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A longitudinal study of the microbial colonisation patterns in a hospital water distribution system and its impact on health

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Submitted in accordance with the requirements for the degree of Masters by Research

York St John University

School of Science Technology and Health

May 2025

Declaration

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List of abbreviations/acronyms

Abbreviations/acronyms	Full meaning
PW	Potable Water
HTM 0401	Health Technical Memorandum 04-01
TMM	Traditional microbiology methods
OPPPs	Opportunistic premise plumbing pathogens
PCR	Polymerase chain reaction
WDS	Water Distribution System
HCAI	Health Care Associated Infections
UKHSA	United Kingdom Health Security agency
SDEC	Same Day Emergency Care
MAC3	MacConkey no3
PCA	Pseudomonas Cetrimide Agar
VIA	Vancomycin Imipenem Amphotericin B
TVC	Total Viable Count
TNC	Too Numerous to Count
PP	Pin Prick
CG	Confluent Growth
API NE 20	Analytical Profile Index Non-Enterobacteriaceae
EUCAST	European Committee on Antimicrobial Susceptibility Testing
AST	Antimicrobial Susceptibility Testing
EPS	Extracellular Polymeric Substances
ICU	Intensive Care Unit
NICU	Neonatal Intensive care Unit
PICU	Paediatric Intensive Care Unit
UTI	Urinary Tract Infection
BSI	Blood Stream Infections
ASDR	Age Standardised per Rate
VAP	Ventilator Associated Pneumonia
LMIC	Low- and Middle-Income Countries
XDR	Extensively Drug Resistant
CF	Cystic Fibrosis

Abstract

Water delivered to taps is not sterile and harbours many microbial species necessitating prior treatments. Water companies use different methods to eliminate and control microbes prior to the point-of-use and hospitals have further requirements for water safety and infection control purposes. Despite these measures, pathogens still find their way into water and represent a threat to vulnerable patients. Thirteen opportunistic premise plumbing pathogens (OPPPs) found in water have been proposed but only two are routinely tested for by current methods (i.e., HTM 04-01).

A total of 345 water samples were collected from a new hospital building opening to patients in 2025 and analysed using traditional microbiological methods (TMM) to identify bacterial species. This included culture techniques and polymerase chain reaction (PCR) amplification to confirm bacterial identity at the genus-species level. The most commonly identified species recovered across 11 sampling occasions were various *Pseudomonas* genera (*P. stutzeri* (most frequently isolated)), *P. alcaligenes*, *P. luteola*, and *P. oryzihabitans*), *Burkholderia cepacia*, *Alcaligene faecalis*, *Aeromonas* spp., *Acinetobacter* spp., and *Stenotrophomonas maltophilia*.

To investigate if more time-efficient and accurate methodology was available, next-generation sequencing was performed using the Oxford Nanopore MinION long-read sequencing technology. Analysis was performed on an additional sampling occasion and directly compared using filtration, DNA extraction, and PCR on 16S rRNA gene regions. Results indicate that MinION identified more bacterial species present in extracted DNA from sampled water than TMM. MinION sequencing identified eight additional bacterial genera, including *Sphingomonas* spp., *Cupriavidus* spp., and *Elizabethkingia* spp., with all classified as potential opportunistic pathogens. This demonstrates limitations in TMM which restricts isolation and identification due to unmet growth conditions.

Overall, there was a good match between both methodologies and coupling nucleotide sequencing with existing TMM could enhance microbiology detection processes, facilitating earlier interventions and limiting the impact of microbial contamination on healthcare systems.

Table of Contents

Declaration	ii
Acknowledgement	iii
List of abbreviations/acronyms	iv
Abstract	v
Table of Figures	viii
List of Tables	ix
1 Introduction	1
1.1. Potable Water	1
1.2 Water regulations	2
1.3. Opportunistic Pathogens	3
1.4 Bacterial Biofilms	5
1.4 Hospital equipment	6
1.6. Bacterial Colonisation	7
1.7. Water Safety Testing	8
1.8. Water-Associated Healthcare Pathogens	11
<i>Pseudomonas</i> species	11
<i>Stenotrophomonas maltophilia</i>	13
<i>Burkholderia cepacia</i>	14
<i>Mycobacterium</i> species	15
<i>Methylobacterium</i> spp.	16
<i>Aeromonas</i> spp.	17
<i>Acinetobacter</i> spp.	17
<i>Cupriavidus</i> spp.	17
<i>Sphingomonas</i> spp.	18
1.9. Detection of OPPP in UK Hospitals	21
1.10. Project Background	22
2. Materials and Methods	24
2.1. Overview of Water Sampling Process	24
2.2. Bacterial Maintenance and Growth Media	24
2.3. Bacterial Collection	25
2.4. Water Collection	25
2.5. Water Processing	27
2.6. Colony Selection and Primary Identification	27
2.7. Biochemical Tests	28
2.7.1. Oxidise Test	28

2.7.2 Analytical Profile Index 20 NE	28
2.8. Antimicrobial Susceptibility Testing	28
2.9. Polymerase Chain Reaction (PCR) Primers and Assay Conditions	29
2.10. Electrophoresis	31
2.11. Water Seeding and DNA Extraction	31
2.12. Nucleotide Sequencing	31
3. Results	33
Part 1. Characterisation of Bacteria by Traditional Microbiology Techniques	33
3.1 Water Processing Primary Identification and Enumeration	33
3.2 Identification Using Patch Plates	37
3.3 Natural Water vs. Treated Water (Whernside and Well Water)	38
3.4 Further Identification Using Biochemical tests (API 20NE)	40
3.5 Polymerase Chain Reaction (PCR)	40
3.6 Collected Species Identification from Hospital Isolates	41
3.7 Antimicrobial Susceptibility Testing	42
3.8. Summary from Part 1.	45
Part 2. Molecular Characterisation of Bacterial Colonisation Patterns.	46
3.9. Introduction to Long-Read Next Generation Sequencing for Microbial Identification.	46
3.10. Sample Preparation for MinION Sequencing.	46
3.11. Sample Validation by PCR Analysis	47
3.12. Analysis of Reads by Kraken2 and Minimap2	49
3.13. Summary from Part 2.	52
4. Discussion	53
5. Limitations	66
6. Conclusion	68
Bibliography	Error! Bookmark not defined.
Appendix	82
Appendix 1* Hospital floor plan	82
Appendix 1.1 The first-floor plan	83
Appendix 1.2 The ground floor plan	84
Appendix 2* Minion pie charts	85

Table of Figures

Figure 1. Water sample pre-flush protocol.....	9
Figure 2. Figure 4. Examples of bacterial growth on 0.45µm nitrocellulose filter membranes on R2A following a 48h incubation period at 37°C.....	34
Figure 3. Distribution of colony growth classification.....	35
Figure 4. Distribution of colony growth classification.....	35
Figure 5. Proximal samples from outlet location CG3001/HW, over three sampling occasions on 0.45µm Nitrocellulose filter membranes on R2A.....	36
Figure 6. Further identification on selective / differential media (Mac3, PCA, VIA) using patch plates.....	37
Figure 7. Wild water vs. well water vs. industrial water vs hospital water patch plated on MAC3.....	39
Figure 8. Well water samples on selective/differential media patch plates.....	40
Figure 9. 16S rRNA gene amplification.....	47
Figure 10. Sequence analysis to genus-level identification using EPI2ME software and Kraken2.....	50
Figure 11. Sequence analysis to genus-level identification using EPI2ME software and Minimap2.....	51
Figure 12. <i>Pseudomonas aeruginosa</i> and other clinically relevant species biofilm interactions.....	57

List of Tables

Table 1. Blood stream infections of <i>Pseudomonas</i> species between 2014 and 2018.....	12
Table 2. Bloodstream infections by <i>S. maltophilia</i> species in the UK from 2014-2018.....	13
Table 3. Bloodstream infections by a genus of other gram-negative, closely related microorganisms in the UK from 2014-2018.....	14
Table 4. Mortality and morbidity of OPPP's, with typical setting, risks.....	18
Table 5. Reference strains and their origin.....	25
Table 6. Outlet type, code and room name.....	26
Table 7. Antibiotics and their concentration are used in antimicrobial susceptibility testing according to EUCAST guidelines and their Zones Of Inhibition (ZOI). Resistant (R), Intermediate resistance (IR).....	29
Table 8. 16S RNA reverse and forward primers, reference cultures, and PCR conditions used in molecular identification.	30
Table 9. Isolation and location of OPPP's from the outlets of Scarborough Hospital during the construction phase (no public access) from 17th of June 2024-11th of December 2024.....	42
Table 10. Table 10. Antimicrobial Susceptibility Profiles of Bacterial Isolates.....	43
Table 11. Antibacterial suceptibility testing on isolated and identified strains.....	44
Table 12. Antibacterial suceptibility testing on isolated and identified strains of <i>Stenotrophomonas maltophilia</i>	45
Table 13. DNA extraction from filters prior to 16S gene amplification.....	48
Table 14. Comparison between reference cultures vs results from Kraken2 vs Minimap2.....	50
Table 15. MinION reads and bacteria identified from the Minimap2 channel with barcode order.....	52

1 Introduction

1.1. Potable Water

Potable/wholesome water must not contain any chemicals, microorganism's, parasite or substance, whether alone or in combination, at a concentration or value that would constitute a potential danger to human health (*Information note on Regulation 4 - Drinking Water Inspectorate*, 2022). Wholesome water is delivered to our taps for hygiene and consumption. Water delivered to the point of use for these purposes is not required to be sterile and is a natural reservoir for many microorganisms ('Guidelines for Drinking-water Quality FOURTH EDITION WHO Library Cataloguing-in-Publication Data Guidelines for drinking-water quality-4th ed', 2011).

UK water companies have a statutory duty to provide “clean” water, and the microbial quality of water is maintained by water treatment processes including by chlorination and routine testing for indicator organisms between reservoirs and outlets. Wholesome water has multiple other public health safety indicators including taste, chemicals and the presence of metals and it must be of satisfactory taste and meet threshold values for the presence of contaminants harmful to health, including metals (mainly mercury and lead), compounds such as nitrates and arsenic, and microbes (WHO, 2017). Water companies are legally obligated to reduce pathogenic microbes to the acceptable level defined by national or international water regulations (Lechevallier, 2024). However, the range of microorganisms tested is extremely narrow. For example, testing for *Escherichia coli* is completed in service reservoirs, and coliforms and Enterococci testing is conducted at consumer taps (*Guidance on implementing the Water Supply (Water Quality) Regulations - Drinking Water Inspectorate*, 2016). Not all coliforms indicate faecal contamination considering that Coliforms are a broad group of Gram-negative, lactose-fermenting bacteria that include genera such as *Escherichia*, *Klebsiella*, *Enterobacter*, and *Citrobacter*. While *E. coli* is a strong and specific indicator of recent faecal contamination, other coliforms—particularly *Klebsiella* and *Enterobacter*—can originate from environmental sources such as soil, vegetation, and biofilms in water systems (*Thermotolerant coliforms | Australian Drinking Water Guidelines*, 2011). To determine water as safe from microbial contamination, samples of 100 ml of water must be free from these species respectively (“The Water Supply (Water Quality) Regulations 2016,” 2016). When these microorganisms are found in the sample, it would require immediate remedial action to avoid a public health incident (Weinbren, 2020). Therefore, there are water regulations set legally

binding standards for the quality, monitoring and supply of drinking water to protect public health and ensure water is safe and acceptable for consumption.

1.2 Water regulations

The regulation of drinking water in the United Kingdom is underpinned by a robust legal framework designed to safeguard public health, maintain high water quality, and assign clear legal responsibilities to those in control of various premises. Drinking water regulations apply differently to public buildings, hospitals, and households within the UK, reflecting the varied risk profiles, usage patterns, and legal responsibilities across these settings. While households rely on water companies to supply wholesome water to the domestic tap, public buildings and hospitals are subject to stricter monitoring and duty-holder obligations under regulations such as Approved Code of Practice L8 (ACOP L8) and Health Technical Memorandum 04-01 (HTM 04-01), requiring proactive risk assessments, sampling regimes, and remedial actions to ensure water remains safe at the point of use.

All hospitals in the United Kingdom are legally required to implement the guidance set out in Health Technical Memorandum (HTM) 04-01: Safe Water in Healthcare Premises, which mandates specific design, installation, commissioning and operational measures for hot and cold-water systems to mitigate the risk of *Legionella*, *Pseudomonas aeruginosa* and other opportunistic waterborne pathogens. These water systems are engineered for stringent microbial control, with hot water stored at or above 60 °C and circulated to maintain at least 50°C at clinical outlets, and cold mains kept below 20°C, all distributed through copper, stainless steel, and high-grade polymer networks subject to targeted retrofits every 10–15 years to mitigate corrosion and biofilm formation (Department of Health, 2016b).

As hospitals have HTM 04-01, public buildings have approved code of practice (ACOP) L8 as guidance in order to control *Legionella* bacteria in water system. Public Buildings are referred in this project as “industrial” for example universities and other large premises for public use. ACOP L8 typically mirror these storage set-points from HTM 04-01 (HSE, 2013). To deliver mixed water at 50–55°C via circulation loops designed primarily for user convenience rather than pathogen suppression; their 20–60-year-old systems of copper, CPVC, or galvanised steel require periodic (*Guide G: Public health and plumbing engineering (2014)* | CIBSE, 2014). No additional tests are required for public buildings.

In contrast to healthcare and other public facilities which controls temperatures due to health and safety and regulations requirements domestic supplies lack active thermal regulation or

recirculation. Temperature control can be variable dependent on the pipes/valves chosen for the household and many households have simple hot and cold-water system (*Guide G: Public health and plumbing engineering (2014) | CIBSE, 2014*). Domestic water typically has a more simple water distribution system but it could still have a dead legs (section that is barely used) and stagnation. Regardless, it may present lower risk than hospital water because; 1. the people using it are typically healthier and less vulnerable to infection, and 2. the systems themselves are usually smaller, simpler, and have fewer areas where water can stagnate and support bacterial growth.

Therefore, especially in hospitals, despite water management systems, many other species of bacteria and other microorganisms that reside in potable water remain undetected by the currently required microbial tests (Riccardi, Rotulo and Castagnola, 2019). Hospital water systems serve high-risk populations such as the elderly, immunocompromised, or those with chronic illnesses, where even low levels of opportunistic pathogens can pose a threat (Papadakis *et al.*, 2025).

1.3. Opportunistic Pathogens.

Opportunistic pathogens (OP) are microorganisms that generally do not cause illness in healthy individuals but can provoke serious infections when the host's immune system is impaired ('Opportunistic Pathogens: A Hidden Threat to Human Health', 2025). Alongside posing infection risk, OP's share similar traits, including their habitat is a natural water reservoir and treatments are often complex (Yiek *et al.*, 2021) especially in the augmented care units (*NHS England » Health Technical Memorandum 04-01: Safe water in healthcare premises, 2016*). While some of them may be easy to treat but prolong patients stay in hospitals, others are multidrug-resistant where treatments are lengthy with accompanying toxicity and not always successful. In addition, they have the ability to form biofilms (see below) in pipe premises, making them even less susceptible to antibiotics. The reason why these opportunistic pathogens are found in drinking water is that reservoirs the water is supplied to us are natural habitat for many microorganisms.

Water not only provides a good habitat but is playing important role in their preservation. Preservation of microbes is by providing a stable environment that support cellular integrity and mitigates external stressors, playing a crucial role in maintaining membrane structure and biomolecular stability through bond water interactions, which are essential for microbial survival even under desiccated conditions (Manzanera, 2021). In aqueous systems, water buffers against thermal fluctuations such as freeze thaw cycles, reduces oxidative damage that

can occur during dehydration and acts as a diluent for toxic agents and solute stressors, lowering their impact on microbial cells (Manzanera, 2021). Additionally, water facilitates electrostatic screening, protecting cells from ion-induced membrane disruption. In environments with moderate to high relative humidity, vapour-phase water supports prolonged viability of both spores and vegetative cells. Microbes have been shown to persist in diverse natural reservoirs—including freshwater, seawater, brines, permafrost, and hypersaline fluid inclusions—sometimes for millennia, underscoring water’s role as a medium for long-term microbial preservation. This phenomenon is particularly relevant in environmental microbiology, hospital water safety, and microbial diagnostics (Hallsworth, 2022).

Water not only preserves microbes but also helps their transmission, where even small spills can spread over large areas and breach the integrity of equipment that should be free of microbial contamination. This is especially important in a hospital environment, where the use of water plays a substantial role in patients’ hygiene, cleanliness, sanitation, and waste management, alongside general consumption (Decker and Palmore, 2014). Pathogens and opportunistic bacteria in water can spread through direct or indirect contact, either through ingestion and aspiration of contaminated water or inhalation of aerosols (Kanamori, Weber and Rutala, 2016). Even more healthcare-associated infections are connected to water-containing hospital equipment (Yiek *et al.*, 2021). In a hospital building environment, several factors may unintentionally facilitate waterborne pathogens, including water temperatures suitable for bacterial growth and the intricate design and age of the water system, which can lead to corrosion, stagnation, and biofilm formation (Ji *et al.*, 2015). Numerous water reservoirs in healthcare facilities, including potable water, have been connected to water-related healthcare-associated infection (HCAI) outbreaks (Perkins *et al.*, 2019).

A report by the National Health Service Scotland described an outbreak in Glasgow. In 2018, between January to September, there were 23 bloodstream infection cases from eleven different microorganisms connected to a contaminated water system across the Royal Hospital for Children and Queen Elizabeth University Hospital (National Health Service Scotland, 2019). These infections were identified using traditional microbiology and molecular techniques to the water distribution system. Testing revealed the water system was contaminated. To resolve the outbreak, point of use filters were installed in showers and handwash basins and drain a decontamination process was undertaken (National Health Service Scotland, 2019).

Bacteria and other microorganisms can also adhere to surfaces, (e.g., door handles, medical equipment and inside the lumen of drinking water pipes), forming symbiotic cluster

communities protected by biofilms. Many microorganisms can coexist as a consortium in the same biofilm in polymicrobial biofilms (Jose Alves *et al.*, 2014).

1.4 Bacterial Biofilms

Biofilms protect the microbial cells from disinfectants (e.g., chlorine) and drinking water conditions make good growth conditions for bacterial growth (Sanchez-Vizuet *et al.*, 2015). Biofilms are structured communities of microorganisms, encased within a self-produced extracellular matrix (ECM) made of extracellular polymeric substances (EPS), composed primarily of polysaccharides, proteins, lipids, and extracellular DNA. Biofilms promote the exchange of antibiotic resistance genes by concentrating cells and stabilising extracellular DNA, thereby enhancing horizontal gene transfer via conjugation, transformation, and transduction. The matrix retains DNA released from lysed cells, increasing natural competence and facilitating uptake by neighbouring bacteria, while the dense, multispecies architecture supports direct plasmid transfer through conjugative pili and broad interspecies exchange. This protected niche amplifies the dissemination of resistance determinants across diverse taxa, turning biofilms into hotspots for antibiotic resistance evolution and persistence (Liu, Prentice and Webber, 2024). ECM masks pathogen-associated molecular patterns, impairs complement activation and phagocytic uptake, and physically restricts diffusion of antibodies and antimicrobial peptides, while embedded cells downregulate immunogenic surface proteins and upregulate stress-response pathways. Together, these features transform biofilms into protected reservoirs that both locate new human niches and persist against innate and adaptive immune defences (Roilides *et al.*, 2015). Beside avoidance of immune defences ECM anchors the cells, facilitates communication through quorum sensing, and provides protection against environmental stresses and antimicrobial agents (Francolini and Donelli, 2010).

Quorum sensing is a cell-to-cell communication mechanism in bacteria that enables coordinated behaviour based on population density. It involves the production and release of small signalling molecules, known as autoinducers, which accumulate in the environment as the bacterial population grows. When a critical concentration of these molecules is reached, they bind to specific receptors—either membrane-bound or cytoplasmic—triggering signal transduction pathways that alter gene expression across the community. Gram-negative bacteria typically use freely diffusible acyl-homoserine lactones (AHLs) (Ng and Bassler, 2009). Conceptual framework of quorum sensing has expanded beyond simple population-density sensing, showing that bacteria can use it to collectively interpret environmental cues—akin to a

“wisdom of the crowds” effect—thereby improving decision-making accuracy under fluctuating conditions (Moreno-Gámez, Hochberg and van Doorn, 2023).

Biofilms are far more problematic in healthcare premises because they establish persistent reservoirs of opportunistic pathogens in fixtures that generate aerosols and directly contact vulnerable patients. Hospital’s sink drains and traps, for example, harbour dense, disinfectant-resistant biofilms that routinely seed *Legionella*, *Pseudomonas aeruginosa* and other nosocomial agents into splash zones and ambient air, contributing to outbreaks of healthcare-associated infections traced to organisms residing below drain lines (Whitacre, 2025).

As established, water systems can provide a favourable environment for bacterial proliferation, however biofilms add to shield microbial cells from disinfectants such as chlorine (Sanchez-Vizuite *et al.*, 2015). This is especially important in a hospital environment, where the use of water plays a great role in patients’ hygiene, consumption, cleanliness, sanitation, and waste management (Decker and Palmore, 2014). Biofilms can develop on both living tissues and inert surfaces, including medical devices, plumbing systems, and natural aquatic environments. In healthcare settings, they are of particular concern because they can harbour opportunistic pathogens, contribute to persistent infections, and resist conventional cleaning and disinfection measures, making them a critical focus for infection prevention and control strategies (Maillard and Centeleghe, 2023). These biofilms and OP’s carry a considerable concern in hospitals are healthcare-associated infections are connected to water-containing hospital equipment (Yiek *et al.*, 2021).

1.5. Hospital equipment

Medical devices and support systems that incorporate water as part of their function—either in open reservoirs, semi-closed circuits, or internal fluid pathways. These include heater-cooler units, haemodialysis machines, neonatal incubators, dental unit waterlines, fluid warmers, nebulizers, water baths, and water traps. While essential for patient care, such equipment can inadvertently serve as reservoirs or amplifiers of microbial contamination if not properly maintained (Yiek *et al.*, 2021).

Microbial contamination arises when water within these devices supports the growth of opportunistic pathogens. Warm temperatures, stagnant flow, and biofilm formation create ideal conditions for microbial persistence and proliferation. Contaminated water can lead to healthcare-associated infections (HAIs) through various transmission routes: direct contact (e.g., hydrotherapy), aerosol inhalation (e.g., nebulizers or cooling units), ingestion (e.g., contaminated ice), or indirect contact via improperly disinfected surfaces and tubing (*D. Water* |

Infection Control | CDC, 2017). However, even with manufacturer's guidelines for maintenance, routine use can poise infection risks, particularly to immunocompromised patients (England, 2025).

1.6. Bacterial Colonisation

Bacterial/host colonisation is in most cases facilitated by biofilms. Enabling bacterial communities to adhere to mucosal or abiotic surfaces, form microcolonies, and sense host-derived signals that trigger dispersal of planktonic cells primed for attachment and invasion (Liu, Prentice and Webber, 2024). The complex design and material choices in healthcare plumbing further exacerbate this risk. Networks of copper, CPVC (Chlorinated Polyvinyl Chloride) and stainless steel—subject to corrosion, micro abrasion and leaching—provide ideal niches for biofilm maturation, while regulated hot- and cold-water set-points and routine use of disinfectants and antibiotics in the environment exert selective pressure favouring multidrug-resistant strains. As a result, hospital biofilms not only persist despite aggressive cleaning protocols but also become hot spots for the exchange and amplification of resistance determinants among diverse taxa (Franco *et al.*, 2020).

Many clinical infections have been connected to biofilms, and evidence implies that biofilms contribute to pathogenesis, particularly in chronic infections (Vestby *et al.*, 2020). Biofilm infections are very difficult to eradicate because of cells within biofilm had alerted gene expression, antimicrobial protection, evasion of immune system, reduced metabolism and persisted cell formation leading to device or chronic tissue associated infections (Lichtenberg *et al.*, 2023). Comparing to planktonic bacteria that exist as free-floating single cell infections, considering their resistance mechanisms are absent, typically respond to targeted treatment, easier to treat. Biofilms can also act as reservoirs, intermittently releasing planktonic cells that cause recurrent acute episodes (*WOUND INFECTION IN CLINICAL PRACTICE Principles of best practice*, 2022). Therefore, biofilms within hospital water systems represent a significant and persistent burden, serving as reservoirs for opportunistic pathogens, increasing the risk of healthcare-associated infections (Capelletti *et al.*, 2016).

Bacteria and their biofilm that reside in natural reservoirs are often referred to as waterborne pathogens and can populate Water Distribution Systems (WDS) to the point of customer use (Yiek *et al.*, 2021). The biofilm formation repeatedly forms and resides in plumbing premises, have been referred to as Opportunistic Premise Plumbing Pathogens (OPPP's) (Weinbren, 2020). Additionally, they are more likely to cause infections in healthcare facilities compared to the general population (Yiek *et al.*, 2021). Previously, it was assumed that water-associated

acquired infections in hospitals spread from wash stations or drains to patients and that bacteria moved from patients to the sink and taps, which formed biofilm and then spread to other patients who used this facility (Weinbren, 2020). Hospital water and wastewater systems are now recognised as complex, interconnected microbial habitats capable of sustaining diverse opportunistic pathogens (Gettler, Smith and Lewis, 2023).

Before 2012, the regulatory HTM 04-01 contained requirements for *Legionella* spp. only, although after 2012 another species, *Pseudomonas aeruginosa*, was added to HTM 04-01. However, even before 2012, there was already substantial evidence demonstrating *Pseudomonas aeruginosa* as a water-related infection transmission risk. Furthermore, while an update of HTM 04-01 was underway in 2011/2012, a *P. aeruginosa* water-related outbreak caused the death of 4 neonates (Wise, 2012). With media assistance, this was “the last drop that overflows the glass” which helped to support the changes. At the Royal Jubilee Maternity Hospital in Belfast, *P. aeruginosa* has been identified as the pathogen that caused the outbreak. Three premature neonates died from bloodstream infections; a further three premature neonates were infected, and one died later. In other research, analysis of five neonates found *P. aeruginosa* on the skin (Wise, 2012).

To evaluate the association of *P. aeruginosa* with taps from the neonate unit, 494 parts were dismantled from flow straighteners and thirty taps. Traditional microbiology techniques involving selective and non-selective agar were used alongside molecular methods (variable tandem repeat analyses). In addition, biofilms were visualised by scanning electron microscopy. All this resulted in finding biofilms of *P. aeruginosa* as a possible contamination source of infections (Walker *et al.*, 2014). This led to urgent bio-decontamination being carried out (Wise, 2012). There were many other water-related *P. aeruginosa* outbreaks; however, the Belfast hospital incident pushed the Department of Health to amend the already existing legally enforced document, HTM 04-01, to add *P. aeruginosa*, alongside existing *Legionella* during water testing in hospital premises. In the same document, there was mention of three gram-negative pathogens (*Mycobacteria*, *Stenotrophomonas maltophilia* and *Burkholderia cepacia*); however, water testing for them has not been enforced.

1.7. Water Safety Testing

HTM 04-01, provides guidance for the construction and management of water on healthcare premises. The document focuses on measures to control *Legionella* spp. and *P. aeruginosa*. There is also a brief mention of other species that are related with water-associated infections like *Stenotrophomonas maltophilia*, *Mycobacteria* spp. and *Burkholderia cepacia* in order to

minimise bacterial colonisation in the hospital water distribution system (WDS). Hospital water is tested for *Legionella* spp. and *P. aeruginosa* every 6 months. The test for *P. aeruginosa* has to yield 0 colonies in 100 ml; the testing is repeated in 6 months. If results show 1 to 10 Colony-Forming Units (cfu) per 100 ml, the water safety group will carry out the use of a water risk assessment, and the water outlet will be simultaneously retested (Figure 1). If the results yield more than 10 cfu/100 ml, further sampling will be carried out with an engineering survey to identify if the contamination is systemic or localised, and modifications will be carried out to improve water quality and ensure patient safety.

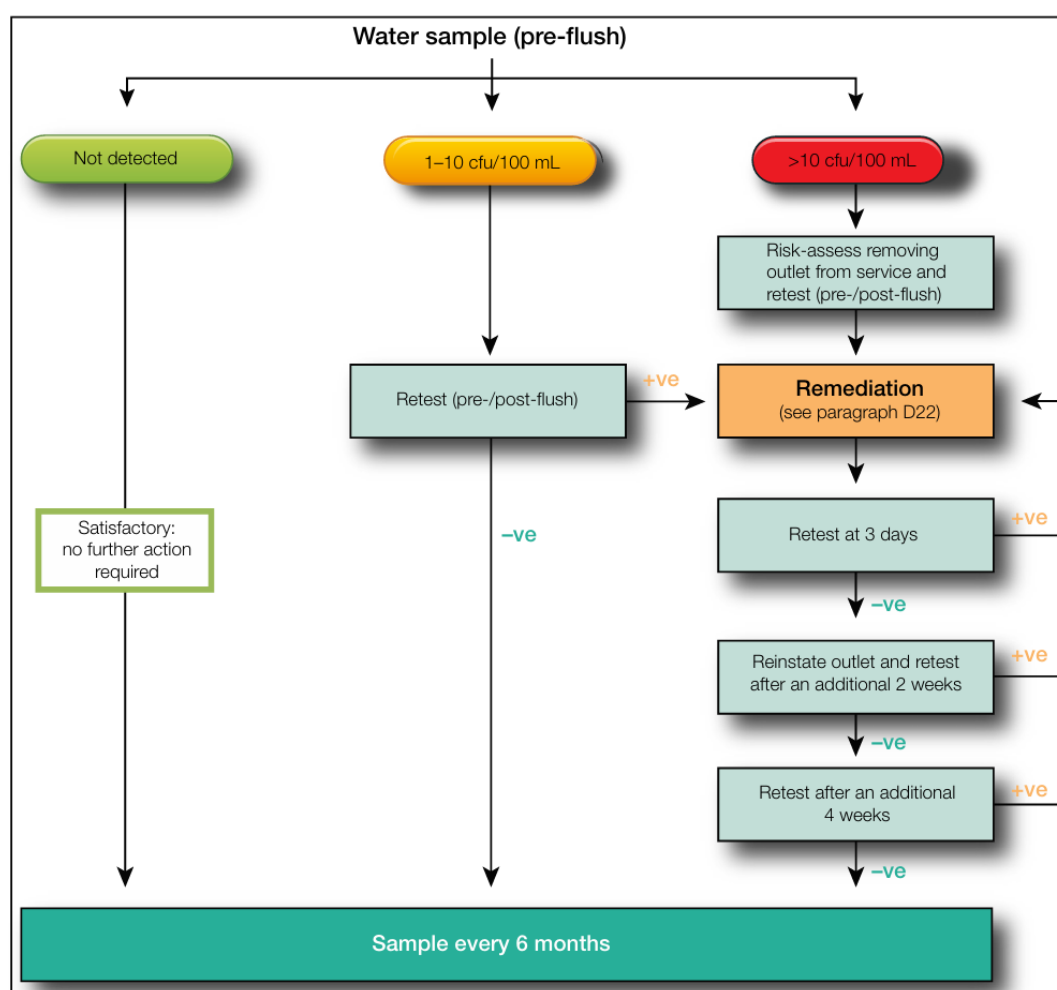


Figure 1. Water sample pre-flush protocol. The green pathway shows if no bacteria is detected, the orange shows if 1-10 cfu/100 ml is detected and necessary actions are taken, and the red pathway shows necessary actions and interventions if 100 ml has more than 10 (Department of Health, 2016a).

To confirm if water contamination is local or systemic, proximal (collected immediately upon tap activation) and distal (collected after 2 minutes of flushing) then water samples are

collected and analysed (Department of Health, 2016a). The proximal water sample, if positive, could indicate that the contamination is local up to 2 metres upstream (Figure 1). However, if the distal water sample was positive (after flushing through the tap), indicating systemic water contamination, the whole WDS would need to be assessed, including the water supply and storage process. Based on the result, the right intervention will be used to treat the water further (Ling *et al.*, 2018). Another simple technique of checking microbiological quality used today is high bacterial count/total viable count (TVC) (Diez-Gonzalez, 2014). Total viable count (TVC) testing offers a rapid, quantitative estimate of live microorganisms—bacteria, yeasts, and moulds—in water samples, providing a broad snapshot of system contamination and overall water quality. As a non-selective method, TVC captures a diverse microbial population, and dual-temperature incubations at 22 °C and 37 °C distinguish ambient environmental flora from organisms capable of surviving at body temperature. By monitoring TVC trends over time, facilities can detect early increases in microbial load before specific pathogens emerge, enabling proactive maintenance such as flushing, filtration, or disinfection adjustments (J. Wang *et al.*, 2023). However, due to a lack of research, it is still unclear if the high TVC count is indicative of the presence of bacterial pathogens. Furthermore, this technique may be used to take counts before disinfection and 48 hours after the disinfection procedure to check its efficacy (Department of Health, 2016a).

HTM 04-01 only covers the control of a small number of OPPP's, *Legionella spp.* and *P. aeruginosa*. For *Stenotrophomonas maltophilia* testing and water sampling should be carried out as per *P. aeruginosa*. *Mycobacteria spp.* have only been mentioned in this document, but no routine testing has been suggested. The full list of OPPPs as described by Weinbren (2020) are:

- *Legionella spp.*,
- *Pseudomonas aeruginosa*,
- *Stenotrophomonas maltophilia*,
- *Burkholderia spp.*,
- *Mycobacteria spp.*,
- *Aeromonas spp.*,
- *Acinetobacter baumannii*,
- *Sphingomonas spp.*,
- *Cupriavidus spp.*,
- *Methylobacterium spp.*,
- *Ralstonia spp.*,

- *Alcaligenes faecalis*,
- *Elizabethkingia* spp.,

These OPPPs can be frequently found in pipes, taps, showerheads, and other water-related facilities within a range of residential, commercial, and industrial settings (Falkinham, Pruden and Edwards, 2015; Hilborn *et al.*, 2015; Inkster *et al.*, 2024).

1.8. Water-Associated Healthcare Pathogens

All of these bacterial species share the following traits. 1. They can cause high mortality and morbidity in a vulnerable individual. 2. Their preferred habitat is a water reservoir, and 3. They can cause nosocomial infections (Yiek *et al.*, 2021).

Pseudomonas species

Pseudomonas spp. is an abundant and very adaptable genus found in a handful of habitats, such as soil, plants, animals, and water (fresh, marine, and drinking water) (Kahli *et al.*, 2022). There are 25 species of *Pseudomonas* that can cause opportunistic infections and can be divided into two groups: fluorescent species (including *P. fluorescens*, *P. aeruginosa*, and *P. putida*) and non-fluorescent species/alcaligenes group (*P. alcaligenes*, *P. stutzeri*, etc.) (Kumar *et al.*, 2019).

Pseudomonas spp, are a clinical concern because of resistance to antibiotics: *Pseudomonas putida* and *Pseudomonas fluorescens* have been implicated in opportunistic infections, often showing resistance to multiple β -lactams and fluoroquinolones (Molina *et al.*, 2014).

Furthermore, *Pseudomonas stutzeri*, while generally more susceptible, has exhibited multi-drug resistance in immunocompromised patients (Bisharat, Gorlachev and Keness, 2012).

Pseudomonas aeruginosa is the cause of significant mortality and morbidity, with antibiotic resistance being a contributory factor, making *P. aeruginosa* a global public health threat (Qin *et al.*, 2022). *Pseudomonas aeruginosa* (Table 1) bloodstream infections have 30-day mortality rates of 27–39 percent, driven by factors such as severe sepsis, pneumonia, and delays in effective antimicrobial therapy (Kang *et al.*, 2003).

Antibiotic resistance in the genus *Pseudomonas*—particularly *Pseudomonas aeruginosa*—is a major global health concern due to its intrinsic defences (low outer membrane permeability, efflux pumps, inducible AmpC β -lactamase) and its ability to acquire additional resistance determinants. Recent hospital surveillance has shown high rates of multidrug resistance (MDR), with a Zambian tertiary care study reporting 73.6% MDR prevalence in *P. aeruginosa* isolates

from nosocomial infections, frequently linked to carbapenem hydrolysing β -lactamase genes, such as *blaOXA-51* (Mukomena *et al.*, 2024). Similarly, a 2023 study found 58.4% MDR prevalence, with the highest resistance to meropenem and ciprofloxacin (both 23.4%), underscoring the erosion of key therapeutic options (Shbaita *et al.*, 2023). Carbapenem-resistant *P. aeruginosa* (CRPA) is increasingly reported worldwide, with a recent meta-analysis highlighting its global spread and association with metallo- β -lactamases (VIM- Verona integron-encoded metallo- β -lactamase; IMP; Imipenemase metallo- β -lactamase; NDM - New Delhi metallo- β -lactamase) and OXA-type enzymes (Ramatla *et al.*, 2025). *P. aeruginosa* can rapidly adhere to wet surfaces and create biofilm, thus *P. aeruginosa* has a natural reservoir in the built environment in infrastructure such as taps, sinks, and any equipment in contact with potable water in healthcare settings (Qin *et al.*, 2022).

A report from Public Health England showed that from all 65 000 gram-negative bacteraemia's total number of reported cases were over 290 000 between 2014 and 2018. From which *P. aeruginosa* has shown gradual increase. Over a 5-year period (2014-2018), approximately 23,000 bloodstream infections were reported by Public Health England (PHE) (Table 1). The clinical importance of this genus can be seen in Table 1, which also shows that the most isolated species of all *Pseudomonas* genus isolates is *P. aeruginosa*, representing over 80% of all *Pseudomonas* cases.

Table 1. Blood stream infections of *Pseudomonas* species between 2014 and 2018. As a leading cause for infection being *P.aeruginosa*, but there are also *P. stutzeri*, *P. putida*, and *P. fluorescens* (Public Health England, 2019).

	2014		2015		2016		2017		2018	
	No.	%	No.	%	No.	%	No.	%	No.	%
<i>Pseudomonas</i> spp.	3,885	100	4,211	100	4,424	100	4,887	100	4,745	100
<i>P. aeruginosa</i>	3,171	82	3,428	81	3,556	80	4,024	82	3,778	80
<i>P. alcaligenes</i>	4	<1	7	<1	8	<1	5	<1	4	<1
<i>P. citronellolis</i>	0	0	0	0	0	0	1	<1	2	<1
<i>P. fluorescens</i> group*	56	1	38	1	44	1	30	1	30	1
<i>P. koreensis</i>	1	<1	2	<1	1	<1	8	<1	2	<1
<i>P. libanensis</i>	0	0	0	0	0	0	1	<1	1	<1
<i>P. luteola</i>	2	<1	4	<1	8	<1	11	<1	9	<1
<i>P. mendocina</i>	7	<1	5	<1	6	<1	17	<1	13	<1
<i>P. oleovorans</i>	0	0	1	<1	5	<1	1	<1	2	<1
<i>P. otitidis</i>	1	<1	0	0	0	0	0	0	2	<1
<i>P. paucimobilis</i>	63	<1	56	1	65	1	62	1	54	1
<i>P. putida</i> group**	87	2	103	2	140	3	172	4	163	3
<i>P. stutzeri</i>	94	2	92	2	84	2	81	2	84	2
<i>P. thomasii</i>	0	0	1	<1	1	<1	0	0	0	0
<i>Pseudomonas</i> spp., other named	78	2	44	1	34	1	21	<1	19	<1
<i>Pseudomonas</i> spp., sp. not recorded	320	8	428	10	468	11	449	9	380	8

* *P. fluorescens* and *P. tolaasii*

** *P. putida*, *P. fulva*, *P. monteilii*, *P. moselii*, and *P. oryzae*

Stenotrophomonas maltophilia

Observations over 5-year period (2014-2018), *S. maltophilia* infections present around 2,000 cases, less than 1% of total burden, however clinical relevance remains significant because of resistance to multiple antibiotics, especially in immunocompromised population and water associated outbreaks. The second most common cause of water-associated gram-negative infections is *S. maltophilia*. *S. maltophilia* is increasingly recognized as an emerging multi-drug-resistant (MDR) opportunistic pathogen, and several national agencies (e.g., CDC, Public Health England/UKHSA, ECDC) highlight it as a pathogen of concern because of its intrinsic resistance and limited treatment options (*EUCAST: Guidance on Stenotrophomonas maltophilia - updated November, 2024.*, 2024). *S. maltophilia* exhibits intrinsic resistance to most other antibiotic classes, including β -lactams, carbapenems, and aminoglycosides, mediated by multiple resistance mechanisms such as L1 metallo- β -lactamases, L2 cephalosporinases, and multidrug efflux pumps. Trimethoprim-sulfamethoxazole (SXT) remains the recommended first line therapy, with the most consistent *in vitro* activity and clinical outcome data. In accordance

with CLSI and EUCAST guidance, other agents are generally not tested unless specifically requested for cases of intolerance, resistance, or complex infection (Rhoads, 2021).

Table 2 shows isolated *Stenotrophomonas* spp. from the bloodstream in the UK, indicating that the most common species from the *Stenotrophomonas* genus is *S. maltophilia*. Furthermore, Table 3 indicates other closely related organisms like *Brevibacterium* spp. and *Burkholderia* spp. accounted for 45% and 39% of bloodstream infections (Public Health England, 2019).

Table 2. Bloodstream infections by *S. maltophilia* species in the UK from 2014-2018.

Reported infections from 2 different *Stenotrophomonas* spp., as *S. maltophilia* as a leading cause for infection, and *Stenotrophomonas* infections where species were not recorded (Public Health England, 2019).

	2014		2015		2016		2017		2018	
	No.	%	No.	%	No.	%	No.	%	No.	%
<i>Stenotrophomonas</i> spp.	516	100	464	100	483	100	477	100	434	100
<i>S. acidaminiphila</i>	0	0	1	<1	0	0	3	1	2	<1
<i>S. maltophilia</i>	473	92	456	98	479	99	469	98	427	98
<i>Stenotrophomonas</i> spp., sp. not recorded	43	8	7	2	4	1	5	1	5	1

Burkholderia cepacia

Across the period 2014-2018, cases of *B. cepacia* complex (Bcc) infections are around 250 cases which is under 1% of all bacteraemia cases (Public Health England, 2019). While uncommon, Bcc remains clinically significant in immunocompromised patients and is associated with environmental reservoirs, including hospital water systems and medical products (Public Health of England, 2018).

In the past, *B. cepacia* was considered as a single bacterial species. Since the 1990s, researchers have identified 23 species that are genetically and phenotypically related as known as *Burkholderia cepacia* complex (BCC). All members of this complex are now recognised as opportunistic pathogens (Tavares *et al.*, 2020). These opportunistic pathogens are widespread and often reside in aqueous environments including drinking water (Zanetti, De Luca and Stampi, 2000). *B. cepacia* complex (BCC) infections are associated with significant morbidity and mortality (Table 4), particularly in immunocompromised patients and those with underlying lung disease such as cystic fibrosis (*About Burkholderia cepacia complex | B. cepacia | CDC*, 2025). Their intrinsic resistance to multiple antimicrobial classes and ability to cause severe, rapidly progressive pneumonia make them a critical concern for infection prevention and control in healthcare environments (*About Burkholderia cepacia complex | B. cepacia | CDC*, 2025).

Table 3 highlights gram-negative bacteria which contribute to bacteraemia, with the highest number of infections from *Burkholderia* spp. occurring in 2015 and 2016, before the number of infections decreased to 49 in 2017 and 2018.

Table 3. Bloodstream infections by a genus of other gram-negative, closely related microorganisms in the UK from 2014-2018. *Brevundomonas* spp. and *Burkholderia* as the two-leading gram-negatives (Public Health England, 2019).

	2014		2015		2016		2017		2018	
	No.	%	No.	%	No.	%	No.	%	No.	%
Related organisms	94	100	121	100	112	100	112	100	109	100
<i>Brevundimonas</i> spp.	40	43	38	31	36	32	38	34	43	39
<i>Burkholderia</i> spp.	31	33	64	53	62	55	49	44	49	45
<i>Comamonas</i> spp.	13	14	12	10	9	8	9	8	10	9
<i>Ralstonia</i> spp.	8	9	4	3	4	4	10	9	2	2
<i>Shewanella</i> spp.	2	2	3	2	1	1	6	5	5	5

Mycobacterium species

In contrast, management guidelines for *Mycobacterium* spp. are difficult to establish, as it is resistant to disinfection, an ability to form clumps and biofilm (Lechevallier, 2024). It has been documented that the natural environment for both *Mycobacterium tuberculosis* and non-tuberculosis mycobacteria is aquatic and terrestrial (Vaerewijck *et al.*, 2005). *Mycobacteria* have often been detected in treated drinking water and plumbing systems, meaning there is a significant source of *Mycobacteria* exposure in drinking water (Dowdell *et al.*, 2019). Nontuberculous mycobacteria (NTM) have been repeatedly detected in treated drinking water and building plumbing systems worldwide, making them a significant and persistent source of human exposure. Surveys have reported NTM in 11–38% of raw water samples and 20–50% of treated municipal water samples (Vaerewijck *et al.*, 2005), with detection rates in hospital plumbing often exceeding 60% of outlets tested (Feazel *et al.*, 2009). Common species include *Mycobacterium avium* complex (MAC), *M. kansasii*, and *M. xenopi*, which are well adapted to survive in low-nutrient, low-oxygen environments and can persist within biofilms and free-living amoebae (Vaerewijck *et al.*, 2005). Their resistance to chlorine and other disinfectants allows them to survive conventional water treatment and recolonise distribution systems, including healthcare facilities (Dowdell *et al.*, 2019). Exposure occurs primarily through inhalation of aerosols from showers, taps, and medical devices, ingestion of contaminated water, or contact with broken skin, with immunocompromised patients, those with chronic lung disease, and individuals with indwelling devices at highest risk (Daley *et al.*, 2020). Clinically, NTM infections are challenging to treat due to intrinsic and acquired antimicrobial resistance. Pulmonary MAC disease typically requires a three-drug regimen

(macrolide + ethambutol + rifamycin) for at least 12 months after culture conversion, often extending to 18–24 months (Daley *et al.*, 2020). Rapid-growing species such as *M. abscessus* may require prolonged combination therapy with both intravenous and oral agents, sometimes exceeding 18 months, and are associated with higher treatment failure rates (Daley *et al.*, 2020). Unfortunately, drug toxicities are common: macrolides can cause hepatotoxicity and QT prolongation; rifamycins cause hepatotoxicity and drug–drug interactions; ethambutol can lead to optic neuritis; and aminoglycosides such as amikacin carry risks of nephrotoxicity and ototoxicity (Daley *et al.*, 2020). Non-tuberculous mycobacterial (NTM) disease in immunocompromised patients carries a more than three-fold increased risk of death compared with immunocompetent hosts (HR 3.54) (Table 4), highlighting substantial morbidity in susceptible populations (Chai *et al.*, 2022). Therefore, *Mycobacteria* is growing concern is the common manifestations of pulmonary, skin, and soft tissue infections in people with pre-existing health conditions and immunocompromised and immunosuppressed individuals (Turner, 2016). For these infections, treatment is usually complex with multi-drug treatments that come with linked toxicity and are administered for lengthy periods (Pennington *et al.*, 2021).

Methylobacterium spp.

Methylobacterium can also be found in drinking water and on taps and showers.

Methylobacterium spp. can often be missed because of the slow growth and is usually an indicator of the presence or absence of *Mycobacterium* spp. in showers, especially showerheads (Falkinham *et al.*, 2016). The relationship between *Methylobacterium* spp. and *Mycobacterium* spp. in premise plumbing biofilms has been highlighted as a potentially useful ecological indicator for water safety monitoring. In a study of household plumbing systems from patients with *Mycobacterium avium* pulmonary disease, there was a striking mutual exclusivity between the two genera. such that when *Methylobacterium* was detected in a biofilm sample, *Mycobacterium* was absent, and vice versa (Falkinham *et al.*, 2016). . Both are slow growing, oligotrophic bacteria adapted to low nutrient, treated water environments, (Vaerewijck *et al.*, 2005). They appear to occupy overlapping ecological niches, leading to competitive exclusion. Possible mechanisms include niche competition for limited carbon sources, biofilm microenvironment modification (e.g., pH, nutrient gradients, extracellular polymeric substances), and antagonistic interactions through inhibitory metabolites or surface-associated factors (Sofia Afonso Dias *et al.*, 2023).

Aeromonas spp.

Aeromonas species are Gram-negative bacteria commonly found in freshwater, brackish water, and drinking-water systems, where they may persist within biofilms in distribution networks and hospital plumbing. They are recognized as opportunistic pathogens, associated with gastrointestinal illness following ingestion of contaminated water and with skin, soft tissue, and systemic infections after traumatic exposure. Their intrinsic resistance mechanisms, including chromosomal β -lactamase production, and their capacity to cause severe disease in immunocompromised patients underscore their relevance to water safety and infection prevention strategies (Pessoa *et al.*, 2022). Emerging opportunistic waterborne organisms show more variable outcomes, often with limited data. *Aeromonas* spp. infections exhibit a 1-year mortality of 26.5 percent (Table 4), with advanced age, ICU admission, and malignancy identified as independent risk factors (Nolla-Salas *et al.*, 2017).

Acinetobacter spp.

Acinetobacter spp. are widely distributed in soil, surface water, sewage, and drinking-water systems, where they may persist in biofilms and survive under nutrient-limited conditions (Carvalho, Silva and Teixeira, 2021). Although community-acquired infections are rare, certain species, particularly *Acinetobacter baumannii*, are recognised as major opportunistic pathogens in healthcare settings (Narciso-Da-Rocha *et al.*, 2013). They are associated with ventilator-associated pneumonia, bloodstream infections, wound infections, and urinary tract infections, especially in critically ill or immunocompromised patients (*Acinetobacter infection: Epidemiology, microbiology, pathogenesis, clinical features, and diagnosis - UpToDate*, 2024). Their remarkable ability to acquire and accumulate antimicrobial resistance mechanisms, including carbapenemases, has led to the emergence of multidrug-resistant and pan-resistant strains (Narciso-Da-Rocha *et al.*, 2013). The environmental ubiquity of *Acinetobacter* spp. and their potential to colonize hospital water systems underscore their importance in water safety and infection prevention strategies ('*Acinetobacter* Background document for the WHO Guidelines for drinking-water quality', 2025). *Acinetobacter baumannii* infections in hospitalised patients have a 13.6 percent all-cause inpatient mortality, which rises significantly with multi-drug-resistant (MDR) and carbapenem-resistant (CRAB) strains (aOR – adjusted odds ratio 1.61–1.68) and leads to acute organ dysfunction (Appaneal *et al.*, 2022).

Cupriavidus spp.

Cupriavidus spp. natural habitat is soil and water, however it has been increasingly detected in healthcare water systems (Inkster *et al.*, 2022). Although rarely reported as human pathogens,

they have been implicated in opportunistic infections, particularly in immunocompromised patients. Clinical presentations include bloodstream infections, pneumonia, and device-associated infections, often linked to contaminated hospital water supplies (Inkster, 2021). Outbreak investigations have demonstrated colonization of hospital plumbing and outlets, with *Cupriavidus pauculus* identified as the cause of bacteraemia in paediatric haemato-oncology patients, underscoring its potential as an opportunistic premise plumbing pathogen (Enfeksiyonlarda *et al.*, 2024). Their persistence in biofilms and resistance to disinfectants highlight the importance of surveillance and control measures in water safety management. Bacteria from the *Cupriavidus* genus can withstand high temperatures (Yootoum *et al.*, 2023), as well as displaying heavy metal resistance (González-Sánchez *et al.*, 2025). Importantly, as a rare waterborne pathogen, it can still cause infection and its presence should be viewed cautiously (Inkster *et al.*, 2022).

Sphingomonas spp.

Sphingomonas spp. while most often found in sterile sites including distilled water, sterile drug solutions, and haemodialysis fluid, can also reside in common surfaces such as shower curtains (Zellmer *et al.*, 2017). Lower-virulence OPPPs (see Table 4) (e.g., *Sphingomonas*, *Methylobacterium*, *Cupriavidus*, and *Alcaligenes faecalis*) still matter clinically and operationally. Biofilm resilience, device affinity, and disinfection tolerance enable persistent colonisation, induce sporadic severe sepsis in high-risk patients, and reside in hard-to-eradicate environmental reservoirs. This often necessitates alternative prevention strategies via engineering controls, device reprocessing assurance, and targeted surveillance, rather than reliance on culture-based case detection alone (Inkster *et al.*, 2021).

Table 4. Mortality and morbidity of OPPP's, with typical setting, risks. ASDR (age standardised per rate), VAP Ventilator-associated pneumonia, BSI blood stream infections. ICU intensive care unit, HR hazard ratio, LMIC low- and middle-income countries, NICU neonatal Intensive care unit, PICU paediatric intensive care unit, XDR (extensively drug resistant)

OPPP'S	Typical setting and infections	Reported mortality (metric)	Morbidity and risk drivers	References
<i>Legionella spp.</i>	Aerosol exposure; Severe pneumonia; community, travel and healthcare associated	3.1 % crude fatality (England/Wales 2023; burden highest in over 70s (ASDR 8.15/100,00 globally (ASDR 0.86/100,00)	Severe pneumonia with respiratory failure/shock; risk increases with age, smoking, comorbidity; aspiration risk in hospitals	(Zhong <i>et al.</i> , 2025)
<i>Pseudomonas aeruginosa</i>	ICU, VAP, BSI, device related	27-39% 30-day mortality in BSI	Mortality driven by severe sepsis, pneumonia, delayed effective therapy; higher with organ failure and inappropriate empirics	(Frem <i>et al.</i> , 2023)
<i>Stenotrophomonas maltophilia</i>	Indwelling devices, haematology/oncology, ICU	31–46% 28–30-day mortality in BSI cohorts	High-risk with neutropenia, high SOFA, pulmonary infection; mortality reduced by prompt CVC removal and appropriate therapy	Gezer <i>et al.</i> , 2024)
<i>Burkholderia cepacia</i> complex	ICU, CLABSI; CF pulmonary disease	41% 30-day BSI mortality; in-hospital up to 64% (ICU cohort)	“Cepacia syndrome” with rapid pulmonary decline in CF; mortality linked to septic shock and delayed line removal	(Özdemir <i>et al.</i> , 2023)
Non tuberculosis <i>Mycobacteria</i>	Immunocompromised; pulmonary and device-associated	Higher mortality in immunocompromised vs. immunocompetent (HR 3.54)	Prolonged disease course; host immunosuppression and species (e.g., M. abscesses) complicate therapy	(Chai <i>et al.</i> , 2022)
<i>Aeromonas spp.</i>	GI disease; polymicrobial extraintestinal infections; hospital and community	26.5% all-cause at 1 year (adult cohort)	Older age, ICU admission, and malignancy independently increase mortality	(Nolla-Salas <i>et al.</i> , 2017)

<i>Cupriavidus spp.</i>	Water-associated outbreaks; device-related; immunocompromised	Rare but can be fatal in outbreaks (e.g., <i>C. pauculus</i>)	Emerging OPPP in UK hospitals; device/invasive care exposure; variable resistance patterns	(Inkster <i>et al.</i> , 2021)
<i>Acinetobacter spp.</i> (<i>A. baumannii</i>)	ICU, VAP, BSI; neonates and LMICs disproportionately affected	13.6% inpatient overall; higher with MDR/CRAB and severe organ dysfunction	Inadequate empiric therapy, shock, respiratory/blood source, and resistance push mortality higher	(Appaneal <i>et al.</i> , 2022)
<i>Sphingomonas spp.</i>	Low-virulence OPPP; device-associated bacteraemia; faucet/plumbing colonisation	Generally low mortality: series with 0% deaths reported	Can cause severe sepsis in immunocompromised; persistent colonisation of hospital plumbing; recolonisation after fixture replacement	(Zellmer <i>et al.</i> , 2017)
<i>Methylobacterium spp</i>	Endoscope/water-associated; immunocompromised	Mortality not well quantified	Biofilm, disinfectant/temperature tolerance; slow growth delays detection and control	(Kovaleva, Degener and Van Der Mei, 2014)
<i>Ralstonia spp.</i>	NICU/PICU outbreaks; contaminated water/devices	Up to 61% 30-day mortality in paediatric outbreak (<i>R. mannitolilytica</i>)	Carbapenem resistance, invasive devices, prolonged ICU stay; environmental water heaters implicated	(Rajni <i>et al.</i> , 2023)
<i>Alcaligenes faecalis</i>	UTI, pneumonia, diabetic foot; often polymicrobial	Mortality variably reported; XDR emergence noted	Rising resistance complicates therapy; infections across multiple sites; prior IV antibiotics common	(Huang, 2020)
<i>Elizabethkingia spp.</i>	ICU, immunocompromised; neonatal meningitis	30.1% pooled mortality (meta-analysis)	High with inappropriate initial therapy, ICU/mechanical ventilation, liver disease; limited susceptibilities	(Seong <i>et al.</i> , 2020)

1.9. Detection of OPPP in UK Hospitals

Prompt identification of bacteraemia's or other infections caused by opportunistic premise plumbing pathogens (OPPPs) facilitates more effective treatment. However, preventing such infections remains the preferred and most impactful strategy. Early detection of OPPP infections, particularly in high-risk healthcare settings, is strongly associated with reduced mortality, morbidity, and healthcare costs. For severe bacterial infections such as sepsis, each hour of delay in effective antimicrobial therapy after onset of hypotension increases mortality by approximately 7.6% (Kumar *et al.*, 2006). While in bacterial meningitis, initiating antibiotics within one hour of hospital arrival can halve mortality compared to delays beyond three hours (Eisen *et al.*, 2022). In hospital-acquired pneumonia, timely targeted therapy shortens ICU stay by 2–4 days and reduces ventilator days by ~25% (Zaragoza *et al.*, 2020). Surveillance systems that detect infections before full clinical onset can identify outbreaks days to weeks earlier than conventional reporting, reducing total cases by 10–30% (Meckawy *et al.*, 2022). Rapid recognition of OPPP's, through environmental monitoring, early clinical suspicion, and prompt laboratory confirmation, can prevent progression to severe disease, limit transmission, and improve patient outcomes.

The United Kingdom Health Security Agency (UKHSA) aims to review existing data and set upcoming goals to lower Gram-negative bloodstream infections. However, these require different interventions for Gram-negative bloodstream infections to be targeted and controlled effectively (Public Health England, 2022). Nevertheless, several Gram-negative bacteria in drinking water associated with hospital waterborne infections globally, currently lack water standardised testing procedures, hindering effective surveillance, risk assessment, and implementation of targeted infection control measures. After the Glasgow incident in 2018, national guidance was issued for the detection of waterborne outbreaks, only recommending water testing when some of the OPPPs were found in clinical specimens (Inkster *et al.*, 2024). With antibiotic resistance on the rise, prevention of infection is better than treatment, which can sometimes lead to complications and even death.

Biological causes of infections that originate from water distribution networks may still not be recognised by infection control and prevention guidelines. Despite the fact that in the United States, drinking water is responsible for roughly 7.15 million infections each year and results in associated expenses of more than \$3.33 billion (Collier *et al.*, 2021). These outbreaks are primarily attributable to biofilms within water distribution systems (WDS), which represent the main source of waterborne healthcare-associated infections (HAIs) in both residential and

hospital settings (Hayward *et al.*, 2024). Many of the studies described above have used different methods to detect OPPPs, ranging from traditional microbiology techniques (that remain the core method in hospital pathology departments), including culture, microscopy, and biochemical testing, to molecular techniques, such as nucleic acid-based tests (e.g., polymerase chain reaction (PCR)), mass spectrometry, and nucleotide sequencing (Laupland and Valiquette, 2013). Indeed, the past few years has seen nucleotide sequencing become the new gold standard in bacterial identification. Specifically, targeted sequencing of the 16S ribosomal RNA, provides a bacterial ‘fingerprint’, where each 16S sequence is unique to each species (Pichler *et al.*, 2018). However, currently there are no single testing platforms that could detect a panel of OPPP’s in water samples. This is a clear indication of the general lack of understanding of which microorganisms are present in potable water. When coupled with the limited testing regulations which currently exist for water supplies, this knowledge gap may substantially impact patients in the hospital environment, particularly post-surgery and during the recovery of immunocompromised patients.

1.10. Project Background

In 2022, work began on a £47 million scheme for a new building at Scarborough Hospital with plans to open in late 2024. The new build is a two-storey building that will house Same-Day Emergency Care (SDEC) on the ground floor and a critical care medical unit on the first floor, which will treat more vulnerable patient groups, including immunocompromised patients, that often require several days or weeks in the hospital. This new build will have all new plumbing infrastructure and a water distribution system that is piped directly to the building and will not pass through any other (older) distribution networks.

A project was designed between the Disease Detection and Prevention research group at York St John University and Scarborough Hospital to investigate the microbial water quality of this new build during the construction period prior to opening to staff and the public. Water samples were to be collected monthly prior to staff and patient occupancy and every two weeks once the building was opened for use in the next phase. However, due to issues with building control sign-off there were substantial delays to the hospital opening to patients and public, preventing sampling during the post-occupancy phase.

The aims of this longitudinal study were to:

- Identify and understand the range of microbial species that live in WDS over time in a new hospital before and after being used as a healthcare facility.
- Observe changes to microbial species over the test period of seven months while they establish themselves in WDS.
- Understand how the presence or absence of OPPP's could further inform risk assessment and water control strategies.
- Contribute to environmental sustainability goals by reducing the amount of flushing and decontamination required in hospital water systems.

2. Materials and Methods

2.1. Overview of Water Sampling Process

During the construction of the new Scarborough Hospital building, water samples were collected from four locations (administration, same day emergency department (SDEC), intensive care unit (ICU), and augmented care. Three outlets on the ground floor and twelve outlets on the first floor were selected for water sampling (see appendix 1*). The aim was to monitor changes to Opportunistic Premise Plumbing Pathogens (OPPP) colonisation over a defined period of seven months (June – December 2024).

For eleven sampling occasions, the interval between the first and the second sampling was 4 weeks. Following this, collections were two or three weeks apart, due to being still construction site locked/security restricted zone, dependent on the availability of hospital staff to provide escort during the sampling (see Table 9). From 15 outlets, one cold water outlet and 14 outlets were mixer outlets, and therefore from these outlets, cold and hot water samples were collected (see Table 6). This was interrupted on the eleventh sampling occasion due to a technical fault with the boiler system where only cold water was available. After the first collection, the pipe/water was disinfected with 1000 ppm Silver Hydrogen Peroxide, carried out by construction team. The second collection occurred while the pipes were still being disinfected, and the third sample collection was after pipe/water disinfection had finished.

Ethics were applied for through York St John University School of Science, Technology, and Health and granted for this project ([ETH2324-0206](#)), where sampling would be carried out at New Scarborough Hospital and processed at York St John University Laboratories.

2.2. Bacterial Maintenance and Growth Media

Different media were used for primary bacterial recovery, identification, maintenance and storage. R2A (Oxoid CM0906) was designed as a water recovery medium in 1985 and is used in this project to recover bacteria from water (Reasoner and Geldreich, 1985). Nutrient (Oxoid CM0001) agar was used for maintenance, and Tryptic Soy Broth (Oxoid CM0129) with 15% glycerol (Fisher Scientific) was used for storing at -80°C.

For isolating target species, the selective media MacConkey No. 3 (MAC3) (Oxoid CM0115) was used for the isolation of gram-negative bacteria. Furthermore, for isolation of *Pseudomonas* spp., *Pseudomonas* ceftrimide agar (PCA) (CM0570) was used, and Vancomycin Imipenem

Amphotericin B (VIA) agar developed by (Kerr *et al.*, 1996) was used for *Stenotrophomonas maltophilia* isolation.

2.3. Bacterial Collection

Reference strains from culture collections were used for assay validation and identification/ comparison protocols against strains collected from water samples (see Table 5).

Table 5. Reference strains and their origin. All bacterial strains served as positive controls, except for *Escherichia coli*, which served as a negative control.

Species	Strain code	Origin	Reference
<i>Pseudomonas Stutzeri</i>	NCTC 12262/ ATCC 17588	Spinal fluid	(Palleroni <i>et al.</i> , 1970)
<i>Pseudomonas Aeruginosa</i>	NCTC 12903/ ATCC 27853	Blood culture	(Medeiros <i>et al.</i> , 1971)
<i>Stenotrophomonas maltophilia</i>	NCTC 10257/ ATCC 13637	The oropharyngeal region of the patient with mouth cancer	(HUGH and RYSCHENKOW, 1961)
<i>Acinetobacter Baumannii</i>	ATCC 17978	Fatal meningitis	(Baumann, Doudoroff and Stanier, 1968)
<i>Aeromonas Hydrophilia</i>	NCTC 8049/ ATCC 7966	Tin of milk with a fishy odor	(Stanier, 1943)
<i>Alcaligenes Faecalis</i>	NCTC 11953/ ATCC 8750	Not stated	(Rehfuss and Urban, 2005)
<i>Burkholderia Cepacia</i>	NCTC 10743/ ATCC 25416	Plant-derived foodstuff – onion (<i>Allium cepa</i>)	(Hotta <i>et al.</i> , 1992)
<i>Escherichia coli</i>	NCTC 12241/ ATCC 25922	Clinical isolate	(Kim <i>et al.</i> , 2005)
<i>Elizabethkingia Meningoseptica</i>	NCTC 10016/ ATCC 13253	Spinal fluid from a premature infant	(Webster and Hugh, 1979)

2.4. Water Collection

Water samples were collected in sterile 100 ml bottles with sodium thiosulphate 18 mg/l as stated in HTM 04-01. The collection was carried out within the new Scarborough Hospital from 15 different outlets (see Table 6). Three 100 ml samples were collected from each tap: proximal (collected immediately upon opening the tap, medial (collected immediately following the proximal sample), and distal (collected after flushing the tap for 3 minutes). As a comparative experiment, water from two natural sources (Yorkshire Dales National Park and well water from a town in the south of Serbia) was also collected to compare to industrial (large public building controlled by ACOP L8 not HTM 04-01) and hospital in order to see the difference between

treated hospital water, treated water from public building as known as industrial from York St John University, against natural untreated water collected from Whernside, Yorkshire Dales and untreated underground (well) water.

Table 6. Outlet type, code and room name. On the ground floor in the A&E department, three mixed water outlets (SDEC opposite the reception area, at trolley bay, and dirty utility). The first floor (FF) mixed water outlets were seven hand wash basins and four showers from staff changing rooms, a bed bay area, two different utility rooms, and two single rooms in the hospital, one outlet, cold tap, from utility room (to see location, use room code in appendix 1*).

Tap NO.	Floor	Room code	Room name	Outlet type
1	Ground floor	SDEC	Same Day Emergency Care	Mixed Tap, hand wash basin
2	Ground floor	UTC 003	Trolley Bay	Mixed Tap, hand wash basin
3	Ground floor	SMS 004	Dirty Utility	Mixed Tap, hand wash basin
4	First floor	STF 020	Staff Change	Mixed Tap, hand wash basin in the main change area
5	First floor	STF 020	Staff Change	Shower
6	First floor	AMS 008	Staff Change	Mixed Tap, hand wash basin
7	First floor	AMS 008	Staff Change	Shower
8	First floor	CG 2020	Level 1, 4 Bed Bay	Mixed Tap, hand wash basin
9	First floor	CG 2020	Level 1, 4 Bed Bay	Shower in en-suite
10	First floor	CG 2033	Level 1, Dirty utility	Mixed Tap, hand wash basin
11	First floor	CG 3016	Level 3, Dirty utility	Mixed Tap, hand wash basin
12	First floor	CG 3016	Level 3, Dirty utility	Cold Tap, general sink
13	First floor	CG 3001	Level 3, Single Room	Mixed Tap, hand wash basin in the main room
14	First floor	CG 3023	Level 3, Single Room	Mixed Tap, hand wash basin in the main room
15	First floor	CG 3023	Level 3, Single Room	Shower

2.5. Water Processing

Following sample collection, samples were immediately transported to the laboratory at York St John University, and 100 ml was filtered using a three-place manifold (Pall Corporation) connected to an aspirator (Aspeed professional suction equipment) and 0.45µm nitrocellulose filter membranes (Pall Corporation). The filters were placed onto R2A (Oxoid CM0906) agar and incubated at 37°C for 48 (+/-2) hours. These were used for TVC and for primary observations of different morphology types. Medial filters were frozen at -80°C for later direct molecular identification.

On the eleventh sampling occasion, an additional fourth sample was collected from each tap before the distal sample for further molecular diagnostics. After collection, additional samples were processed in the laboratory as stated above, and filters were used for immediate DNA extraction. DNA was used in PCR reactions and MinION (Oxford Nanopore) nucleotide sequencing.

2.6. Colony Selection and Primary Identification

Following incubation, the enumeration of the Total Viable Count (TVC) was recorded. Bacterial colonies were selected for patch plating based on their characteristics: size, shape, growth type, and colour. Colony morphology exhibited either Pin Prick (PP) or confluent growth (CG); however, counts exceeding 250 colonies per plate were deemed as Too Numerous to Count (TNC). Colonies exhibiting distinct morphologies were isolated from the original plate and subjected to patch plating (see Figure 6), in which sterile wooden toothpicks were used to inoculate the designated location on the selective and differential media with a single colony.

The following selective agars were chosen for identification. MacConkey No3- MAC3 (Oxoid CM0115) for gram-negatives; *Pseudomonas* Cetrimide Agar-PCA (Oxoid CM0570) *Pseudomonas spp.* PCA; *Stenotrophomonas maltophilia* VIA agar (Kerr *et al.*, 1996).

To preserve the remaining bacterial colonies following patch plating, the membrane was incubated for an additional 24 hours. Following incubation, Cellulose membranes were aseptically wiped using sterile swabs and stored at -80°C in Tryptic Soy Broth (Oxoid CM0129) with 15 % Glycerol (TSB+15%G) (Fisher Scientific). Subsequently, for bacterial maintenance and clonality, one bacterial colony was sub-cultured on Nutrient Agar (Oxoid CM0003) and incubated for 24 (+/-2) hours at 37°C. The same principle was used to store them at -80°C.

2.7. Biochemical Tests

Biochemical tests, the oxidase test and the Analytical Profile Index 20 Non-Enteric (API 20 NE, bioMerieux, see section 2.6.2 for further details) were used to aid the identification process further.

2.7.1. Oxidise Test

As a differential test, oxidase strips (Millipore) performed as per manufacturer instructions were used for the detection of cytochrome oxidase activity, where several bacterial colonies were wiped off the Petri dish plate with a diagnostic strip paper zone, where change of colour indicated a positive reaction, and no change in colour indicated in negative reaction.

2.7.2 Analytical Profile Index 20 NE

API 20 NE is a series of standardised biochemical tests that analyse biochemical activities in 48 hours. API 20 NE was selected as it provides to identification of Gram-Negative rods, non-enteric and non-fastidious bacterial species. Bacterial solutions were prepared by suspending the colonies in distilled water to a density of 0.5 McFarland turbidity standard used on API strips NE 20 (bioMerieux). API strip readings are taken after 24 and, if required, 48 hours of incubation, as directed in the manufacturer's instruction manual (bioMerieux, 2003).

2.8. Antimicrobial Susceptibility Testing

Susceptibility to anti-pseudomonal antibiotics was tested using the disc diffusion method. Antimicrobial susceptibility testing (AST) was performed on all primarily identified bacterial species following the European Committee on Antimicrobial Susceptibility Testing (Matuschek, Brown and Kahlmeter, 2014). Bacterial inoculation was made to a 0.5 McFarland standard, and a confluent lawn was prepared onto Mueller Hinton II agar (Becton Dickinson and Company code). A standardised antibiotic disc dispenser was used to distribute the designated antibiotic discs (Oxoid) (See Table 7) for each bacterial species and incubated at 37 °C. Duplicate AST was performed for each isolated and identified bacterial strain. Plates were subsequently assessed following EUCAST guidelines after a 24-hour incubation period at 37°C (*euca*st: *Clinical breakpoints and dosing of antibiotics*, 2025). *P. aeruginosa* ATCC 27853 strain was used as a susceptible control.

Table 7. Antibiotics and their concentration are used in antimicrobial susceptibility testing according to EUCAST guidelines and their Zones of Inhibition (ZOI). Resistant (R), Intermediate Resistance (IR).

Antibiotic name	Amount in µg	Abbreviation	Zone of inhibition,mm		
			R	IR	S
Ceftazidime	10	CAZ 10	<17	18-50	>50
Ciprofloxacin	5	CIP 5	<26	27-50	> 50
Aztreonam	30	ATM 30	<16	17-50	> 50
Amikacin	30	AK 30	<15	/	> 15
Trimethoprim/Sulfamethoxazole	25	STX 25	<16	17-50	> 50
Piperacillin-Tazobactam	36	TZP 36	<18	19-50	50
Imipenem	10	IMP 10	<20	21-50	50

2.9. Polymerase Chain Reaction (PCR) Primers and Assay Conditions

PCR was carried out on all recovered bacteria. The crude heating method was used for DNA extraction, where aseptically in a 1.5 ml Eppendorf, 100 µl of PCR-grade water, and 5-10 colonies (size dependent) were resuspended. The resuspended mixture was subsequently subjected to a heat treatment at 100 °C to induce membrane disruption for 10 min, and centrifugation for 10 min at 13,000 x g (Thermo Scientific Micro CL 17R) separated the pellet and supernatant, where the supernatant contained extracted DNA. 16S RNA primers (see Table 3) and PCR were used for further identification. All reactions were carried out in a 25 µl final volume. 7 µl of 5x TAQ polymerase master mix (Thermo Fisher Scientific) and 1 µl of 10 mol concentration of forward and reverse primers (Table 8), with the final molarity of primers in the PCR solution being 0.4 µM. 15 µl of PCR grade water and 1 µl of extracted DNA to make 25 µl altogether (BIO–RAD T100™ Thermal Cycler) were used in PCR. Positive and negative controls were used in each reaction.

Table 8. 16S RNA reverse and forward primers, reference cultures, and PCR conditions used in molecular identification.

Reference strain	Primers	PCR conditions	References
<i>Pseudomonas. aeruginosa</i> NCTC10743	F - GGGGGATCTTCGGACCTCA R - TCCTTAGAGTGCCCACCCG	95°C for 2 min, 94 for 20 sec, 54°C for 20 sec, 72°C for 40 sec, 72°C for 1min	(Spilker <i>et al.</i> , 2004)
<i>Pseudomonas</i> <i>genus specific</i>	F – GACGGGTGAGTAATGCCTA R - CACTGGTGTTCTTCCTATA	95°C for 2 min, 94 for 20 sec, 58°C for 20 sec, 72°C for 40 sec, 72°C for 1min	(Spilker <i>et al.</i> , 2004)
<i>P. stutzeri</i> NCTC12262	F – GTGGGGGACAACGTTTC R - TCAGTGTCAGTATTAGC	94°C for 5min, 40 cycles of 2min at 94°C, 2min at 57°C, 3 min at 72°C, 10 min at 72°C	(Sikorski, Teschner and Wackernagel, 2002)
<i>S. maltophilia</i> ATCC 13637	F – CAGCCTGCGAAAAGTA R – TTAAGCTTGCCACGAACAG	95°C for 5 min, 30 cycles of 10 sec at 95°C, 58°C for 10 sec, 72°C for min, 72°C for 2 min	(Whitby <i>et al.</i> , 2000)
<i>A. baumannii</i> ATCC17978	F – CAGCTCGTGTCGTGAGATGT R – CGTAAGGGCCATGATGACTT	94°C FOR 4 min, 30 cycles for 35 sec, 55°C for 45 sec, 72 for 40 sec, 72 for 4 min	(Abdul-Hussein <i>et al.</i> , 2019)
<i>A. hydrophilia</i> NCTC8049	F – ACGCAGGCGGTTGGATAAGT R – GGCACAAAGGACAGGGGT	94°C for 3min, 35 cycles of 1min at 94°C, 1min 54°C, 1 min 72°C and 5 min at 72°C	(Mendes-Marques, Hofer and Leal, 2013)
<i>A. faecalis</i> NCTC11953	F – ACTGCTGACGGTATCTGCAG R – TACTAAGGCCTAACGGCCCC	95°C for 30s, 36 cycles 95°C, 64 °C for 1 min, 72°C for 1 min, 72°C for 5 min.	(Nakano, Niwa and Nishimura, 2013)
<i>B. cepacia</i> NCTC10743	F – GCAGGCTAGAGTATGGCA R – GTTACTAAGGAAATGAATCCC	95°C for 10min, 35 cycles of 30 s at 95°C, 30 s at 55°C, 1min 72°C and 10 min at 72	(Fu <i>et al.</i> , 2022)

2.10. Electrophoresis

Following PCR, the amplicon products were subjected to agarose gel electrophoresis (Flowgen bioscience tank), 1x Tris-Borate-EDTA buffer on 1.2% ultra-pure agarose gel with 1x gel red. For size determination of the PCR product, a 100bp ladder was employed, with a power supply of 150V (using nanoPAC 500, Cleaver Scientific Ltd.) for 45 minutes to an hour. An Orange G loading dye (6x) (Alpha Aesar) was incorporated into the PCR product for DNA separation and visualisation. Following electrophoresis, band visualisation was performed using the iBright Imager 1500CL (Smart Digital Imaging, Invitrogen by Thermo Fisher Scientific).

2.11. Water Seeding and DNA Extraction

For bacterial DNA extraction from reference cultures, water-seeded filters, that will be used for MinION sequencing and for either water-seeded or sample-collected DNA extraction were processed with a Zymo DNA extraction and purification kit (Zymo Research, 2022). 24-hour incubated subcultures of the reference strains (5-10 size-dependent colonies were selected for inoculation of 100 ml autoclaved distilled water) were used in the first 9 samples: the remaining samples consisted of 100 ml filtered water collected from each tap (Table 6).

2.12. Nucleotide Sequencing

Filter-extracted DNA and water-seeded DNA from reference cultures and each tap were barcoded, and PCR was performed on the 16S gene (see Table 4). Following the barcode application and PCR amplification, from 50µl of the PCR product, the 45µl of barcode samples pooled together, and 5µl was used for electrophoresis visualisation. DNA quantification in the sample was performed using a BioDrop spectrophotometer. After a further purification process using freshly prepared 80% ethanol and a magnetic rack, the pellet was then resuspended with 15µl elution buffer. 3µl of this solution was used with elution buffer to make up 11µl, which was further mixed with rapid adapter solution to make a DNA library. This DNA library was further mixed with sequencing buffer and library beads for nucleotide sequencing as outlined in the 16s Barcoding Kit 24 V14 (SQK-16s114.24) (Oxford nanopore technologies, 2024). Nucleotide sequencing was performed in-house by the author using Oxford Nanopore MinION Mk1c platform and analysed using EPI2ME software. The author conducted all sequencing procedures, including library preparation and data acquisition.

The prepared barcoded 16S library was added to a Flow Cell R10 Version (Oxford Nanopore Technologies) before sequencing on the MinION Mk1c as directed in Protocol Rapid Sequencing DNA-16s Barcoding Kit 24 V14 (SQK-16s114.24) (Oxford Nanopore Technologies, 2024). The EPI2ME software facilitated the analysis of MinION-generated data, using Kraken2 and Minimap2, which applied distinct algorithms on identical datasets.

Data was collected from the MinION Mk1c was transferred onto external drive and then saved onto OneDrive. Analyses were carried out using EPI2ME software 16S rRNA workflow, utilising Kraken2 and Minimap2 (alignment program) to map sequences against a large reference database.

3. Results

Sections 3.1 to 3.7 provide results from water sampling at Scarborough Hospital using more traditional microbiology methods (filtration, culture, biochemical tests, molecular identification methods (PCR) and antibiotic sensitivity testing). Sections 3.8 and 3.9 provide metagenomics results from MinION nucleotide sequencing.

Part 1. Characterisation of Bacteria by Traditional Microbiology Techniques

Water samples were filtered and the nitrocellulose membranes incubated on R2A agar for 48 hours (Section 3.1). Where possible two examples of each colony type were subsequently plated on three selective media: MAC3, PCA, VIA agar, followed by a second 24-hour incubation period (Section 3.2). Growth from the selective media was further cultured and the bacteria used in subsequent AST and PCR (sections 3.7 and 3.5 respectively) and stored at -80°C.

3.1 Water Processing Primary Identification and Enumeration

Following primary incubation on R2A agar, the colony-forming units were enumerated and observations made on distinct colony types. However, many samples were classified as confluent or too numerous to count, demonstrating high bacterial numbers present in 100 ml samples (Figure 2b and 2d).

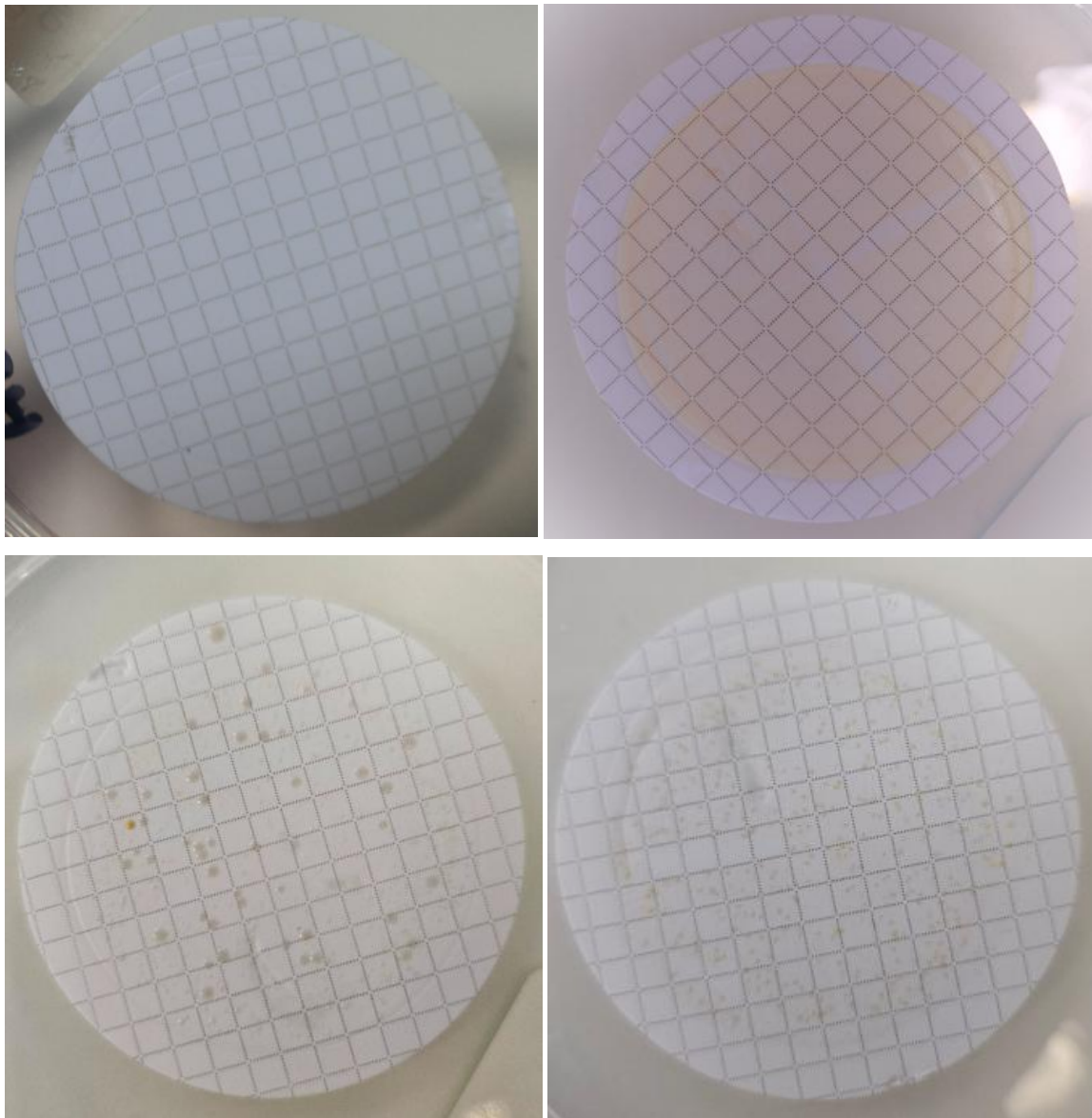


Figure 2. Examples of bacterial growth on 0.45µm nitrocellulose filter membranes on R2A following a 48h incubation period at 37°C. a) No growth, autoclaved distilled water; b) Proximal sample demonstrating confluent growth; c) Distal sample demonstrating a countable number of colonies and some of the different morphologies seen on a membrane following incubation; d) Proximal sample showing too numerous to count growth on.

A total of 330 water samples were collected (proximal and distal). Of these 173 (52.42%) had confluent growth, 130 (39.4%) were classified as too numerous to count. Only 23 (8.18%) had a number of colonies that could be counted Figure 3.

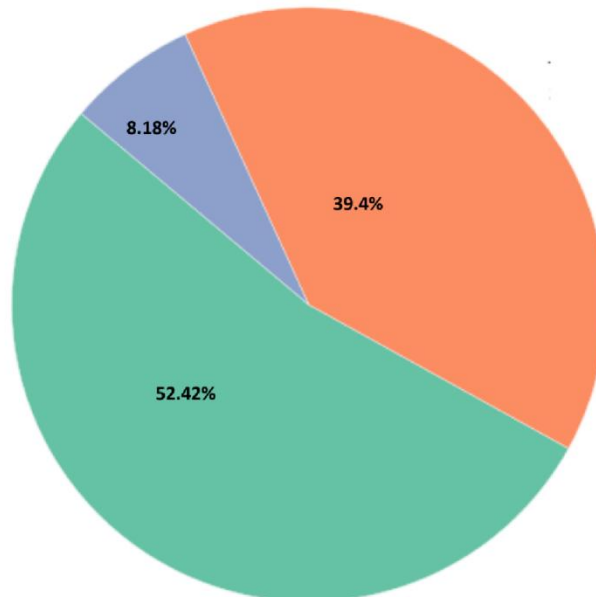


Figure 3. Distribution of colony growth classification. Confluent growth is presented with green, too numerous to count is presented with orange and filters with countable colonies are presented with blue

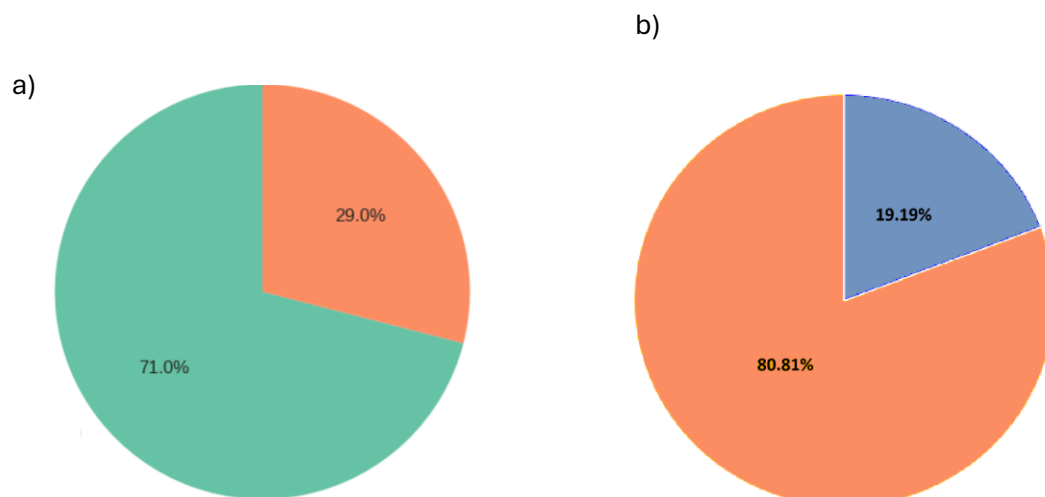


Figure 4. Distribution of colony growth classification. a) Proximal samples where green represents confluent growth and orange represent TNC. b) Distal samples where orange shows TNC and countable colonies are presented with blue.

91% percent of all membranes collected had a confluent growth or too numerous to count, 71% of proximal samples had a confluent growth (Figure 3a), 80.81% of distal samples produced colonies too numerous to count Figure 3b), and from the countable membranes, 82.60% were of distal samples.

Considering this is routine work, dilutions in this study were not carried out knowing that most validated protocols (e.g., ISO, EUCAST, USP) recommend counting plates with 30–300 colonies. Plates outside this range are excluded to maintain statistical reliability and reproducibility (Becker, 2022).

On the second sampling, the WDS underwent silver ionisation treatment as part of the construction team's procedures to ready the building for opening to the public. Figure 5 illustrates the variation observed between proximal samples from the same outlet during the first 3 sampling events. The figure below (figure 6) shows the change to bacterial recovery at this time. Week 1 (prior to silver ionisation treatment) there is confluent growth (figure 6.a), week 2 demonstrates a reduction from confluent growth to countable (figure 6.b) week 3, shows the subsequent return to confluent growth (figure 6.c).

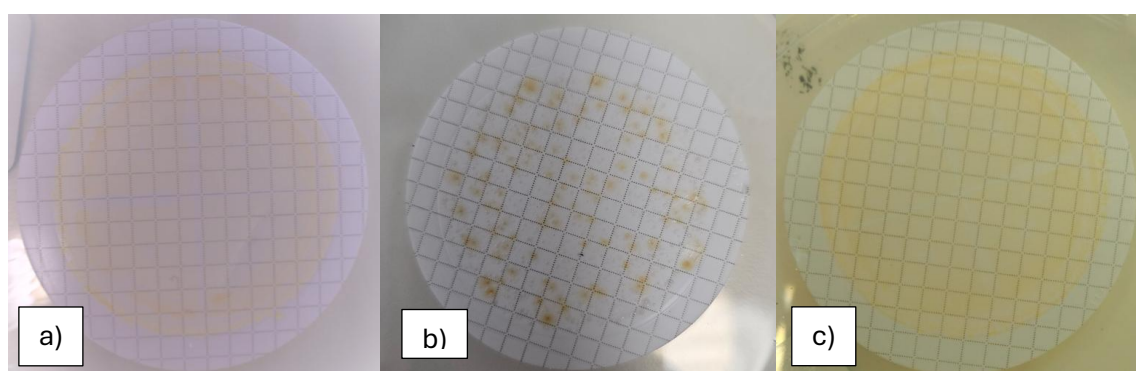


Figure 5. Proximal samples from outlet location CG3001/HW, over three sampling occasions on 0.45µm Nitrocellulose filter membranes on R2A. Following 48h incubation period on 37°C Proximal sample filters 48h incubation from the first week a), the sampling occasion week 1 filtered sample indicate confluent growth pre silver ionisation b), the sampling occasion week 2 filtered sample indicate countable colonies whilst silver ionisation c) sampling occasion week 3 filtered sample shows return to confluent growth post silver ionisation.

Reduction in bacterial diversity and population can also be seen in Table 9, where from the first sampling, bacteria were isolated from 7 outlets. In the subsequent sampling, while silver ionisation was in process, the number of outlets from which bacteria were isolated decreased to 5. This decline continued in the third sampling, where silver ionisation was completed, with bacteria isolated from only 4 outlets, all of which contained only *Pseudomonas* spp. (Table 9).

Following the initial sampling, the majority of filters exhibited either TNTC (too numerous to count) or confluent growth. After silver ionisation treatment, colony counts decreased and a reduced diversity of bacterial species was isolated. However, subsequent monitoring indicated a gradual re-establishment of bacterial populations over time.

3.2 Putative identification using patch plates and selective/differential media

Further identification was carried out by patch plating. After 48 hours of incubation on R2A agar, colonies displaying distinct morphologies were isolated from the original plate and subsequently patched onto selective/differential agars—MAC3, PCA, and VIA (Figure 6). No growth was observed on plates a), c), and e), whereas growth was detected on plates b), d), and f) at positions 2E, 2F, and 4E, 4F across all selective media.

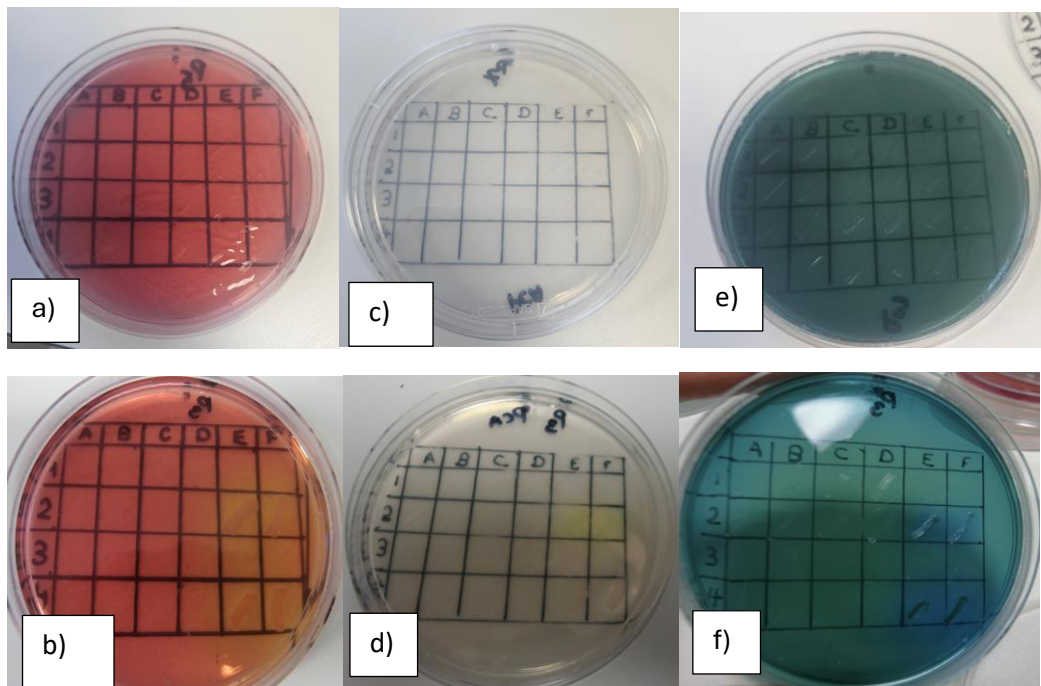


Figure 6. Further identification on selective / differential media (Mac3, PCA, VIA) using patch plates. A sterile toothpick was used to lift colonies from the nitrocellulose membrane and “patch” onto the same position of each selective / differential agar; a) No growth MAC3; b) Bacterial growth can be seen on the bottom right-hand side of the Mac3 plate indicated by colourless colonies and yellowing of the media indicating putative target species; c) No growth on PCA; d) Green/yellow growth on the right hand indicates the presence of a putative *Pseudomonas* spp.; e) No growth on VIA; f) Bacterial growth on VIA agar and colour change from

green to blue indicating *S. maltophilia*. A notable finding is the presence of putative *Pseudomonas* spp. In the same position on the grided patch plate as putative *S. maltophilia*.

3.3 Natural Water vs. Treated Water (Whernside and Well Water)

As a comparison to the treated water collected from the hospital outlets, a comparison study looked to establish the differences in morphologies seen on selective/differential media from natural water sources rather than treated water sources.

Water was collected from Whernside (one of the 'three peaks' in the Yorkshire Dales National Park) at 500 meters altitude, which can be compared to hospital water collected at a similar time. Natural water on the MAC3 plate is indicative of faecal flora (Figure 6a). The pink colouration in Figure 6a is indicative of enteric bacteria (though no confirmation steps were taken). Water was also collected from a domestic well (the back garden of a domestic home). Patch-plated well water was indicative of coliforms and gram-negative bacteria (Figure 6b).

As an alternative to water samples from the hospital but still representing treated water from a large building (and consequential large water distribution system), water samples were collected from York St John University outlets. In the treated university water, all 24 patch-plated spaces were indicative of heavy growth of gram-negative bacteria (Figure 6c). In hospital water from 24 used places on the patch plate, only 4 spaces showed gram-negative growth in Figure 6d.

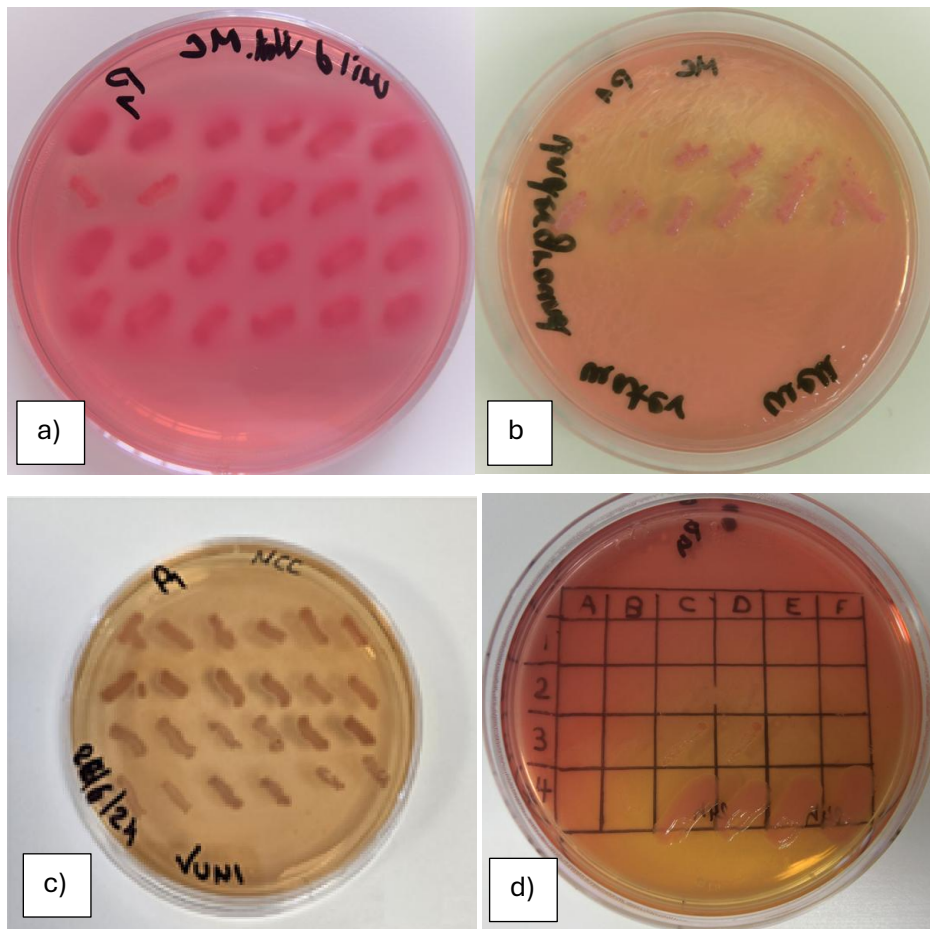


Figure 7. Wild water vs. well water vs. industrial water vs hospital water patch plated on MAC3. a) Wild water from the Whernside; b) Well water from Serbia, c) The industrial water patch plate, d) the Hospital water patch plate. The notable feature here is that the growth from natural sources indicates the possible recovery of faecal bacteria, whereas the plate from well water indicates coliforms and gram negatives, WDS has only an indication for gram-negative bacteria. Patch plated well water has shown growth on MAC3, PCA and VIA agars.

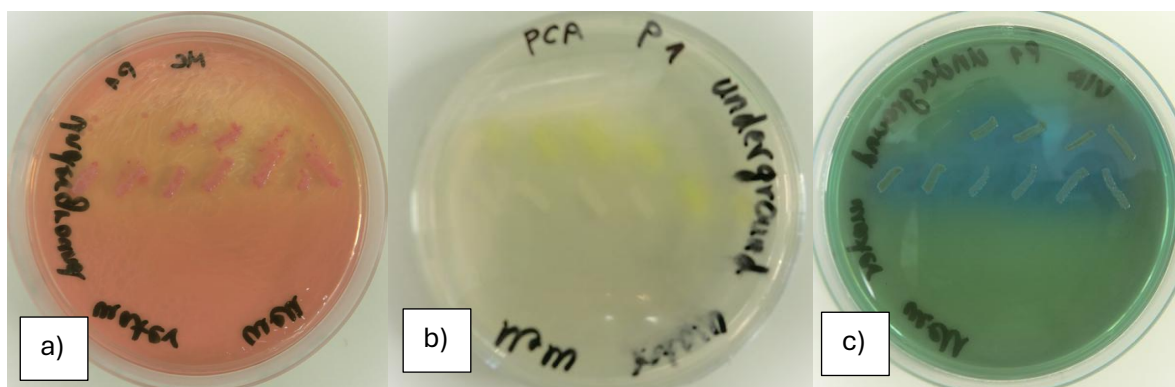


Figure 8. Well water samples on selective/differential media patch plates. A sterile toothpick was used to lift colonies from the nitrocellulose membrane and “patch” onto the same position of each selective / differential agar. a) MAC3 growth indicates gram-negative bacteria, b) PCA fluorescent and white growth indicates the presence of two different putative *Pseudomonas* spp.; c) VIA growth with a blue colour change on the agar indicates *S. maltophilia*. A notable finding is the presence of putative *Pseudomonas* spp. In the same position on the grided patch plate as putative *S. maltophilia*.

3.4 Further Identification Using Biochemical Tests (API 20NE)

API 20NE (bioMérieux) strips were used for further bacterial identification from 15 carefully selected outlets. This system was able to identify a range of bacterial species that could not be identified using selective / differential media. API 20NE identified, *P. alcaligenes*, *P. fluorescens*, *P. luteola*, *P. oryzihabitans*, *Delftia acidovorax*, *R. picettii*, *Ochrobactrum anthropi*, *Acenitabacter lwoffii*, from fourteen different outlets from over 11 sampling occasions. The API 20 NE system was used to identify additional bacterial isolates to species level that had not been identified using selective agars. This was of benefit for species that primers for PCR were unavailable for this study.

3.5 Polymerase Chain Reaction (PCR)

Further identification of what is left unidentified isolates was performed using 16S rRNA PCR. PCR was also applied to confirm the identification of bacterial species that had already been determined by API 20 NE and selective agars. 16s RNA, PCR was conducted on 300 samples utilising the forward and reverse primers listed in Table 5, along with the specified PCR conditions for each individual species. PCR has validated API 20NE results for all microorganisms for which primers were supplied. *Pseudomonas* genus specific primers were used on (*P. Fluorescens*, *P. oryzihabitans*, *P. alcaligenes*, *P. luteola*). Besides confirming API results, four additional bacterial species have

been identified: *Acinetobacter* spp, *Aeromonas* spp, *Pseudomonas* spp and *A. faecalis* (Table 9).

3.6 Collected Species Identification from Hospital Isolates

The results from the use of API 20NE and/or PCR analysis revealed that species of bacteria belonging to the OPPP list (Weinbren, 2020) were isolated from the water samples collected from Sacrborough Hospital. A total of fifty-five *Pseudomonas* species was isolated in total, eight *S. maltophilia*, 18 *Burkholderi cepacia*, two *Acinetobacter* spp., one *Aeromonas* spp. and eight non targeted species (seven *Ochrobactrum anthropi* and one *Delftia acidovorans*). It was observed that specific outlets could have the same species isolated from sequential sampling occasions (Table 9).

3.7 Primary identification summary

In accordance with HTM 04-01 requirements, despite the implementation of routine flushing and regular water quality monitoring—undertaken on the basis of information provided by the building contractors—persistent elevations in Total Viable Counts (TVC), comprising unidentified bacterial populations, continue to be detected within the hospital distribution system. This indicates that current control measures are insufficient to achieve compliance with the guidance thresholds and necessitates further investigation and remedial action to mitigate potential risks to patient safety.

Table 9. Isolation and location of OPPP's from the outlets of Scarborough Hospital during the construction phase (no public access) from 17th of June 2024-11th of December 2024.

Bacteria isolated from samples over the 11 sampling occasions are *Burkholderia Cepacia* Complex (BCC), *Alcaligenes faecalis* (Af), *P. luteola* (Pl), *P. oryzihabitans* (Po), *Ralstonia picettii* (RP), *Acinetobacter lwoffii* (Alw) and some non-targeted species *Ochrobactrum anthropic* (Oa) and *Delftia acidovorans* (Da). Pse is for *Pseudomonas* spp., Aer is for *Aeromonas* spp. Acb is for *Acinetobacter* spp. MT means multiple target (MT) species isolated in week 1, it was *P. alcaligenes* (Pal) and *P. fluorescens* (Pf) in sampling occasions 10 and 11 on outlets 10 and 11. Isolated species were *S. maltophilia* (Sm) and *P. aeruginosa* (Pa). It can be seen in this table that specific outlets had the same species (or other species) of OPPP isolated on numerous occasions.

Outlet no.	Outlet code	Sampling 1 17.06.2024	Sampling 2 15.07.2024	Sampling 3 29.07.2024	Sampling 4 19.08.2024	Sampling 5 02.09.2024	Sampling 6 23.09.2024	Sampling 7 07.10.2024	Sampling 8 21.10.2024	Sampling 9 04.11.2024	Sampling 10 18.11.2024	Sampling 11 11.12.2024
1	SDEC	MT		Pal		Oa				Oa		
2	UTC003	Ps	Ps	Ps	Ps	Ps	Ps	Ps	Ps		Pse	
3	SMS004	Pal			Af1				Oa			Af
4	STF020					Pl	BCC	Pa				
5	STF020SH											
6	AMS008	BCC	Af1		Af2		RP	BCC	BCC			
7	AMS008SH	Ps									RP	
8	CG2020											BCC
9	CG2020SH		BCC			Oa				Pl		
10	CG2033		BCC	Ps	Pl					BCC	MT	MT
11	CG3016	Ps	Ps	Pse							MT	MT
12	CG3016GS					Da			Pal			
13	CG3001						Oa			BCC	Ps	Oa
14	CG3023						Oa			Po		
15	CG3023SH	Pal			Alw	Pse	Aer	Pf	Acb			

Colour coded key for Table 9.

<i>Pseudomonas fluorescens</i> (Pf)	<i>Pseudomonas oryzihabitans</i> (Po)	<i>Stenotrophomonas maltophilia</i> (Sm)	<i>Burkholderia Cepacia</i> complex (BCC)
<i>Pseudomonas aeruginosa</i> (Pa)	<i>Acinetobacter lwoffii</i> (Alw)	<i>Ralstonia picettii</i> (RP)	<i>Pseudomonas</i> spp.(Pse.)
<i>Pseudomonas stutzeri</i> (Ps)	Multiple target species isolated (MT)	Other non targeted species (O)	<i>Acinetobacter</i> spp.(Acb.)
<i>Pseudomonas alcaligenes</i> (Pal)	<i>Alcaligenes faecalis</i> 1 (Af1)	<i>Delftia Acidovorans</i> (Da)	<i>Aeromonas</i> spp.(Aer.)
<i>Pseudomonas luteola</i> (Pl)	<i>Alcaligenes faecalis</i> 2 (Af2)	<i>Ochrobactrum anthropi</i> (Oa)	water sample collected, no recovery

3.8 Antimicrobial Susceptibility Testing

All bacteria identified by API 20NE and later on confirmed by PCR underwent antimicrobial susceptibility testing (AST) using EUCAST methodology version 14.0 for year 2024.

All isolates, *S. maltophilia* being exception, underwent AST with a selection of six anti-pseudomonal antibiotics that are typically used clinically for the range of bacteria isolated from the water samples of this study and with breakpoints identified in the EUCAST breakpoint table version (v 14.0). *Stenotrophomonas maltophilia* was tested using trimethoprim-sulfamethoxazole (STX) only, also identified by the EUCAST breakpoint table version (V 14.0).

A total of 100 strains were tested, and overall resistance to antibiotics was low. However, resistance and intermediate resistance were identified in some strains. 4% of all strains tested were identified as resistant, and 20% of all strains tested were identified as intermediate resistant shown on Table 10.

Table 10. Antimicrobial Susceptibility Profiles of Bacterial Isolates. Summary of resistance (R), intermediate resistance (IR), and sensitivity (S) patterns for *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Stenotrophomonas maltophilia*, and *Burkholderia cepacia complex* isolated from hospital water outlets. Percentages represent the proportion of strains within each species exhibiting the specified susceptibility category to tested antibiotics. R = resistant; IR = intermediate resistant; S = sensitive; IMP = imipenem; TZP = piperacillin-tazobactam; CAZ = ceftazidime; CIP = ciprofloxacin; ATM = aztreonam; STX = trimethoprim-sulfamethoxazole; AK = amikacin.

Species	No. of strains	No. of outlets	% Resistant	% Intermediate resistant	% Sensitive
All isolates (total)	100	15 over 11 collections	4% (all species)	20% (all species)	76%
<i>Pseudomonas aeruginosa</i>	10	5	10%	10%	80%
<i>Pseudomonas fluorescens</i>	2	2	0%	50%	50%
<i>Stenotrophomonas maltophilia</i>	8	2	0%	100%	0%
<i>Burkholderia cepacia</i>	18	9	67% (AK)	67% (CIP, ATM)	33%

A total of ten *Pseudomonas aeruginosa* strains (see table 11) were isolated from five different outlets. These ten strains, two from the same outlet were resistant to one antibiotic, Imipenem.

The same strain was also identified as intermediate resistance to four further antibiotics (TZP, CAZ, CIP, ATM). All other strains were sensitive to all antibiotics.

Table 11. Antibacterial susceptibility testing on isolated and identified strains. Where *P.a* stand for *Pseudomonas aeruginosa*, *P.f* for *Pseudomonas Fluorescence*, *B.c* for *Burkholderia cepacia*. Where green present sensitive (S) strains, orange Intermediate resistance (IR), and red presents resistant strains (R). dark gray means out of tested strains, no strains have shown any R/IR or S to this antibiotic.

	Identified bacteria								
Antibiotics	<i>P. aeruginosa</i> out of 10 strains			<i>P. fluorescence</i> out of 2 strains			<i>B. cepacia</i> out of 18 strains		
Resistance profile	S	IR	R	S	IR	R	S	IR	R
CAZ 10	8	2		1	1		18	18	
CIP 5	8	2		1	1		6	12	
ATM 30	8	2		1	1		6	12	
AK 30	10			2			6		12
TZP35	8	2		1	1			18	
IMP 10	8		2	1	1		18	18	

A total of two *P. fluorescens* were isolated from two outlets one from the first sampling occasion and the other from seventh sampling occasion and two different outlets, one (50% of all *P. fluorescens*) was identified as having intermediate resistance to CAZ, IMP, TZP, CIP and ATM. Both of these strains were isolated from two different outlets. All remaining *P. fluorescens* (the other 50%) were sensitive to all antibiotics.

Table 12. Antibacterial susceptibility testing on isolated and identified strains of *Stenotrophomonas maltophilia*. Where green present sensitive (S) strains, orange Intermediate resistance (IR), and red presents resistant strains (R).

Bacteria	Total No.	STX		
		S	IR	R
<i>Stenotrophomonas maltophilia</i>	8	/	8	/

All isolates, *S. maltophilia* being exception, underwent AST with a selection of six anti-pseudomonal antibiotics that are typically used clinically for the range of bacteria isolated from the water samples of this study and with breakpoints identified in the EUCAST breakpoint table version (v 14.0). *Stenotrophomonas maltophilia* was tested using trimethoprim-sulfamethoxazole (STX) only, also identified by the EUCAST breakpoint table guideline version (V 14.0).

A total of eight *S. maltophilia* strain have been isolated from two different outlets over the last two sampling occasions. 100 % of *S. maltophilia* were identified as having intermediate resistance to the antibiotic STX. No sensitive or resistant species were identified (Table 12).

Furthermore, a total of 18 *B. cepacia* have been isolated from nine different outlets. Twelve (67%) were identified as having intermediate resistance to CIP and ATM and resistance to AK. All remaining 6 (33%) isolated bacteria have been identified as sensitive to all antibiotics as seen on Table 11.

3.9. Summary from AST.

A total of thirty-three *P. stutzeri*, ten *P. alcaligenes*, seven *O. anthropy*, two *Acinetabacter spp.*, one *Delfia Acidovorans* and one *Aeromonas spp.* isolated from outlets were sensitive to all antibiotics. Considering that no bacterial resistance has been identified in the first half of the sampling occasions, it is noteworthy that in the second half *B. cepacia* and *P. aeruginosa* have begun to develop antimicrobial resistance.

Part 2. Molecular Characterisation of Bacterial Colonisation Patterns.

3.10. Introduction to Long-Read Next Generation Sequencing for Microbial Identification.

The 16S RNA gene (~1.5kb) is regarded as a bacterial fingerprint and has been utilised in molecular microbiology for bacterial identification, by either PCR amplification with genus-specific primers, or by nucleotide sequencing approaches (Winand *et al.*, 2019). Where traditional microbiology struggles with slow growing bacteria, uncommon phenotypes, inappropriate growth conditions, and a lack of media/testing selectivity which can prolong (or prevent) the identification process, sequencing of the 16S rRNA gene has become widely exploited (Winand *et al.*, 2019). In addition, recent research illustrates that the targeting of 16S variable areas using short-read sequencing technologies (operational taxonomic units and/or single-nucleotide polymorphisms) cannot attain the taxonomic resolution provided by sequencing the complete (~1.5 kb) gene (Johnson *et al.*, 2019). Compared to other methods, Nanopore sequencing offers a significant advancement in DNA sequencing technology, enabling the generation of exceptionally long-read sequence data at a lower cost and increased accuracy (*Nanopore Sequencing: Principles, Platforms and Advantages - CD Genomics*, 2025). Recent studies have shown the MinION platform as a portable, rapid, economical, approach to monitoring the microbiome, such as assessing the surface water of a representative river in Cambridge, UK for bacterial content (Urban *et al.*, 2021).

3.11. Sample Preparation for MinION Sequencing.

Samples for nucleotide sequencing were carefully selected. The first nine were reference strains, prepared as water-seeded samples and used as sequencing controls. These reference cultures were followed by the medial water samples from the final sampling occasion (the eleventh). The medial sample was obtained from all 15 outlets between Proximal and Distal samples and subsequently used for DNA extraction and nucleotide sequencing (Table 13). Therefore, a total of 24 samples were examined in this stage of the study

After filtration of these twenty-four water samples, filters were subjected to genomic DNA extraction (Zymo DNA extraction kit). The extracted DNA were then barcoded and 16S rRNA gene amplification performed by PCR. After amplification all samples were pooled together, purified, and adapters ligated. This process was required for attaching DNA to the MinION sequencing pores. The final step was adding sample to library beads solution before application onto a flow cell attached to the MinION Mk1c device.

3.12. Sample Validation by PCR Analysis

Prior to flow cell application and DNA sequencing, the products produced from the 16S rapid barcoding kit for genus-level bacterial identification were verified by standard PCR. Following PCR amplification of the whole 16S rRNA gene sequence using species specific primers outlined in Table 4, electrophoresis was employed to verify the size of 16S rRNA gene PCR products. The 16S rRNA gene is approximately 1.5kb in length, with the main PCR product amplified from reference bacterial strains matching the expected size (Figure 9). The first 9 samples were from water-seed reference cultures where bands were clearly visible, indicating a high concentration of pure bacterial DNA. Outlet samples (order of samples can be seen in Table 13) CG 2033, UTC003, and CG3023 are less visible compared to the reference cultures however, other samples are not visible at all.



Figure 9. 16S rRNA gene amplification. PCR was carried out to amplify the 16S RNA gene from barcoded DNA samples prior to MinION sequencing. 5µl of PCR products were mixed with 5µl orange, fluorescent dye for electrophoresis visualisation. The first position is occupied by a 100 bp ladder, followed by a negative control (DNA extracted from a filter through which autoclaved water was strained) and nine water-seeded reference cultures, each with a designated outlet (see Table 13) for the corresponding barcode and designated outlet).

Table 13. DNA extraction from filters prior to 16S gene amplification. DNA was isolated using a Zymo DNA extraction kit, quantified using a BioDrop spectrophotometer, and the 16S rRNA sequence amplified by PCR. PCR products were also quantified by BioDrop spectrophotometry.

No.	Sample	Concentration of DNA Exacted from Filters using the Zymo kit	Volume of DNA added for 16S PCR	Concentration of Amplified PCR Products
1.	<i>P. aeruginosa</i>	65.15 µg/ ml	1 µl	788.5 µg/ ml
2.	<i>S. maltophilia</i>	57.56 µg/ ml	1 µl	676.7 µg/ ml
3.	<i>A. hydrophilia</i>	60.28 µg/ ml	1 µl	653.5 µg/ ml
4.	<i>P. stutzeri</i>	29.60 µg/ ml	2 µl	659.6 µg/ ml
5.	<i>B. cepacian</i>	108.3 µg/ ml	1 µl	797.3 µg/ ml
6.	<i>A. faecalis</i>	110.3 µg/ ml	1 µl	697.8 µg/ ml
7.	<i>E. meningoseptica</i>	137.5 µg/ ml	1 µl	699.6 µg/ ml
8.	<i>E.coli</i>	70.58 µg/ ml	1 µl	681.0 µg/ ml
9.	<i>A. baumannii</i>	43.61 µg/ ml	1.5 µl	675.7 µg/ ml
10.	GF/SDEC/HW	9.731 µg/ ml	10 µl	698.4 µg/ ml
11.	FF/CG 2020/HW	5.631 µg/ ml	10 µl	694.4 µg/ ml
12.	FF/AMS 008/SH	3.491 µg/ ml	10 µl	740.5 µg/ ml
13.	FF/STF O20/HW	2.75 µg/ ml	10 µl	641.6 µg/ ml
14.	FF/CG 3016/HW	2.70 µg/ ml	10 µl	709.7 µg/ ml
15.	FF/CG 2020/SH	1.735 µg/ ml	10 µl	648.2 µg/ ml
16.	FF/CG 3016/GS	2.65 µg/ ml	10 µl	681.9 µg/ ml
17.	FF/CG 2033/HW	2.996 µg/ ml	10 µl	700.4 µg/ ml
18.	GF/UTC 003/HW	5.307 µg/ ml	10 µl	698.6 µg/ ml
19.	GF/SMS004/HW	2.195 µg/ ml	10 µl	697.5 µg/ ml
20.	FF/CG 3023/HW	2.426 µg/ ml	10 µl	700.5 µg/ ml
21.	FF/AMS 008/HW	4.049 µg/ ml	10 µl	686.6 µg/ ml
22.	FF/CG 3023/SH	2.366 µg/ ml	10 µl	699.7 µg/ ml
23.	FF/STF 020/HW	1.775 µg/ ml	10 µl	687.3 µg/ ml
24.	FF/CG 3001/HW	3.088 µg/ ml	10 µl	1.615 µg/ ml
Total DNA after barcoding and PCR purification steps				436.3 µg/ ml

3.13. Analysis of Reads by Kraken2 and Minimap2

After sample preparation and verification, the multiplexed and barcoded 16S rRNA amplicons were applied to the flow cell (V10) attached to MinION Mk1C device. The MinION sequencing run was carried out over 24h before stopping the sequencing run for data analysis.

The MinION findings were analysed using the EPI2ME software. EPI2ME, 16S RNA workflow used comprises two analysis pipeline options, Kraken2 and Minimap2. Sequences were uploaded and processed using both tools and evaluated to ascertain the most appropriate approach for in depth analysis. Both Kraken2 and Minimap2 obtained results by employing different algorithms to analyse 16S RNA whole genome sequencing at the bacterial genus level.

Kraken2 and Minimap2 analysed the same dataset. The first nine samples contained water-seeded reference cultures, which were used to determine the accuracy of the sequence alignments and identification. Both Kraken2 and Minimap2 analysed and matched datasets to the genus level. Kraken2 identified samples 1-9 as: Barcode 1 as *Pseudomonas*; Barcode 2 as *Stenotrophomonas*; Barcode 3 as *Aeromonas*; Barcode 4 as *Pseudomonas* and *Stutzerimonas*; Barcode 5 as *Burkholderia* and *Paraburkholderia*; Barcode 6 as *Alcaligenes*; Barcode 7 as other; Barcode 8 as *Citrobacter*, and Barcode 9 as *Acinetobacter* (Figure 11). However, considering that the Barcode 8 water sample was seeded with *E. coli* reference culture (Table 11), and Barcode 5 was seeded with *Burkholderia cepacia* reference culture, not *Paraburkholderia*, several discrepancies were noted between expected sequences (reference cultures) and sequences identified by Kraken2. Furthermore, Barcode 4, which was seeded with *P. stutzeri* (genus level) should have been recognised as *Pseudomonas*, rather than characterised as both *Pseudomonas* and *Stutzerimonas* genus.

In contrast, Minimap2 successfully recognised most of the barcodes designated reference cultures correctly (Figure 12), although Barcode 4, corresponding to *P. stutzeri* reference strain was identified as *Stutzerimonas*, instead of *Pseudomonas* genus (Table 12). Considering that every other bacterial species was identified correctly using Minimap2 algorithm, any sequence matching to *Stutzerimonas* in the samples relating to hospital taps, will be considered as *P. stutzeri*.

Table 14. Comparison between reference cultures vs results from Kraken2 vs Minimap2

Reference culture	Kraken2	Minimap2
<i>P.aeruginosa</i>	<i>Pseudomonas</i>	<i>Pseudomonas</i>
<i>S.maltophilia</i>	<i>Stenotrophomonas</i>	<i>Stenotrophomonas</i>
<i>A.hydrophilia</i>	<i>Aeromonas</i>	<i>Aeromonas</i>
<i>P.stutzeri</i>	<i>Pseudomonas</i> + <i>Stutzerimonas</i>	<i>Stutzerimonas</i>
<i>B.cepacia</i>	<i>Burkholderia</i> + <i>Paraburkholderia</i>	<i>Burkholderia</i>
<i>E.meningoseptica</i>	Other	<i>Elizabethkingia</i>
<i>E.coli</i>	<i>Citrobacter</i>	<i>Enterobacter</i>
<i>Acinetobacter baumannii</i>	<i>Acinetobacter</i>	<i>Acinetobacter</i>

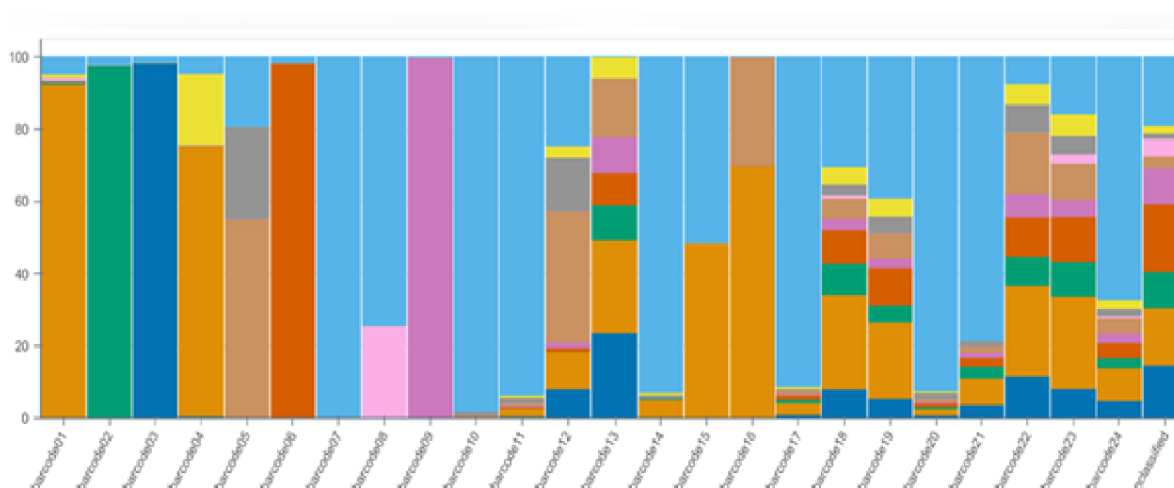


Figure 10. Sequence analysis to genus-level identification using EPI2ME software and Kraken2. Barcode number is displayed on the bottom X-axis in the same order as outlined in Table 13. Relative abundance on displayed on the left Y-axis. *Aeromonas* is dark blue, *Pseudomonas* is orange, *Stenotrophomonas* is green, *Alcaligenes* is red, *Acinetobacter* is purple, *Burkholderia* is brown, *Citrobacter* is pink, *Paraburkholderia* is grey, *Stutzerimonas* is yellow, other bacteria are blue.

Taking into account Traditional Microbiology Methods, already identified bacteria from outlets that have identified multiple targeted species CG2033 and CG3016. Species isolated from both outlets were *S. maltophilia*, *P. aeruginosa* and *P. stutzeri*. These outlets have shown high reads for these specific bacteria on the Minimap2 as well, with the exception of *S. maltophilia*, with very low reads (under 10). Bacteria isolated from outlet SMS 004 using Traditional

Microbiological Methods (TMM), are *A. faecalis*, *B. cepacia* from outlet CG2020 (Table 9), which have also shown over 10 reads on the Minimap2/MinION nucleotide sequencing (Table 15).

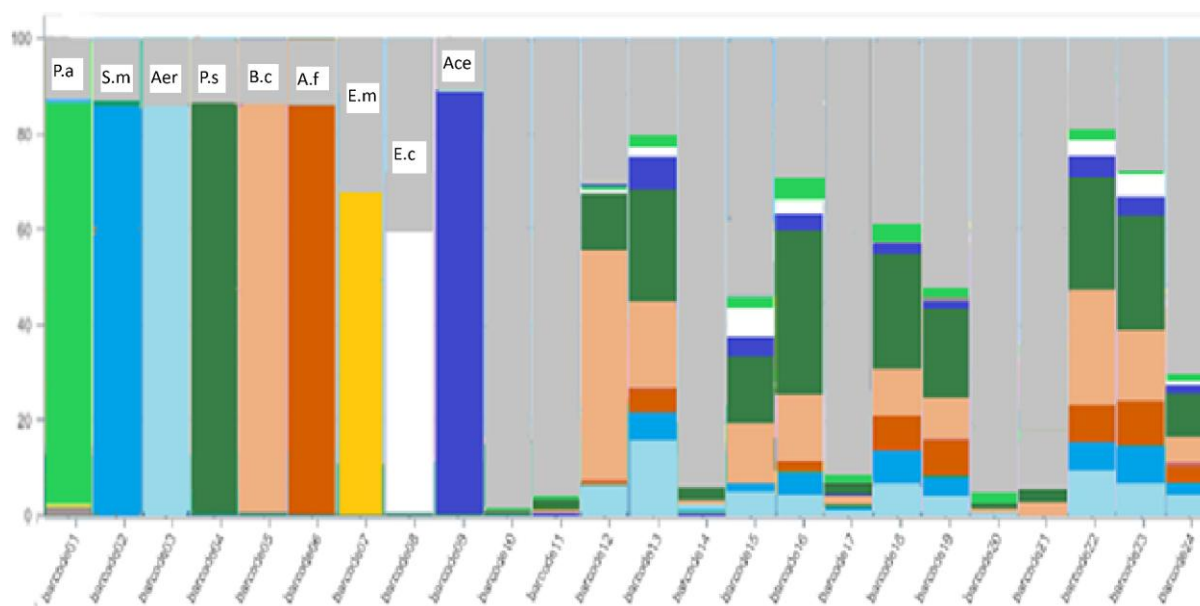


Figure 11. Sequence analysis to genus-level identification using EPI2ME software and Minimap2. Barcode number on the bottom X-axis, displayed in the same order from Table 13, compared against relative abundance on the left Y-axis. *Pseudomonas* (Pa) is Light green, *Stenotrophomonas* (S.m) is blue, *Aeromonas* (Aer) is light blue, *Stutzerimonas* (Ps) is dark green, *Burkholderia* (B.c) is peach colour, *A. faecalis* (Af) is light brown, *Elizabethkingia* (Em) is Yellow, *Escherichia/Enterobacter* (Ec) is white, *Acinetobacter* (Ace) is indigo blue, Other is grey.

Sequence reads identified by Minimap2 also displayed high reads from other targeted species that may have been missed by Traditional Microbiology Methods (TMM), such as *Acinetobacter*, *Aeromonas*, and other strains whose growth conditions could not be met using traditional plating methods. These included *Sphingomonas*, *Cupriavidus*, *Methylobacterium* and other non-targeted species such as *Acidovorax*, *Aquabacterium* and *Herminomonas* (Table 15).

Table 15. MinION reads and bacteria identified from the Minimap2 channel with barcode order (also see Table 13). First nine water-seeded reference cultures are positive controls. No.1 *P. aeruginosa* – bright green; no.2 *S. maltophilia* – blue; no.3 *Aeromonas hydrophilia* – light blue; no.4 *P. stutzeri* – dark green; no. 5 *B.cepacia* – peach; no. 6 *A. faecalis* – reddish/brown; no.7 *E. meningoseptica* – orange; no.8 *E.coli* – white; no. 9 *Acinetobacter baumannii* – dark blue; other species identified without corresponding positive controls/reference cultures were: *Acidovorax* and *Delftia* spp.- pink; *Shingomonas* spp. – yellow; *Cupriavidus* and *Methylobacterium* spp – white. Every other non-targeted species is shown as white, with no or under 10 reads per species shown as grey; followed by 15 outlets and bacteria identified within the sample (see Appendix 1* for their location).

No.	Outlet	Total reads	Pse.	Stutz.	Steno/Xani	Aero.	Burkh.	Acido.	Sphing.	Alcali.	Cupr.	Eliz.	Acin/Morx	Entero	Delf.	Methyl	Aqua.	Herm.
1	<i>P.aeruginosa</i>	168293	161641															
2	<i>S.maltophilia</i>	301394		300369														
3	<i>A.hydrophilia</i>	313654			313563													
4	<i>P.stutzeri</i>	195483	192658															
5	<i>B.cepacia</i>	214846				214639												
6	<i>A.faecalis</i>	275570							275457									
7	<i>E.meningoseptica</i>	41138										40873						
8	<i>E.coli</i>	234337												234198				
9	<i>A.baumannii</i>	186614											186558					
10	SDEC	2051	5	7			4	324	59		29		36				287	1077
11	UTC003	3210	106	880	267	257	333	391	97	289		42	87	19+31				247
12	SMS004	728	14	157	33	35	72	69	31	66			16	10+7			179	
13	STF020	1138		323	103	93	191	43	33	130	21	16	53	60+22				
14	STF020SH	220	16	59	15	41	46			12			18					
15	AMS008	969	31	43	174+29	38	37	253	151	22	74		12					
16	AMS008SH	867		112		73	499	31	58	10			12	11+2			19	
17	CG2020HW	3317	10	90		21	32	1306	631				12				1029	60
18	CG2020SH	83		14			12		17				4				11	
19	CG2033	5355	102	125	58	55	77	4221	404	70		11	13	10+1		39	59	43
20	CG3016	2302	102	33			17	1739	101		43					10	110	62
21	CG3016GS	72		29			12											
22	CG3001	3984	219	442	114+55		284	567	769	180	142	30	103	32+20		14	467	118
23	CG3023	6553	34	123	137+37	54	68	2380	929	54	242	10	23	11+11	25	14	2025	34
24	CG3023SH	839	21	222	61	90	225		14	80		13	47	28+14				

3.14. Summary from Part 2.

Comparative analysis of two EPI2ME 16S rRNA workflow channels using reference strains water seeded as positive control, indicates that MiniMap2 yielded more accurate alignment and identification results. The MiniMap2 has correctly identified all reference cultures except *P. stutzeri*, which was misidentified as the closely related *Stutzerimonas* spp. Fifteen additional samples from outlets underwent DNA identification using positive control identification. MinION has also identified in those fifteen samples additional bacterial DNA from following genus *Acidovorax* spp., *Sphingomonas* spp., *Cupriavidus* spp., *Methylobacterium* spp., and *Delftia* spp.

4. Discussion

Water supplied to taps for hygiene and consumption it is not sterile and serve as a natural reservoir and living environment for various microorganisms. Currently, the UK drinking water standards must be met that water is free of faecal contaminant indicators (*E. Coli* and coliforms) and UK water companies have a statutory duty to test water supplies on a regular basis. These microorganisms typically do not induce illness in healthy individuals possessing a functional immune system. However, in the hospital environment, these microorganisms may present a risk, especially in individuals with compromised skin barriers, such as burn patients, or those with weakened immune systems, such as cancer patients, may be susceptible to water-associated microorganism infections (Kanamori, Weber and Rutala, 2016).

Additional water safety regulations in place for healthcare facilities (HTM 04-01) also include risk mitigation and testing for *Legionella* species and *Pseudomonas aeruginosa*. This limited list of bacteria that are regulated by water companies and healthcare facility managers means that the remaining OPPPs may not be fully understood or recognised in the water distribution systems of healthcare premises (Weinbren, 2020). Considering that sampling was carried out within construction phase without patient driven antibiotic use, a water distribution system can still undergo measurable changes in its antimicrobial resistance (AMR) profile due to biofilm mediated persistence, co-selection pressures, stagnation effects, and occasional external contamination. Continuous monitoring, ideally combining culture based and molecular methods is essential to detect and manage these shifts (Alfahl *et al.*, 2024). All isolates were confirmed to species level; however, it is only presumed that they represent the same strain and are clonal, as no additional testing has been performed to verify strain identity. Further in-depth phenotypic analysis and genomic characterisation is required to precisely determine the clonality of the isolates and fully understand their spectrum of pathogenicity, resistance profile, and risk to specific patient groups.

Water sampling at Scarborough Hospital was completed on eleven sampling occasions between June to December 2024 and yielded results that could have patient implications in the future. Aside from the water treatment intervention implemented before and during the second sampling event, no further contextual information was provided. Input from construction team was minimal and lacked detail. The only confirmed operational activity was routine flushing of water outlets. Two methods were used to analyse 345 samples, traditional microbiology methods (TMM) and a nucleotide sequencing metagenomics method, although both methods have their own benefits and limitations. Included in the 345 samples were an additional 15

samples collected during the eleventh sampling occasion, then analysed only by nucleotide sequencing.

For traditional microbiology methods from 330 samples collected, all demonstrated bacterial growth on the membrane following incubation. From a total of 15 outlets routinely monitored during the seven-month study period, 14 outlets yielded an OPPP. However, one outlet (outlet number 8) did not contain a detectable OPPP over the 11 sampling occasions (Table 9). Other outlets have yielded a range of OPPP's but notably species from the *Pseudomonas* genus (*P. stutzeri*, *P. alcaligenes*, *P. luteola*, *P. fluorescens*, *P. oryzae* and *P. aeruginosa*), were isolated from a range of different outlets. This genus of bacteria is known to be causes of bloodstream infections (Table 1).

The most frequently isolated member of this genus isolated during this study was *Pseudomonas stutzeri* which was isolated in the first sampling occasion from the new hospital building in June 2024. *P. stutzeri* was present in both proximal and distal samples from the ground floor, the hand wash outlet in the trolley bay area, and two outlets on the first floor, along with both distal samples from the shower staff change and the utility. Over the study period the bacterium was isolated 15 times from 5 outlets. A study by Bisharat, Gorlachev and Keness (2012) demonstrated that in 10 years of monitoring hospital laboratory data and number of isolates recovered from clinical specimens (urine, wounds, blood cultures), there were 103 incidences of *P. stutzeri* infections in patients from 2000 to 2010. However, it was noted that it rarely caused invasive infections and was more susceptible to antibiotics compared to *P. aeruginosa*. Nevertheless, ten elderly patients died with underlying comorbidities where infections were usually found on wounds and urine. The 10 year monitoring was carried out in Israel, Clalit Healthcare Services no further information was provided (Bisharat, Gorlachev and Keness, 2012).

In a hospital outbreak in 2016, *P. stutzeri* was isolated from the wounds of nine patients, where sterile dressing products caused the infection (Choi *et al.*, 2017). *P. stutzeri* is not usually known to cause a serious infection (e.g., bacteraemia), but any healthcare-associated infection is undesirable and costly to the patient and healthcare provider (Ranieri *et al.*, 2020). A 20-year retrospective observational study in public hospitals in Queensland, Australia showed a rise in *P. stutzeri* infections. Data source included looking into public hospital admissions, statewide laboratory surveillance and vital statistic database and between 2000 and 2019, clinical sources of infections were skin and soft tissue, lower respiratory tract and abdominal sources. 228 cases of *P. stutzeri* bloodstream infections were identified; 49 cases (21.5%) were

nosocomial infections, and there were thirty-one patient deaths (13.6%). Respiratory infections associated with *P. stutzeri* also had a higher mortality rate, with 48 individuals (21%) dying from the infection (Horcajada *et al.*, 2024). These studies highlight the potential for *P. stutzeri* to substantially impact patients and healthcare systems if undetected and untreated.

During the first sampling week of this current study, five OPPPs were isolated from seven different outlets including *P. stutzeri* from three different outlets. On the second sampling occasion, silver ionisation disinfection was in process, and there was a reduction in the total viable counts (when countable), along with a noticeable change in the species isolated. However, *P. stutzeri* was able to maintain its presence in two of the three outlets it was recovered from in the first sampling occasion. Notably, outlet 2 showed consistent isolation of *P. stutzeri* on eight consecutive occasions, despite numerous actions taken by the construction company to appropriately prepare the water system for staff and patient use. *P. stutzeri* was able to withstand the use of silver ionisation and regular chlorination eradication procedures. The almost continual presence of *P. stutzeri* within hospital plumbing and WDS, demonstrate that this bacterial species can maintain colonisation in WDS despite extensive measures actioned to control the microbial population in water.

Table 9 documents the isolation of OPPP's from the 15 outlets and the changes in species recovery before, during and after silver ionisation across sampling week 1, sampling week 2 (during ionisation), and sampling week 3. In sampling week 3 there was an elimination of all other OPPP's with only those belonging to the *Pseudomonas* genus detectable. However, by sampling occasion 4, different species of OPPP's began to reemerge from the water samples. Notably, was after this first decontamination attempt *P. aeruginosa* was identified, which is a serious pathogen and regulated through HTM 04-01.

Other *Pseudomonas* spp., were also identified in the hospital water outlets, including *P. alcaligenes*, *P. fluorescens*, *P. luteola*, and *P. oryzae*. Over the study period, *P. alcaligenes* was isolated five times in four different outlets. In the first week on both floors in distal samples of three outlets ground floor, hand wash outlet in the utility, the handwash outlet near SDEC, and one of the shower outlets in a single room on the first floor. *P. alcaligenes* were also found in the third (outlet near SDEC) and the eighth (general sink where there was only a cold-water outlet from a dirty utility) sample collections. Previously, *P. alcaligenes* was classified as closely related *P. aeruginosa*, noted to also causes bloodstream infections and in rare cases, infections can result in death. In addition, a strain isolated from a patient in Hospital in Japan and

identified by whole genome sequencing was found to be resistant to most monobactams and cephalosporins (Suzuki *et al.*, 2013).

P. fluorescens was isolated on two sampling occasions from two different outlets. The first sampling occasion from a distal sample, a hand wash outlet near SDEC on the ground floor and on the seventh sampling occasion from a distal sample, the first floor CG 3023, a shower outlet scales (Scales *et al.*, 2014). *P. fluorescens* optimal temperature is around 25 to 30 °C, and it can survive very low temperatures, down to 4 °C. It can also adapt to grow at 37°C when required (Oba *et al.*, 2017). This wide growth parameter may provide *P. fluorescens* with a survival and growth advantage in UK water systems where there is a large seasonal fluctuation in temperature. As seen during this research, recovered *P. fluorescens* from the first sampling occasion could only grow at room temperature; however, recovered *P. fluorescens* from sampling occasion seven grew at 37°C (Table 9). In addition, *P. fluorescens* is not known to be virulent, however numerous different studies have reported infections connected to drinking water in immunocompromised patients (Wong *et al.*, 2011; Liu *et al.*, 2021), with two longitudinal bloodstream infection outbreaks caused by *P. fluorescens* (Oba *et al.*, 2017).

Furthermore, in this study period of seven months, *P. luteola* was isolated three times from three different outlets, on sampling occasions four, five and nine. *P. oryzihabitans* was yet another *Pseudomonas* spp. that was recovered once in a single outlet on the first-floor room coded CG 3023 (the hand wash outlet) during the 9th sampling occasion. All these bacteria isolated from hospital outlets have the same characteristics in that they rarely cause infections in healthy individuals. However, if found in hospital environment they may cause an outbreak within vulnerable patient groups, with water a potential common source of infection as it is distributed around the hospital for both patients and staff usage. Overall, although *Pseudomonas* spp, *P. stutzeri*, *P. fluorescens*, *P. alcaligenes*, *P. luteola*, and *P. oryzihabitans* rarely cause serious complications and infections, regardless of treatment responsiveness, they can still delay the recovery of elderly and immunocompromised patients, and in some cases cause premature death, emphasising the need for improved monitoring processes.

Among the species isolated from the WDS was *P. aeruginosa*, one of the most concerning opportunistic pathogens. *P. aeruginosa* was found in both proximal and distal samples in the last two sample collections on the first floor, both utility rooms (CG2033 and CG3016) and both HW outlets. *P. aeruginosa* is designated as an opportunistic pathogen. It can cause hospital and community-acquired infections in immunocompromised patients, and also those with chronic obstructive pulmonary disease, burns, traumatic injuries, sepsis, malignancies, and ventilator-

associated pneumonia (Qin *et al.*, 2022). Another well-known ability of *P. aeruginosa* is the formation of biofilm. *P. aeruginosa* can establish symbiotic relationships with various microorganisms, encompassing bacteria, fungi, and viruses, potentially increasing its pathogenicity and infection risk (Figure 12) (Cendra and Torrents, 2021).

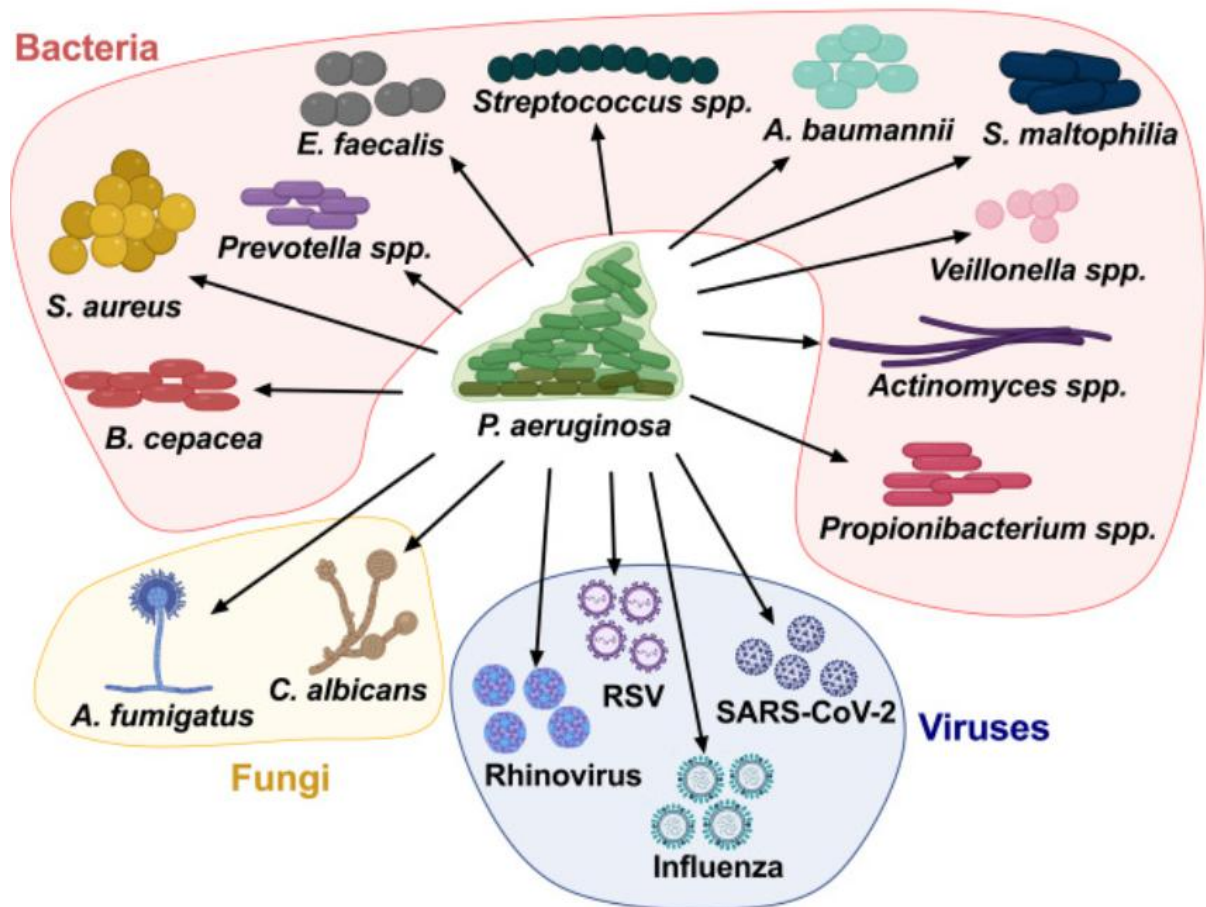


Figure 12. *Pseudomonas aeruginosa* and other clinically relevant species biofilm interactions. *P. aeruginosa* ability to interact and coexist with other “partners in crime” with numerous clinically relevant microorganisms (*B. cepacia*, *A. baumannii*, *S. maltophilia*) including other bacteria, viruses and fungi (Cendra and Torrents, 2021).

Some of the species seen in Figure 12 were isolated during this study using TMM during weeks 10 and 11 (Table 9) (*P. aeruginosa*, other *Pseudomonas* spp. and *S. maltophilia*). The other species mentioned in Figure 12 were identified by nucleotide sequencing with high reads of DNA isolated from outlet UTC 003. Biofilm can be the source of great genetic microbial diversity, colonisation stability and horizontal gene transfer (Rather, Gupta and Mandal, 2021). As such, the presence of multiple species could be a risk to the water distribution supply and patient

safety. Intriguingly, *S. maltophilia* was also identified in the well water sample alongside two *Pseudomonas spp*, consistent with findings from the 10th and 11th hospital sampling occasions, suggesting a strong symbiotic relationship. Beside having *S. maltophilia* as a common biofilm tenant, the additional species found in well water were *P. aeruginosa* and *P. putida*, although, in hospital water these were *P. stutzeri* and *P. aeruginosa*. This is a strong indication for the establishment of multispecies biofilms, generating a complex microbial community that may be more difficult to treat.

Pseudomonas aeruginosa frequently engages in complex interkingdom and interdomain interactions that influence its persistence, pathogenicity, and resistance, therefore polymicrobial communities could be clinically important. In polymicrobial biofilms, *P. aeruginosa* can coexist with fungi such as *Candida albicans* and *Aspergillus fumigatus*. These relationships are often competitive, where *P. aeruginosa* secretes phenazines and siderophores that inhibit fungal growth by disrupting membrane integrity and limiting iron availability. However, it can also be synergistic in chronic infections, where biofilm cohabitation enhances tolerance to antimicrobials and host defence mechanisms. In the case of *Aspergillus*, iron competition is a key driver of interaction, with both organisms adapting their metabolism to persist under nutrient-limited conditions, which may also occur in WDS (Cendra and Torrents, 2021).

Interactions with human viruses, including SARS-CoV-2, are increasingly recognised in the context of secondary or co-infections. COVID-19-associated lung damage, immune dysregulation, and altered epithelial barriers create a permissive environment for *P. aeruginosa* colonisation and biofilm formation (Bajire and Shastry, 2024). In patients with chronic respiratory diseases such as COPD, co-infection with *P. aeruginosa* and SARS-CoV-2 has been linked to more severe disease courses, prolonged inflammation, and poorer outcomes. Viral infection can impair mucociliary clearance and modulate immune signalling, indirectly facilitating bacterial persistence, while *P. aeruginosa* biofilms further exacerbate inflammation and tissue injury (Bajire and Shastry, 2024). These findings underscore the importance of considering *P. aeruginosa* not only as an isolated pathogen, but as part of a dynamic microbial network that could include additional bacteria, fungi, and viruses. Such interactions have direct implications for infection control strategies in both clinical and environmental water-associated settings.

Considering that HTM 04-01 aims to mitigate the presence of *P. aeruginosa* in hospital water (Figure 1), the isolation of this bacteria only a week before for the hospital was due to open, is an

indicator that this pathogen can emerge suddenly in a WDS and therefore continued vigilance in water testing is required. Importantly, consideration is also needed around the reason for biofilm formation; namely it is a bacterial response to harsh environmental conditions, where bacteria secrete protective exopolysaccharides, fibrins, lipoproteins extracellular DNA, proteins and polysaccharides known as extracellular polymeric substances (EPS) (Flemming *et al.*, 2024). EPS adhere to the surface of objects found in water distribution systems, including the surface of pipes, and they also adhere onto tissue surfaces in the human body (Zhao, Sun and Liu, 2023). The highest concentration of EPS is found above the cells, likely as a protective mechanism against chlorine stress (Flemming *et al.*, 2024).

The EPS is regulated by signalling molecules, collectively known as quorum sensing (QS), that serve as intracellular communicators, where the communal behaviour of biofilms is regulated by these bacterially secreted molecules (Lv *et al.*, 2024). A recent study has shown that bacteria can interact by QS to coordinate gene expression, allowing them to respond collectively to environmental changes, including antibiotic stress (Wang *et al.*, 2023). Driven by exposure to chemical stressors, such as antibiotics, heavy metals, disinfectants, herbicides, and pesticides, QS plays an important role in enhancing horizontal gene transfer of antibiotic resistance genes by enhancing cell membrane permeability, pilus formation, plasmid replication and cellular competence (M. Wang *et al.*, 2023). Thus, further consideration must be given to better understanding the process of QS and how it can be targeted to improve infection control processes.

In this research, alongside *P. aeruginosa*, the presence of its “partner in crime”, *S. maltophilia*, within several outlets (10 and 11), suggests the presence of mature multi-microbial biofilm formation (also confirmed by nucleotide sequencing in Figure 11). Unfortunately, water is not regularly tested for this “emerging multidrug-resistant global opportunistic pathogen” (Brooke, 2012). There have been numerous reported cases of hospital water associated *S. maltophilia* outbreaks causing infections (Cervia, Ortolano and Canonica, 2008; Guyot, Turton and Garner, 2013; Yashar *et al.*, 2024). In two Turkish hospitals over a period of two years from 2021-2022, *S. maltophilia* infections exhibited an overall mortality rate of 56%, with bacteraemia and pneumonia subgroups showing rates of 60% and 53%, respectively, emphasising the need to consider the presence of this species as a potential infection risk (Hasbek *et al.*, 2025).

Over the study period, *S. maltophilia* was isolated four times from two outlets. It is a worldwide concern associated with a major fatality: case ratio and is often recovered with *P. aeruginosa*, from polymicrobial infections, most commonly respiratory infections. Considering that both *S.*

maltophilia and *P. aeruginosa* were found in the same outlets during this current study (CG2033 and CG3016), it is highly likely that they have formed a polymicrobial biofilm. *S. maltophilia* presence is of great concern for all augmented care patients, especially if these biofilms are formed in lungs of cystic fibrosis patients, where they commonly develop antibiotic resistance and become difficult to treat leading to increased mortality rates (Høiby *et al.*, 2017; Galdino *et al.*, 2023). Indeed, this biofilm property could be the reason behind the intermediate resistance to STX antibiotic shown by *S. maltophilia* in this study. One of the significant clinical challenges in the treatment of infections caused by *S. maltophilia* is the inherent multidrug resistance of the bacteria (Mojica *et al.*, 2022).

Burkholderia cepacia DNA was also detected by nucleotide sequencing, within outlets 10 and 11 during the last sample collection. However, due to the technical limitations of the characterisation processes employed, it is unclear if the bacterial DNA was in the water or it was from viable cells within the biofilm. *B. cepacia* was identified using API 20 NE (bioMérieux) strips and over the study it was isolated nine times from six different outlets, including from the proximal sample in the handwash outlet on the first floor on the first and second sampling occasions, then again in the sixth, seventh, eighth and ninth and eleventh occasions. This is another example of a bacterium identified in WDS that could also cause infections in patients. One example is a pseudo-outbreak in Kentucky Regional Acute Care Hospital, where five *B. cepacia* infections over ten months were reported related to urological operating rooms (Eldridge *et al.*, 2022). In another hospital across a year, *B. cepacia* caused complex bloodstream infections (see Table 3) in eight infected cases, where three suffered from recurrent infections, and three subsequently died. The source of infection was identified as water-based heaters and remedial action was taken to test all heaters and then initiate removal, because all of the heater-derived cultures were *B. cepacia* positive (Rhee *et al.*, 2022).

In this current study, all isolated and API NE 20 identified *B. capecia* were tested for AST. The bacteria from the first water sampling were susceptible to all antibiotics used. However, further down the sampling collection, bacteria isolated from the last few sampling occasions gained resistance to AK and developed IR to CIP and CAZ. Resistance to Amikacin indicates activation by mutation to the RND 3 efflux pump, suggesting the *B. cepacia* recovered from water samples exhibited intrinsic resistance to aminoglycosides (Rhodes and Schweizer, 2016). Considering that the first two *B. cepacia* recovered from the first two sampling occasions showed sensitivity to all antibiotics, it suggests that water treatments might drive slowly developing resistance to antibiotics. An additional study has shown that different chemical stressors, including

antibiotics, heavy metals, disinfectants, herbicides, and pesticides, act as catalysts for the development of antimicrobial resistance (Wang *et al.*, 2023).

In hospital premises, biofilm formation in WDS is a serious matter, and its removal or elimination is required by the hospital Estates team. Considering that bacteria are deeply imbedded and shielded by EPS, which serves as a protective barrier against external threats while allowing biofilms to demonstrate impressive adaptive abilities, this renders their elimination a challenging undertaking (Li *et al.*, 2023). The biofilm treatment required in this case was combined treatment chemical and mechanical removal. Mechanical removal includes mechanical scrubbing to break down biofilm. Chemical removal with common agents like chlorine, bromine ozone (Li *et al.*, 2023), however in this case copper silver ionisation was also used in combined therapy, which provided only temporary relief from bacterial contamination.

Other isolated bacterial species of potential interest were *Acinetobacter lwoffii*, *Alcaligenes faecalis*, and *Aeromonas spp.* *Acinetobacter lwoffii* was identified once by API 20 NE strips in the fourth sampling occasion from the shower in room CG3023. PCR of the 16S rRNA gene identified *Acinetobacter spp.* on eight sampling occasions taken from the same outlet. In the United Kingdom, *Acinetobacter spp.* are increasingly recognised as potential pathogens, and bacteraemia caused by *Acinetobacter baumannii* is associated with *Acinetobacter* infections. Although both species induce comparable human infections and inhabit the same ecological niche, *A. lwoffii* has, to date, retained susceptibility to antibiotics, which contrasts with *A. baumannii*, however all *Acinetobacter* should be viewed as problematic within WDS and should be routinely monitored for (Darby *et al.*, 2024).

Over the study period *Alcaligenes faecalis* was isolated four times from two different outlets, where it was recovered during the second, fourth and eleventh sampling occasions. All water isolates identified in this research study were AST tested, and no resistance was found. However, recently, *A. faecalis* has become less susceptible to routinely used antibiotics, and this trend is expected to continue. Some of the most frequent infections are soft tissue and skin infections, urinary tract infections, middle ear infections and bacteraemia. Infections caused by *Alcaligenes faecalis* that are extremely resistant to many drugs have arisen, and infection risk should be minimised by careful surveillance and control processes (Huang, 2020).

Aeromonas spp. was isolated only once from one outlet on the sixth sampling occasion. 16S rRNA PCR identified this waterborne pathogen, which is considered an emerging enteric pathogen. It possesses the ability to proliferate in water distribution systems, particularly within

biofilms, where they may exhibit resistance to chlorination. *Aeromonas* spp. is associated with human diseases, including skin, muscle and soft tissue infections, and is known to cause septicaemia and gastroenteritis (Igbinosa *et al.*, 2012). In a hospital in India, from 36 confirmed *Aeromonas* infections, five patients died, contributing 13.88% of the mortality rate, highlighting the infection risk carried by this bacterial class (Veeran *et al.*, 2022).

Delftia acidovorans has been isolated by TMM only once in the fifth sampling occasion and identified by API NE 20 in the fifth sampling occasion from outlet CG3016. In the eleventh sampling occasion from outlet CG3023, *Delftia* spp. was identified by nucleotide sequencing, however no detection was made by TMM. From case studies and case series, infections caused by *Delftia acidovorans* are reportedly not persistent and predominantly impact male patients with preexisting comorbidities, including malignancies. It is often found in polymicrobial cultures along with *P. aeruginosa* and *S. maltophilia*. Isolates are known to exhibit resistance to gentamicin, while fewer isolates (but still over 50% of them) demonstrated colistin resistance (Højgaard *et al.*, 2022). Another study has shown *Delftia acidovorans* as rare infections but is linked to neonatal sepsis (Alam *et al.*, 2023), and mortalities, predominantly within elderly populations exhibiting several coexisting medical conditions (Lu and Huang, 2024). *Delftia acidovorans* has also previously been connected to water-related pseudo-outbreak (Yassin *et al.*, 2020). In an 8-year retrospective study where 26 patients were diagnosed with *D. acidovorans* infections, a mortality rate of 11.5% was observed, primarily among elderly patients and those with malignant disease (Lu and Huang, 2024). Subsequently, there is a reasonable argument for *Delftia acidovorans* to be added onto the OPPP's list, as it perfectly fits the profile of OPPP's.

Monitoring the water microbiome by reliably and rapidly identifying the “tenants”, will help prevent healthcare-acquired infections and avoid the overuse of antibiotics. Furthermore, it will reduce unnecessary and very costly WDS flushing, producing a benefit to both hospitals and patients. Worryingly, in recent studies, it has been noted that some gram-negative bacteria have started to develop chlorine resistance, reducing the effectiveness of one of the main treatment strategies. As resistant gram-negative bacteria are often found in aqueous environments, residing in private, commercial, and industrial plumbing systems, improving our understanding of their impact on human health is becoming increasingly important (Falkinham, Pruden and Edwards, 2015).

From all 360 samples collected only 30 of them were used for nucleotide sequencing (NS), all from the eleventh (last) sampling occasion. These 30 samples were collected, filtered and DNA

was extracted and used for MinION long-read nucleotide sequencing. All results gathered from the MinION were then uploaded and analysed using the supplied EPI2ME Workflow for 16S rRNA sequencing and the Kraken2 and Minimap2 sub-workflows. These workflows differ in their analysis approach, with Kraken2 using Kmer-based algorithms. Kmers can have intrinsic biological significance, and their distribution and frequency can provide insights into genomic characteristics such as repetitive elements, functional regions, genomic variation, and DNA damage and repair mechanisms (Moeckel *et al.*, 2024). In contrast, Minimap2 performs split-read alignment, utilising concave gap costs for extensive insertions and deletions and incorporates novel heuristics to minimise erroneous alignments (Li, 2018). These altered processing and alignment strategies may account for the different identification outcomes for certain bacteria noted between Kraken2 and Minimap2 pipelines. However, the Kmer approach used by Kraken2 reportedly has approximately 10-15% error rates while carrying out direct RNA or cDNA sequencing on contigs or closely related whole chromosomes spanning hundreds of megabases (Li, 2018). Therefore, bacteria that are closely related may not be discernible as the correct species. For example, the *Pseudomonas* spp. *P. stutzeri* reference culture was identified as *Stutzeromonas* instead of *Pseudomonas* spp. (Table 15), where identification was carried out only at a genus level, partially accounting for some of the discrepancies noted here, and providing a note of caution around interpreting microbial colonisation based on sequencing approaches alone. Thus, a combined approach between TMM and molecular methodologies is recommended during future screening and diagnostic pathways.

Beside already confirmed species that were characterised by both TMM and NS, additional bacteria were identified by MinION nucleotide sequencing from the 11th sampling occasion in various outlets, such as *Sphingomonas* spp., *Cupriavidus* spp., *Methylobacterium* spp., *Elizabethkingia* spp.. However, due to culturing limitations, certain bacteria could not be identified by TMM; hence, no viable bacteria have been isolated to confirm or deny MinION results. MinION nucleotide sequencing identified high reads of *Sphingomonas* spp. from all outlets except STF 020 SH and CG 3016 GS. Moreover, research from 2017 using genome sequencing indicated prolonged colonisation of *Sphingomonas koreensis* within hospital plumbing systems, which subsequently acted as a reservoir for occasional infections in immunosuppressed patients (Zellmer *et al.*, 2017). Infections of this pathogen have been isolated from the hospital environment, such as respirators, haemodialysis apparatus. Infections are both community-acquired and nosocomial, and one very rare isolation was made from the cerebrospinal fluid. A range of diseases have been recorded attributed to *Sphingomonas* spp., including endophthalmitis, septic pulmonary embolism, sepsis, septic

arthritis, peritonitis, and meningitis caused by *Shingomonas paucimobilis*, adding to the growing body of evidence indicating this species as a pathogen of concern (Göker *et al.*, 2017).

Cupriavidus spp. was identified by MinION nucleotide sequencing in six outlets (SDEC, STF 020, AMS 008, CG 3016, CG 3001, CG 3023) out of fifteen. Intriguingly, all of them were hand-wash outlets and Scarborough hospital is not the only one in the UK where *Cupriavidus* spp. have been found. Ten hospitals in the UK had their water tested and reported in a 2022 study, where four identified *Cupriavidus* spp.. The most prevalently isolated species was *Cupriavidus pauculus*. Importantly, the other six hospitals demonstrated the presence of additional opportunistic premise plumbing pathogens, including *Acinetobacte* spp., *Sphingomonas* spp. *Pseudomonas* spp. (*P. aeruginosa*, *P. fluorescens*), *Elizabethkingia meningoseptica*, *Stenotrophomonas maltophilia*, and two possible opportunistic premise plumbing pathogens *Acidovorax* spp., *Delftia acidovorans* (Inkster *et al.*, 2022) – most of which were also identified within this study at Scarborough hospital. Public Health England has records from 2015 to 2020 (over a five-year period), confirming fourteen cases of *C. pauculus* bacteraemia, where three cases were Glasgow-associated, and the other nine were also from additional hospitals in Scotland (Inkster *et al.*, 2021). The need to continue the careful monitoring and tracking of potential OPPPs within hospital WDS is becoming increasingly apparent.

This confirms that while infrequent, these infections should not be disregarded as a potential hospital reservoir *Methylobacterium* spp, were identified from four out of eleven outlets by nucleotide sequencing at the eleventh sampling occasion. *Methylobacterium* species are becoming an emerging OPPP that causes infections associated with catheters, bloodstream infection, and peritoneal infection. All these infections were linked vulnerable group of patients to the usage of contaminated water. Infections could vary from mild fever to pneumonia, peritonitis and bacteraemia, and have the potential to cause substantial health impacts (Szwetkowski and Falkinham, 2020).

The current study identified *Elizabethkingia* spp. by sequencing 16S rRNA from six of fifteen outlets. Severe infections can occur in immunocompromised or vulnerable populations, including individuals undergoing chemotherapy, transplantation, or premature infants (Fahmy *et al.*, 2024). *Elizabethkingia* species present risks for both community and healthcare-associated outbreaks because of antimicrobial resistance, where resistance develops during treatment, restricting antimicrobial alternatives and potentially resulting in treatment failure and mortality (Fahmy *et al.*, 2024). As such, this bacterium warrants more careful screening within high-dependency and acute hospital and care facilities.

Another bacteria that was identified by MinION nucleotide sequencing in the WDS, which is not currently on the OPPPs list, is *Acidovorax* spp.. From fifteen outlets, it was present in eleven as identified by sequencing. Previously, *Acidovorax* spp.. has been recorded to cause infections in several patient groups. These aerobic, gram-negative, non-fermenting, rod-shaped bacteria, are normally characterised as a plant pathogen, but can also cause infections in humans. As it is also found in water reservoirs, this makes *Acidovorax* a potential candidate for an OPPP (Javaid *et al.*, 2021). A case study described a 55-year-old patient with type II diabetes mellitus, who developed sepsis caused by the *Acidovorax avenae* organism, as identified by PCR (Shetty *et al.*, 2005). The purified flagellin protein of *A. avenae* serves as a virulence factor, capable of activating the innate immune receptors in human macrophages and fibroblasts. Their activation induces cytokine secretion, potentially resulting in a range of inflammatory diseases, including providing a potential sepsis risk (Javaid *et al.*, 2021). Another *Acidovorax* spp., *A. oryzae*, has caused catheter-related bacteremia in a 48-year-old immunocompetent patient (Orsborne *et al.*, 2014). Overall, these reports, along with the findings in this study, suggest *Acidovarax* should also be treated as a potential threat to patient health if identified within hospital water systems.

Other bacterial species identified during the last water collection and not known to cause any human infections were: *Herminiimonas* spp. and *Aquabacterium* spp.. *Herminiimonas* spp. was isolated from seven outlets with reasonable count rates (<10¹⁷), suggesting it is a potentially common species in WDS. However, in other studies *Herminiimonas* spp. was found from multiple drinking water sources, tap water, bottled water, the Greenland ice, and arsenic-contaminated industrial wastewater sludge, demonstrating a wide-residency pattern within diverse water sources (Kämpfer *et al.*, 2013). Importantly for human health, some of *Herminiimonas* spp have the ability to oxidise arsenate, degrade urea, and are reportedly resistant to heavy metals and antibiotics (Koh *et al.*, 2017). As it is often found within biofilms, possible horizontal gene transform within the biofilm that *Herminiimonas* spp may share, should also be regarded as a potential concern. This may result in the rise of new resistant or pathogenic species as a result, supporting findings from this study indicating a high presence within hospital WDS. Similarly, *Aquabacterium* spp. was identified by nucleotide sequencing from nine out of fifteen outlets. *Aquabacterium* spp. are highly abundant in drinking water, however, to date there is limited information linking *Aquabacterium* to human health, but more research is needed to examine any contribution to biofilm formation or QS.

5. Limitations

Bacterial identification in clinical and environmental microbiology commonly relies on many principal methodologies. However, two most common techniques used to identify bacteria are traditional culture-based techniques and nucleotide sequencing approaches. While both methods are foundational to diagnostic workflows and microbial surveillance, each presents inherent limitations that must be considered when interpreting results or designing comprehensive detection strategies.

Microbial detection in water samples utilising traditional, culture-based methods relies on the growth of microorganisms on designated culture media. Traditional methods are highly dependent culturing microorganisms on specific media (Houpikian and Raoult, 2002). Media used in this study cannot support growth for all OPPPs and the method will always remain challenging for OPPP species-specific identification using this technique only. Thus, development of a more rapid and thorough method for microbial identification is warranted.

Traditional microbiology methods are time-consuming due to incubation periods, and it is challenging to meet the specific growth conditions required for each bacterial genus and even more species. Examples include *Elizabethkingia meningoseptica* that will grow on MacConkey agar but not on MacConkey agar No. 3 (MAC3) as used in this study, preventing its identification by TMM (Inkster *et al.*, 2024). Furthermore, fastidious bacterial genera like *Methylobacterium* will only grow on blood agar, and due to slow growth in many cases this bacterium can be missed (Kovaleva, Degener and Van Der Mei, 2014). Furthermore, *Shingomonas* spp. will not grow on MAC3 but will only grow on blood agar (El Beaino *et al.*, 2018). Additional consideration is needed regarding results using R2A media, which is not a differentiating agar. Due to the high TVCs produced on this media, only 2 colonies with the same apparent morphology types were lifted and patch plated. This left many colonies untested and therefore not every member of the water microbial community would have been screened and identified. Another limitation was that only 100ml of water was tested for the presence of OPPPs and additional low abundance species may have been missed.

Furthermore, YSJ University laboratories permit only Category 2 microorganisms to be grown. Culturing of *Legionella* and other biosafety level 2+ bacteria could not be performed due to health and safety reasons. However, these bacteria may be identified by molecular methods and nucleotide sequencing. With sixteen different species identified within the hospital WDS in this study, alongside the ten standard OPPPs, there were four additional bacterial species identified, two of which are known to cause infections in patients (*Acidovorax* spp and *Delftia*

spp.), and another two that no human pathogenicity has been recorded (*Aquabacterium* spp. and *Herminiimonas* spp.). They were identified within the 15 different outlets with significant reads (over 10).

Recovery of multiple bacterial species using TMM from a single sample was infrequent. This is likely attributable to competitive growth dynamics, where dominant or faster-growing organisms suppress the growth of others (Valiei *et al.*, 2024). Furthermore, the selective nature of the culture media used may favour specific taxa while inhibiting others. In addition, some species may have been present at concentrations below the detection threshold for culture-based methods. It is therefore possible that greater species diversity was present in the original samples but remained undetected in the subsequent analyses (Schmitz *et al.*, 2024). Molecular techniques (e.g., 16S rRNA gene sequencing), were also carried out to reveal additional taxa not recovered under the initial culture conditions (Li, 2018).

Although 16S rRNA sequencing is widely used and dependable method for bacterial identification, it does not inherently determine viability. However, due to high read counts, their presence in water is highly likely. Furthermore, as MinION identifies bacteria from extracted 16S rRNA sequences of genomic DNA by nucleotide sequencing, it is unknown if the reads are from extracted DNA of viable bacteria or if the DNA was present in the water following release from bacteria during prior water treatments. Therefore, it is still unclear if all the bacteria identified using nucleotide sequencing would be able to establish an infection in a patient, or if bacterial DNA detected in the water samples was released from non-viable bacteria before or during genomic DNA extraction, and thus unlikely to cause infection. Another limitation of MinION sequencing arises from the use of software EPI2ME Minimap2 workflow, which only facilitates recognition at the bacterial genus level. It is well established that not all bacterial species within the same genus possess identical risk factors and treatment responses. Another limitation is the current 15% error rate (Li, 2018). Recent advancements in Oxford Nanopore Technology sequencing, specifically the introduction of the R10.4 flow cell, have resulted in a substantial improvement in data quality (Chiou *et al.*, 2023). Nevertheless, there exists other software or bioinformatic approaches which may yield more favourable alignment and identification outcomes (Lao *et al.*, 2023).

In clinical and environmental microbiology, several non-culturing and non-genomic techniques are employed to identify bacterial species, particularly when rapid turnaround or culture-independent analysis is required. However, for drinking water, immunoassays are often the most practical non-genomic, non-culturing option, particularly for routine monitoring of

indicator organisms like *E. coli*. However, confirmatory testing or enrichment may still be necessary depending on regulatory thresholds and sample complexity (Bursle and Robson, 2016). If additional time and resources had been available, it would have been advantageous to investigate the potential for identifying other bacterial species, beside *E. coli*, using immunoassay-based detection methods, accompanied by an evaluation of their sensitivity and accuracy.

6. Conclusion

In a hospital setting, when high TVC are identified in a water sample, it may result in immediate ward closure and elicit remedial action of the distribution system. However, a lack of research on hospital water distribution systems means that it is unknown if a high TVC is indicative of bacterial pathogen presence – chiefly because not all bacteria are considered harmful (Australia, 2025). Importantly, ward closure and remedial action interrupt the patient care pathway and are a financial burden to the NHS and hospital environment. This warrants further investment in improving understanding of the links between bacterial species resident within hospital WDS and threats to human health.

Currently, there is no standardised methodology for the optimal identification of multiple opportunistic waterborne pathogens in water (Inkster *et al.*, 2024). By developing and installing a robust testing platform for identifying the presence of waterborne pathogens (or lack of), a healthcare facility could develop a risk assessment plan and water management strategy that both decreases patient risk of infections and has a cost-saving benefit. It is generally acknowledged that as treatment-resistant gram-negative bacteria continue to emerge, new screening, treatments, and monitoring processes are required to ensure any future outbreaks are prevented or rapidly contained. MinION nucleotide sequencing could provide a solution to enable faster and more accurate data to be derived. This study has shown promising results for incorporating NGS techniques into the bacterial identification pathway in hospitals. However, future research is needed to find out if these OPPP's cause infection in at-risk or compromised patients, or if they are simply transient and of no substantial concern. Regardless, further consideration must be given to the identification and monitoring of waterborne pathogens. The MinION system could detect the presence of the DNA of OPPP's but these could be non-viable cells with little to no clinical relevance. In which case the traditional method of microbiology remains relevant, as it will only identify through culturing live cells capable of expanding and contributing to infection risk. A potential solution could be through detection of an OPPP using nucleotide sequencing followed by selective sampling and analysis by traditional microbiology

techniques for the confirmation of species recovery, prior to any costly remediation taking place.

Benefits for faster and accurate bacterial water microbial identification include shorter hospital stays, cutting NHS spending on patient care, and reducing the requirement for costly, and potentially unnecessary, treatments – both for the patient and in the hospital WDS.

Understanding water microbiome formation, and preventing infection instead of fighting it, may prolong the use and slow down the bacterial evolutionary fight with antibiotics, especially now when many bacteria have developed resistance to some already known and commercially used antibiotics (*Antibiotic Resistance in Hospitals Is Worse Than We Thought*, 2025). This combined detection approach recommended by this study may provide microbiology researchers with more time to find treatment alternatives for tackling future infections. Importantly, this study provides crucial evidence of the need for hospital management and infection control teams to develop more robust screening processes to better understand the potential OPPPs present in WDS and the impact on infection risk.

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Appendix

Appendix 1* Hospital floor plan

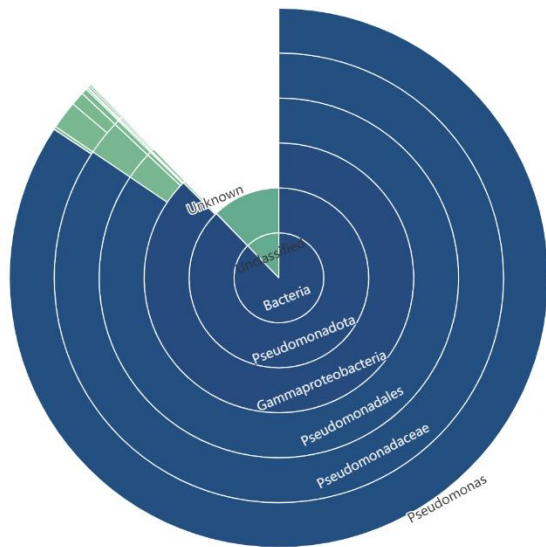
Level 01 Proposed Plan



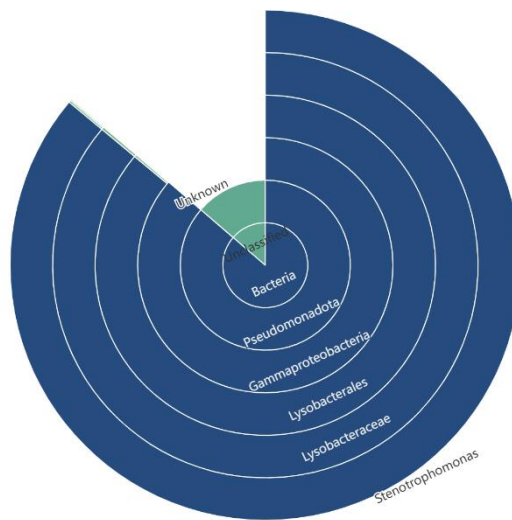
Appendix 1.2 The ground floor plan



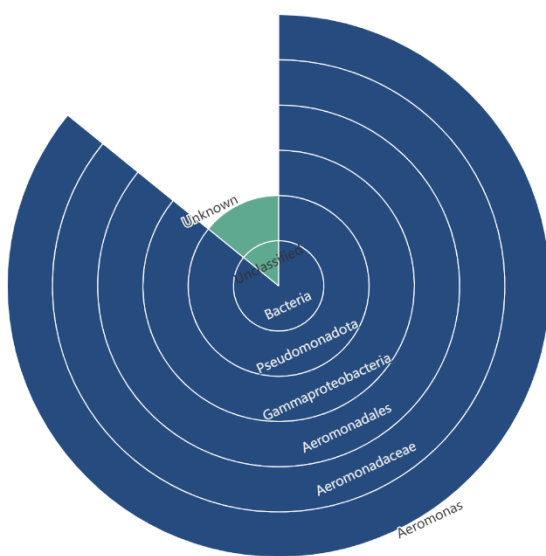
Appendix 2* Minion pie charts



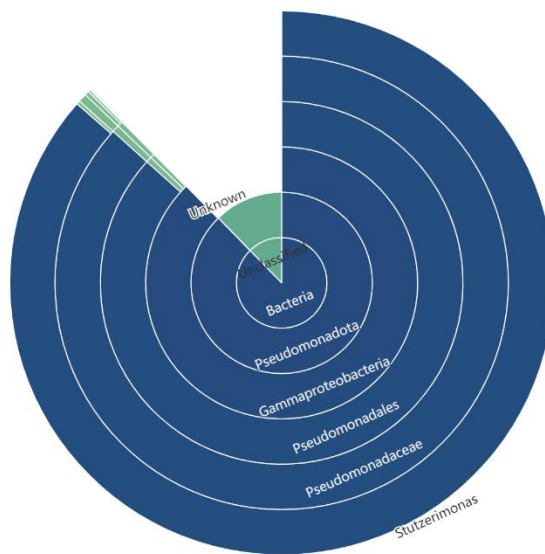
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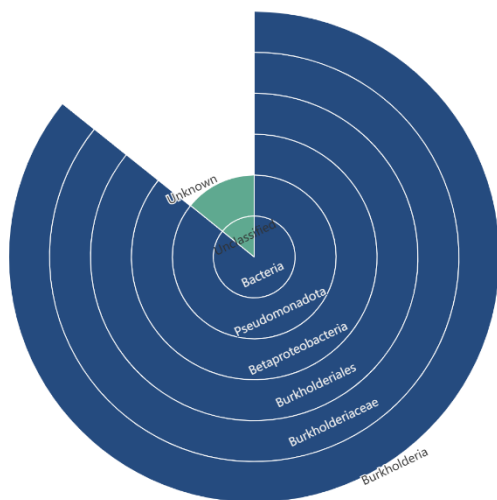
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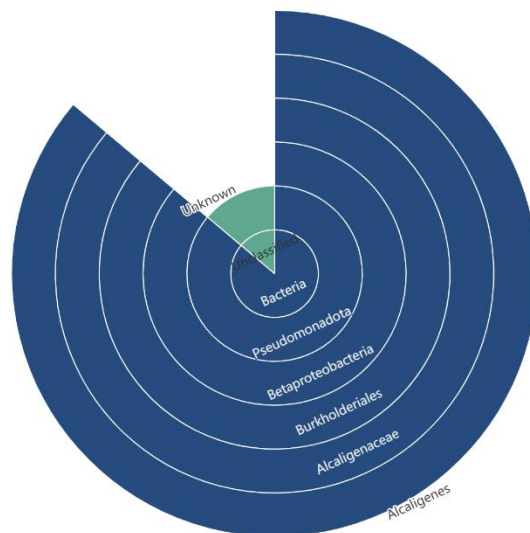
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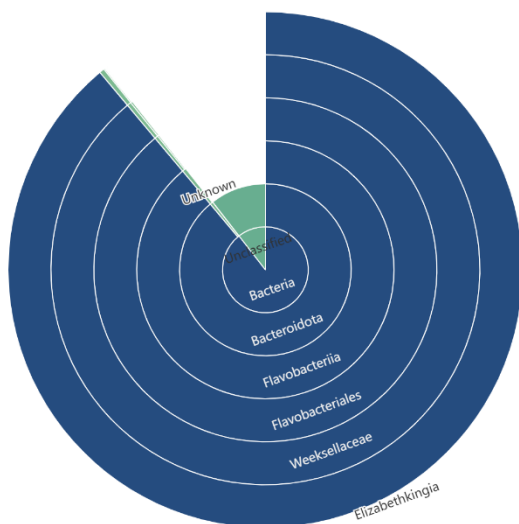
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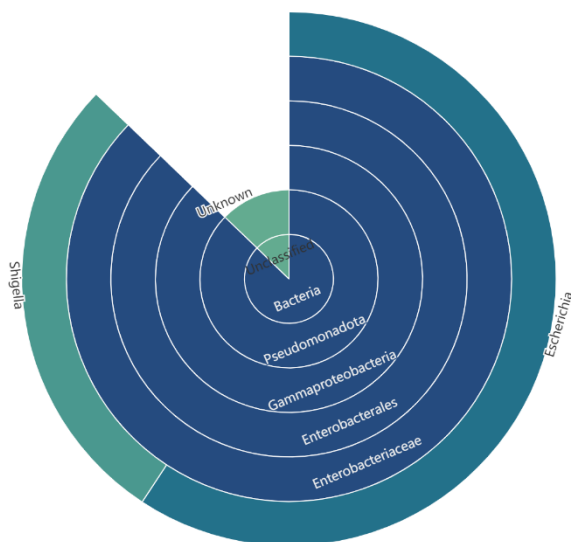
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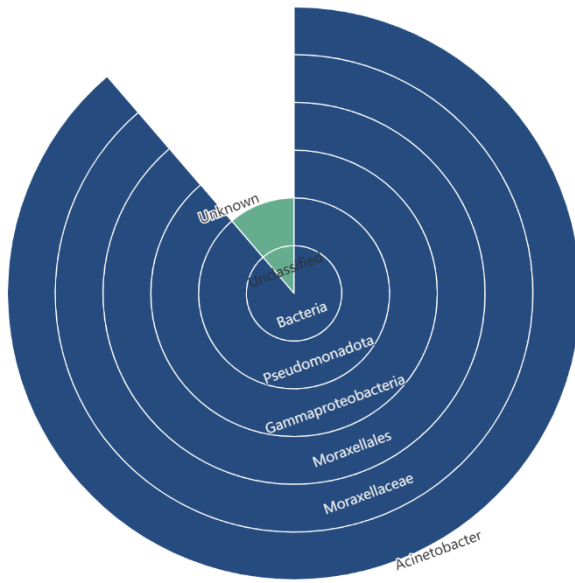
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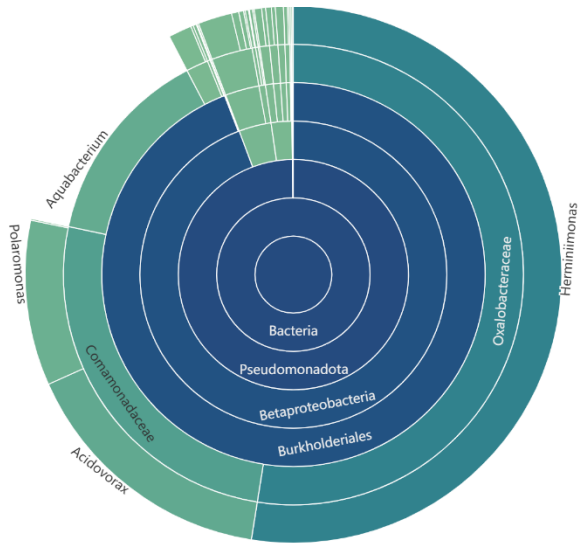
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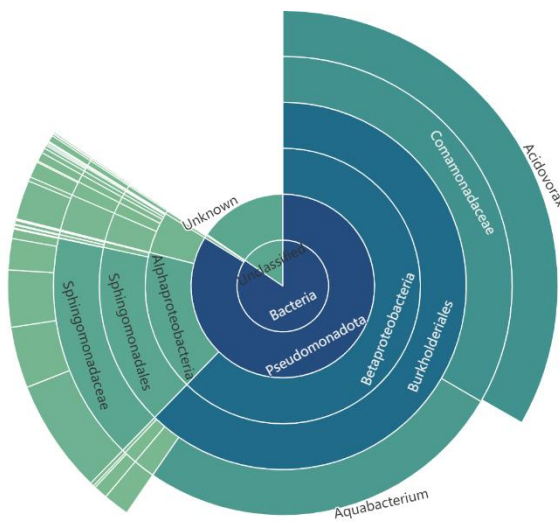
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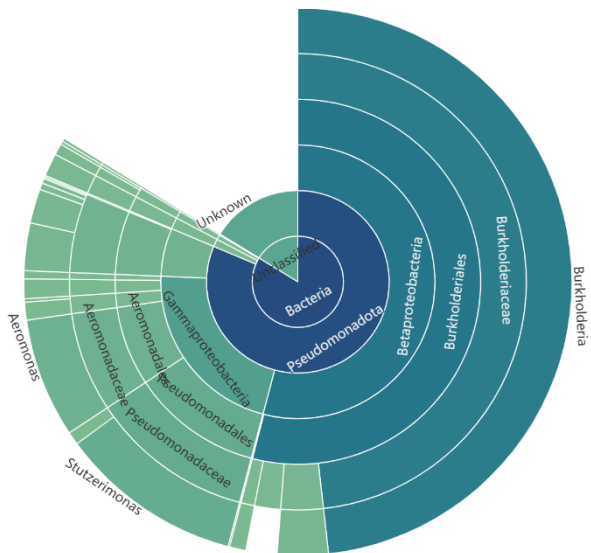
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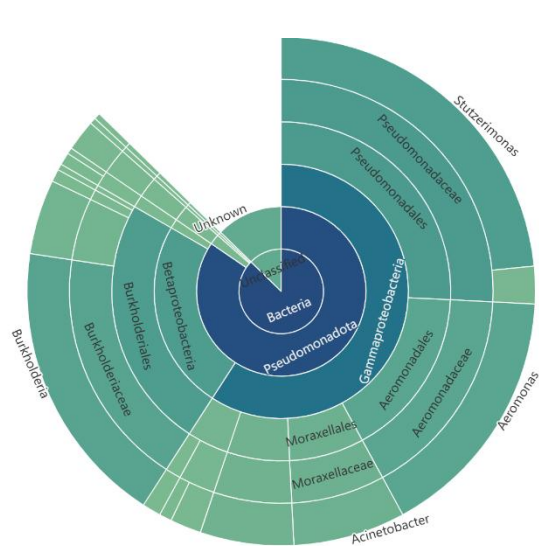
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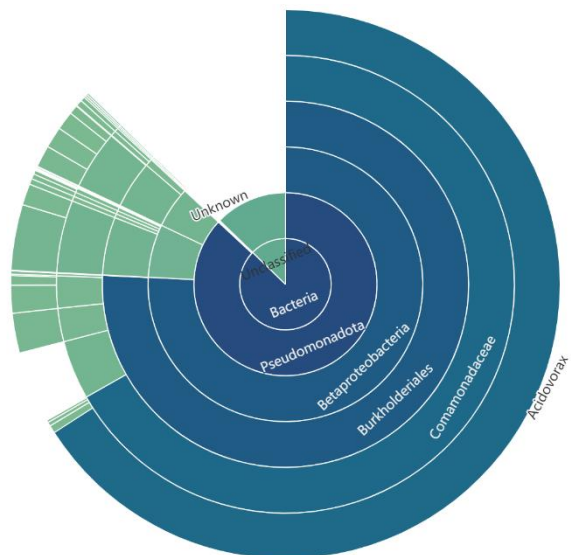
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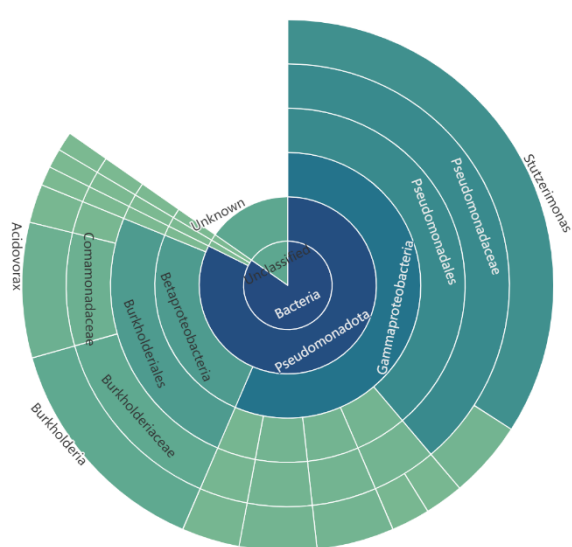
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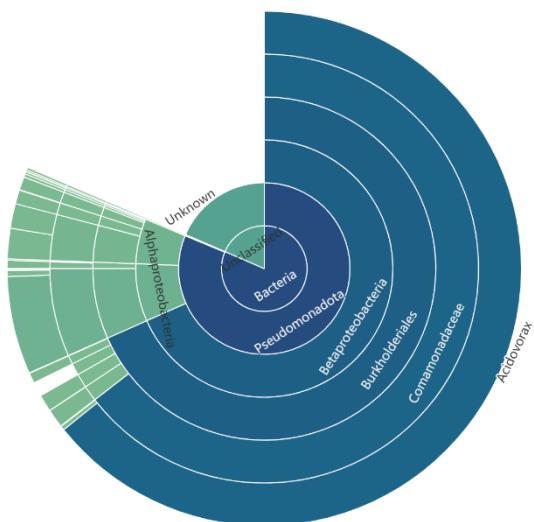
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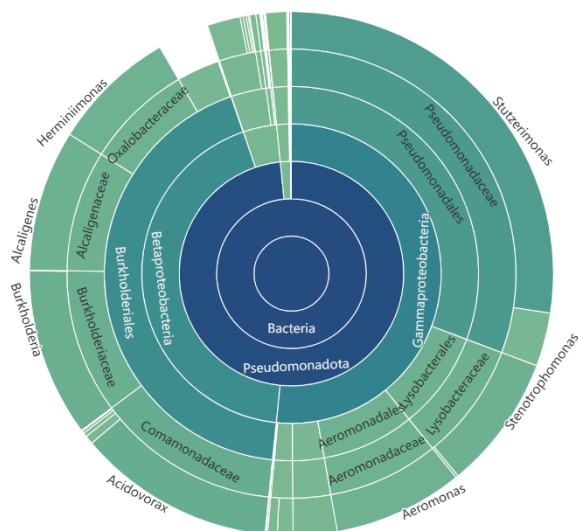
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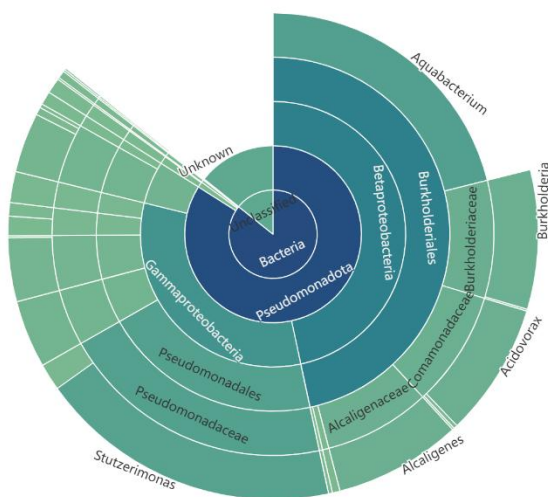
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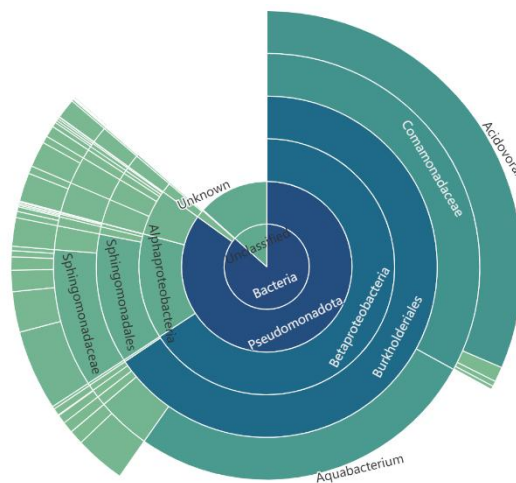
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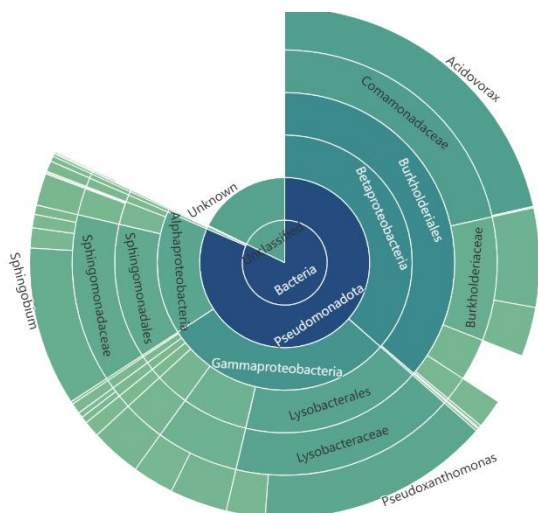
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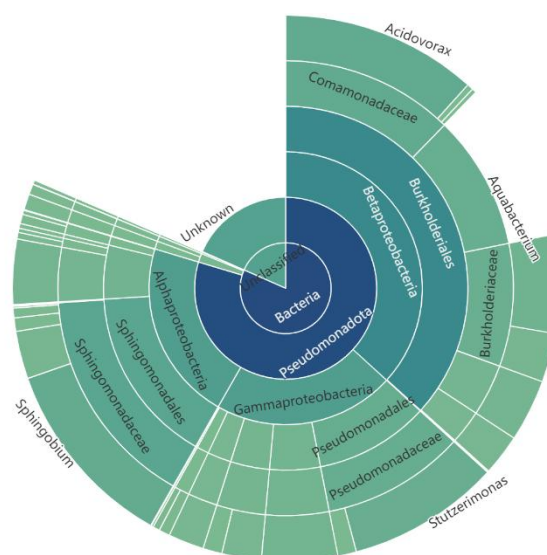
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Barcode 22



Barcode 23



Barcode 24

