programmable process controller initiates treatment cycles. At the start of each cycle, the process controller sends a signal to the actuator to close the valve on the wastewater outflow pipe. After a 30-s delay, a signal activates the catholyte dosing pump for 3.5 min, and catholyte is pumped into the pipework below the washbasin U-bends until the pipework and U-bends are completely filled to a level 5 cm above the washbasin drain outlets. Catholyte is left *in situ* for 10 min, after which time the process controller opens the valve, voiding catholyte to the wastewater stream. The valve is then closed, and after a 30-s delay, the process controller activates the anolyte dosing pump for 3.5 min and the cycle proceeds as per catholyte dosing. After 10 min, the anolyte is voided to waste, completing the cycle. ECA, electrochemically activated.

Identification of bacterial isolates

Bacterial identification was determined using the Vitek MS Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry system (Vitek, bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions.

Electron microscopy

At the end of the study, selected U-bends were cut longitudinally and sections were examined for biofilm, without prior fixation, by scanning electron microscopy [13].

Statistical analysis

Statistical analyses were performed using GraphPad Prism v.5 (GraphPad Software, San Diego, CA, USA). Statistical significance was determined using unpaired, two-tailed Student's *t*-test with 95% confidence intervals. Statistical significance of more than two sets of data was determined using one-way analysis of variance.

Results

Automated U-bend decontamination

A novel large-scale automated U-bend decontamination system was developed and installed at the Accident & Emergency Department at DDUH which permitted each U-bend, drain and associated wastewater pipes of 10 washbasins to be completely filled sequentially with catholyte followed by anolyte (Figure 2). Empirical experiments were undertaken with the system to determine the optimal concentrations of each ECA for effective decontamination of the 10 U-bends in a relatively short time period. The previous proof of concept study used 450 ppm of anolyte and 40 ppm of catholyte, while for the larger system, this was increased to 800 ppm anolyte and 80 ppm of catholyte. The contact time between the solutions and the pipework was increased from 5 min to 10 min. Sampling was also changed from using a single access port U-bend to U-bends with two access ports (Figure 1). This permitted six selected sites to be sampled in rotation, reducing mechanical removal of biofilm from repetitive sampling as ECA-treated U-bends were sampled 1040 times (Table I).

Table I
Average quantitative bacterial counts from 10 washbasin U-bends subjected to automated treatment with electrochemically activated solutions (ECA) and the corresponding counts from six untreated U-bends

Agar medium	U-bend	Average bacterial counts in cfu/swab from ECA-treated ($N = 62$ cycles, 620 swabs) and control ($N = 372$ swabs) U-bends	SD	Range of bacterial counts in cfu/swab	*P*-value
CBA	Treated	73.4	258.2	$0–4.6 \times 10^3$	<0.0001
	Untreated	2×10^5	4×10^5	$0–4 \times 10^6$	
R2A	Treated	122.5	371.3	$0–5.8 \times 10^3$	<0.0001
	Untreated	3.3×10^5	1.1×10^6	$0–1.8 \times 10^7$	
PAS	Treated	15.3	184.5	$0–3.4 \times 10^3$	<0.0001
	Untreated	2.7×10^4	1.2×10^5	$0–1.4 \times 10^6$	
		Average bacterial counts in cfu/swab 24 h after ECA treatment ($N = 42$ cycles, 420 swabs) and control ($N = 252$ swabs) U-bends[a]			
CBA	Treated[a]	53.2	127.6	$0–1 \times 10^3$	<0.0001
	Untreated	2.1×10^5	4.3×10^5	$500–3.2 \times 10^6$	
R2A	Treated[a]	91.7	277.6	$0–3.5 \times 10^3$	<0.0001
	Untreated	2.9×10^5	6.1×10^5	$1.3 \times 10^3–5 \times 10^6$	
PAS	Treated[a]	15.6	119	$0–1.7 \times 10^3$	<0.0001
	Untreated	2.6×10^4	1.1×10^5	$0–1.4 \times 10^6$	

CBA, Columbia blood agar; R2A, Reasoner's 2A agar; PAS, *Pseudomonas aeruginosa* selective agar; SD, standard deviation; cfu, colony-forming units.
[a] The average bacterial counts in cfu/swab were determined for the 10 ECA-treated U-bends and the six untreated U-bends 24 h after treatment for 42 of 62 ECA treatment cycles.

All 10 test washbasins were exposed to three weekly decontamination cycles (Monday, Wednesday and Friday) over five months (62 cycles), which was almost double the number of cycles assessed in the previous proof of concept study. Six additional washbasins located elsewhere in DDUH were used as controls. Swab samples were taken from the internal surfaces of the U-bends, and semi-quantitative bacterial counts were determined on CBA, R2A and PAS. The average bacterial density from the six untreated U-bends during the study on CBA, R2A and PAS was 2×10^5 (SD 4×10^5), 3.3×10^5 (SD 1.1×10^6) and 2.7×10^4 (SD 1.2×10^5) cfu/swab, respectively (Table I). For the 10 ECA-treated U-bends over 62 cycles, the average bacterial density on CBA, R2A and PAS was 73.4 (SD 258.2), 122.5 (SD 371.3) and 15.3 (SD 184.5) cfu/swab, respectively (Table I). The average reduction in viable counts from ECA-treated U-bends was >3 log or a 99.9% reduction. Reductions in average bacterial counts from treated U-bends on all media relative to the counts from control U-bends were highly significant ($P<0.0001$) (Table I). There was no significant difference in average bacterial counts on all media between the 10 individual treated U-bends over the study period ($P>0.4$). Additional U-bend samples taken from all 10 treated U-bends 24 h after treatment for 42 of 62 decontamination cycles showed no significant increase ($P>0.1$) in average bacterial counts on all media (Table I).

Bacterial species identified from U-bends

The range of bacterial species identified from treated and control U-bends throughout the study is shown in Table A (see online supplementary material). Although the bacterial density in treated U-bends was consistently significantly lower than controls, the diversity of species identified was greater due to a greater number of Gram-positive bacterial species comprising several species of staphylococci (Table A see online supplementary material). Gram-negative bacterial species identified from treated and control U-bends were similar. *P. aeruginosa* was recovered from all U-bends during the study. The average *P. aeruginosa* count from treated U-bend samples was 15 (SD 185) cfu/swab ($N = 620$ samples); however, only 12% (74/620) of samples yielded *P. aeruginosa*, and of these, only 2% yielded >10 cfu/swab. In contrast, 78% (290/372) of swab samples ($N = 372$) from control U-bends yielded *P. aeruginosa*, and of these, 58% yielded >1000 cfu/swab.

Biofilm on ECA-treated and control U-bends

Following completion of the ECA treatment phase, the U-bends from several ECA-treated and control washbasins were removed and cut in longitudinal sections. Visual examination of the control U-bends revealed patchy, slimy biofilm on the inner surfaces, which extended to the region connecting to the washbasin drain outlet (Figure 1). In contrast, ECA-treated U-bends were visually free from biofilm (Figure 1). Electron microscopy of several sections of the inner surfaces of control U-bends confirmed the presence of dense biofilm and its absence in ECA-treated U-bends (Figure A, see online supplementary material).

Biofilm on washbasin drain outlet surfaces

At the end of the study period, a visual examination of washbasin drain outlets revealed biofilm within the outlets of all control washbasins and its absence in treated washbasin

drain outlets (Figure B, see online supplementary material). Neutralized swab samples taken from the drain outlets of six treated washbasins yielded average bacterial densities of 1 cfu/swab (range 0–5) on CBA agar. No bacteria were recovered on PAS agar. The corresponding average bacterial densities from control washbasin drain outlets were 4.1×10^3 (range 120–5.6×10^3) on CBA and 874.2 (range 5–2.7×10^3) cfu/swab on PAS. Additional swab samples were taken from the surface of each washbasin immediately adjacent to the drain outlets, and no bacteria were recovered from samples from the six test washbasins on CBA or PAS media. In contrast, 3.6×10^3 (range 30–8.6×10^3) cfu/swab was recovered on CBA and 1.2×10^3 (range 0–6.2×10^3) on PAS from the control washbasin surface samples.

Adverse effects on washbasin wastewater network

No adverse effects were observed following regular inspection of the washbasins, U-bends or associated wastewater pipework during and at the end of the study, and no leaks were identified.

Discussion

Proof of concept for effective and consistent decontamination of washbasin U-bends by automated sequential treatment with catholyte followed by anolyte was demonstrated in a previous study using a single domestic pattern washbasin located in a hospital washroom [8]. The present study developed a novel automated ECA treatment system to decontaminate 10 hospital pattern washbasin U-bends, drain outlets and proximal wastewater pipes simultaneously in a busy hospital department. The results of the study demonstrate that the large-scale system has a comparable decontamination efficacy to the pilot system, as both resulted in a >3 log reduction in bacterial counts in treated U-bends relative to controls ($P<0.0001$). However, with the large system, >3 log reductions were achieved simultaneously in 10 separate U-bends in a busy hospital clinic, demonstrating that this approach has good potential for application in hospital departments and wards equipped with multiple washbasins. In the pilot study, *P. aeruginosa* was not recovered from the ECA-treated U-bend. The finding of low densities of *P. aeruginosa* in some ECA-treated U-bends within the larger system is not surprising because of its larger and more extensive network of pipes servicing 10 washbasins. All control and ECA-treated U-bends were positive for *P. aeruginosa* at some point during the study, indicating that it is endemic within the wastewater network. Similarly, Cholley *et al*. sampled 28 U-bends over eight weeks and found that all were colonized at least once by *P. aeruginosa* [1]. In the present study, and in the pilot study, bacterial counts recovered immediately after ECA treatment and 24 h later were similar on all media tested, which demonstrated that biofilm within the pipework did not recover rapidly from ECA treatment [8]. A limitation to the present study is that the authors did not demonstrate that this approach would help to control an actual hospital outbreak associated with contaminated U-bends.

A variety of Gram-negative bacterial species other than *P. aeruginosa* were identified in ECA-treated and control U-bends (Table A, see online supplementary material). However, a greater range of Gram-positive species was identified from treated U-bends due to the recovery of several staphylococcal species that were not identified in the controls (Table A, see online supplementary material). Staphylococci are common skin commensals that inevitably get transferred into U-bends during handwashing. The recovery of staphylococci from treated U-bends, albeit in low numbers, could be due to their presence being masked by high densities of Gram-negative bacteria within the control samples.

The presence of Gram-negative bacteria in washbasin wastewater pipework constitutes a greater risk of infection due to their motility. A recent study using green fluorescent protein-tagged *Escherichia coli* found that bacteria inoculated into a U-bend supplied with nutrients reached the drain outlet in one week [9]. In the present study, $>10^3$ cfu bacteria/swab was found within the visible biofilm in untreated washbasin drain outlets as well as on the washbasin surface in front of the outlets. In contrast, ECA-treated washbasins showed neither visible biofilm nor yielded detectable bacterial contamination within or adjacent to the drain outlets (Figure B, see online supplementary material). These findings show the efficacy of ECA decontamination to control biofilm within the drain outlet as well as the U-bend, impeding its ability to potentially contaminate the patient environment.

The majority of previous approaches to control hospital outbreaks linked to contaminated U-bends and drains have involved pouring chemicals down the drain outlets and/or replacing the washbasin and/or associated pipework [2,3,6,10]. Vergara-López *et al*. installed manual shut-off valves into sink drainage pipes, followed by 30-min treatment with a quaternary ammonium compound and subsequent flushing with hot water to control a *Klebsiella oxytoca* hospital outbreak [5]. A number of valves had to be manually operated prior to manual addition of the disinfectant, which may lead to air being trapped in the pipework, shielding some areas from disinfection. In contrast, the ECA decontamination system developed and tested in this study is automated and backfills the pipework from below each U-bend, reducing the likelihood of air being trapped. A recent study showed that sink-to-sink transmission can occur via a common wastewater pipe [9]. The approach used in this study minimizes opportunities for transmission of organisms between U-bends connected by common wastewater pipework, as the system decontaminates drains, U-bends and pipework.

In conclusion, microbial contamination of multiple hospital washbasin U-bends and drain outlets can be consistently minimized by automated ECA treatment.

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Conflict of interest statement
None declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jhin.2018.01.012.

References

[1] Cholley P, Thouverez M, Floret N, Bertrand X, Talon D. The role of water fittings in intensive care rooms as reservoirs for the colonization of patients with *Pseudomonas aeruginosa*. Intensive Care Med 2008;34:1428—33.

[2] Hota S, Hirji Z, Stockton K, Lemieux C, Dedier H, Wolfaardt G, et al. Outbreak of multidrug-resistant *Pseudomonas aeruginosa* colonization and infection secondary to imperfect intensive care unit room design. Infect Control Hosp Epidemiol 2009;30:25—33.

[3] La Forgia C, Franke J, Hacek DM, Thomson Jr RB, Robicsek A, Peterson LR. Management of a multidrug-resistant *Acinetobacter baumannii* outbreak in an intensive care unit using novel environmental disinfection: a 38-month report. Am J Infect Control 2010;38:259—63.

[4] Breathnach AS, Cubbon MD, Karunaharan RN, Pope CF, Planche TD. Multidrug-resistant *Pseudomonas aeruginosa* outbreaks in two hospitals: association with contaminated hospital waste-water systems. J Hosp Infect 2012;82:19—24.

[5] Vergara-López S, Domínguez MC, Conejo MC, Pascual Á, Rodríguez-Baño J. Wastewater drainage system as an occult reservoir in a protracted clonal outbreak due to metallo-β-lactamase-producing *Klebsiella oxytoca*. Clin Microbiol Infect 2013;19:E490—8.

[6] Leitner E, Zarfel G, Luxner J, Herzog K, Pekard-Amenitsch S, Hoenigl M, et al. Contaminated handwashing sinks as the source of a clonal outbreak of KPC-2-producing *Klebsiella oxytoca* on a hematology ward. Antimicrob Agents Chemother 2015;59:714—6.

[7] Chapuis A, Amoureux L, Bador J, Gavalas A, Siebor E, Chrétien ML, et al. Outbreak of extended-spectrum beta-lactamase producing *Enterobacter cloacae* with high MICs of quaternary ammonium compounds in a hematology ward associated with contaminated sinks. Front Microbiol 2016;7:1070.

[8] Swan JS, Deasy EC, Boyle MA, Russell RJ, O'Donnell MJ, Coleman DC. Elimination of biofilm and microbial contamination reservoirs in hospital washbasin U-bends by automated cleaning and disinfection with electrochemically activated solutions. J Hosp Infect 2016;94:169—74.

[9] Kotay S, Chai W, Guilford W, Barry K, Mathers AJ. Spread from the sink to the patient: in situ study using green fluorescent protein (GFP)-expressing *Escherichia coli* to model bacterial dispersion from hand-washing sink-trap reservoirs. Appl Environ Microbiol 2017;83:pii:e03327—16.

[10] Stjärne Aspelund A, Sjöström K, Olsson Liljequist B, Mörgelin M, Melander E, Påhlman L. Acetic acid as a decontamination method for sink drains in a nosocomial outbreak of metallo-β-lactamase-producing *Pseudomonas aeruginosa*. J Hosp Infect 2016;94:13—20.

[11] Kizny Gordon AE, Mathers AJ, Cheong EYL, Gottlieb T, Kotay S, Walker AS, et al. The hospital water environment as a reservoir for carbapenem-resistant organisms causing hospital-acquired infections — a systematic review of the literature. Clin Infect Dis 2017;64:1435—44.

[12] Fusch C, Pogorzelski D, Main C, Meyer C-L, el Helou S, Mertz D. Self-disinfecting sink drains reduce the *Pseudomonas aeruginosa* bioburden in a neonatal intensive care unit. Acta Paediatr 2015;104:e344—9.

[13] O'Donnell MJ, Boyle M, Swan J, Russell RJ, Coleman DC. A centralised, automated dental hospital water quality and biofilm management system using neutral Ecasol maintains dental unit waterline output at better than potable quality: a 2-year longitudinal study. J Dent 2009;37:748—62.

[14] Boyle MA, O'Donnell MJ, Miller A, Russell RJ, Coleman DC. Control of bacterial contamination of washbasin taps and output water using Ecasol: a one-year study. J Hosp Infect 2012;80:288—92.

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Whole-genome sequencing identifies highly related *Pseudomonas aeruginosa* strains in multiple washbasin U-bends at several locations in one hospital: evidence for trafficking of potential pathogens via wastewater pipes

E.M. Moloney [a], E.C. Deasy [a], J.S. Swan [b], G.I. Brennan [c], M.J. O'Donnell [a], D.C. Coleman [a,*]

[a] *Microbiology Research Unit, Division of Oral Biosciences, Dublin Dental University Hospital, University of Dublin, Trinity College, Dublin, Ireland*
[b] *Facilities Department, Dublin Dental University Hospital, Dublin, Ireland*
[c] *National MRSA Reference Laboratory, St. James's Hospital, Dublin, Ireland*

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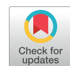

SUMMARY

Background: Hand washbasin U-bends have increasingly been associated with nosocomial outbreaks by Gram-negative bacteria, including *Pseudomonas aeruginosa* which is virtually ubiquitous in U-bends. Wastewater networks servicing U-bends are potential highways for trafficking pathogenic bacteria.
Aim: To use *P. aeruginosa* to investigate trafficking of bacteria between hospital washbasin U-bends.
Methods: Twenty-five washbasin U-bends in five locations in Dublin Dental University Hospital (DDUH) were investigated for trafficking of *P. aeruginosa*: 10 in Clinic 2 (C2), 10 in the Accident & Emergency Department (A&E) and five in three other locations. In addition, washbasin tap samples (N=80) and mains and tap water samples (N=72) were cultured for *P. aeruginosa*. Selected *P. aeruginosa* isolates recovered over 29 months underwent whole-genome sequencing, and relatedness was interpreted using whole-genome multi-locus sequence typing and pairwise single nucleotide polymorphism (SNP) analysis.
Findings: *P. aeruginosa* was recovered from all U-bends but not from taps or water. Eighty-three U-bend isolates yielded 10 sequence types (STs), with ST560 and ST179 from A&E, C2 and two other locations predominating (70%). ST560 was also recovered from a common downstream pipe. Isolates within ST560 and ST179 were highly related regardless of source. ST560 was divided into Cluster I (N=25) and Cluster II (N=2) with average allelic differences and SNPs of three and zero, and two and five, respectively. The 31 ST179 isolates exhibited an average allelic difference and SNPs of three and 12, respectively.
Conclusion: Highly related *P. aeruginosa* strains were identified in multiple U-bends in several DDUH locations, indicating trafficking via the wastewater network.

* Corresponding author. Address: Microbiology Research Unit, Division of Oral Biosciences, Dublin Dental University Hospital, University of Dublin, Trinity College, Lincoln Place, Dublin 2, Ireland. Tel.: +353 1 6127276; fax: +353 1 6127295.
E-mail address: david.coleman@dental.tcd.ie (D.C. Coleman).

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Introduction

Handwashing is vital to reduce the spread of infection. Ironically, while the presence of hand washbasins in hospitals promotes handwashing, it also provides increased associated risk of infection [1].

In hospitals, the wastewater pipe network is a complex and lengthy system servicing sanitary fixtures throughout the facilities. Wastewater pipes are constantly damp which encourages biofilm growth [2]. Wastewater networks open to the environment throughout hospital buildings in areas occupied by patients and staff at washbasin, sink and shower drains. Wastewater traps are a fundamental part of sanitary fixtures including washbasins, sinks, baths, showers and toilets, and prevent sewer gas entering buildings from wastewater pipes. Traps are situated below the drain outlet and consist of shaped pipework (e.g. U-bends) that retain water, forming a seal against the ingress of sewer gas [3]. This water stagnates when the fixtures are idle, and biofilms form readily within the retained water section of the trap and can extend to the fixture drain outlet [3]. Micro-organisms present in these biofilms can contaminate the washbasin and the surrounding environment, particularly if tap water directly impacts the drain causing splashing and aerosol formation [3–5].

Many studies have described nosocomial outbreaks caused predominantly by Gram-negative bacteria, associated directly or indirectly with contaminated washbasin and sink drains [1,4,6–9]. Furthermore, many recent reports have highlighted the importance of washbasin and sink drains in the nosocomial transmission of carbapenemase-producing Enterobacteriaceae, an emerging health threat globally [9].

Previous studies have demonstrated that bacteria present in washbasin and sink drains can be aerosolized by the impact of tap water flow, and can contaminate the washbasin, taps and local environmental surfaces [1,10,11]. An in-vitro study using a monoculture of a laboratory strain of *Escherichia coli* expressing green fluorescent protein (GFP) showed that biofilm in a sink U-bend model system grows upwards towards the sink drain outlet, and that subsequent splatter contaminates the bowl and surrounding area [5]. This study also showed trafficking of *E. coli* to adjacent sinks via common wastewater pipes. Deasy *et al*. [3] reported the growth of biofilm between washbasin U-bends and drain outlets in a hospital setting. It is not surprising that bacteria should spread from the U-bends of adjoining sanitary fixtures via common pipework, and over time perhaps via the wastewater pipe network to the U-bends of distantly located fixtures.

This study investigated whether individual strains of bacteria are distributed throughout a washbasin wastewater pipe network in order to provide evidence for strain trafficking in a hospital setting. For this purpose, *Pseudomonas aeruginosa* isolates from washbasin U-bends were used as a marker organism because this is among the most frequently encountered bacteria identified from hospital washbasin U-bends. The genetic relatedness of *P. aeruginosa* isolates from multiple washbasin U-bends at adjacent and distant sites in one hospital was investigated using whole-genome sequencing (WGS). The study also investigated whether regular decontamination of washbasin U-bends affects the population structure of *P. aeruginosa*.

Methods

Hand washbasins

Twenty-five ceramic hand washbasins at Dublin Dental University Hospital (DDUH) were investigated. Twenty-four were hospital-pattern (HP) washbasins with offset drain outlets [3,12]. One domestic pattern (DP) washbasin had the drain located directly below the tap water flow. Each HP washbasin tap had a thermostatic mixing valve set to provide water at 38°C. The DP washbasin had a manual mixer tap. All washbasins were used for handwashing alone with Tork extra mild liquid soap (SCA Hygiene Products Ltd, Dunstable, UK). All were in frequent use each day on weekdays, and fitted with identical polypropylene U-bends with two integrated sampling ports, as described in detail elsewhere [3]. Unscrewing a cap from each port permitted access to the U-bend interior for sampling.

Washbasins were selected to represent the diversity of large clinics and other areas in different DDUH locations. Ten HP washbasins were located in the Accident & Emergency Department (A&E; equipped with 11 washbasins in total) on the ground floor and 10 HP washbasins were located in Clinic 2 (C2; equipped with 15 washbasins in total) on the second floor. C2 and A&E were refurbished in August 2017 and 2016, respectively, with identical new washbasins, taps, U-bends and wastewater pipes [3]. Cold water was provided to washbasin taps from a 15,000-L tank supplied with mains water, which also supplied a calorifier providing hot water to the taps. Hot and cold water supplied to DDUH washbasins is treated with residual anolyte (2.5 ppm), an electrochemically activated disinfectant solution composed predominately of hypochlorous acid [13]. Both clinics operate as outpatient facilities on weekdays. Additional HP washbasins from different locations in DDUH included one in the Central Sterile Services Department (CSSD; equipped with one washbasin) on the first floor and three in West Clinic (WC; equipped with six washbasins) on the ground floor. The DP washbasin was in a staff bathroom on the third floor, distant from clinics (Figure S1, see online supplementary material).

The 10 washbasins investigated in C2 are located in five bays, each with three washbasins; two washbasins in each bay were included in this study. The U-bend of each C2 washbasin was connected via a 1-m vertical pipe to one of a series of five horizontal wastewater pipes, each of which serviced individual bays. Each pipe discharged water into an individual vertical pipe, which passed through the building into the basement (Figure S1, see online supplementary material). Three of the five vertical pipes connected to a larger common horizontal wastewater pipe connected to the municipal sewer at the building perimeter. The other two vertical pipes connected to a separate common horizontal wastewater pipe that discharged

wastewater to the municipal sewer at a separate outlet. The U-bends of each A&E washbasin were connected via 1-m vertical pipes to a common horizontal wastewater pipe that discharged water into a vertical pipe connected to the same large common wastewater pipe servicing C2. The CSSD washbasin discharged water into one of the vertical wastewater pipes servicing C2. The washbasin U-bends in WC and the DP washbasin discharge wastewater to the sewer system at different outlets to C2 and A&E (Figure S1, see online supplementary material).

Since their installation in August 2016, A&E washbasin U-bends have undergone automated decontamination three times each week, involving sequential treatments with two electrochemically activated (ECA) solutions generated from brine: catholyte (80 ppm NaOH) with detergent properties and anolyte (632 ppm HOCl) with disinfectant properties [3]. This involves completely filling U-bends with ECA solutions sequentially for 10 min each, facilitated by closing an electronic valve on the common wastewater outflow pipe [3]. Other DDUH washbasin U-bends were not decontaminated.

Testing water and taps for P. aeruginosa

Seventy-two 1-L water samples, eight from the washbasin cold water supply, eight from the mains supply and 56 from washbasin taps (including 16 each from A&E and C2), were tested for *P. aeruginosa*. Samples were taken in sterile bottles, neutralized with 0.5% sodium thiosulphate [14] and vacuum filtered through 0.45-μm filters (Sartorius, Göttingen, Germany), followed by incubation on *P. aeruginosa* selective agar (PSCN). Swab samples of 20 representative DDUH washbasin taps (including five each from A&E and C2) were sampled four times at 6-month intervals with swabs dipped in sodium thiosulphate (0.5%) and cultured on PSCN.

Recovery of P. aeruginosa from U-bends

U-bends were flushed with water prior to sampling. *P. aeruginosa* was recovered by swab sampling U-bend interiors via sampling ports using sterile cotton swabs (Venturi Transystem, Copan, Italy) dipped in 0.5% (w/v) sodium thiosulphate solution [3,13]. C2 U-bends were sampled once weekly for 52 weeks (*N*=520), whereas A&E U-bends were sampled immediately after decontamination, and 24 h and 48 h after decontamination for 52 weeks (*N*=1560). Average bacterial densities were calculated from these samples.

Swab tips were suspended in 1 mL of sterile phosphate buffered saline, vortexed and serially diluted, and 100-μL aliquots were spread in duplicate on Colombia blood agar (CBA), Reasoner's 2A (R2A) agar and PSCN as described elsewhere [3]. Presumptive *P. aeruginosa* isolates were recovered on PSCN, purified and identified using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF-MS) [3]. Isolates were stored at -80°C in Microbank cryovials (Prolab Diagnostics, Neston, UK). Unless otherwise stated, a single isolate from each sample was stored (see below).

Study design and isolate selection

C2 was selected as a model clinic to investigate the population of *P. aeruginosa* in washbasin U-bends (*N*=10) by WGS. A 6-month time frame was established for the selection of *P. aeruginosa* isolates to be sequenced (February–July 2018) to reduce WGS costs. Overall, 55 isolates were sequenced (Table S1, see online supplementary material). These included five *P. aeruginosa* isolates from at least three independent U-bends recovered monthly for the 6-month period (*N*=30). An additional 17 *P. aeruginosa* from one U-bend (B2D3) consisted of isolates recovered at intervals of at least 1 week over the 6 months. The remaining eight isolates consisted of separate *P. aeruginosa* isolates recovered in February 2019 from one B2D3 sample following completion of the sampling period.

Twenty-one *P. aeruginosa* isolates from ECA-treated A&E U-bends were investigated (Table S1, see online supplementary material). Isolates recovered over a longer sampling period (January 2017–March 2019) were selected for WGS because the majority of ECA-treated U-bend samples failed to yield *P. aeruginosa* [3]. Isolates from three time points were investigated: immediately after ECA treatment (*N*=7), 24 h after ECA treatment (*N*=7) and 48 h after ECA treatment (*N*=7).

Additional isolates from other locations in DDUH were included in this study: three from separate WC U-bends (June and July 2017), two from a CSSD U-bend (May and June 2017) and two from the DP washbasin U-bend (May and August 2017). Three additional isolates recovered in May 2019 from the main common wastewater collection pipe servicing C2 and A&E at the point of discharge into the municipal sewer were also investigated. In total, 83 U-bend isolates and three additional wastewater pipe isolates were selected for sequencing from DDUH.

A selection of *P. aeruginosa* comparator isolates from separate washbasin U-bends from two other Irish hospitals (*N*=9), from a dental chair water reservoir from a clinic outside of DDUH (*N*=2) and isolates previously recovered from dental suction systems (*N*=7) [15] were investigated as comparator isolates. The *P. aeruginosa* reference strains PA01 [16] and ATCC15442 [17] were also included. In total, 104 environmental isolates and two reference isolates were sequenced.

Whole-genome sequencing

Genomic DNA was extracted from *P. aeruginosa* using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Crawley, UK). Sequencing libraries were prepared using the Nextera DNA Flex Kit (Illumina, Eindhoven, The Netherlands), and paired-end reads were generated using the MiSeq Reagent Kit v2 (500 cycles) (Illumina) using the MiSeq sequencing platform. All isolate sequences passed quality metrics of average Q30 >30 with an average read coverage of $\geq$50X. Read qualities were checked using Galaxy software tools [18] and, where necessary, reads with a Phred score of <30 were trimmed using Trimmomatic software [18].

Genome assembly and analysis

The BioNumerics v7.6 (Applied Maths, Sint-Martens-Latem, Belgium) suite of software applications were used to analyse WGS data. FASTQ files were uploaded to BioNumerics, the raw reads were de-novo assembled and contigs were generated using SPAdes v6.4 [19]. The BioNumerics *P. aeruginosa* whole-genome (wg) multi-locus sequence typing (MLST) scheme

consisting of 15,136 loci was used for assembly-free and assembly-based allelic detection. The MLST profile of each isolate was determined using PubMLST (BioNumerics). Pairwise single nucleotide polymorphism (SNP) analysis was used. SNP filter exclusion parameters were set to remove potential indel-related SNPs (SNPs occurring within 12 bp), positions with ambiguous base calls, and SNPs in repeat regions. Minimal spanning trees (MSTs) were generated using BioNumerics, based on Kruskal's algorithm.

Statistical analysis

Statistical significance was determined using an unpaired, two-tailed Student's *t*-test with 95% confidence interval using GraphPad Prism v.5 (GraphPad, San Diego, CA, USA).

Results

P. aeruginosa from C2 and A&E U-bends

P. aeruginosa was recovered from all U-bends in C2 and A&E during the study. C2 U-bends were not decontaminated, whereas A&E U-bends were decontaminated three times each week with ECA solutions. The average bacterial densities from the 10 A&E U-bends showed a >4.4 log reduction on all media (CBA, R2A and PSCN) immediately after disinfection compared with the corresponding average bacterial densities from the 10 C2 U-bends over the 12-month study. Reductions were highly significant ($P<0.0001$) on all media. The average bacterial densities in C2 U-bends (520 samples, all *P. aeruginosa* positive) on CBA and PSCN were 1,862,000 (±678,076) colony-forming units (cfu)/swab and 1,547,000 (±807,633) cfu/swab, respectively. The corresponding average bacterial densities in A&E U-bends immediately after ECA treatment (520 samples, 6% *P. aeruginosa* positive) were 28.6 (±57.13) and 13.54 (±77.63) cfu/swab, respectively.

The approximate abundance of *P. aeruginosa* relative to other bacteria in U-bends from each clinic was determined. Over a period of 5 weeks, representatives of the different colony types recovered on CBA from A&E U-bends were identified using MALDI-TOF-MS. *P. aeruginosa* accounted for 58% of all identifiable colony types. Similarly, over a period of 2 weeks, *P. aeruginosa* accounted for 32% of all identifiable colony types from C2 U-bends.

P. aeruginosa STs in DDUH U-bends

Sequencing of 55 *P. aeruginosa* isolates selected from C2 U-bends yielded four STs (ST179, ST252, ST298 and ST560). ST179 and ST560 accounted for 49.1% (27/55) and 34.5% (19/55) of isolates, respectively (Table S1, see online supplementary material). C2 U-bend B2D3 was sampled weekly during the same period and 17 isolates from separate samples belonged to ST179 [nine isolates, average allelic difference of 1 (range 0–2)] and ST560 [eight isolates, average allelic difference of 1 (range 0–2)]. Eight isolates from one B2D3 sample belonged to ST179 [average allelic difference of 2 (range 0–7)]. Analysis of all C2 ST560 (*N*=19) and ST179 isolates (*N*=27) showed that isolates within each ST were very closely related [average allelic difference of 1 (range 0–4) and 2 (range 0–14), respectively].

Sequencing of 21 *P. aeruginosa* isolates selected from seven A&E U-bends yielded six STs, including ST308 (*N*=7), ST560 (*N*=4), ST773 (*N*=4), ST296 (*N*=3), ST179 (*N*=2) and ST27 (*N*=1). The four ST560 and two ST179 isolates exhibited an average allelic difference of 14 (range 0–35) and 0–7 allelic differences, respectively. Overall, the allelic difference range for the two most abundant STs recovered from C2 and A&E, ST179 and ST560, exhibited an average allelic difference of 3 (range 0–17) and 10 (range 0–64), respectively.

Seven *P. aeruginosa* isolates from three other washbasin U-bends in CSSD (*N*=2), WC (*N*=3) and the staff bathroom (*N*=2) yielded four STs (ST27, ST179, ST253 and ST560) (Table S1, see online supplementary material). Three isolates belonging to ST253 and ST560 were recovered from the main common wastewater pipe receiving wastewater from A&E, C2 and CSSD at the point of discharge to the municipal sewer.

P. aeruginosa STs among comparator isolates

The 11 *P. aeruginosa* isolates investigated from three other healthcare facilities (including nine isolates from washbasin U-bends in two acute hospitals) yielded eight STs (ST17, ST253, ST282, ST298, ST313, ST348, ST390 and ST395) (Table S1, see online supplementary material). Only two of these (ST253 and ST298) were identified in DDUH. The ST298 isolate from Hospital 2 exhibited 135 allelic differences to the ST298 isolates (*N*=6) from C2 (ST298 was not identified in A&E U-bends). Furthermore, the two ST253 isolates identified in the common wastewater pipe servicing C2 and A&E exhibited 53 allelic differences to the single ST253 isolate from Hospital 2. *P. aeruginosa* recovered from dental suction systems (*N*=7) yielded two STs including ST1320 [*N*=6; average allelic difference of 4 (range 0–11)] and ST2865 (*N*=1).

Population structure of DDUH and comparator P. aeruginosa

An MST based on wgMLST profiles was generated showing the STs of all isolates investigated (Figure 1a). Overall, eight *P. aeruginosa* STs were identified among 83 isolates from DDUH U-bends and three from the common wastewater pipe (ST27, ST179, ST252, ST253, ST296, ST298, ST308, ST560 and ST773). One of the predominant DDUH STs, ST179 (*N*=31), exhibited an average allelic difference of 3 (range 0–17), indicating that these isolates were very closely related (Figure 1a). Isolates of the second predominant ST, ST560 (*N*=27), exhibited an average allelic difference of 7 (range 0–64), suggesting that these isolates were more diverse. However, two isolate clusters were evident within ST560: Cluster I (*N*=25; average allelic difference of 3 (range 0–21)] and Cluster II (*N*=2; no allelic differences) (Figure 1a).

ST560 and ST179 isolates were also investigated by pairwise SNP analysis; these isolates exhibited an average of nine (range 0–66) and 12 SNPs (range 0–38), respectively (Figure 1b,c). ST560 Cluster I isolates exhibited an average of two SNPs (range 0–8) (including isolates from C2, A&E, CSSD and the common wastewater pipe), whereas the two ST560 Cluster II isolates (from A&E) exhibited five SNPs. Cluster II was differentiated from Cluster I by 59 SNPs (Figure 1b). ST179 isolates (including isolates from C2, A&E and staff bathroom) exhibited an average of 12 SNPs (range 0–38) (Figure 1c). Two

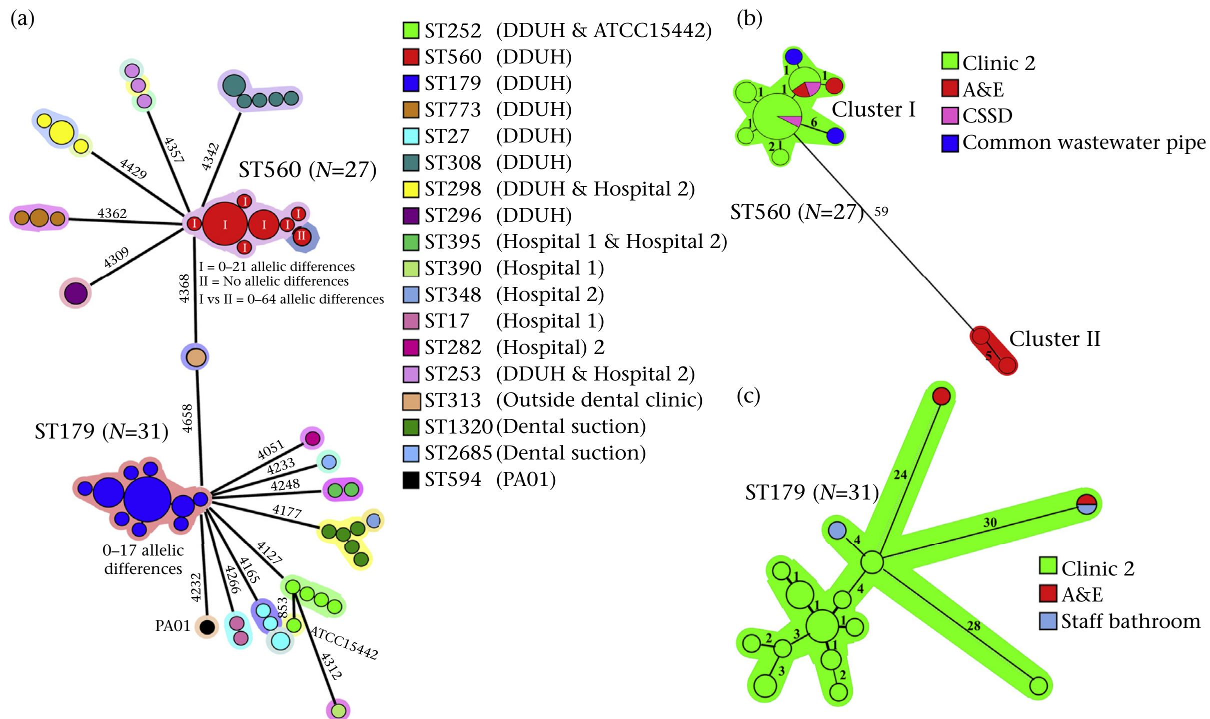

Figure 1. Minimum spanning trees (MSTs) based on whole-genome multi-locus sequence typing (wgMLST) and single nucleotide polymorphism (SNP) data of *Pseudomonas aeruginosa* isolates. (a) An MST based on wgMLST data of all 106 isolates investigated showing the relationships between the sequence types (STs) identified including the PA01 and ATCC15442 reference strains. Numbers on the branches show allelic differences between ST isolate groupings. The colour-coded key to the right of the figure identifies STs and the origin of isolates within each ST. Eighty-three isolates (ST179, ST252, ST298, ST560, ST308, ST773, ST296 and ST27) were recovered from U-bends at Dublin Dental University Hospital (DDUH) and three isolates (ST253 and ST560) were recovered from the main common wastewater pipe servicing washbasins in Clinic 2 (C2), the Accident & Emergency Department (A&E) and the Central Sterile Services Department (CSSD). ST179 and ST560 accounted for 37.3% (31/83) and 32.5% (27/83) of the total DDUH isolates sequenced. ST560 isolates had two distinct clusters: Cluster I consisted of 25 isolates, whereas Cluster II consisted of two isolates. ST179 and ST560 were not identified among comparator isolates investigated. The allelic threshold of relatedness for *P. aeruginosa* isolates was set at <14 allelic differences, as suggested previously [29]. (b) An MST based on the SNP analysis of the 27 ST560 DDUH isolates recovered from C2, A&E, CSSD and the main common wastewater pipe. The threshold of isolate relatedness was set at <37 SNP differences, as suggested previously [31]. The isolates formed two distinct groups, Clusters I and II, differentiated by 59 SNPs. The average number of SNPs within the 25 isolates of Cluster I was two (range 0–8), whereas the two Cluster II isolates differed by five SNPs. Isolates within each of the two clusters revealed by SNP analysis corresponded to the same isolates identified within the two ST560 clusters identified by wgMLST analysis. These findings confirmed that isolates within each cluster were very closely related. (c) An MST based on the SNP profiles of the 31 ST179 DDUH isolates recovered from C2, A&E and the staff bathroom. Isolates differed by an average of 12 (range 0–38) SNPs.

isolates from U-bends in A&E and the staff bathroom exhibited no SNPs (Figure 1c).

Effects of A&E U-bend decontamination on the P. aeruginosa population structure

Of the 21 isolates sequenced from A&E, four STs were identified immediately after U-bend decontamination (ST296, ST308, ST560 and ST773), four were identified 24 h after disinfection (ST27, ST179, ST308 and ST560) and three were identified 48 h after disinfection (ST296, ST308 and ST773). Isolates from ST308, ST296, ST773 and ST560 were identified between two or more of the sampling time points. ST308 was the only ST recovered at all three time points, and the seven isolates exhibited an average allelic difference of 4 (range 0–8).

Testing DDUH water for P. aeruginosa

P. aeruginosa was not detected in the potable mains water supply (*N*=8), the anolyte-treated water supply to washbasin taps (*N*=8) or washbasin tap water (*N*=56). Swab sampling of 20 DDUH washbasin taps including five each from C2 and A&E on four occasions each also failed to detect *P. aeruginosa*.

Discussion

Washbasin U-bends and drains have increasingly been identified as reservoirs for nosocomial infections [5,6,8,20]. Here, *P. aeruginosa* was used as a marker organism for washbasin U-bend contamination, where it is virtually ubiquitous, and many reports have linked nosocomial transmission of *P. aeruginosa* to contaminated U-bends [20–22]. WGS was used

to investigate the distribution of *P. aeruginosa* STs in washbasin U-bends in DDUH focusing on two separate clinics on different floors, each with a common water supply, similar usage and equipped with identical washbasins, U-bends and wastewater pipes. The wastewater pipes from each clinic discharged into common outflow pipes connected to the municipal sewer (Figure S1, see online supplementary material). C2 U-bends were not decontaminated during the study, whereas A&E U-bends were decontaminated three times each week. Consequently, the burden of *P. aeruginosa* in A&E U-bends immediately after decontamination was significantly reduced with a >4.4 log reduction in overall bacterial counts over 12 months relative to the corresponding bacterial counts in C2 U-bends. Nonetheless, *P. aeruginosa* was recovered from every washbasin investigated during the study. A previous study investigated the *P. aeruginosa* population diversity at two wastewater sampling sites in a French hospital using MLST, and identified 15 different STs from 30 samples [23]. Here, the diversity of *P. aeruginosa* in C2 U-bends yielded only four STs among 55 isolates, of which ST179 and ST560 predominated (83.6%). Six STs were identified among 21 *P. aeruginosa* isolates from A&E U-bends, with ST179 and ST560 accounting for 28.6%. All STs identified have been recovered previously from the environment, and all except ST296 have been associated with clinical infections [24–28].

In this study, the allelic and SNP thresholds of relatedness for *P. aeruginosa* isolates were set at <14 allelic differences and <37 SNPs, as suggested previously [29–31]. Isolates within ST179 and ST560 were very closely related based on wgMLST and SNP analyses regardless of the location of recovery (Figure 1 and Figure S1, see online supplementary material). ST560 represented 32.5% of all DDUH isolates investigated and was only recovered from C2, A&E and CSSD U-bends, and the main wastewater pipe common to all three. The average allelic differences and SNPs within ST560 isolates were 7 (range 0–64) and 9 (range 0–66), respectively (Figure 1). However, on closer inspection, the 27 ST560 isolates were grouped into two clusters: Cluster I (N=25) and Cluster II (N=2). Cluster I isolates exhibited average allelic and SNP differences of 3 (range 0–21) and 2 (range 0–8), respectively, whereas Cluster II isolates exhibited no allelic differences and five SNPs (Figure 1a,b). Similarly, ST179 accounted for 37.3% of all DDUH isolates investigated and was only recovered from C2, A&E and staff bathroom U-bends. The average allelic differences and SNPs within ST179 isolates were 3 (range 0–17) and 12 (range 0–38), respectively (Figure 1a,c). Interestingly, an ST179 isolate (E24Aug) from an A&E U-bend and an ST179 isolate (LP3F2) from the staff bathroom U-bend exhibited zero SNPs (Figure 1 and Table S1, see online supplementary material). These U-bends are located at opposite ends of DDUH, separated by a distance of approximately 132 m. These results revealed the presence of very closely related *P. aeruginosa* isolates in washbasin U-bends in several different areas of DDUH (i.e. C2, A&E, CSSD and staff bathroom) and in one of the main wastewater outflow pipes. C2 washbasin U-bend B2D3 was selected to investigate the diversity of isolates in an individual U-bend. Seventeen isolates recovered at intervals of at least 1 week belonged to ST179 (N=9) and ST560 (N=8), and isolates within each ST were very closely related [both with an average allelic difference of 1 (range 0–2)]. These findings reveal the persistence and stability of isolates in an individual U-bend, at least during the 6-month period when isolates were sequenced. At the end of the study, eight isolates from one sample from B2D3 belonged to ST179 and exhibited an average allelic difference of 2 (range 0–7). Isolates from A&E U-bends were included to investigate the effects of regular U-bend decontamination on the diversity of *P. aeruginosa*. The abundance and prevalence of *P. aeruginosa* in A&E U-bends was significantly lower than non-decontaminated U-bends elsewhere in DDUH and the range of STs was slightly higher (6 vs 4); however, the majority of ST560 (25/27) and all ST179 (N=31) isolates from the former were very closely related to isolates from the latter (Figure 1b,c).

P. aeruginosa isolates in U-bends could have originated from the supply water, taps, water discharged down U-bends and wastewater pipes. Hot and cold water supplying washbasin taps in DDUH has been treated continuously with residual anolyte (2.5 ppm) since 2012 [13]. During this study, *P. aeruginosa* was not isolated from washbasin taps, mains water, the anolyte-treated washbasin water supply or tap output water. A previous year-long study from DDUH also failed to detect *P. aeruginosa* in anolyte-treated washbasin tap water or taps from the same sites [13]. Anolyte readily penetrates biofilms in water systems and this is very likely to be a significant factor contributing to the failure to detect *P. aeruginosa* in washbasin water and taps in the present and previous studies [3,13,14]. These findings suggest that supply water was unlikely to be a significant source of *P. aeruginosa* in U-bends; otherwise, a wider range of STs would be expected. It is highly unlikely that the residual anolyte (2.5 ppm) used to treat washbasin tap water in DDUH had any significant effect on bioburden in DDUH U-bends due to high densities of bacteria recovered from non-decontaminated U-bends. A previous study demonstrated that small amounts of organic matter (i.e. 1 mg/mL of bovine serum albumin) completely neutralize the free available chlorine present in 100 ppm anolyte, which is a concentration 40 times higher than that used to treat washbasin water in DDUH [32]. *P. aeruginosa* can be carried transiently on the hands [33]. However, if handwashing was a frequent contributor of *P. aeruginosa* to U-bends, a far wider range of STs would be anticipated. The detection of highly related strains in U-bends in four separate DDUH locations (C2, A&E, CSSD and staff bathroom) and the main wastewater outflow pipe indicates that the wastewater pipe network is a more likely contributor to U-bend contamination. Interestingly, ST560 and ST179 isolates were recovered from U-bends in adjacent clinical bays in C2. Washbasins in individual bays in C2 do not share common proximal wastewater pipes; common pipework occurs more distally in the network, suggesting that trafficking of isolates occurs from more downstream regions (Figure S1, see online supplementary material). The low diversity identified in *P. aeruginosa* from U-bends in this study may be associated with ECA decontamination of A&E U-bends. Large volumes of spent ECA solutions are discharged following decontamination, which likely reduces the burden of *P. aeruginosa* in downstream pipework, and this may reduce trafficking [3].

Trafficking of bacteria in wastewater pipes could occur by wastewater flow, bacterial motility and air currents. Water discharged down washbasin drains can traffic bacteria in U-bends and pipes to distal sites in the network. As mentioned above, the model U-bend and wastewater system supplied with nutrients in the absence of tap water flow demonstrated that an *E. coli* strain expressing GFP exhibited an average growth of

1 inch/day along the pipework [5]. Flagellar motility has been shown to be an essential element in the ability of *P. aeruginosa* to form biofilms on surfaces and tissues [34]. Air currents occur in wastewater pipes both by air flow down into the sewer and within wastewater networks [35]. The flow of water in pipes results in a partial vacuum that draws air behind the flow of water. All three methods likely contribute to the dissemination of related strains in wastewater networks.

In conclusion, previous studies suggested that washbasin U-bends and associated fittings are potential highways for trafficking potentially pathogenic bacteria [5,11]. This study confirmed this using high-resolution WGS typing for the first time, and demonstrated the distribution of highly related strains of *P. aeruginosa* in multiple washbasin U-bends in different locations in a hospital setting. Consideration should be given to effective decontamination of wastewater pipes in hospitals, at least in critical areas. The use of ECA solutions for this purpose has already yielded encouraging results [3,36].

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Conflict of interest statement
None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2019.11.005.

References

[1] Hota S, Hirji Z, Stockton K, Lemieux C, Dedier H, Wolfaardt G, et al. Outbreak of multidrug-resistant *Pseudomonas aeruginosa* colonization and infection secondary to imperfect intensive care unit room design. Infect Control Hosp Epidemiol 2009;30:25–33.

[2] Dancer SJ. Controlling hospital-acquired infection: focus on the role of the environment and new technologies for decontamination. Clin Microbiol Rev 2014;27:665–90.

[3] Deasy EC, Moloney EM, Boyle MA, Swan JS, Geoghegan DA, Brennan GI, et al. Minimizing microbial contamination risk simultaneously from multiple hospital washbasins by automated cleaning and disinfection of U-bends with electrochemically activated solutions. J Hosp Infect 2018;100:e98–104.

[4] Breathnach AS, Cubbon MD, Karunaharan RN, Pope CF, Planche TD. Multidrug-resistant *Pseudomonas aeruginosa* outbreaks in two hospitals: association with contaminated hospital waste-water systems. J Hosp Infect 2012;82:19–24.

[5] Kotay S, Chai W, Guilford W, Barry K, Mathers AJ. Spread from the sink to the patient: in situ study using green fluorescent protein (GFP)-expressing *Escherichia coli* to model bacterial dispersion from hand-washing sink-trap reservoirs. Appl Environ Microbiol 2017;83: e03327–16.

[6] Snitkin ES. Contamination of hospital plumbing: a source or a sink for antibiotic-resistant organisms? Contamination of Hospital Plumbing Invited Commentary. JAMA Netw Open 2019;2:e187660.

[7] Mombini S, Rezatofighi SE, Kiyani L, Motamedi H. Diversity and metallo-β-lactamase-producing genes in *Pseudomonas aeruginosa* strains isolated from filters of household water treatment systems. J Environ Manage 2019;231:413–8.

[8] Hopman J, Meijer C, Kenters N, Coolen JPM, Ghamati MR, Mehtar S, et al. Risk assessment after a severe hospital-acquired infection associated with carbapenemase-producing *Pseudomonas aeruginosa*. JAMA Netw Open 2019;2:e187665.

[9] Kizny Gordon AE, Mathers AJ, Cheong EYL, Gottlieb T, Kotay S, Walker AS, et al. The hospital water environment as a reservoir for carbapenem-resistant organisms causing hospital-acquired infections — a systematic review of the literature. Clin Infect Dis 2017;64:1435–44.

[10] De Geyter D, Blommaert L, Verbraeken N, Sevenois M, Huyghens L, Martini H, et al. The sink as a potential source of transmission of carbapenemase-producing Enterobacteriaceae in the intensive care unit. Antimicrob Resist Infect Control 2017;6:24.

[11] Aranega-Bou P, George RP, Verlander NQ, Paton S, Bennett A, Moore G, et al. Carbapenem-resistant Enterobacteriaceae dispersal from sinks is linked to drain position and drainage rates in a laboratory model system. J Hosp Infect 2019;102:63–9.

[12] Department of Health, UK. Health building note (HBN) 00-10 Part C: sanitary assemblies 2013. London: DoH; 2013. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/148497/HBN_00-10_Part_C_Final.pdf [last accessed May 2019].

[13] Boyle MA, O'Donnell MJ, Miller A, Russell RJ, Coleman DC. Control of bacterial contamination of washbasin taps and output water using Ecasol: a one-year study. J Hosp Infect 2012;80:288–92.

[14] O'Donnell MJ, Boyle M, Swan J, Russell RJ, Coleman DC. A centralised, automated dental hospital water quality and biofilm management system using neutral Ecasol™ maintains dental unit waterline output at better than potable quality: a 2-year longitudinal study. J Dent 2009;37:748–62.

[15] O'Donnell MJ, Tuttlebee CM, Falkiner FR, Coleman DC. Bacterial contamination of dental chair units in a modern dental hospital caused by leakage from suction system hoses containing extensive biofilm. J Hosp Infect 2005;59:348–60.

[16] Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, et al. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature 2000;406:959.

[17] Wang Y, Li C, Gao C, Ma C, Xu P. Genome sequence of the non-pathogenic *Pseudomonas aeruginosa* strain ATCC 15442. Genome Announc 2014;2: e00421–14.

[18] Afgan E, Baker D, Van den Beek M, Blankenberg D, Bouvier D, Čech M, et al. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. Nucleic Acids Res 2016;44:W3–10.

[19] Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 2012;19:455–77.

[20] Lowe C, Willey B, O'Shaughnessy A, Lee W, Lum M, Pike K, et al. Outbreak of extended-spectrum β-lactamase–producing *Klebsiella oxytoca* infections associated with contaminated hand-washing sinks. Emerg Infect Dis 2012;18:1242.

[21] Ferranti G, Marchesi I, Favale M, Borella P, Bargellini A. Aetiology, source and prevention of waterborne healthcare-associated infections: a review. J Med Microbiol 2014;63:1247–59.

[22] Decker BK, Palmore TN. The role of water in healthcare-associated infections. Curr Opin Infect Dis 2013;26:345.

[23] Slekovec C, Plantin J, Cholley P, Thouverez M, Talon D, Bertrand X, et al. Tracking down antibiotic-resistant *Pseudomonas aeruginosa* isolates in a wastewater network. PLoS One 2012;7:e49300.

[24] Ruiz-Roldán L, Bellés A, Bueno J, Azcona-Gutiérrez JM, Rojo-Bezares B, Torres C, et al. *Pseudomonas aeruginosa* isolates from Spanish children: occurrence in faecal samples, antimicrobial

resistance, virulence, and molecular typing. Biomed Res Int 2018;2018:8060178.

[25] Hilliam Y, Moore MP, Lamont IL, Bilton D, Haworth CS, Foweraker J, et al. *Pseudomonas aeruginosa* adaptation and diversification in the non-cystic fibrosis bronchiectasis lung. Eur Respir J 2017;49:1602108.

[26] Kocsis B, Toth A, Gulyas D, Ligeti B, Katona K, Rokusz L, et al. Acquired *qnrVC1* and *blaNDM-1* resistance markers in an international high-risk *Pseudomonas aeruginosa* ST773 clone. J Med Microbiol 2019;68:336–8.

[27] Subedi D, Vijay AK, Kohli GS, Rice SA, Willcox M. Comparative genomics of clinical strains of *Pseudomonas aeruginosa* strains isolated from different geographic sites. Sci Rep 2018;8:15668.

[28] Domitrovic TN, Hujer AM, Perez F, Marshall SH, Hujer KM, Woc-Colburn LE, et al. Multidrug resistant *Pseudomonas aeruginosa* causing prosthetic valve endocarditis: a genetic-based chronicle of evolving antibiotic resistance. Open Forum Infect Dis 2016;3:ofw188.

[29] Dekker JP, Frank KM. Next-generation epidemiology: using real-time core genome multilocus sequence typing to support infection control policy. J Clin Microbiol 2016;54:2850–3.

[30] Schürch AC, Arredondo-Alonso S, Willems RJL, Goering RV. Whole genome sequencing options for bacterial strain typing and epidemiologic analysis based on single nucleotide polymorphism versus gene-by-gene-based approaches. Clin Microbiol Infect 2018;24:350–4.

[31] Snyder LA, Loman NJ, Faraj LJ, Levi K, Weinstock J, Boswell TC, et al. Epidemiological investigation of *Pseudomonas aeruginosa* isolates from a six-year-long hospital outbreak using high-throughput whole genome sequencing. Eurosurveillance 2013;18:pii:20611

[32] Boyle MA, O'Donnell MJ, Russell RJ, Coleman DC. Lack of cytotoxicity by Trustwater Ecasol^{TM} used to maintain good quality dental until waterline output water in keratinocyte monolayer and reconstituted human oral epithelial tissue models. J Dent 2010;38:930–40.

[33] Widmer AF, Wenzel RP, Trilla A, Bale MJ, Jones RN, Doebbeling BN. Outbreak of *Pseudomonas aeruginosa* infections in a surgical intensive care unit: probable transmission via hands of a health care worker. Clin Infect Dis 1993;16:372–6.

[34] Vater SM, Weiße S, Maleschlijski S, Lotz C, Koschitzki F, Schwartz T, et al. Swimming behavior of *Pseudomonas aeruginosa* studied by holographic 3D tracking. PLoS One 2014;9:e87765.

[35] Gormley M, Aspray TJ, Kelly DA, Rodriguez-Gil C. Pathogen cross-transmission via building sanitary plumbing systems in a full scale pilot test-rig. PLoS One 2017;12:e0171556.

[36] Swan JS, Deasy EC, Boyle MA, Russell RJ, O'Donnell MJ, Coleman DC. Elimination of biofilm and microbial contamination reservoirs in hospital washbasin U-bends by automated cleaning and disinfection with electrochemically activated solutions. J Hosp Infect 2016;94:169–74.