Monitoring and Detecting *Legionella* within Evaporative Cooling Towers

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Abstract

Legionella pneumophila, a waterborne bacterium, is the primary cause of Legionnaires' disease, a severe form of pneumonia contracted through the inhalation of contaminated aerosols. While this study focuses on *L. pneumophila*, other species, such as *Legionella longbeachae*, can also cause the disease, particularly in Australia and New Zealand. Cooling towers are a predominant source of Legionnaires' outbreaks, as they support the growth of both sessile and planktonic microbial communities and facilitate their aerosolisation. Therefore, microbial growth within these structures must be rigorously controlled through a variety of control measures. Currently, the efficacy of these control measures is assessed by routine monitoring of bulk water samples using culture-based methods. However, this reliance on planktonic samples combined with culture methods, typically delays remedial actions, allowing outbreaks to occur. This thesis aims to minimise the delay between the detection of *Legionella* and the implementation of remedial actions to prevent outbreaks. It hypothesises that biofilm samples could potentially serve as lead indicators for the rapid detection of *L. pneumophila,* thereby enabling timely corrective measures. Additionally, this research explores how biofilm samples enhance our understanding of interactions between biofilms, planktonic communities, and physicochemical parameters, potentially revealing key factors and trends that influence *Legionella* growth and lead to outbreaks.

To investigate whether biofilm samples could serve as lead indicators for *Legionella* detection, a novel and optimised biofilm sampling technique was developed and subsequently employed. This method was complemented by independently assessed emerging detection techniques and applied across four strategically selected operational cooling towers over an 18-month period. Additionally, the impact of microbial concentration, viability, and bacterial community structure was examined using flow cytometry and 16S rRNA bacterial community analysis, respectively. This approach significantly enhanced the likelihood of identifying precursor events indicative of *Legionella* presence.

The research revealed that biofilm samples exclusively identified *L. pneumophila* during the study period, with *Legionella* spp. found in higher concentrations in biofilms compared to bulk water. This underscores biofilms as a crucial ecological niche for *Legionella* spp. Although bacterial concentrations, viability, and physicochemical parameters were found to be insufficient predictors of *Legionella* proliferation, the composition of the bacterial community within biofilms played a pivotal role. Biofilms, while sharing a core set of taxa with bulk water, exhibited a unique community dominated by low-abundance and rare taxa that significantly influenced microbial interactions and ecological dynamics. These dynamics include the introduction of additional organic compounds and nitrogen, as well as the potential reduction of competitive bacterial populations, which can foster the growth of *L. pneumophila.* These findings suggest that biofilms can serve as a lead indicator for *L. pneumophila,* highlighting the need for more comprehensive monitoring policies, practices, and regulations. Such measures should include the assessment of biofilm samples alongside traditional planktonic samples. Prioritising biofilm samples provides a more accurate and holistic understanding of the dynamics of *Legionella*, revealing crucial insights into the microbial communities that foster conditions conducive to the presence of *L. pneumophila.* Therefore, integrating biofilm sampling into current

monitoring strategies will not only enhance our understanding of *Legionella* proliferation but also allow for the earlier implementation of remedial actions. Ultimately, this approach is expected to reduce the risk of *Legionella* outbreaks, thereby safeguarding public health.

Acknowledgements

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Abbreviations and Nomenclature

Symbol	Definition
BYCE	Buffered Charcoal Yeast Extract
CFU	Colony Forming Units
DNA	Deoxyribonucleic Acid
ddPCR	Digital Droplet Polymerase Chain Reaction
EMA	Ethidium Monoazide
EPS	Extracellular Polymeric Substance
GVPC	Glycine Vancomycin Polymyxin B Cycloheximide
GU	Genomic Unit
HPC	Heterotrophic Plate Counts
HSE	Health and Safety Executive
ICC	Intact Cell Count
IMS	Immunomagnetic Separation
LEfSe	Linear Discriminant Analysis Effect Size
Ml	Milliliters
MPN	Most Probable Number
MWY	Modified Wadowsky Yee
NHS	National Health Service
PERMANOVA	Permutational Multivariate Analysis of Variance
PMA	Propidium Monoazide
PI	Propidium Iodide
PVC	Polyvinyl chloride
qPCR	Quantitative Polymerase Chain Reaction
P	Correlation Coefficient (Rho)
rRNA	Ribosomal RNA
R^2	Coefficient of Determination
TCC	Total Cell Count
UM	Micrometers
USA	United States of America
$\chi 2$	Chi-squared test
W	Sum of the ranks of the first sample

Chapter 1: Introduction

1.1 *Legionella* and Legionellosis

The genus *Legionella* comprises a diverse group of rod-shaped, Gram-negative, intracellular, waterborne bacteria, naturally occurring in freshwater and engineered water systems (Tan et al., 2021). With over 60 identified species and more than 70 distinct serogroups recognised to date (LPSN, 2024), *L. pneumophila,* particularly serogroup 1, stands out as the predominant species responsible for legionellosis, accounting for 90% of all cases (Viasus et al., 2022).

Legionellosis consists of two infections caused by *Legionella* bacteria: firstly, Legionnaires' disease, a severe form of pneumonia, which was first discovered after an outbreak that occurred in 1976 at the American Legion convention; and secondly, Pontiac fever, a milder, flu-like illness (Viasus et al., 2022). Legionellosis is contracted when aerosols contaminated with *Legionella* are inhaled. In the case of Legionnaires' disease, the inhaled *Legionella* bacteria travel to the lungs where they specifically infect alveolar macrophages. This interaction leads to the death of these immune cells, subsequently triggering a widespread immune response characterised by significant inflammation. In contrast, Pontiac fever represents a distinctly milder condition, resembling an upper respiratory tract infection with flu-like symptoms. Unlike Legionnaires' disease, Pontiac fever is generally self-limiting, with symptoms typically resolving on their own within a few days (Jomehzadeh et al., 2019).

The case fatality rate of Legionnaires' disease in Europe and the USA is approximately 10% (Burillo, Pedro-Botet and Bouza, 2017). However, the incidence and mortality rates are influenced by several factors, including chronic lung diseases, smoking, immunosuppression, older age, sex, diabetes, alcoholism, cancer, and cardiovascular diseases (Phin et al., 2014). The expansion of urban infrastructure has led to an increasing reliance on engineered water systems, most importantly cooling towers (National Academies of Sciences, Engineering, and Medicine, 2020). Combined with an increase in urban and ageing populations and changes in climate (Paschke, Schaible and Hein, 2019), Legionnaires' disease cases are rising in Europe and the USA, with reported cases increasing from 1.6 per 100,000 in 2010 to 3.9 per 100,000 in 2020 in the USA (CDC, 2020) and from 1.8 per 100,000 in 2018 to 2.2 per 100,000 in 2019 in Europe (Samuelsson et al 2017). Consequently, there is an urgent need for a deeper understanding of *Legionella* survival and proliferation, effective monitoring methods, and management strategies to enhance public health protection.

1.2 Engineered Water Systems

Engineered water systems, including potable water distribution systems, cooling towers, shower heads, and spa pools, which are significant sources of *L. pneumophila* and outbreaks of Legionnaires' disease in the UK (Table 1). These environments are particularly conducive to producing aerosols capable of containing *L. pneumophila* (Goutziana et al., 2008; Collins et al., 2017; Leoni et al., 2018; Paranjape et al., 2020b). In addition, decorative fountains and water features have also been identified as potential reservoirs for *L. pneumophila* (Smith et al., 2015).

Table 1: *Documented Legionnaires' Disease Outbreaks in the UK, Including Sources, Case Numbers, and Fatalities*

Year	Location	Source	Cases	Fatalities	Citation
1985	Stafford	Hospital water system	68	22	(O'Mahony et al., 1990)
1989	London	Unknown cooling source	33	5	(Watson et al., 1994)
2002	Barrow-in-Furness	Air-conditioning unit	179	7	(Bennett et al., 2014)
2006	Hereford	Spa pool	3	0	(Foster, Gorton & Waller, 2006)
2012	Stoke on Trent	Spa pool	Multiple (data not specified)	Data not specified	(Leoni et al., 2018)
2012	Edinburgh	Industrial cooling towers	92	3	(Irons et al., 2013)
2017	England and Wales	Public and Private Water Systems	604	Data not specified	(UK Health Security Agency, 2017)

Evaporative cooling towers pose a particular concern as they create conditions that promote both sessile and planktonic microbial growth, supported by temperatures between 25-35°C, a near-neutral pH, exposure to sunlight, and a nutrient supply enriched by continuous aeration (Di Gregorio et al., 2017). Cooling towers are the most frequent confirmed source of *L. pneumophila* aerosolisation, contributing to 28% of all outbreaks (Di Pippo et al., 2018). Additionally, cooling towers can disperse *Legionella* as far as 12 km (Paniagua et al., 2020), compounding the risk, especially when situated near densely populated areas, hospitals, or care homes.

Evaporative cooling towers are pivotal to urban water systems, serving as a cost-effective and energy-efficient cooling solution for a wide range of systems and processes, including those in power generation and the petrochemical industries. As heat exchange devices, they circulate water at elevated temperatures from industrial processes over a pack material. Air flows in the opposite direction to the water, promoting heat transfer and resulting in heat loss. The cooled water is then collected in the basin, to be either recirculated within the system or discarded into the sewage system (Figure 1). The pack material, typically made of polyvinyl chloride (PVC), is crucial to these systems as it provides an extensive surface area, increasing the contact time between water and air, which in turn enhances the cooling process's efficiency.

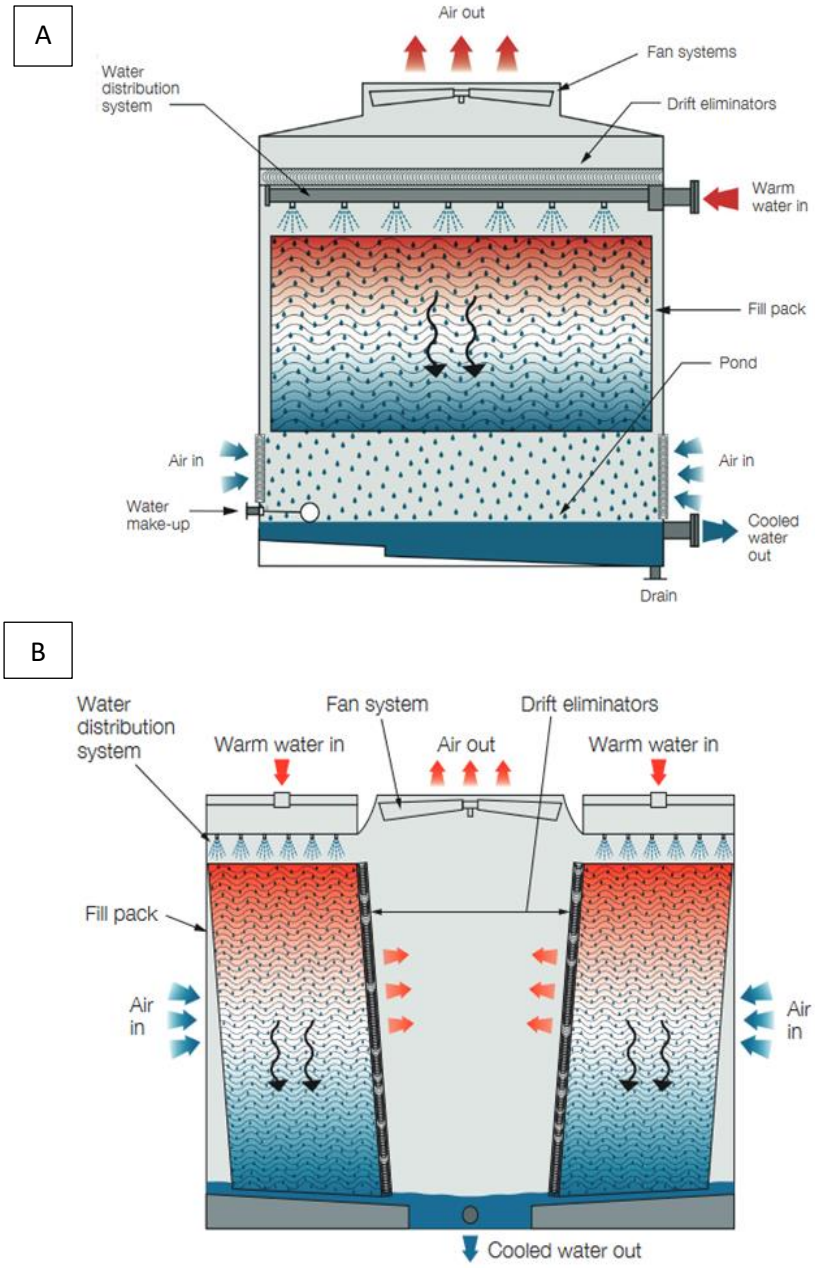

***Figure 1**: Schematic of cooling towers (Health and Safety Executive, 2020). Panel A is an induced draught counter flow cooling tower. Panel B is an induced draught double crossflow cooling tower.*

1.3 Monitoring and Preventing *L. pneumophila* Outbreaks

Given that cooling towers are a significant source of large outbreaks of Legionnaires' disease worldwide, several governments have enacted policies to prevent such outbreaks. In the United Kingdom, the control and management of *Legionella* within cooling towers are overseen by the Health and Safety Executive (HSE). The regulations mandate a comprehensive water safety plan, which includes a risk assessment, water treatment program, ongoing monitoring and maintenance, and meticulous record-keeping (Health and Safety Executive, 2020). These water treatment programs are specifically designed to prevent Legionnaires' disease outbreaks, thereby saving lives and reducing financial costs. For instance, a notable outbreak in Edinburgh in 2012 resulted in four deaths (Othieno et al., 2014) and incurred medical expenses amounting to £725,800 for the National Health Service (NHS) (BBC News, 2013). Failure to adhere to these regulations can lead to enforcement actions by the HSE, ranging from a notification of contravention to improvement notices or even prohibition notices. Notably, the majority of improvement notices issued over the past decade have been for failing to effectively implement written control schemes (Crook et al., 2020).

Water treatment programs typically involve the continuous or shock dosing of disinfectants, often utilising oxidising agents like sodium hypochlorite or a combination of oxidative and non-oxidative agents, to inhibit microbial growth, including that of *Legionella*. To assess the effectiveness of these control measures and to detect any system malfunctions, routine monitoring is employed. This monitoring includes testing for *Legionella* through culture methods and evaluating microbial concentrations using Heterotrophic Plate Counts (HPC) (Sciences et al., 2020). Current action levels for *Legionella* dictate that concentrations below 100 CFU/litre require no immediate action, levels between 100 and 1,000 CFU/litre necessitate a reassessment of the control program and an adjustment in biocide dosage, and levels above 1,000 CFU/litre demand immediate remedial action (Health and Safety Executive, 2020). Following the HSE protocol summarised in (Figure 2), bulk water samples undergo routine monitoring methods and, if the microbial counts exceed these action levels, remedial action is required. While current monitoring methods provide some information on the efficacy of the biocide used, *Legionella* culture takes between 10-14 days to acquire results (Walker and McDermott, 2021), causing a delay in any remedial action and thus outbreaks still occur. Therefore, there is an urgent need and growing literature on using emerging detection techniques that can offer more immediate results, allowing for quicker response times and preventing outbreaks.

Emerging detection methods for *Legionella*, such as most probable number (MPN) and quantitative polymerase chain reaction (qPCR), offer significant advantages over traditional culture methods, particularly with their speed and sensitivity. However, while these emerging detection methods are superior, they have not been extensively tested or researched using biofilms as a sample type within operational cooling towers. This is a critical gap, given that ecological niches like biofilms are crucial for the survival and proliferation of *Legionella* in engineered environments. Although the importance of biofilms is recognised, their potential, especially within cooling towers, to act as indicators has not been explored.

This study hypothesises that biofilms, when paired with emerging detection technologies, could potentially detect *Legionella* spp. earlier and more reliably than traditional bulk water samples, acting as "lead indicators" for *L. pneumophila*. Such a strategy promises a more proactive approach, allowing for earlier detection and timely intervention. Additionally, investigating the complex interactions between *Legionella*, microbial communities, and physicochemical parameters within biofilms may reveal supplementary indicators of *Legionella* presence, enriching our understanding and enhancing strategies for managing this bacterium. Adopting this comprehensive approach could markedly decrease outbreak occurrences, thereby bolstering public health protection.

Building on this hypothesis, this thesis investigates the potential of biofilm samples from operational cooling towers to enable the early detection of *L. pneumophila* using emerging detection techniques. It also aims to characterise bacterial communities within these samples, fostering a proactive approach to remedial action. The research begins with a comprehensive literature review to identify current knowledge gaps, forming the foundation for the study's aims and objectives. A novel biofilm sampling technique is introduced, incorporating advanced methods such as flow cytometry, quantitative polymerase chain reaction, and 16S rRNA bacterial community analysis. These methods provide a dual focus allowing for both the precise detection of *L. pneumophila* and an in-depth examination of microbial community dynamics. The findings are critically analysed in relation to existing literature, highlighting their implication for improving public health policies and practices. Ultimately, this research contributes to safeguarding public health by offering innovative solutions for monitoring and managing *Legionella* in engineered environments.

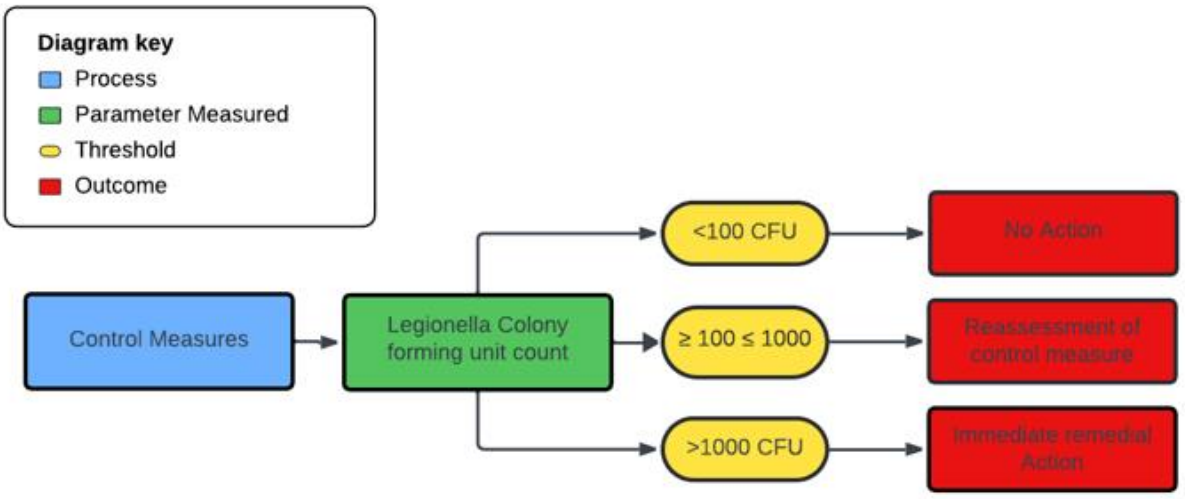

***Figure 2**: Flow diagram illustrating the decision-making process based on Legionella colony-forming units (CFU) counts. The process begins with control measures (blue), followed by measuring Legionella CFU counts (green). Threshold levels (yellow) guide decisions*

Chapter 2: Literature Review

2.1 *Legionella* Ecology

Legionella species are Gram-negative, rod shaped, oxidase-negative, urease-negative, and strictly aerobic species of bacteria. *Legionella* species survive in low-nutrient freshwater ecosystems; however, the concentration of nutrients that the bacterium requires to grow and proliferate to high quantities is rarely found in aquatic systems. Therefore, although *Legionella* are ubiquitous waterborne bacteria, they are present at low concentrations in freshwater sources, representing less than 1% of the resident bacterial population (Fliermans et al., 1981).

Legionella species are primarily detected in engineered water systems such as cooling towers, water distribution systems, pools, and fountains. These engineered water systems promote favourable growth conditions such as water stagnation (low flow rates), warm temperatures (between 25 and 50 °C) (Huang et al., 2010) and the presence of amoebae and biofilms, which provide nutrients and protection complicating disinfection efforts (WHO, 2007; Shaheen, Scott and Ashbolt, 2019). Since *Legionella* species are fastidious and slow-growing organisms with specific nutrient requirements (particularly iron and cysteine), the lack of these nutrients in the water of engineered systems limits the potential for planktonic growth (Buse et al., 2017), causing *Legionella* to associate itself with free-living amoebae and biofilms (Abdel-Nour et al., 2013) to facilitate nutrient acquisition.

2.1.1 *Legionella* Life Cycle

Legionella species are well-known as intracellular parasites, with their propagation within engineered water systems often occurring through their interactions with protozoa (Rowbotham, 1980; Fields, Benson and Besser, 2002; Kanarek, Bogiel and Breza-Boruta, 2022) For *L. pneumophila,* using co-culture and co-isolation techniques, around thirty species of protozoa have been experimentally confirmed as its host (Boamah et al., 2017), with many host species yet to be characterised (Paranjape et al., 2020b). The life cycle of *L. pneumophila* can be summarised in four general stages outlined in Figure 3. In the first stage, microbial grazers such as protozoa must ingest *L. pneumophila* cells through either coiling phagocytosis or pinocytosis. In the second stage, *L. pneumophila* are captured in a vacuole, called the phagosome, following initial engulfment. Typically, the phagosome will be used to degrade and recycle biological materials such as prey cells, for nutrition and growth of host cells. The lysosome fuses with the phagosome, causing it to release hydrolytic enzymes and create an acidic pH, changing the phagosome into a phagolysosome, which will degrade the biological material within it (Oliva, Sahr and Buchrieser, 2018).

In the case of *L. pneumophila,* a range of 300+ effector proteins secreted by the Dot/Icm Type IV secretion system modifies vesicular trafficking, inhibiting the fusion of the lysosome to the phagosome and recruiting vesicles from the endoplasmic reticulum, as well as mitochondria (Segal, Shuman & Purcell, 1998; Isberg, O'Connor & Heidtman, 2009). These effector proteins create a hospitable growth environment within the host's cell phagosome, known as the *Legionella*-containing vacuole, allowing *L. pneumophila* to enter the replicative phase (Isberg, O'Connor & Heidtman, 2009; Shaheen, Scott and Ashbolt, 2019).

During the third stage, *L. pneumophila* will replicate and consume the nutrients of the host cell. Once the nutrients are consumed, the bacteria differentiate into a transmissive form and then to a dormant form called the mature infectious form. The mature infectious form is virulent, flagellated and resistant to stress. Finally, in the fourth stage, *L. pneumophila* mature infectious form cells are released from the *Legionella*-containing vacuole to the cytosol of the host cells, where they induce host cell lysis to be released back into the bulk water to find a new intracellular host where *L. pneumophila* reverts to its replicative form, starting a new cycle (Oliva, Sahr and Buchrieser, 2018).

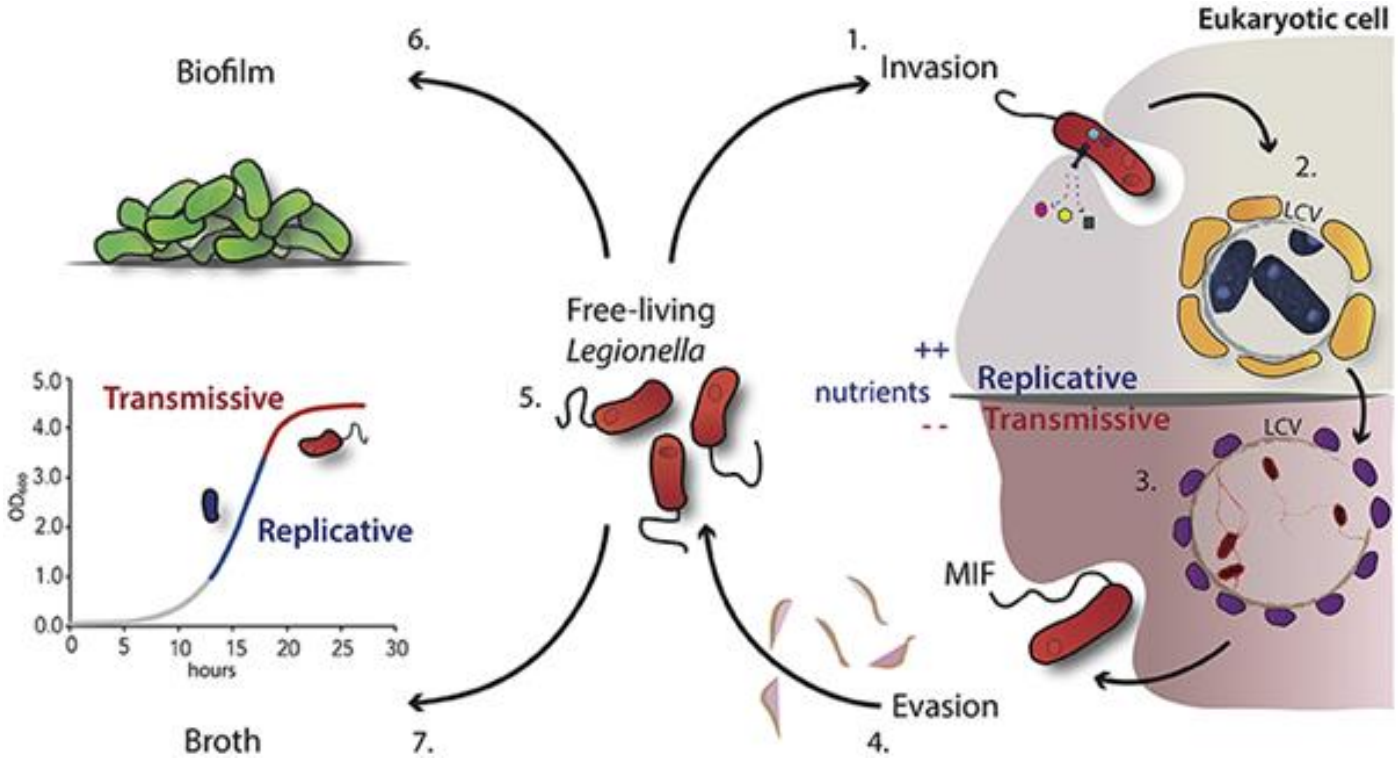

***Figure 3**: Schematic overview of the Legionella species during its growth cycle (Oliva, 2018). 1). Uptake of Legionella by host cell. 2). after internalisation the Legionella evade the phagosome-lysosome fusion. 3). Legionella replicate and consume the host nutrients. 4). Legionella escape the membrane and release into the extracellular environment. 5). Legionella may repeat the cycle. 6). Alternatively, Legionella may be associated within biofilms.*

2.1.2 *Legionella* Association with Free-Living Amoeba

Engineered water systems are challenging environments with biocide presence, rapid temperature changes and a competing complex microbial community. The ability of *Legionella* to act as an intracellular parasite of protozoan species including free-living amoeba, ciliates and nematodes, allows the bacteria to gain protection from biocides and emerging temperature changes, but also acquires nutrients to reduce competition within the microbial community (Shaheen, Scott and Ashbolt, 2019). Free-living amoeba and ciliates can exist in both trophozoite and cyst forms. The trophozoite stage enables active feeding and reproduction of both planktonic and biofilm associated bacteria, while the cyst form offers significant resistance to environmental stresses, including physical and chemical disinfection but can also harbour *Legionella* and other pathogens, providing an additional layer of protection to these bacteria (Greub and Raoult, 2004). Nematodes, though less studied in relation to *Legionella*, are another potential host that could contribute to the bacteria's persistence in engineered water systems. Hemmerling et al. (2023) have indicated that *Legionella* can colonize nematodes, leveraging them as protective reservoirs in environments where other protozoan hosts might be less prevalent.

This cystic survival mechanism leads to the persistence of hard-to-eradicate *Legionella*, posing a major barrier to achieving effective microbial control in engineered water systems, significantly increasing the risk of Legionnaires' disease outbreaks. Common water disinfection protocols, such as chlorination, monochoramine, chlorine dioxide and ozonisation treatments have been shown to be ineffective at completely eliminating amoebae cysts that harbour *L. pneumophila* (Loret et al., 2005). The primary reasons for this resistance include the impermeable nature of the cyst wall and the ability of protozoa to shield *L. pneumophila* from direct exposure to biocides. Moreover, free-living amoeba host cells have been shown to resuscitate viable but non-culturable *L. pneumophila* cells again significantly increase the risk of Legionnaires' disease outbreaks (Garcia et al., 2007). Therefore, to address these challenges, research has focused on developing long-term disinfection protocols capable of targeting potential hosts harbouring *L. pneumophila* (Critchley and Bentham, 2009; Scheikl et al., 2016) and also understand the microbial interactions between protozoa and bacteria.

Since various microbial taxa are fundamental for the *L. pneumophila* life cycle, it has been suggested that monitoring free-living amoebae can help predict the possible imminent occurrence of *Legionella* in engineered water systems (Shaheen and Ashbolt, 2021). However, *Legionella*-protozoan interactions are increasingly recognised as more complex than previously presented in the literature (Rowbotham, 1980; Fields, Benson & Besser, 2002).

Free-living amoebae have a preferential predation mechanism, causing them to feed on *Legionella* only when alternative bacterial food sources are in low abundance. Furthermore, several studies have indicated that numerous protozoan species can avoid consuming *Legionella* through various mechanisms, with others consuming *Legionella* in a digestive vacuole (Shaheen and Ashbolt, 2021). Given this selective nature of protozoan species, and the challenging environment of engineered water system, *Legionella* must also be capable of surviving outside host cells, such as within biofilms (Surman et al., 2001; Fields, Benson & Besser, 2002).

As *Legionella* primarily survives and proliferates within free-living amoeba there is a substantial focus on understanding the biological and chemical interactions that can promote or limit the ability of *Legionella* to establish and replicate within some of their protozoan hosts (Rowbotham, 1980; Fields, Benson & Besser, 2002; Cavallaro et al., 2022). This concentrated focus may overlook the broader ecological versatility of *Legionella*, since *Legionella* can form micro colonies within laboratory-derived multi-species biofilms devoid of amoeba (Taylor, Ross and Bentham, 2009). Therefore, *Legionella* likely employs several feeding strategies to gain the required nutrients for growth from microorganisms in surrounding biofilms. Examples include algae and Cyanobacteria within aquatic systems and bacterial mat communities growing in thermal vents (Taylor, Ross and Bentham, 2009). This understanding of *Legionella* ecology and life cycle, particularly within the context of an engineered water system, is critical to understanding *L. pneumophila* outbreaks and establishing appropriate monitoring and control schemes.

2.1.3 *Legionella* Association with Biofilms

Biofilms are complex microbial communities encased in extracellular polymeric substances (EPS) and facilitate symbiotic interactions to exchange nutrients, provide shelter from many environmental stresses by acting as a physicochemical barrier, and can contribute to the depletion of biocide concentration (Costerton, 1995; Lappin-Scott,

Burton and Stoodley, 2014; Flemming, 2020). As biofilms offer many advantages for microbial growth, it is estimated that 95% of all microorganisms within drinking water pipelines are attached to the walls of biofilms, and most microbial interactions occur within these biofilms (Flemming et al., 2016). Furthermore, biofilms are a significant microbial source that can surpass planktonic microbial numbers by three to four logarithmic units within drinking water pipes (Flemming et al., 2016). Biofilm microbial concentrations could be considerably higher in cooling tower systems due to cooling towers supporting temperatures between 25 and 35 °C, a pH close to neutral, exposure to sunlight and continuous aeration (Di Gregorio et al., 2017); however, cooling towers biofilms remain unstudied. Also, biofilm and planktonic communities are known to be significantly different in microbial composition in other engineered water systems (Wang et al., 2014; Buse et al., 2017), but this relationship has yet to be explored within the pack of cooling tower systems.

In the case of *L. pneumophila,* pure cultures have been reported to form biofilms under well-defined experimental conditions using a nutrient-rich buffered yeast extract medium (Pécastaings et al., 2010). However, under oligotrophic conditions, like those within many engineered water systems, it has been demonstrated that *Legionella* incorporates itself in pre-established multispecies biofilms as secondary colonisers where sufficient nutrient content is entrapped, and bacterial competition is high (Declerck, 2010). *L. pneumophila* within a biofilm can obtain nutrients through its interactions with protozoan species, directly from other living microorganisms and through necrotrophic feeding from decaying microbial cells, which are reported to comprise nearly 50% of a biofilm (Temmerman et al., 2006).

One of the most critical functions of biofilm for *L. pneumophila* is attracting microbial grazers (free living amoeba) since most of the microbial concentration is found within the biofilm, providing a plentiful food source (Huws et al., 2005). *L. pneumophila* exploits this to replicate within the free-living amoeba, as mentioned above. Furthermore, species within the biofilms can either promote or inhibit *L. pneumophila* (Abu Khweek and Amer, 2018). For example, *Flavobacterium* and *Cyanobacteria* can facilitate *Legionella* growth by providing a source of nutrients or synthesising capsular material, which supports adherence. Conversely, *Pseudomonas aeruginosa* inhibits *Legionella* growth by producing a bacteriocin (protein or peptide that is toxic to other bacteria) (Stewart, Muthye and Cianciotto, 2012).

Biofilms play an essential role in the dissemination of *Legionella* in engineered water systems, as biofilms are characterised by the initial attachment of the bacteria to the surface, development of microcolonies, maturation, and, finally, detachment of cells or parts of biofilms. The detachment and dispersal can occur either through the shedding of daughter cells as a result of nutrient levels, shedding of biofilm aggregates because of flow effects, or destruction of biofilms due to biocide use (Salgar-Chaparro et al., 2020). This results in high numbers of *L. pneumophila* mobilising into the bulk water, which may be aerosolised causing a Legionellosis outbreak (Declerck, 2010). Furthermore, since the current standard is to take bulk water samples, it is most likely that any *Legionella* detected is already at high concentrations and has been aerosolised before remedial actions can take place.

This complex relationship between *Legionella*, biofilms and protozoa are often overlooked, with no standardised sampling regime monitoring any aspect of cooling tower biofilms (Pereira, Silva and Melo, 2021). As a result, any data collected is not representative of the actual numbers of *Legionella* within a system, as current techniques only sample

and monitor organisms in their planktonic state. Furthermore, it is likely that any recorded planktonic microbes originate from an established biofilm, making bulk water a clear lag indicator. Therefore, any actions taken due to such sampling are too late.

2.2 Cooling Tower Design and Operation

Evaporative cooling towers are essential heat exchange devices widely utilised in industrial and commercial settings to dissipate waste heat into the atmosphere. These systems provide a cost-effective and energy-efficient cooling solution, as discussed in Section 1.2, but also present unique challenges due to the conditions they create, which can promote microbial growth, including *L. pneumophila*. Cooling towers are composed of several key components, including spray nozzles, which evenly distribute warm water over the fill; a fill or "pack", typically made from polyvinyl chloride (PVC) or polypropylene, which increases the surface area for heat exchange between water and air; a basin, where water collects; a fan that draws or forces air through the system; and drift eliminators, which capture water droplets that may escape with the airflow. If these droplets escape that may carry *L. pneumophila,* posing significant public health risk by contributing to outbreaks of Legionnaires' disease. As illustrated in Figure 4, heat from industrial processes is transferred to water within a heat exchanger.

This heated water is sprayed through the nozzles and flows downward along the fill. As the water flows down the fill, ambient air is drawn into the tower by a fan, creating a counterflow where the heat from the water is absorbed into the air by three primary mechanisms evaporation, conduction and convection. The water then collects in the basin for reuse. Any water lost through evaporation, windage, or blowdown is replaced by make-up water to maintain consistent operation.

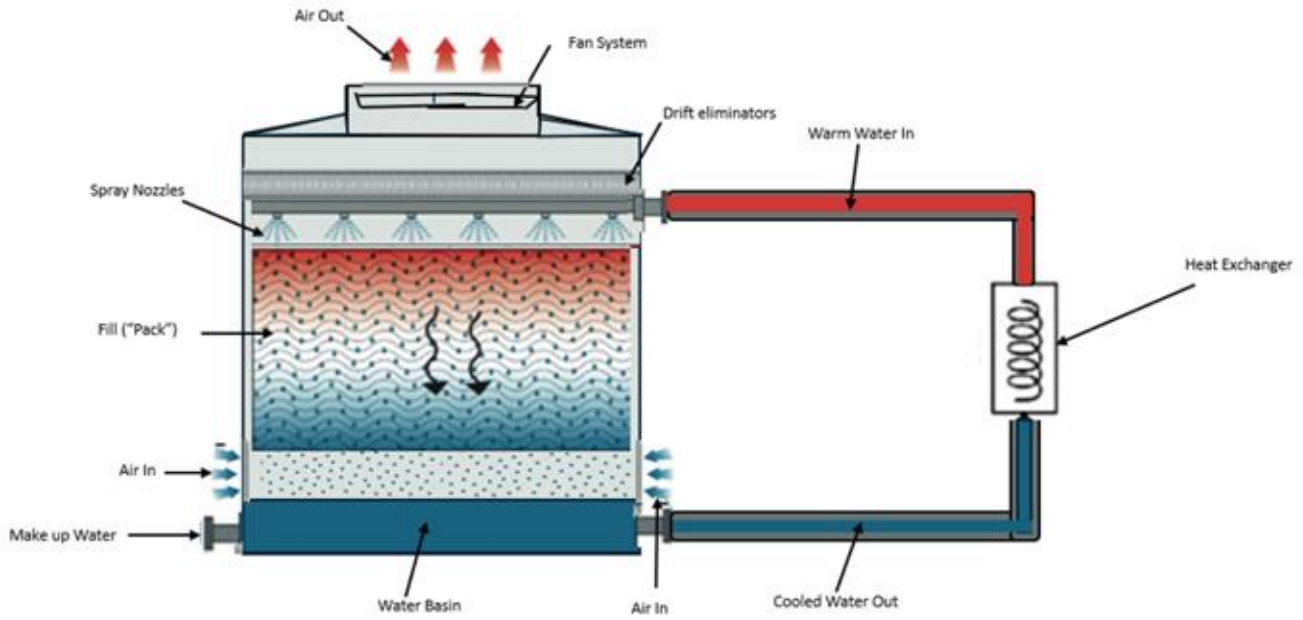

***Figure 4**: Schematic of an induced draught counterflow cooling tower, illustrating key components and processes. Warm water from industrial processes enters the system via spray nozzles and flows downward through the fill ("pack") to maximise surface area for heat exchange. Air is drawn upward by the fan system while drift eliminators reduce the release of water droplets. Cooled water collects in the water basin and is recirculated through a heat exchanger. Make up water compensates for losses due to evaporation, drift and blowdown. Image adapted from (Health and Safety Executive, 2020).*

Manmade systems, such as cooling towers create favourable conditions for the growth and proliferation of *Legionella*. The operating environment of cooling towers creates favourable conditions for bacterial development due to multiple influencing factors. Operational practices, such as the choice of water source, water flow, stagnant zones (e.g., dead legs), improper or irregular maintenance, aging infrastructure, and the ineffective or inconsistent use of biocides (Zahran et al., 2018), can significantly contribute to the risk of *Legionella* colonisation.

Water quality parameters, including heterotrophic plate counts, high turbidity, pH, conductivity, calcium hardness and the presence of iron are inferred to promote *Legionella* growth within cooling towers (Yamamoto et al., 1992). Additionally, total organic carbon has been shown to positively correlate with *Legionella* qPCR detection (Donohue et al., 2024). Biological factors, such as biofilms, algae, and protozoa, further favour the multiplication of *Legionella* (Fitzhenry et al., 2017; Casini et al., 2018). Finally, meteorological parameters, such as ambient temperature, humidity, and seasonal fluctuations, can also influence the occurrence and growth of *Legionella* (Brigmon et al., 2020).

Although several factors have been suggested to support *Legionella* growth, it is important to note that most studies have focused on hot and cold-water distribution systems or plumbing systems, rather than cooling towers. Furthermore, the limited studies on cooling towers have not extensively examined biofilm samples, which play a crucial role in bacterial proliferation. Prior research also shows conflicting results in many cases underscoring the complexity of *Legionella* proliferation and the need for further examination. For instance, while two studies reported higher *Legionella* proliferation during summer and autumn using PCR and culture methods, respectively (Yamamoto et al., 1992; Salvador and Garcia, 2011), a more recent study by Campana et al. (2023) found no clear seasonal trend. Similarly, Campana et al. (2023) and Kyritsi et al. (2018) observed a positive correlation between heterotrophic plate count and *Legionella* count, but other studies (Whiley et al., 2019) did not. Kyritsi et al. (2018) also identified a positive correlation between *Legionella* count and pH, conductivity, and hardness, whereas Serrano-Suárez et al. (2013), Campana et al. (2023), Xu et al. (2024) and did not observe such associations.

2.3 Current *Legionella* Detection Techniques

The culturing of *Legionella* using the ISO 11731 protocol is considered the 'gold standard' for detecting and quantifying *Legionella* bacteria. This method is low-cost and identifies and enumerates viable *Legionella* species and serogroups, producing results in standard colony forming units per litre, with extensive historical use and a wealth of data to support its effectiveness (ISO, 1998). These results and historical data are particularly important as they allow for direct comparison against current HSE risk levels and thresholds, thereby guiding the necessity and urgency of remedial actions and ensuring compliance with regulatory standards (HSE, 2014; Walker and McDermott, 2021). Updates to the ISO 11731 protocol have introduced the application of buffered charcoal yeast extract (BYCE) agar, Modified Wadowsky Yee (MWY) agar and glycine, vancomycin, polymyxin B, cycloheximide (GVPC) agar (ISO, 2017). MWY has been demonstrated as the best medium for potable water samples with GVPC agar being more efficient in detecting *Legionella* than BCYE medium within samples with high concentrations of competing microorganisms (Scaturro et al., 2020). BYCE and GVPC contain activated charcoal as a scavenger of radicals and peroxides, to which the genus *Legionella* is particularly sensitive. Additionally, these media are supplemented with a-ketoglutarate for its

antioxidant properties, L-cysteine and iron pyrophosphate to stimulate *Legionella* growth. GVPC also contains the addition of four selective compounds: glycine, polymyxin B sulfate, vancomycin hydrochloride and cycloheximide, these antibiotics are used to reduce competitive microbial flora (Casino *et al.,* 2022).

The current standard has many shortcomings, including a long turnaround time from 10 to 14 days, an inability to identify viable but non-culturable cells and low reliability due to competing microorganisms that can outcompete *Legionella*, leading to underestimation or even false negatives (Díaz-Flores et al., 2015). To reduce the risk of competing microorganisms affecting results, water samples undergo several pre-treatment steps, such as filtration or centrifugation, followed by acid and heat treatment that can interfere with *Legionella* growth (Whiley and Taylor, 2016). Afterwards, culture requires expert-trained technicians for the identification of *Legionella*, leading to interpretation differences in colony forming units (CFU) counts (Lucas, Taylor Jr and Fields, 2011). Furthermore, the subsequent use of latex agglutination tests on cultured samples, aimed at serotyping *Legionella*, presents additional challenges. These tests can suffer from limited specificity and potential cross-reactivity with non-*Legionella* bacteria, complicating the accurate identification and serotyping of *Legionella* strains, leading to misinterpretation of the presence and risk of specific *Legionella* serogroups (Fields, Benson and Besser, 2002).

The Most Probable Number (MPN) method is a liquid culture technique fully validated for detecting *Legionella* in potable and related water systems. Unlike traditional culture methods, MPN does not require pre-treatment steps to eliminate competing microorganisms, and it indicates *Legionella* presence through a colorimetric change (Sartory et al., 2017). The method's advantages include ease of use, higher reliability, and sensitivity, while also offering a faster turnaround—typically taking half the time compared to culture methods to produce results (Boczek et al., 2021; Monteiro, Robalo and Santos, 2021). However, MPN is prone to false positives in cases of turbid or heavily soiled water, where the colour change can be obscured, leading to false positives (Niu, Zhang and Zhang, 2022). Additionally, while faster, MPN still requires up to seven days to yield results, which may delay immediate corrective actions (Barrette, 2019).

Given the substantial turnaround times of 10-14 days for culture methods and 7 days for the MPN method to produce results, there is a significant delay in implementing corrective actions. This delay poses a risk as the microbial status, including *Legionella*, of the system can escalate, potentially leading to outbreaks within this timeframe (Yamaguchi et al., 2017). Consequently, the urgency for deploying rapid detection technologies has become paramount, aiming to drastically shorten the time required for detecting *Legionella* and enabling swift remedial action. The effectiveness of such rapid response measures would be further enhanced by the identification of a 'lead indicator,' which could serve as an early warning system for proactive management of *Legionella* outbreaks and system failures. Unfortunately, a reliable lead indicator for *Legionella* presence has yet to be identified, underscoring the need for continued research and development in this critical area of public health.

2.4 Emerging *Legionella* Detection/ System Performance Techniques

Promising rapid detection techniques for monitoring the presence of microbial concentration, microbial viability and *Legionella* are emerging, including the use of flow cytometry, quantitative polymerase chain reaction (qPCR), immunomagnetic separation and lateral flow. These techniques claim to have increased sensitivity, high reliability, high throughput, and rapid turnaround time compared to current methods. Thus, these methods have been suggested as suitable complementary or replacement method to culture for (i) routine surveillance, (ii) monitoring changes in *Legionella* concentration over time and (iii) emerging corrective action (Walker and McDermott, 2021). This rapid response could decrease feedback time and potentially reduce biocide costs and ecological benefits, ultimately reducing *Legionella* outbreaks.

Flow cytometry is a rapid, non-specific method adept at detecting microbial concentrations and viability within cooling towers by quantifying both intact and compromised cell counts, including those unable to grow on traditional growth media—thus encompassing approximately 99% of environmental bacteria (Wade et al., 1997). This technique utilises intercalating dyes, such as SYBR Green, to stain all bacteria in a sample, and Propidium Iodide (PI), which penetrates only cells with damaged membranes, to make this distinction (Nescerecka et al 2016). Such capabilities enable near real-time assessment of biocide treatment efficacy, demonstrating log reductions in microbial counts depending on the biocide used (Helmi et al., 2018). Furthermore, flow cytometry's utility extends to analysing cell concentrations within drinking water distribution systems, examining both biofilm and planktonic samples (Schleich et al., 2019; Fish et al., 2020; Gabrielli, Turolla and Antonelli, 2021). Expanding its application, flow cytometry incorporates 'fingerprinting' of microbial communities through machine learning, creating unique profiles that offer insights into the composition and functional state of these communities, including shifts that may indicate the presence or absence of *L. pneumophila* (Kyritsakas, Boxall and Speight, 2023). Despite these advancements, it is critical to note that even with fingerprinting, flow cytometry cannot specifically identify *L. pneumophila.* This limitation underscores the necessity for complementary methods to achieve targeted pathogen detection.

Immunomagnetic separation (IMS) presents a rapid detection method for *Legionella*, leveraging magnetic beads coated with antibodies that specifically target *Legionella* cells. This enables the selective separation of the cells from samples for focused analysis, delivering results within two hours with impressive sensitivity and specificity (96.6% and 100%, respectively), and demonstrating a detection efficiency of 97.8% compared to traditional culture methods (Bedrina et al., 2013). Despite these advantages, IMS has not become widely adopted in general practice, largely due to the limited availability of commercial products and the method's inherent complexity, which requires numerous procedural steps. A particular challenge is posed by the bulk water samples from cooling towers, which are often coloured and require filtration. These conditions can complicate the IMS process and lead to false positives (Keserue et al., 2021). Additionally, the 'recoverability' of *Legionella* post-separation might be compromised during the filtration process, further complicating its practical application (Bedrina et al., 2013).

Two bench top studies conducted by (Füchslin et al., 2010; Keserue et al., 2012) using pure *Legionella* strains have showed that if flow cytometry is combined with immunomagnetic separation, this combination of methods can detect

Legionella with a high recovery rate. Furthermore, recent research has successfully validated and applied a combined flow cytometry and immunomagnetic separation method that capable of detecting *L. pneumophila* using spike field samples (Streich et al., 2024). Despite the advantages of combining flow cytometry with immunomagnetic separation for *Legionella* detection, a notable gap in current research is the scarcity of field studies and absence of investigations using biofilm samples.

qPCR emerges as the most promising alternative to traditional methods for *Legionella* detection, characterised by its high negative predictive value and low detection limit (Toplitsch et al., 2021). Its enhanced sensitivity, reliability, and rapid turnaround time, coupled with high throughput capabilities, render qPCR an invaluable tool for pre-selecting negative samples. By implementing qPCR as a negative screening strategy for *Legionella* — wherein only samples testing positive via qPCR proceed to culture — the time needed to pinpoint potential *Legionella* sources can be substantially shortened, facilitating swifter crisis response compared to dependence on conventional culture techniques alone (Collins et al., 2017; Fisher et al., 2020). Further advancing *Legionella* detection, digital droplet PCR (ddPCR) offers significant improvements over standard qPCR, including higher sensitivity, precision, reproducibility, and enhanced tolerance to the PCR inhibitory substances frequently encountered in cooling tower samples (Paruch, 2022). Crucially, ddPCR enables the absolute quantification of DNA without necessitating standard genetic references, offering more accurate and reproducible quantification of bacteria. This feature is beneficial in cooling tower environments where accurate and reliable *Legionella* concentration assessments are critical for effective water management and safety protocols (Wei et al., 2023).

PCR methods have been challenging to incorporate into national legislation since the risk levels are defined by CFU, not by the genomic units (GU) that qPCR produces. These challenges arise because GU shows a weak correlation to CFU counts and is difficult to convert (Eble et al., 2021). Furthermore, qPCR cannot discriminate between living and intact cells without the use of intercalating dyes such as ethidium monazide (EMA) and propidium monoazide (PMA), with EMA having high intrinsic toxicity (Taylor, Bentham and Ross, 2014). Since EMA has high toxicity, PMA has been more commonly used for its greater efficiency and more accurate estimation of viable cells. However, studies have still shown mixed results in PMA's ability to discriminate between live and dead cells (Taylor, Bentham and Ross, 2014; Bonetta et al., 2017). These mixed results are due to differences in PMA concentration, method, and a lack of a reference protocol. Young et al. (2021) described a longitudinal study over 39 months using qPCR on cooling tower planktonic samples. In this study, a cooling tower operator established what was considered "typical" for the cooling tower on-site and set an in-house limit of 5000 GU/L. This value was considered a good indicator of system health and any results above this value prompted corrective action, such as adjustment of biocide use. Since adopting this in-house limit and intervention, no further cases of Legionnaire's disease have been associated with this cooling water distribution system. Therefore, this study indicates that although conversion to GU to CFU per litre is wanted to associate these results with current activity levels, this may not be necessary since future GU action levels could be made based on trend analysis allowing emerging corrective action.

Lateral flow immunochromatographic assays utilise a plastic paddle and capillary flow technology to detect *L. pneumophila* serogroup type 1 antigens in water samples through the binding of a coloured antibody, indicating a positive result with two red lines. These tests are particularly valued for their portability, ease of use, and the ability to deliver results in under an hour (Sun et al., 2013; Walker and McDermott, 2021). Additionally, the COVID-19 pandemic has heightened interest and improved the understanding of lateral flow technologies. However, they often suffer from lower sensitivity, which can limit their effectiveness in some water samples (Párraga-Niño *et al.*, 2018). According to a study by Ezenarro et al. (2020), using lateral flow assays in combination with a filtration technique can overcome the sensitivity limitation and detect *Legionella* concentrations ranging from 10^1 to 10^4 CFU/ml, with a detection limit as low as 4 CFU/ml.

Most peer-reviewed papers assessing emerging detection methods have shown significant advantages over the traditional culture method with few limitations. However, these methods have not been evaluated with biofilm samples, which are the prevailing mode of microbial life, including *Legionella* in engineered water systems (Di Pippo et al., 2018). Furthermore, microbial communities within biofilms are significantly different than planktonic communities, exceeding planktonic numbers by three to four logarithm units and can be a potential source of pathogens (Flemming, 2020). Therefore, because of *Legionella* ecology and biofilms being the prevailing mode of microbial life, current research applying emerging techniques to water samples only are unrepresentative of the true microbial load and *Legionella* concentration found within engineered water systems. It should also be noted that all the emerging detection methods, except qPCR have not been tested using operational cooling towers over a long period of time.

2.5 Microbiome Present Within Engineered Water Systems

Recent advancements in molecular biology, particularly the advent of high-throughput sequencing technologies, have revolutionised our understanding of microbial communities in engineered water systems such as cooling towers (Tan et al., 2015; McDaniel et al., 2021). The use of 16S and 18S rRNA gene amplicon sequencing exemplifies this shift, targeting highly conserved regions of ribosomal RNA genes specific to bacteria and eukaryotes, respectively. These regions are characterised by their conserved sequences interspersed with hypervariable regions, allowing for the accurate classification and taxonomy of diverse microbial species, thus providing a detailed view of microbial diversity and composition (Zhang and Liu, 2019).

Understanding the microbial composition and structure within engineered water systems is increasingly recognised as crucial, not only for its implications on the presence and absence of *L. pneumophila* but also on the operational integrity of these systems, enabling improved management strategies (Flemming et al., 2020). Moreover, by integrating these microbiological insights with water quality parameters and predictive modelling, we gain greater insights into the communities and parameters that promote or inhibit *Legionella* growth (Wang et al., 2014; Buse et al., 2017 This enhanced understanding helps explain why *L. pneumophila* outbreaks occur and how they can be linked with other water quality parameters (Ashbolt, 2015).

2.5.1 Microbial Community and Structure in Water Distribution Systems

Research on community composition within water distribution systems research has found a diverse bacteria phylum (11), encompassing more than 100 species (Mathieu, Paris and Block, 2019). A meta-analysis conducted by Thom et al. (2022) found that the most common phyla included Actinobacteriota, Bacteroidota, Cyanobacteria, Nitrospirota, Planctomycetota and Proteobacteria. Furthermore, biofilms were found to be significantly different from bulk water in terms of taxa present, the abundance of taxa and beta diversity. The bacterial communities within both the biofilm and bulk water are known for their role in biofilm production, organic matter degradation, and nitrogen fixation, which can have potential impacts on water quality, the presence of pathogens and infrastructure integrity (Thom et al., 2022). Since the microbial community is influenced by a variety of factors, studies have examined the effects of temperature, biocide use, and the materials within water distribution systems affect planktonic microbial communities.

A meta-analysis by Thom et al. (2022) also showed that microbial communities in residual disinfectant-free water distribution systems are generally more diverse than those in systems maintaining a disinfectant residual. This was evidenced by a higher Shannon index in both bulk water and biofilm samples, indicating greater species richness and evenness. This increased species richness can correlate with *Legionella* concentrations (Llewellyn et al., 2017), however, systems with a disinfectant residual can also support *Legionella spp.* depending on environmental conditions and microbial interactions (World Health Organization, 2007) albeit in lower abundance. Additionally, systems maintaining a disinfectant residual were found to have a higher abundance of specific taxa, including *Mycobacterium* and *Pseudomonas*. Chlorination and chloramination alter microbial community composition, leading to shifts in bacterial populations. For instance, Hwang et al. (2012) investigated an urban drinking water distribution system subjected to phases of chlorination and chloramination. Their research revealed that certain bacterial families, such as *Cyanobacteria,* Methylobacteriaceae*,* Sphingomonadaceae*,* and Xanthomonadaceae*,* were more abundant during chlorination phases. In contrast, families like Methylophilaceae*,* Methylococcaceae*,* and Pseudomonadaceae were more prevalent during chloramination phases (Hwang et al., 2012).

Notably, these shifts in microbial communities can influence the presence of *Legionella* in ways that depend on both the disinfection process and other factors, such as pipe material and water temperature. For example, a study conducted by Aloraini, Alum, and Abbaszadegan (2023) found that higher water temperatures (32°C vs. 22°C) were associated with an increased abundance of certain bacterial families, including *Mycobacteriaceae* and *Legionellaceae*. The study also demonstrated that cross-linked polyethylene pipes supported a more distinct microbial community than copper or steel pipes.

Pipe materials are also an essential driver of the chemical and biological water quality parameters in plumbing pipe material, allowing biofilm growth and the incorporation of *Legionella*. Most cooling towers are made from stainless steel, cast iron, copper and plastics (Cullom et al., 2020). Iron and steel show increased surface roughness and that gives cells more of a "foothold" on the wall of a pipe and makes it easier for microorganisms to attach (Greenberg, 2021). In addition, the material can also corrode, releasing nutrients from the materials (Cullom et al., 2020; Rakić,

Vukić Lušić and Jurčev Savičević, 2022) have shown increased iron concentrations to enhance *Legionella* growth in tap water. Copper, in contrast, shows antimicrobial properties with several studies confirming the efficacy of copper, either passively leached from plumbing material (Proctor et al., 2017) or actively using copper-silver ionisation to inhibit the growth of *Legionella* (Ashbolt, 2015). Plastic materials are becoming more popular due to their low cost, durability, and lower disinfectant residual. However, studies have shown that plastics such as PVC surfaces are hydrophobic, which favours the attachment and accumulation of *Legionella*. Moreover, PVC also releases organic carbon, whereas metallic pipes do not, causing an increase in *Legionella* within biofilms (Learbuch, Smidt and Van Der Wielen, 2021). Furthermore, plastic materials can also release phosphate, which, when limited, can restrict the growth of microorganisms (Pinel et al., 2020b).

As outlined in Section 2.1.2, the association of *Legionella* with free living amoebae complicates disinfection efforts in engineered water systems. Therefore, there is a growing importance of understanding the protozoa microbiome and their interactions with bacterial communities including *Legionella* and physicochemical parameters. Buse et al. (2013) showed that in both hot and cold drinking water distribution systems contained a high eukaryotic diversity compared to bacterial diversity, which often changed across sample period with temperature having an impact, indicating that the eukaryotic community is unstable. Other studies such as Loret and Greub (2010) and Thomas and Ashbolt (2011) have also reported that the most common FLA in engineered water systems are Acanthamoeba, Vermamoeba, and Naegleria species and have been experimentally confirmed as *Legionella* hosts ((Boamah et al., 2017). FLA have been shown to positively correlate with *Legionella* spp. abundance, as they provide a protective environment and resources for *Legionella* survival and proliferation. However, *Legionella* is not exclusively reliant on FLA for its persistence as studies have demonstrated that *Legionella* can survive and proliferate within biofilms independently of protozoan hosts, using biofilm structures as reservoirs that protect against environmental stress and disinfection.

Despite the significant insights gained from these studies, it is important to note that much of the research on water distribution systems has predominantly utilised scaled systems for analysis. While these studies provide valuable information on microbial community composition, behaviour, and responses to environmental changes, enabling improved management strategies and control measures, they may not fully capture the dynamics present in actual field conditions, especially within cooling towers. Cooling towers, with their unique environmental conditions and operational parameters, offer a distinct ecosystem that could influence microbial communities differently. Furthermore, these studies have not focused on how the dynamic microbial community, alongside physicochemical parameters, may lead to *Legionella* outbreaks, which primarily occur in cooling towers.

2.5.2 Microbial Community and Structure in Cooling Towers

Cooling towers are a major source of Legionnaires' disease outbreaks, owing to an environment that promotes bacterial proliferation, such as high temperatures, surface area, nutrient availability, and the production of aerosols which can be dispersed over long distances. To date, cooling towers have not been studied as extensively as other engineered water systems. A study conducted Llewellyn et al. (2017) using 16S rRNA sequencing, collected a total of 196 bulk water samples from separate cooling towers across the United States within nine different climate regions.

The most abundant bacterial species found were similar to that of water distribution systems, including *Proteobacteria*, *Flavobacterium*, Bacteroidetes and *Cyanobacteria*. Remarkably, the most abundant species present were consistent across each region, indicating little spatial or climate-driven variation. *Legionella* was detected in 84% of all samples, with a higher abundance of *Legionella* found in samples containing more diverse taxa. Pereira et al. (2017) collected bulk water samples from one cooling tower in Germany across two years to determine the planktonic microbiome. The most abundant bacterial phyla were similar to those reported by Llewellyn et al. (2017), including *Proteobacteria*, *Hypomicrobium* and *Rhizobactera* across the two years, with only a difference in genera. This knowledge is essential as it suggests that a core community of phyla is common within cooling towers. Therefore, there may be a universal, selective pressure imposed by the environment or other microorganisms present within the cooling towers, which gives insight into what monitoring, and intervention approaches should be used to reflect the performance of control measures.

A more recent study Paranjape et al. (2020b) combined 16S rRNA sequencing with a linear discriminant analysis effect algorithm (LEFse) on cooling tower bulk water samples and found that the most abundant bacterial phyla to be similar to that reported by Llewellyn et al. (2017) and Pereira et al. (2017). Based on the LEFse analysis, the bacteria *Brevundimonas,* Xanthobacteraceae and *Qipengyuania* taxa were identified as being a predictors of high *Legionella* concentration. Conversely, other bacterial species such as *P. aeruginosa, Sphingomonas* spp. and *Klebsiella pneumonia* are negatively correlated to the presence of *L. pneumophila* (Paranjape, et al., 2020b). Using 18S rRNA sequencing Paranjape et al. (2020a) has shown fungal groups were the most abundant and prevalent taxa with Basidiomycota and Ascomycota classes being the most dominant. Interestingly *Legionella* host cells Acanthamoeba, Vermamoeba, Naegleria and Oligohymenophorea were also present in the cooling towers but with a low abundance (5%) in some cooling towers and 30% for other cooling towers showing amoeba may not be necessary for *Legionella* proliferation. Vermamoeba and Oligohymenophorea are of specific interest as there found to be positively correlated with *Legionella* and high Oligohymenophorea concentrations being linked to the bacteria *Brevundimonas* as a food source.

These few studies have shown that *Legionella* spp. is part of a complex microbiomes whose interactions with other microorganisms may promote or inhibit growth (Thom et al., 2022) but is an under examined area of research. Furthermore, these works only look at the microbiome present bulk water samples likely due to the difficulties in taking biofilm samples since this requires the cooling tower to be shut off and the pipes dismantled. Biofilm samples exceed planktonic numbers by three to four logarithmic units and can be a potential source of continuous microbial corrosion (Di Pippo et al., 2018). Furthermore, the biofilm microbiome differs significantly from a planktonic microbiome (Roeselers et al., 2015). As a consequence, planktonic samples give an incomplete understanding of the microbial community and structure in natural and engineered environments since they are not representative entirely of the cooling tower microbiome.

Similar to water distribution systems a variety of physicochemical factors have an impact on the microbial composition, promoting or inhibiting the growth of *Legionella* or organisms that support or inhibit *Legionella* colonisation. For instance, cooling towers have an average temperature between 20 -35 °C, which is within *L.*

pneumophila growth temperature range (Wadowsky et al., 1985). Furthermore, similar temperatures found within water distribution systems also promote biofilm formation and the presence of potential protozoan host cells (Ashbolt, 2015). Therefore, cooling tower temperatures are an essential determinant for *Legionella* colonisation, residential microbiota and even the ability of *Legionella* to infect free-living amoeba (Buse et al., 2017).

A study conducted by Pereira et al. (2017) established that temperature is a major factor in determining community structure and *Legionella* concentration within cooling tower bulk water. Notably, the study highlighted that an increase from 10 to 22°C from summer to winter months led to a significant shift in community structure, characterised by a marked increase in the abundance of Cytophagales, *Legionella*les, and Sphingobacterales, along with a considerable reduction in species richness.

Another study conducted by Hammer (2018) reveals that with the transition from spring, at an average temperature of 12.7°C, to summer, with temperatures reaching 25.8°C, there is a noticeable shift in the bacterial community composition. Specifically, there's a decline in the prevalence of Streptophyta and *Pseudomonas* spp., which are more abundant in cooler temperatures, giving way to an increased presence of *Legionella* as the temperature rises. Additionally, this temperature-driven transformation is accompanied by a significant enhancement in species richness, indicating that warmer temperatures not only alter community structure by favouring certain bacteria over others but also promote a more diverse microbial ecosystem.

To investigate the effects of temperature on cooling tower biofilm composition and abundance Paniagua et al. (2020) produced a pilot cooling tower consisting of a heated loop (30.7°C) and a cold loop (22.7°C) connected to a water bath. Biofilm samples were collected after a total of 92 days and showed temperature had a significant difference in beta diversity with bacterial families such as Burkholderiaceae, Rhodocyaceae and Microbacteriaceae were predictive in biofilms around 20 °C with Nitrosomonadaceae and Reynellaceae were predictive in biofilm at 30 °C. Furthermore, alpha and beta diversity using Shannon index and Bray dissimilarity index respectively showed that biofilms had a significantly different bacterial community. The study conducted by Pinel et al. (2021) used a pilot cooling tower to investigate the effects of pH (9.0 - 9.6) over 25-35 days on *L. pneumophila growth* and characterise the changes in the bulk water microbiome. *Legionella* and protozoan hosts were found to proliferate at pH 9.0 and 9.4. However, at a pH of 9.6, *Legionella* was below the detection limit (<100 CFU/LI) and correlated with a low abundance of protozoa. Furthermore, at alkaline pH there was an increase of Bacteroidetes, *Sphingopyxis* and *Flavobacterium* abundance.

Low levels of chlorine result in a higher abundance in *Legionella*. However, cooling towers with high levels of chlorine and continuous application showed a higher abundance of *Pseudomonas* and a lower species richness. This suggests that maintaining a free chlorine residual greater than 0.3mg/L is key to prevent the colonisation of *Legionella* in cooling towers (Paranjape et al., 2020b). Current literature has shown that several physicochemical factors, such as water temperature, pH, and disinfection regime, can affect the planktonic microbial communities. Therefore, understanding these conditions is critical to elucidate the risk factors linked to *Legionella* outbreaks. Although several models have been developed and have contributed to a deeper understanding of how a range of abiotic factors affect bacterial composition, as well as genotypic and phenotypic variation, most models only examine one abiotic factor at

a time, without considering the complexity of multiple abiotic factors found within a cooling tower, such as an open circulatory system. Furthermore, other studies that have used operational cooling towers still do not sample biofilms; therefore, the understanding of microbial communities and interactions within them, and how they are impacted by physicochemical factors, is currently limited.

2.6 Current Cooling Tower Management Practices: Implications for Cell Concentration and *Legionella* Control

Current cooling tower management practices are centred on maintaining optimal water quality, avoiding water stagnation (including during system downtime), implementing disinfection regimes, and conducting periodic cleaning and disinfection. Monitoring the effectiveness of these practices, as well as inspecting pipe materials for signs of corrosion and biofilm formation, are critical components. These strategies are integral to controlling cell concentration and managing the risk of *Legionella* proliferation. By focusing on these areas, facility managers can significantly mitigate the factors that contribute to the growth and spread of *Legionella* within cooling tower systems (ASHRAE, 2020; CDC, 2021; HSE, 2014).

In the management of cooling tower systems, monitoring for high HPC is of critical importance as it serves as a general indicator of microbial activity within the water system (WHO, 2007). While it has been posited that elevated HPC levels may facilitate the proliferation of *Legionella*, including *L. pneumophila,* the evidence linking high HPCs directly to increased *Legionella* risk is varied and not definitively conclusive. Despite this uncertainty, the primary and most critical utility of detecting high HPCs lies in its role as a prompt for the adjustment of biocidal regimes. This is essential for maintaining effective system control and preventing the public health risks associated with microbial outbreaks (Pierre et al., 2019).

2.6.1 Oxidising, Non-Oxidising biocides and Alternative Methods for Microbial and *Legionella* Control

Chlorine is widely used for its strong oxidising power in primary disinfection. It reacts with a variety of bacterial cellular components and is able to permeabilise the cytoplasmic membranes, causing leakage of proteins and DNA damage. However, it has a reduced effect on biofilms (Ridgeway and Olson, 1982). High levels of residual chlorine are problematic, as they are responsible for the corrosion of copper, iron, and PVC pipes, and the formation of potentially toxic and carcinogenic disinfection by-products. As secondary disinfectants, chlorine dioxide and chloramine are superior in penetrating biofilms and have been found to be more effective at reducing *Legionella* concentrations than chlorine (Donohue et al., 2019). However, despite these benefits, the main disadvantage of these methods is that they require consistent monitoring to achieve optimal concentrations. Furthermore, both secondary disinfectants increase pipe corrosion, and chloramine can lead to nitrification, which may result in a microbial bloom (Sciuto et al., 2021). Non-oxidising biocides are non- pollutant, less reactive and attack specific targets from the microbial cell metabolism (Tewari, Mehta and Vaishnav, 2019) with the most widely used being 2-2-Dibromo-3-nitrilopropionamide (DBNPA). Since most of these non-oxidising biocides have their own mechanism of action, they are used at higher concentrations and longer contact times to achieve equivalent microbial effectiveness as oxidising biocides (Proner et

al., 2021). Since non-oxidising biocides can promote resistant bacteria, they are often used in alternation with oxidising biocides (Wales and Davies, 2015).

Since biocides can cause corrosion and toxic disinfection by-products, which results in adverse effects on the environment, there is a growing interest in disinfectant-free alternative. High-pressure carbon dioxide has been considered, as this has sterilising effects at much lower temperatures than chlorine while being inert, non-toxic, accessible, and affordable (Martín-Muñoz, Tirado and Calvo, 2022). Ultraviolet light has also been an attractive option for disinfection since no chemicals are added to the water and short wavelength ultraviolet light shows biocidal effects. However, ultraviolet light shows poor biofilm penetration and does not produce residual desired to maintain microbial concentration, including *Legionella*, so other disinfection measures must be used (Sciuto et al., 2021)

2.6.2 Biocide Effect on Microbial Composition, Structure and *Legionella* Presence

To optimise disinfectant efficiency and manage microbial risks more effectively in cooling towers, it is essential to grasp the bacterial dynamics influenced by different biocide regimes. Understanding which biocides are most effective is not just about immediate results; it involves gaining insights into their long-term impact on microbial community composition, including which bacterial species are promoted or suppressed. This knowledge is crucial for comprehending the dynamics of microbial communities and specifically for tackling *Legionella* survival and proliferation. By identifying the effects of various biocides on the bacterial populations that either compete with or facilitate the growth of *Legionella*, we can develop targeted disinfection strategies. Such strategies not only aim at immediate microbial control but also ensure the sustained efficacy of biocides, thereby mitigating the risk of *Legionella* outbreaks and enhancing public health safety.

Prior research has shown that active bacteria can still be present in cooling towers after continuous chlorine dosage (Helmi et al., 2018). Pinel et al. (2020a) showed continuous chlorine disinfection in cooling towers' planktonic waters showed a noticeable decrease in Actinobacteria and an increase in Proteobacteria. After post-disinfection, the cooling tower water bacterial communities were predominantly made up of AlphaProteobacteria made up from the order Obscuribacterales (*Melainabacteria*), Caulobacterales, Sphingomonadales, and Rhizobiales, which are notable for their resilience or adaptation to chlorine exposure, partly due to their biofilm-forming capabilities. Interestingly, these findings suggest that the decay rate of different bacterial orders, rather than their growth potential, is the primary determinant in shaping cooling tower microbiome.

Despite biocides showing a significant effect on the microbiome present, there is little to no studies on the effects of other biocide regimes within cooling towers such as monochloramine, non-oxidising biocide and alternative biocide or comparing their effects. Furthermore, there is no studies looking at the impact of biocide regimes on the microbial community within cooling tower biofilms.

2.7 Literature Review Summary

In freshwater, water distribution systems, shower hoses and laboratory biofilm reactors, *L. pneumophila* can be found in multispecies biofilms, where they benefit from enhanced protection against environmental stresses, increased resistance to disinfectants, improved nutrients availability and facilitated genetic exchange. However, despite these advantages, biofilm samples within cooling towers have never been studied or sampled for *Legionella* detection, to assess microbial concentration and viability, and to inform intervention and management strategies.

Rapid *Legionella* detection methods used with planktonic samples have shown several advantages, including rapid turnaround time and improved sensitivity/ specificity compared to the "gold standard" culture methods, with fewer limitations. These methodologies have remained unexplored in the realm of biofilm samples, especially in the context of operational cooling towers and across a long period of time.

Several studies have shown that the planktonic microbiome, including specific species present within cooling tower bulk water, can either promote or inhibit the proliferation of *Legionella*. No studies have examined biofilms in the same way, despite being an ecological niche that can exceed planktonic numbers by three to four logarithmic units, representing 95% of all microorganisms within an engineered water system. This niche has been shown to be significantly different from bulk water within water distribution systems.

Multiple physicochemical parameters may influence the planktonic microbial concentration, community composition/structure and the presence or absence of *Legionella* within cooling tower bulk water. The impact of these physicochemical parameters on the microbial concentration, community composition/structure and *Legionella* presence or absence in operational cooling towers biofilm have never been investigated over a long term.

Chapter 3: Aims and Objectives

This research aims to minimise the delay between the detection of *Legionella* and the implementation of water safety plans for cooling towers. It seeks to provide novel insight into utilising biofilms as a routine sampling method, alongside a deeper understanding of the interactions between biofilms, planktonic communities, and the environment within cooling towers. This knowledge will support a lead indicator approach for routine monitoring and enable timely corrective actions, ultimately reducing the incidence of *Legionella* outbreaks.

In this study, biofilm samples refer specifically to microbial communities attached to the cooling tower pack, chosen due to its ideal conditions for biofilm growth and proliferation. A novel sampling technique was developed, optimised and employed to collect biofilm samples, enabling consistent and reproducible quantification of microbial parameters such as microbial concentration, viability, *Legionella* spp. concentration and bacterial community structure. Comparing biofilm and bulk water samples will help identify microbial trends and lead indicators for *Legionella* risk, supporting the future development of proactive water safety measures.

Using these parameters, the objectives are:

1. Explore using biofilm samples as lead indicators for *Legionella* detection, microbial concentration, microbial viability and intervention assessment.

2. Assess emerging *Legionella* detection methods for routine monitoring and trend analysis using biofilm and bulk water samples.

3. Compare the microbial communities present in biofilms to that of bulk water and identify trends in composition/structure or specific microorganisms that either promote or hinder *Legionella* growth and survival in cooling towers.

4. Examine the long-term stability of microbial concentration, viability and community structure in relation to seasonal variations, cooling towers structure/design, biocide regime and between consecutive towers cleans.

Chapter 4: Methodology and Methods

4.1 Experimental Overview/Design

This chapter provides a detailed overview of the research design and methodology employed to fulfil the aims and objectives outlined in Chapter 3, as illustrated in Figure 5. Within the experimental site, outbreaks from operational cooling towers are exceptionally rare, a testament to the effectiveness of conservative operational procedures, including stringent use of biocide regimes and regular cleaning throughout the study period. Recognising the low likelihood of observing an outright *Legionella* outbreak, the study strategically focused on monitoring four cooling towers, each with its own unique design, biocide regime, and historical performance. This approach, combined with the use of emerging detection methods over an extended observation period, aimed to enhance the likelihood of identifying *Legionella* precursor events, leveraging the unique attributes of each tower and the advanced capabilities of modern detection technologies to maximise the potential for early detection, despite the typically low incidence rates at the site.

To investigate if biofilm samples could act as lead indicators for *Legionella* detection, a novel biofilm sampling technique was developed and optimised. This approach was motivated by the recognition of biofilms as ecological niches for *Legionella*, potentially offering a more indicative measure of the bacterium's presence and risk levels. Concurrently, various emerging rapid *Legionella* detection methods were independently assessed to evaluate their suitability for field trials. The focus was on these methods' ability to reliably detect *Legionella* in both bulk water and biofilm samples, aiming to leverage their advantages over traditional culture methods for a more effective and timely identification of the bacterium.

To explore the impact of microbial concentration, viability, and characteristics of the bacterial community on *Legionella*'s survival and proliferation, the study meticulously compared biofilm and bulk water samples. This comparison was crucial to understanding how these microbial factors in different environmental niches might facilitate or inhibit *Legionella*'s survival and growth. Achieving this required the analysis of both biofilm and bulk water samples using flow cytometry to quantify microbial concentration and viability, along with 16S bacterial community analysis and ecological indices.

Utilising the longitudinal field study data in combination with cooling tower quality information (physical and chemical) over a two-year period, a comparative analysis was conducted. This analysis spanned across seasonal variation, biocide utilisation, cooling tower structural attributes and cleaning regimes for the identification of *Legionella* lead indicators and facilitated the assessment of interventions.

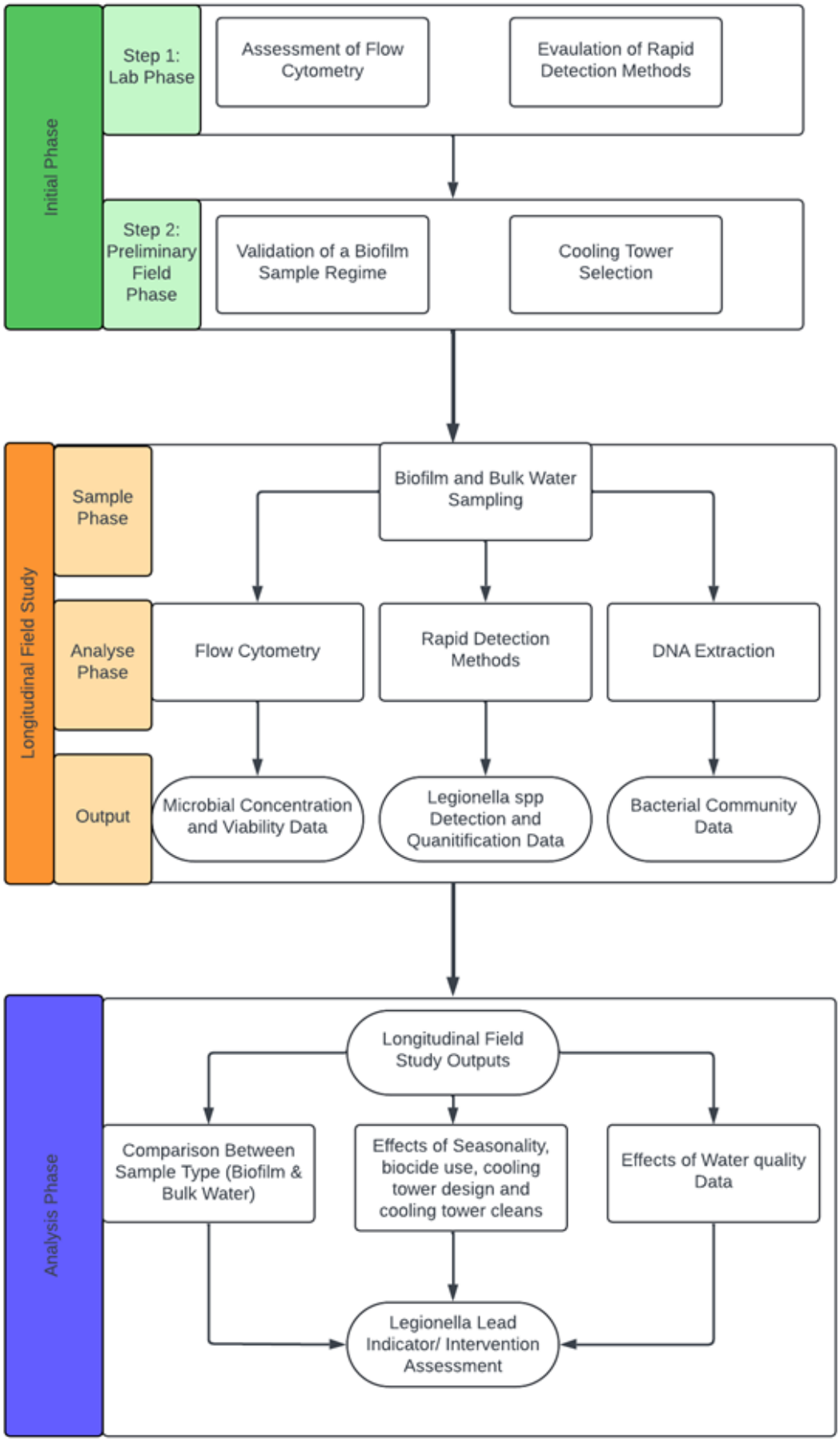

Figure 5*: The diagram illustrates the multi-phase research approach employed to investigate Legionella monitoring in cooling towers, spanning from laboratory validation to field studies and final analysis. The process is divided into three distinct phases, each represented by a colour: green for the Initial Phase, orange for the Longitudinal Field Study, and blue for the Analysis Phase*

4.2 Assessing Lab-Based Flow cytometry and Emerging Rapid Detection Method for Field Suitability

4.2.1 Flow Cytometry Analysis of Lab-Grown Biofilm Samples for Microbial Load Detection

Flow cytometry is utilised independently from other rapid detection methods due to its unique capabilities in assessing cell concentration and viability. This dual-purpose approach is pivotal for two main reasons: firstly, it investigates whether cell concentrations within the water systems create conditions that are conducive to *Legionella* growth, offering insights into environmental factors that may favour or inhibit bacterial proliferation. Secondly, the viability assessment provided by flow cytometry enriches *Legionella* detection efforts by indicating the potential pathogenicity.

To determine the practicality of incorporating flow cytometry for field analysis of biofilm samples, it was essential first to assess its applicability and reliability under conditions akin to those within cooling towers pack. This evaluation was pivotal in deciding whether to employ flow cytometry directly in the field study. A bespoke bench-top system was designed to closely simulate the environmental conditions found in cooling towers pack whilst promoting biofilm growth for rapid assessment. The setup comprised of four PVC coupons suspended within a 1-liter glass beaker filled with a chosen medium, at consistent temperature of 30°C, and shaken consistently throughout the 14-day incubation period. The chosen media all contained a mixture of tap water and BYCE media to promote multispecies biofilms (Stewart, Muthye and Cianciotto, 2012) utilising the natural microbial community and nutrients present.

To ensure the reliability of flow cytometry data in evaluating biofilm samples, a comprehensive approach involving 21 replicates was employed to measure both total and intact cell counts. This was underpinned by two key expectations: that total and intact cell counts would remain consistent across replicates, reflecting uniform conditions for each sample, and that intact cell counts would be lower than total cell count, indicating the presence of non-viable cells. Furthermore, the singlet-doublet ratio was carefully assessed to confirm the effectiveness of our homogenisation process. A high singlet-doublet ratio is indicative of successful disaggregation of samples into individual cells, a critical factor for ensuring the accuracy of flow cytometry analysis by minimizing the potential for clumping artefacts.

4.2.2 Independent Assessment of Emerging Rapid Detection Methods for *Legionella* spp. including *L. pneumophila*

To validate the claims of each method chosen for this study to take into the longitudinal field study, considering factors such as sensitivity, specificity, practicality and reliability, each emerging detection method (Table 2 was rigorously assessed using a known positive *L. pneumophila* serogroup type 1 Lenticule disc (NCTC 12821) following the manufacturing protocols.

These Lenticule discs were selected because they provide a known concentration of viable *L. pneumophila* serogroup type 1, establishing a standardised benchmark. Lenticule discs were prepared in accordance with the manufacturer's instructions. In summary, the Lenticule discs were allowed to equilibrate at ambient temperature for 5-10 minutes, followed by immersion in 100ml of 1/40 Ringer's solution for a 15-minute rehydration period. Subsequently, when the

Lenticule disc become indiscernible, the solution was vortexed to become homogenised and transferred into an appropriate volume of sterile distilled water tailored to each specific method.

***Table 2**: Table to summarise each emerging detection method*

Name	Method	Target	Volume Needed (ml)
Nephros	qPCR	*Leigonella* spp. (Genus)	1000
Genomadix	qPCR	*Legionella pneumophila*	20
Legiped	Immuno-magnetic separation	Legionella spp. (Genus)	9
Hydrosense	Lateral Flow	Legionella spp. (Genus)	250

The primary focus of this study was to evaluate each method's ability to accurately and reliably detect *L. pneumophila* and assess the number of samples it could process within a given timeframe, represented as total throughput. Each method's throughput was initially calculated by dividing the total available sampling time (2,400 minutes) by the sample time required per method. Each sampling session lasted five days, with a maximum of eight working hours per day, totalling 2,400 minutes of available time. Sample time refers to the duration required for a method to complete one batch of tests, based on its specifications or experimental data. To calculate the total throughput, this initial throughput was multiplied by the number of tests that could be processed in a single batch. The total throughput was therefore calculated using the formula:

Total Throughput = (Total Available Time / Sample Time per Batch) × Number of Tests per Batch.

Following this selected method were submitted to further evaluation. This evaluation focused on the method's detection capabilities across three different *L. pneumophila* concentration: ≤ 100 CFU, ≤ 1000 CFU and > 1000 CFU. These concentrations levels were selected to mirror the risk categories defined in HSE guidelines, offering a practical framework for assessing each method's effectiveness in identifying *L. pneumophila* under conditions of varying risk. Subsequently, the methods that succeeded in reliably detecting each concentration of *L. pneumophila* was taken further into the field study.

4.3 Validation of a Novel and Optimised Biofilm Sampling Regime

The development of a robust biofilm sampling method required systematic optimisation to ensure that the sampling regime yielded consistent, reliable, and representative data for subsequent analysis. This section outlines the key elements that were optimised in the method, focusing on the selection of a sampling location within cooling towers and the determination of whether temperature or sample area caused variations in results.

4.3.1 Biofilm Sample Location

To investigate the potential of biofilm samples from cooling tower packs as leading indicators for *Legionella* presence and to aid in intervention efficacy assessment, developing a method for collecting consistent and representative biofilm samples from operational cooling towers was crucial. Many areas within operational cooling towers were

considered for biofilm sampling, including make-up water pipes, spray nozzles, pack, basin walls, dead legs and heat exchanger surfaces. These areas are all recognised for their potential to harbour and promote biofilm growth due to environmental conditions such as ample surface, area, optimal temperature range, nutrient availability and slow flow rate, all of which are known to enhance robust biofilm growth (Flemming, 2020). However, the cooling tower pack uniquely exhibited all of these factors, making it the most strategic choice for biofilm sampling, which was conducted exclusively at this location throughout the study. This decision is also complemented by existing literature that suggests that the cooling tower pack is a key location where *Legionella* aerosolisation can occur, with contaminated aerosols potentially escaping through the cooling tower fan, thereby contributing to outbreak occurrences (Pereira, A., Silva, A.R. & Melo, L.F., 2021). Furthermore, the accessibility of the pack areas compared to other locations within operational cooling towers was a practical advantage, facilitating a more reliable sampling technique.

Other areas that promote biofilm within operational cooling towers, presented significant inaccessibility and authorisation challenges, often requiring invasive procedures such as cutting pipes, shutting down the industrial process, or turning off the operational cooling tower. Most exchangers, at this site in particular, were located in heavily radiated zones, making sampling from these areas not only impractical but also unsafe for routine collection. These procedures would not only incur a massive expense and require additional staff allocation for the company but also would be impossible if the cooling is required for the industrial process. Such distributions would ultimately lead to a less reliable sampling regime and fewer results, further reinforcing the decision to sample exclusively from the cooling tower pack.

4.3.2 Biofilm Sample Optimisation

To ensure the collection of reliable and representative biofilm samples from operational cooling tower packs, the sampling method was optimised by determining the most suitable biofilm sampling location and surface area to use within the cooling tower pack. This optimisation was based on a crucial understanding of how biological variability between replicates could be influenced by specific environmental factors. Key among these factors is temperature gradients, which occurs from the top to the bottom of the pack, and surface area, as a larger surface area offers more ecological niches and gradients of nutrients, oxygen, and other essential factors, and naturally, a higher cell concentration. Therefore, a preliminary sampling regime was designed to systematically assess the impact of both temperature and surface area on biofilm cell concentration, viability and *L. pneumophila*, ensuring that the method captured consistent and representative samples.

To account for the measured temperature gradient between the top and bottom of the pack, biofilm samples were methodically collected from three distinct locations: the top, middle and bottom. The temperature gradient was determined by measuring the temperature (°C) at these locations (n=3) using a digital thermometer inserted 90 cm into the pack. For each measurement the thermometer was allowed to stabilise for 30 seconds before recording and the average temperature for each event was calculated. The deliberate selection of varying surface areas (100 cm^2, 200 cm^2, and 300 cm^2) at a single pack location served distinct purposes. The choice of a 100 cm^2 surface area aimed to capture localised variability in biofilm composition, allowing for a focused examination. Meanwhile, surfaces larger

than 300 cm² were considered impractical for handling within the pack. This rationale ensured a comprehensive yet manageable approach to establishing surface area variations during biofilm sampling, as illustrated in Figure 6 and a 3D printed squared with predefined dimensions was used to standardised and define the surface area for sampling.

Subsequent to their collection from two independent cooling towers (A1 & A2), the only towers authorised for sample collection at the time, the biofilm samples underwent detailed analysis for cell concentration and viability using flow cytometry, as detailed in Section 4.4.3. Furthermore, the efficacy of these samples for *L. pneumophila* detection was assessed, as described in Section 4.2.2. This comprehensive approach not only aimed to delineate the influence of key environmental factors on biofilm formation but also sought to refine the methodology for *Legionella* monitoring within cooling tower ecosystems.

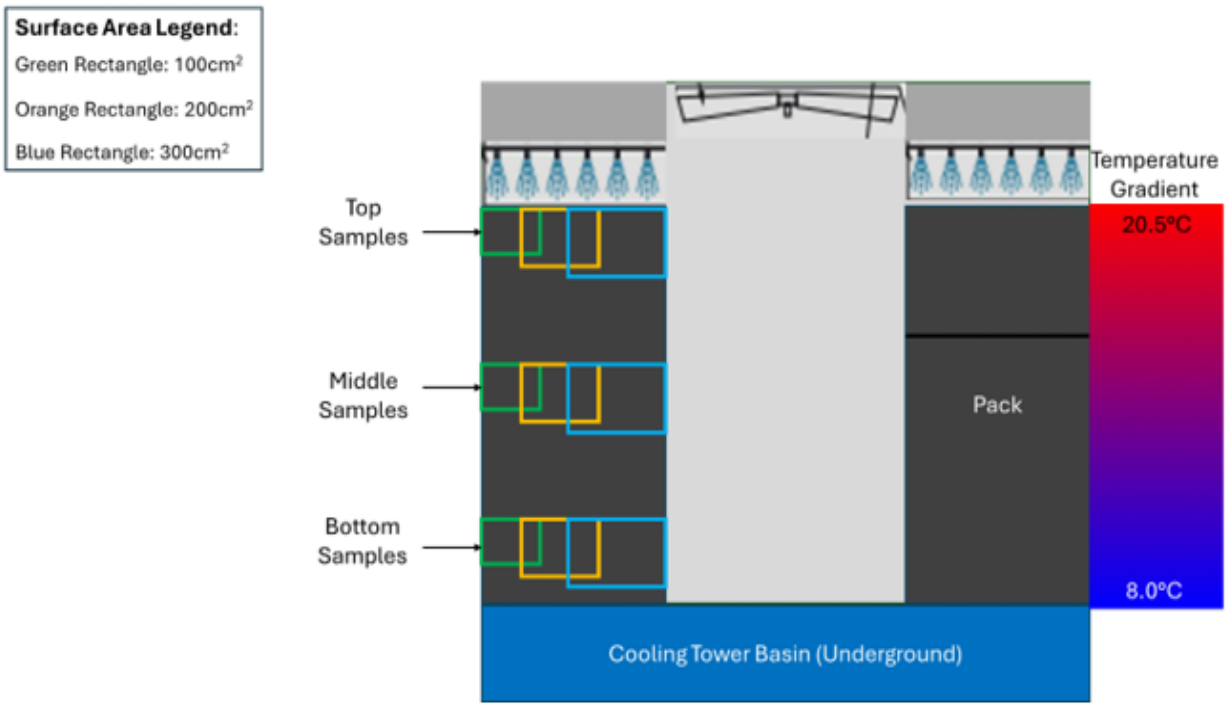

***Figure 6**: Diagram of the cooling tower pack showing top, middle, and bottom sampling locations with corresponding surface areas (100 cm², 200 cm², and 300 cm²) and the temperature gradient (20.5°C at the top to 8.0°C at the bottom).*

4.4 Site Details and Cooling Tower Selection

The sample site, a prominent nuclear site in the United Kingdom known for its nuclear waste processing, storage, and decommissioning activities, houses a diverse array of cooling towers. This site faces unique challenges as samples cannot be taken offsite until after a quarantine period. This prevents the standard CFU count *Legionella* culture method from providing accurate and reliable results that align with the ISO 11731 protocol. Therefore, an onsite lab setup is used, which employs qPCR for *Legionella* detection. This circumstance provided me with the opportunity to take advantage of the onsite lab to analyse samples as promptly as possible.

The selection of cooling towers for this study was strategically driven to cover a broad spectrum of designs and operational practices present at the site. Specifically, the goal was to analyse *L. pneumophila* prevalence across different cooling tower designs (induced draught crossflow or induced draught) and biocide treatment regimens

(oxidising biocide or a combination of oxidising and non-oxidising) within biofilm and bulk water samples. This approach ensures a comprehensive understanding of how varying designs and treatments influence microbial dynamics within these systems. Accessibility was also a factor, ensuring that sampling could be conducted efficiently, safely and reliably. After conducting preliminary visits to several cooling towers on the field site, four towers were chosen and paired based on their design and treatment regime, capturing the desired range of operational practices. These selected towers represent a cross-section of the cooling technologies and biocide strategies employed at site, offering insights into the environmental and operational conditions conducive to biofilm development and *Legionella* proliferation.

To use the novel and optimised biofilm sample technique in combination with flow cytometry and emerging detection methods for obtaining microbial concentration, viability, and *Legionella* detection, four cooling towers were selected for the 18-month period. These cooling towers were chosen based on their biocide regime, engineered structure, water quality information, and accessibility. Each cooling tower was paired with another based on its structural design and biocide regime as detailed in Table 3.

***Table 3**: Cooling Tower Information*

Cooling Tower	**Engineered Structure**	**Biocide Regime**	**Oxidising/Non-Oxidising Biocide**	**Water Quality Data**
A1	Induced Cross Flow	Sonoxide[1] and Sodium Hypochlorite	Oxidising	Hardness, Alkalinity, Chloride, Conductivity, Water Temperature, Pack Temperature
A2	Induced Cross Flow	Sodium Hypochlorite weekly then NX1102 + NX1422 weekly	Combination of Oxidising and non-oxidising	Hardness, Alkalinity, Chloride, Conductivity, Water Temperature, Pack Temperature
B1	Induced Draught Flow	Sodium Hypochlorite	Oxidising	Hardness, Alkalinity, Chloride, Conductivity, Water Temperature, Pack Temperature
B2	Induced Draught Flow	Sodium Hypochlorite + HCS555C	Combination of Oxidising and non-oxidising	Hardness, Alkalinity, Chloride, Conductivity, Water Temperature, Pack Temperature

[1] Sonoxide was a proprietary system that utilises a combination of ultrasound and ultraviolet light to treat the makeup water entering the cooling tower.

4.5 Longitudinal Field Study

4.5.1 Sampling Overview

The longitudinal field study at the field site was strategically designed with the objectives of rigorously evaluating biofilms as potential lead indicators for the presence of *L. pneumophila,* intricately understanding the dynamics within biofilm bacterial communities, and examining how these interactions with physicochemical conditions may either foster or hinder *Legionella* growth. A critical advantage of this longitudinal approach was its ability to capture seasonal variations and maximise opportunities to detect *L. pneumophila,* given its historical rarity at the sample site.

From February 2022 to October 2023, spanning thirteen distinct sampling periods, the campaign was structured to collect a higher number of biofilm samples (n=5) compared to bulk water samples (n=3) during each sampling visit from each accessible tower. This disparity in sampling frequency was deliberate and applied consistently across all sampling periods, as biofilms represent a relatively unexplored area with significant uncertainties regarding their role in the *Legionella* lifecycle and detection capabilities. This approach was guided by the hypothesis that biofilms might offer new insights and be a more sensitive indicator. By collecting more biofilm samples within each tower during each sampling visit, this study had a better opportunity to enhance our understanding of biofilms as both a habitat and indicator for *Legionella*. In contrast, the collection of bulk water samples, though fewer, adhered to a more traditional approach to *Legionella* monitoring, serving as a comparative baseline to the biofilm data.

Both biofilm and bulk water samples were aliquoted for different types of analysis. Five biofilm samples and three bulk water samples per sampling period were analysed using flow cytometry to determine total and intact cell counts. These same five biofilm samples and three bulk water samples were also used for 16S rRNA gene amplicon sequencing to analyse bacterial community composition, with DNA extractions sent to the molecular lab at Sheffield University and stored at -20°C for subsequent analysis. For molecular analysis on-site, a subset of three biofilm samples and three bulk water was further analysed using qPCR platforms: Genesig for *Legionella* species and Genomadix for *L. pneumophila.* This limitation to three biofilm samples for qPCR was due to practical constraints in processing capacity, limited resources and fieldwork time. Standard cooling tower water quality data, including physical, chemical, and microbiological parameters, were also collected throughout the sampling period, as detailed in Table 3.

4.5.2 Biofilm Sampling

Biofilm samples were meticulously collected from the pack of each cooling tower using sterile cotton swabs. This method was specifically chosen to align with established practiced for biofilm collection in other engineered water systems, enabling comparability across studies. After sampling, the swabs were transferred into falcon tubes for transport. The selection of specific sample areas and locations was informed by the findings detailed in Section 4.3, aiming to ensure that the results were both representative of and repeatable within the unique environment of an operational cooling tower.

Upon arrival at the on-site laboratory, each sample tube was filled with sterile water (50ml) and subjected to vigorous vertexing at 3000rpm for thirty seconds. To achieve a thoroughly homogenised biofilm suspension, the tubes were

then rotated 180 degrees and vortexed again for an additional thirty seconds. This methodical preparation process was critical for creating biofilm suspensions that were homogeneously mixed and ready for subsequent analyses, ensuring consistency across the samples collected.

4.5.3 Bulk Water Sampling

Bulk water samples were obtained from each cooling tower following the ISO 11731 protocol (International standard for standardisation, 2017). In brief, water samples were collected using one-litre sterile polypropylene bottles containing 0.01% sodium thiosulfate, either from the cooling tower's basin or from a sampling port when the basin was inaccessible. These bulk water samples were returned to the laboratory, vortexed and either aliquoted for Genomadix or concentrated through a filter (refer to Section 4.6.3) and subsequently subjected to DNA extraction.

4.6 Field Work Sample Analysis

In this phase of the study, emerging detection methods was utilised for the detection of *Legionella* spp. and *L. pneumophila* for field work sample analysis. These methods were selected based on their demonstrated potential, reliability, and throughput, as detailed in Section 4.2. The application of each selected method to analyse the field samples is outlined below, providing a clear link between preliminary assessments and their practical application in the field.

4.6.1 Emerging Detection Methods for *Legionella* and *L. pneumophila*

4.6.1.1 Genomadix qPCR

To analyse the presence and quantify *L. pneumophila* sg1, the Genomadix qPCR (Genomadix Bioscience, Ottawa, Canada, version 1.0.3) system was employed. This system includes a portable DNA analyser, the Genomadix Cube, along with a single-use disposable concentration kit and test cartridge. Briefly, 20 ml of either biofilm suspension or bulk water samples was introduced into the concentration kit, where it was filtered through a 0.45 µm polyethersulfone (PES) Millex-HP filter (Merck Millipore Ltd, Cork, Ireland). The filter was then washed to remove unwanted contaminants, and the captured intact bacteria were eluted from the filter by gentle homogenisation with all solutions provided by the kit. Finally, the eluate was transferred to a test cartridge, which contains qPCR primers, a probe, and an internal positive control, and was placed into the Genomadix Cube. These primers and probes are designed against a highly conserved region of the *L. pneumophila* (mip) gene.

4.6.1.2 Genesig qPCR

To accurately detect and quantify *Legionella* spp., the study employed the Genesig qPCR platform (Primer Design Ltd, Southampton, UK) following their standardised protocol for *Legionella* spp. detection. In preparation, a reaction mix (15 µl) comprising qPCR master mix, *Legionella* species-specific primer/probe mix, internal extraction control primer/probe mix, and RNase/DNase-free water was prepared. This mix was added to DNA samples, including designated positive and negative controls. An additional endogenous control reaction mix (15 µl) was also added to each sample to ensure comprehensive analysis. The prepared samples were then ready for analysis using the Genesig PCR platform. Prior to the qPCR procedure, DNA extraction from both biofilm and bulk water samples was necessary to obtain high-quality genetic material for analysis. This crucial step was performed using the DNeasy PowerWater Kit

from Qiagen, as detailed in Section 4.6.3. Post-extraction, to mitigate potential inhibition from substances present within the samples, each isolated DNA sample underwent a tenfold dilution, ensuring the integrity and reliability of subsequent Genesig analyses.

4.6.2 Flow Cytometry for Microbial Concentration and Viability

To obtain total cell counts (TCC) and intact cell counts (ICC), the staining method outlined by (Fish, et al., 2020) was used for both biofilm and bulk water samples. Briefly, 500 µL sample aliquots were stained with either 5 µL of a 100x SYBR Green solution (Life Sciences, California, USA) for total cell counts or 6 µL of Propidium Iodide mixture (Life Sciences, California, USA) for intact cell counts. After vortexing, the dyed samples were incubated at 37°C for 10 minutes, shielded from light and then analysed using a BD Accuri C6 flow cytometer (BD, Accuri, UK). Data points were captured by the BD Accuri C6 flow software (version 1.0.264.21).

To prepare each dye, SYBR Green 10,000x stock was diluted with dimethyl sulphoxide (DMSO; Fisher Scientific, Fair Lawn, NJ) to a working stock concentration of 100x. To create a Propidium Iodide (PI) mixture, five parts 100x SYBR green and one part of Propidium Iodide was mixed together for a final concentration of 3mM. Negative controls included triplicates of 50ul of sterile water, sterile water stained with SYBR green and sterile water stained with PI.

4.6.3 Sample Concentration & DNA Extraction

To concentrate both biofilm and bulk water samples for more effective molecular analysis and as a preliminary step towards DNA Extraction, 30 mL of biofilm samples and 1000 mL of bulk water samples were passed through a 0.4 µm Polycarbonate Track-Etched filter (Sartorius, Göttingen, Germany, Type 23006) using a vacuum filtration system.

DNA was extracted using the Qiagen PowerWater Kit (QIAGEN GmbH, Hilden, Germany) to comply with site regulations, following the manufacturer's protocol. In this extraction procedure, the filter membranes were carefully placed into 5ml bead-beating tubes containing a specialised bead mix. Following this, a lysis buffer was added to initiate the breakdown of cell walls and release the genomic DNA. Subsequently, a crucial step involving protein and inhibitor removal was meticulously executed to purify the DNA extract. The elution process was then performed using a DNA elution buffer, resulting in the collection of high-quality DNA suitable for a spectrum of downstream molecular applications.

4.7 Bacterial Community Analysis

To elucidate the bacterial composition and structure within biofilms and their interactions that may influence *Legionella* growth, Illumina sequencing was utilised. A critical component of this analysis is the comparison between the microbial compositions of biofilms and bulk water samples. This comparison is designed to reveal both unique and shared microbial characteristics that impact *Legionella*'s ecological niche. Moreover, the investigation extends to understanding how different physicochemical parameters affect biofilms and bulk water, pinpointing environmental conditions that may either promote or hinder *Legionella* proliferation. Adopting such a comparative approach enhances the comprehension of *Legionella*'s survival strategies and identifies conditions that either facilitate or

restrict its growth within engineered water systems. This detailed examination is instrumental in identifying key factors for mitigating *Legionella* risks.

4.7.1 PCR Amplification & Gel Electrophoresis

PCR amplification was undertaken to amplify DNA from biofilm and bulk water samples, setting the stage for thorough genetic analysis. The bacterial 16S rRNA gene was targeted using the forward primer 63F (5′-CAGGCCTAACACATGCAAGTC-3′) and the reverse primer 518R (5′-CGTATTACCGCGGCTGCTCG-3′), incorporating Illumina overhangs as specified by Girvan et al. (2003). Optimised PCR cycle conditions were applied to ensure effective amplification for environmental samples from cooling towers. The reaction mixture included 25 µL of Sigma ReadyMix Taq solution (Sigma-Aldrich, UK), 1 µL of each primer, and 1 µL of DNA template, completed to 50 µL with nuclease-free water. PCR conditions were set to an initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 45 seconds, concluding with a final elongation at 72°C for 10 minutes.

For the verification of the amplified DNA products, a 1% agarose gel was prepared by dissolving 1.8 g of agarose in 180 mL of 1x Tris-acetate-EDTA buffer and adding 9 µL of ethidium bromide. A mixture of 4 µL of the PCR product with 2 µL of 6x SYBR orange was prepared, from which 5 µL was loaded into the gel alongside 5 µL of DNA hyper ladder (Bioline, London, UK), spanning 200-10,037 base pairs. Electrophoresis was conducted at 100V for 50 minutes, with results visualised under UV light using a G: BOX gel imaging system and GeneSnap v6.07 software (Syngene, Cambridge, UK).

4.7.2 Illumina Sequencing Preparation

16S rRNA gene-targeted amplicon sequencing preparation was conducted at the NERC Environmental Omics Facility laboratory within The University of Sheffield, and sequencing was carried out on the Illumina MiSeq platform. Following the amplification of 16S rRNA from environmental samples, PCR products were purified using Promega Pronex beads (Promega, Madison, WI, USA), as per the manufacturer's protocol, to eliminate excess primers and dNTPs. The second multiplex PCR was carried out comprising unique identifier sequences (dual-plexed: Fi5 and Ri7 primers in a unique combination for each sample) and incorporated into each sample. This included 8 µL of amplified DNA, 10 µL of Qiagen multiplex PCR master mix, 1 µL of each Fi5 and Ri7 primer (10 µM), and 1 µL of double-distilled water. The PCR program involved an initial denaturation at 95°C for 15 minutes, followed by 12 cycles of denaturation at 98°C for 10 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension step at 72°C for 5 minutes.

To verify the successful addition of identifier sequences, pre- and post-PCR samples were analysed on a 4200-tape station (Agilent Technologies, Santa Clara, California) to confirm an increase in PCR fragment size. Subsequently, DNA amplicons underwent purification and quantification using the Quantifluor dsDNA system kit (Promega) before being pooled together to a concentration of 150ng. These samples were then diluted to a final concentration of 4nM of DNA before submission for Illumina sequencing at CGR University of Liverpool.

4.8 Data Analysis

To identify and visualise patterns, relationships, and trends that elucidate the dynamics of *Legionella* survival and growth, raw data were compiled into a Microsoft Excel spreadsheet and subsequently imported into R for in-depth analysis. This process aims to provide a comprehensive understanding of the environmental factors influencing *Legionella* presence, offering insights into effective management and mitigation strategies within engineered water systems.

4.8.1 Total Cell Count, Intact Cell Count and Intact to Total Cell Count Percentage

To ensure the accuracy of cell count measurements and to account for any potential background interference contributed by the SYBR Green and PI dyes themselves, a corrective process was implemented. First, the mean cell count obtained from sterile water controls, which served to measure any baseline signal from the dyes, was subtracted from the cell counts recorded during the control runs using SYBR Green and PI. These adjusted control results were then averaged separately for each dye. The final step involved deducting these averaged control counts from the total and intact cell counts of the environmental samples that were stained with SYBR Green and PI, respectively. This procedure ensures that the reported cell counts for environmental samples accurately reflect the cellular content, free from artefacts introduced by the staining dyes.

To account for variations in biofilm surface area water sampled, the adjusted biofilm sample cell counts were normalized using the following equation: TCC or ICC = ((Cell Count)/ (Volume Analysed)) × Total Volume of Sample ÷ Surface Area.

Where the count is the total or intact cell count, volume analysed is the volume of sample that was processed in the flow cytometer (50ul), the total volume in this case was 30ml (30000ul) and surface area was the area which was sample on the pack of a cooling tower. The bulk water sample cell counts were expressed ml-1 by taking the planktonic cell count or intact cell count and multiplying it by 1000. Additionally, to determine the intact-to-total cell count percentage, intact cell counts were divided by the total cell count of the same sample and multiplied by 100.

4.8.2 Emerging Detection Methods for *Legionella* spp. and *L. pneumophila*

To understand the occurrence of *Legionella* species and *L. pneumophila* within each cooling tower across the longitudinal study (two years), results obtained from the Genesig software (Version 2.0.5), which outputs copy numbers, and Genomadix Cube *Legionella* (Version 2.3.2) software, which provides equivalent CFUs, were initially organised in a Microsoft Excel Spreadsheet (Microsoft Office, 2023). To allow for standardised measurements, facilitating accurate comparison across samples and studies, copy numbers obtained from the Genesig software were then converted to copy number per litre. This conversion was followed by a multiplication by 10 to account for the tenfold dilution, as outlined in Section 4.5.3. Meanwhile, the CFUs obtained from Genomadix Cube could be used directly for analysis.

4.8.3 Bioinformatics

To provide a comprehensive overview of the bacterial populations present within biofilms and bulk water, and to elucidate how these communities are shaped by physicochemical parameters, the study utilises bacterial community

profiling and ecological indices. These methodologies are vital for interpreting the intricate data obtained from Illumina sequencing. They not only highlight the bacteria present but also the richness and evenness of species within individual samples and demonstrate how these communities differ across biofilm and bulk water environments under varying physicochemical conditions.

4.8.3.1 Bacterial Community Profiling

The bacterial community profiling sequencing data were processed using the Qiime 2 pipeline (version 2023.9). The demultiplexed forward and reverse fastq files for each sample were imported into Qiime 2 using a manifest file, with the PCR primer sequences trimmed utilising the cutadapt tool (Martin, 2011). Subsequently, these sequences were denoised and grouped into amplicon sequence variants (ASVs) through DADA2. To ensure high read quality for both forward and reverse reads and to eliminate chimeras, the reads were truncated at a quality score of 30. The resulting ASVs were then classified against a trained classifier using the Silva 138 99% OTU full-length sequences database, providing the bacterial taxonomic information in each sample.

To eliminate unknown taxa classified by the Silva database, sequences were imported into the Basic Local Alignment Search Tool (BLAST). Taxa were identified based on stringent criteria: a query coverage of 98–100%, an expectation value (E-value) of less than 0.01, and a percent identity greater than 90%.

4.8.3.2 Ecological Indices

To facilitate the computation of alpha and beta-diversity metrics, a rooted phylogenetic tree based on the ASV representative sequence, as described above, was constructed using multiple alignment programs for amino acid or nucleotide sequences (MAFFT). Additionally, an unrooted phylogenetic tree was constructed using the Fasttree 2 tool. Non-informative regions were masked during this process. Alpha diversity within each sample was assessed using the Observed, Pielous Evenness metrics. To observe beta diversity, unweighted Bray-Curtis's metrics (Bray and Curtis, 1957) were employed. Statistical analysis aimed to identify significant differences in alpha diversity concerning categorical or numerical metadata, utilising the Kruskal-Wallis's test and Ranked Spearman (correlation) test, respectively. For assessing significant differences in beta diversity, PERMANOVA analysis was employed. Lastly, to identify differentially abundant taxa between sample groups, the ANCOM-BC method was applied (Mandal et al., 2015).

4.8.4 Visualisation of Data, Statistical Analysis and Community Analysis

To visualise data, a variety of plots including scatter plots, bar charts, boxplots and dendrograms were generated using R Studio (version 2023.09.1+494) with the ggplot2 package (v3.4.4; Wickham, 2018). All statistical analyses were conducted on the base R Studio platform (version 2023.09.1+494). For R community analysis, Qiime 2 artifacts were imported into R as a phyloseq object using the Qiime2R package (version 0.99.6; Bisanz, 2018).

A variety of non-parametric statistical methods were employed, as the data were not normalised, to analyse differences and relationships based on the characteristics of the dataset. The Kruskal-Wallis test was used to assess significant differences across multiple groups, such as between cooling towers or seasons, providing an initial overview of group variability. For paired comparisons, such as differences between sample types (biofilm and bulk

water) or before and after cleaning events, the Wilcoxon rank-sum test was applied, offering a robust method for non-parametric pairwise analysis. To further explore relationships within the data, the Spearman rank correlation coefficient was calculated to assess associations between variables, such as correlations between sample types or between cell concentration and *Legionella* spp. concentration. For microbial community structure, Permutational Multivariate Analysis of Variance (PERMANOVA) was employed to evaluate differences across sample groups, leveraging its suitability for high-dimensional ecological data.

When significant results were obtained from the Kruskal-Wallis test, post-hoc analyses were conducted to identify specific pairwise differences. For smaller datasets or comparisons, a post-hoc Wilcoxon rank-sum test with Bonferroni correction was used to control for Type I error, ensuring robust conclusions. In cases with multiple comparisons, a post-hoc Dunn test was applied as an alternative approach, providing flexibility in adjusting for multiple comparisons while balancing the risk of Type I and Type II errors. These combined statistical approaches allowed for a comprehensive and nuanced analysis of the dataset, tailored to the specific questions and characteristics of the data.

Chapter 5: Results

5.1 Method Validation

5.1.1 Flow Cytometry – Microbial Concentration and Viability Detection

To validate the flow cytometry protocol outlined in Methodology Section 4.2.1, the technique was employed on lab-grown biofilm samples to measure total and intact cell counts, as well as the singlet-doublet percentage, as depicted in **Figure 7.** All samples were found to be homogenised, with a singlet-doublet percentage exceeding the threshold level of 90% (Pick and Fish, 2024) for both total and intact cell counts. Furthermore, the intact cell counts were lower than the total cell counts, aligning with expectations due to the inherent composition of biofilms, which typically include a mix of viable and non-viable cells. These findings indicate that an acceptable number of events were recorded as individual cells and flow cytometry was suitable for use with biofilm samples, providing a representative and reliable quantification of cell concentration and viability, thereby giving confidence to apply these methods in the field.

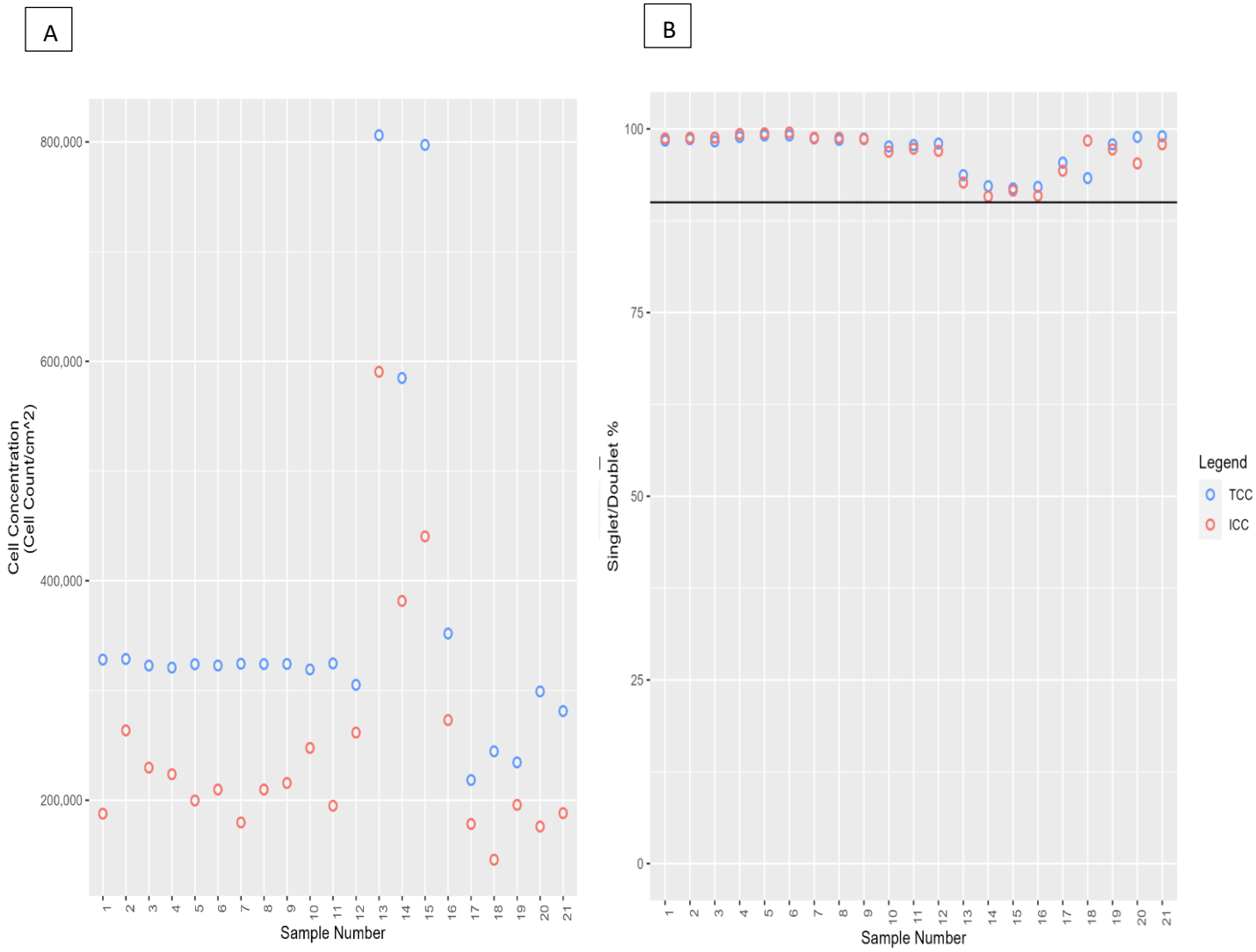

***Figure 7**: Biofilm total cell count (TCC), intact cell count (ICC) and sample homogenisation (singlet/doublet %). Panel A shows the cell concentration and viability, and panel B shows sample homogenisation.*

5.1.2 *Legionella* Detection via Emerging Methods

Table 4 presents a comparative analysis of various emerging methods for detecting *Legionella* in an initial screening, focusing on sample throughput and the percentage of *Legionella* positives identified using a *L. pneumophila* serogroup type 1 Lenticule discs, as detailed in Methodology Section 4.2.2. In this study, total throughput is defined as the number of tests that can be completed within a constrained sampling period (2400 minutes per sample period). Nephros exhibited the highest throughput among the methods evaluated; however, it achieved a relatively low detection success rate of only 6.25% for *Legionella* positives. In contrast, Genomadix despite its lower throughput, achieved a *Legionella* positive detection success rate exceeding 80%. Both Hydrosense and Legiped were unable to detect *Legionella* positives throughout the course of this experiment. Due to its deployment at the field site during the experimental period, Genesig could not be evaluated in lab tests but was still applied as it represents a current industry method.

***Table 4**: Comparison of Legionella Detection Test Results*

Emerging Detection Test	Sample Time (Mins)	Number of Tests	Total Throughput (Tests per session)	Percentage of *Legionella* Positives (%)
Nephros	240	16	160	6.35
Genomadix	75	1	32	83.34
Hydrosense	25	3	288	0
Legiped	75	6	192	0
Genesig	N/A[2]	N/A[1]	N/A[1]	N/A[1]

Given its performance, Genomadix was selected for a subsequent sensitivity analysis involving three known concentrations of *Legionella*, as detailed in Section 4.2.2 and illustrated in Figure 8. The results show that Genomadix consistently detects *Legionella* at various CFU concentrations across multiple replicates (n=10). However, it is crucial to acknowledge that the estimated equivalent CFUs observed deviate significantly from the anticipated concentrations. This deviation is likely attributable to the challenges and limitations in converting genomic units to equivalent CFUs, as described in Section 2.3. Consequently, in the longitudinal study, Genomadix will be utilised for binary detection (presence/absence) and trend analysis, rather than for absolute quantification.

[2] Genesig data are not available as it was already deployed at the field site during the experimental period and could not be evaluated in lab tests.

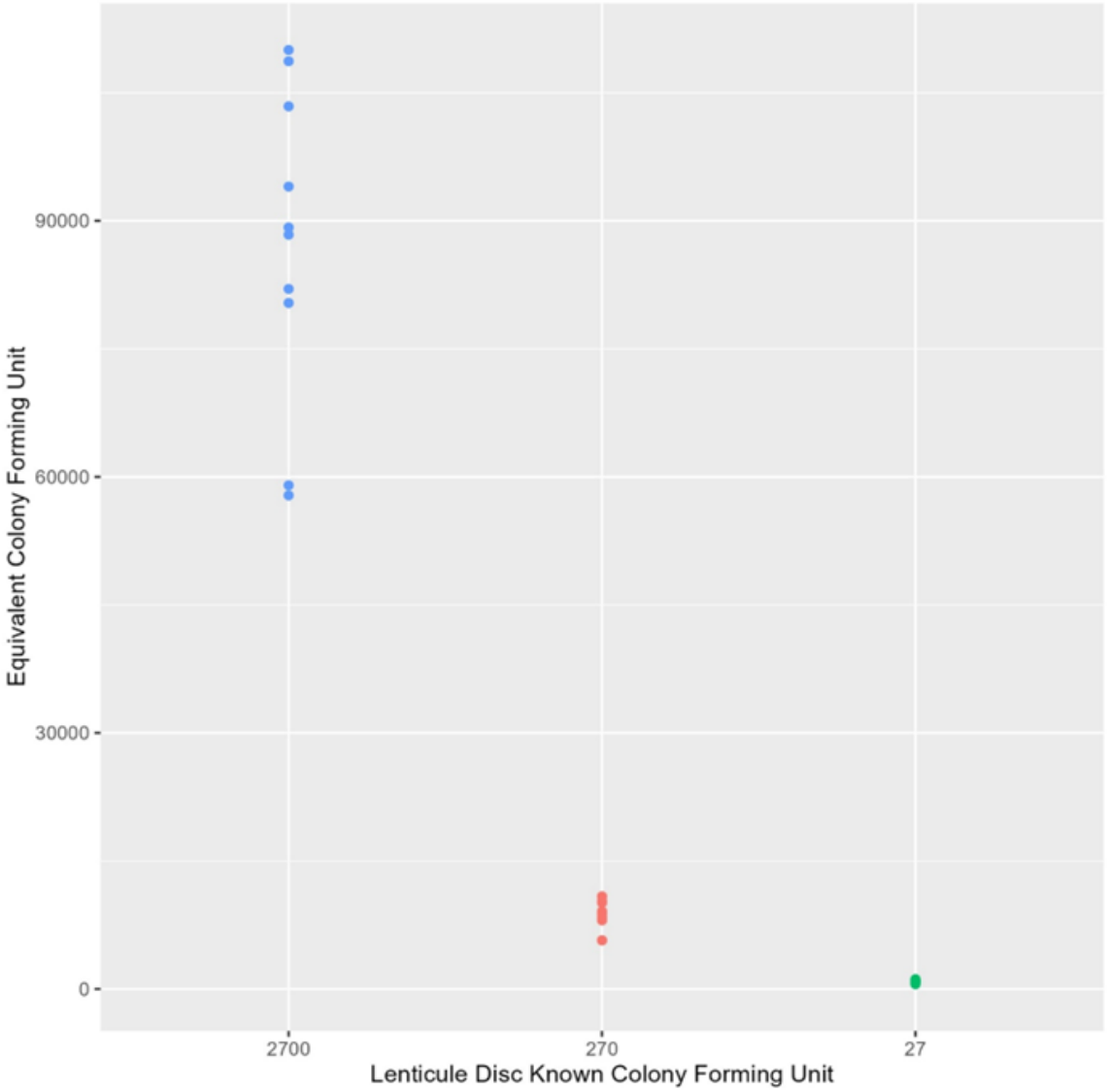

***Figure 8**: Sensitivity analysis of Genomadix for Legionella detection across varying CFU concentrations.*

5.1.3 Novel and Optimised Biofilm Sample Technique

Figure 9 presents biofilm total and intact cell counts at three different locations within the pack for cooling towers A1 and A2. These locations were chosen to represent different temperature zones, as the top of the pack is typically hotter than the middle and bottom due to its proximity to the spray nozzles. For cooling tower A1, the top of the pack had an average temperature of 20.5 °C ± 0.30 °C, the middle of the pack had an average of 16.5 °C ± 0.10 °C and the bottom had an average of 8 °C ± 0.20 °C. Similarly in cooling tower A2, the top of pack had an average temperature of 15 °C ± 0.20 °C, the middle was 13.2 °C ± 0.10 °C and the bottom was 7.5 °C ± 0.10 °C.

It was observed that both biofilm total and intact cell counts remain consistent across the temperature range, despite a higher range noted at the top of the pack. This consistency indicates that variations in temperature within the studied ranges do not significantly affect cell concentration and viability. Confirming this observation, the Kruskal-Wallis test shows no significant differences in cell counts across the temperatures for both towers (A1 TCC, $\chi^2 = 4.5$, p = > 0.05; A1 ICC, $\chi^2 = 2.38$, p = > 0.05; A2 TCC, $\chi^2 = 1.64$, p = > 0.05; A2 ICC, $\chi^2 = 3.3$, p = >0.05). Consequently, these findings led to the optimisation of biofilm sampling by supporting the selection of the most accessible location (The middle of the pack) since temperature fluctuations within these ranges are not a critical factor in biofilm metrics, ultimately allowing for a higher biofilm sample size, which was consistent and reliable throughout the study period enhancing the study robustness.

Figure 10 presents biofilm total and intact cell counts across different surface areas, ranging from 100 to 300 cm², in cooling towers A1 and A2. The analysis shows no significant change in either total or intact cell counts between sampling areas, implying that surface area variations do not markedly affect cell concentration and viability. The Kruskal-Wallis test confirms this, showing no significant differences (A1 TCC, $\chi^2 = 4.62$, p = > 0.05; A1 ICC, $\chi^2 = 5.6$, p = > 0.05; A2 TCC, $\chi^2 = 3.82$, p = > 0.05; A2 ICC, $\chi^2 = 5.6$, p = > 0.05). This justification supports the use of a standardised 100cm² quadrat for biofilm sampling throughout the longitudinal study.

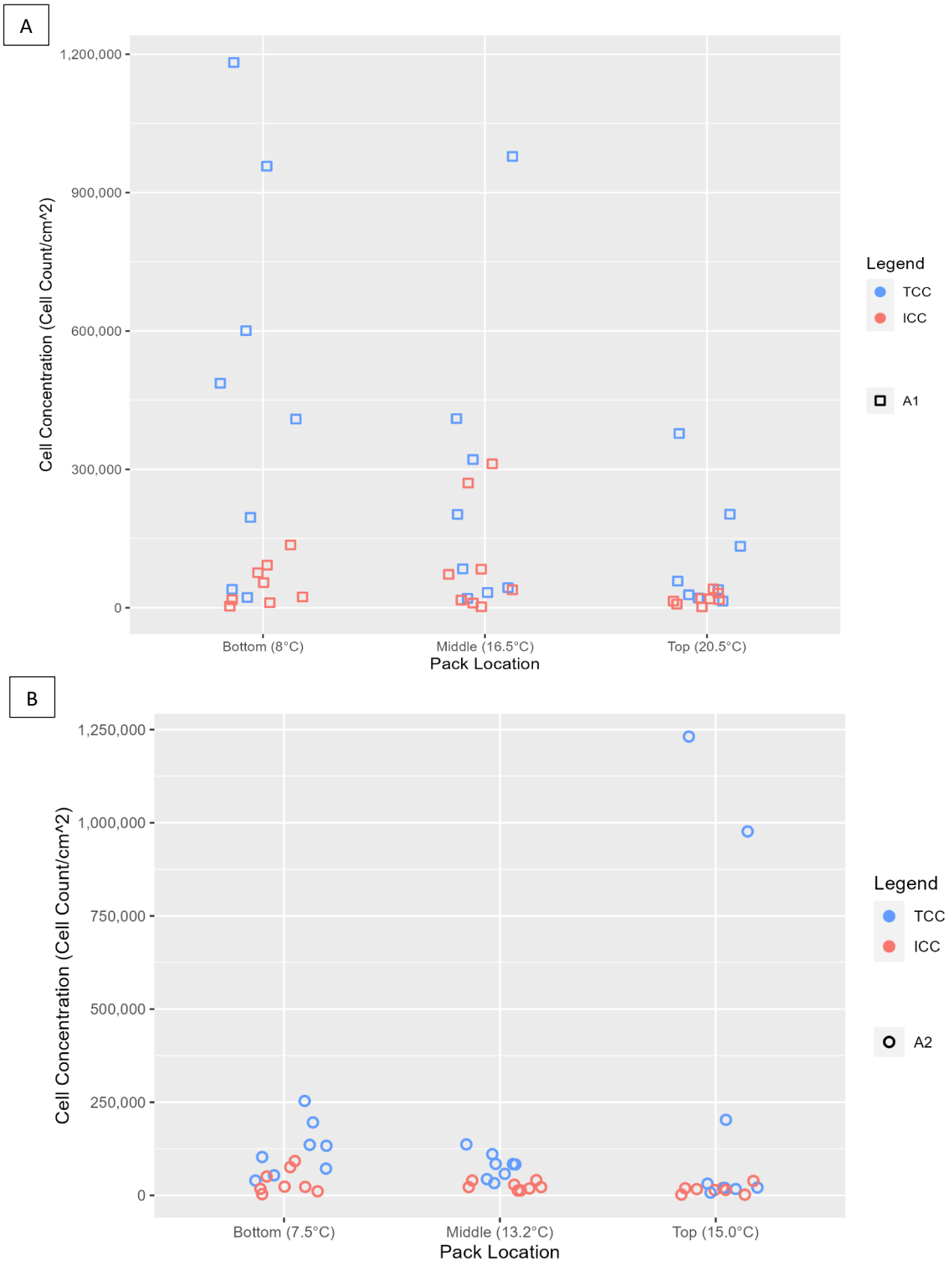

Figure 9: *Impact of temperature variations (represented by pack location) on biofilm total (TCC) and intact cell counts (ICC) at different pack locations (Bottom, Middle, Top) in cooling towers A1 and A2. Temperatures in parentheses represent the average values measured on-site over two months (N=3). Panel A represents cooling tower A1, and Panel B represents cooling tower A2.*

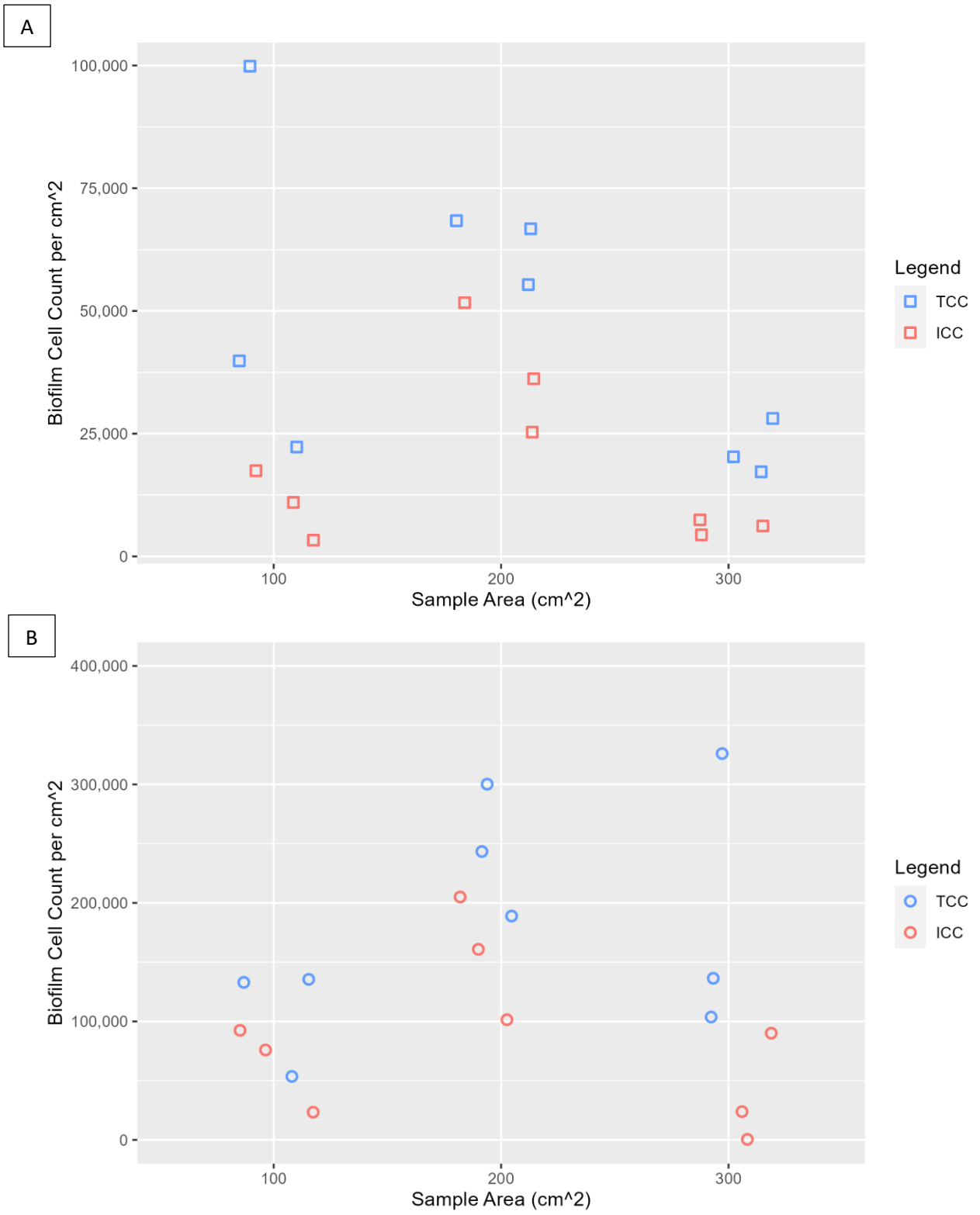

***Figure 10**: Impact of surface area variations on biofilm total and intact cell count. Panel A represent cooling tower A1 and panel B represent cooling tower A2.*

5.2 Longitudinal Study

5.2.1 *L. pneumophila* Detection and Quantification

The comparison of *L. pneumophila* detection across biofilm and bulk water samples from cooling towers A1 and A2 is illustrated in **Figure 11**, using the Genomadix platform that expressed results as equivalent colony forming units (CFU). Notably, *L. pneumophila* was exclusively detected in the biofilm sample of cooling tower A2, with no detection in the bulk water during the sample period. A progressive escalation in equivalent CFU was observed throughout 2023, with increases by a factor of approximately 2.59 from April to July, then by a factor of approximately 2.23 from July to September, and finally by a factor of approximately 1.88 from September to October, reaching its highest point. Importantly, cleans conducted in June and August were followed by a period where no *L. pneumophila* was detected in either cooling tower.

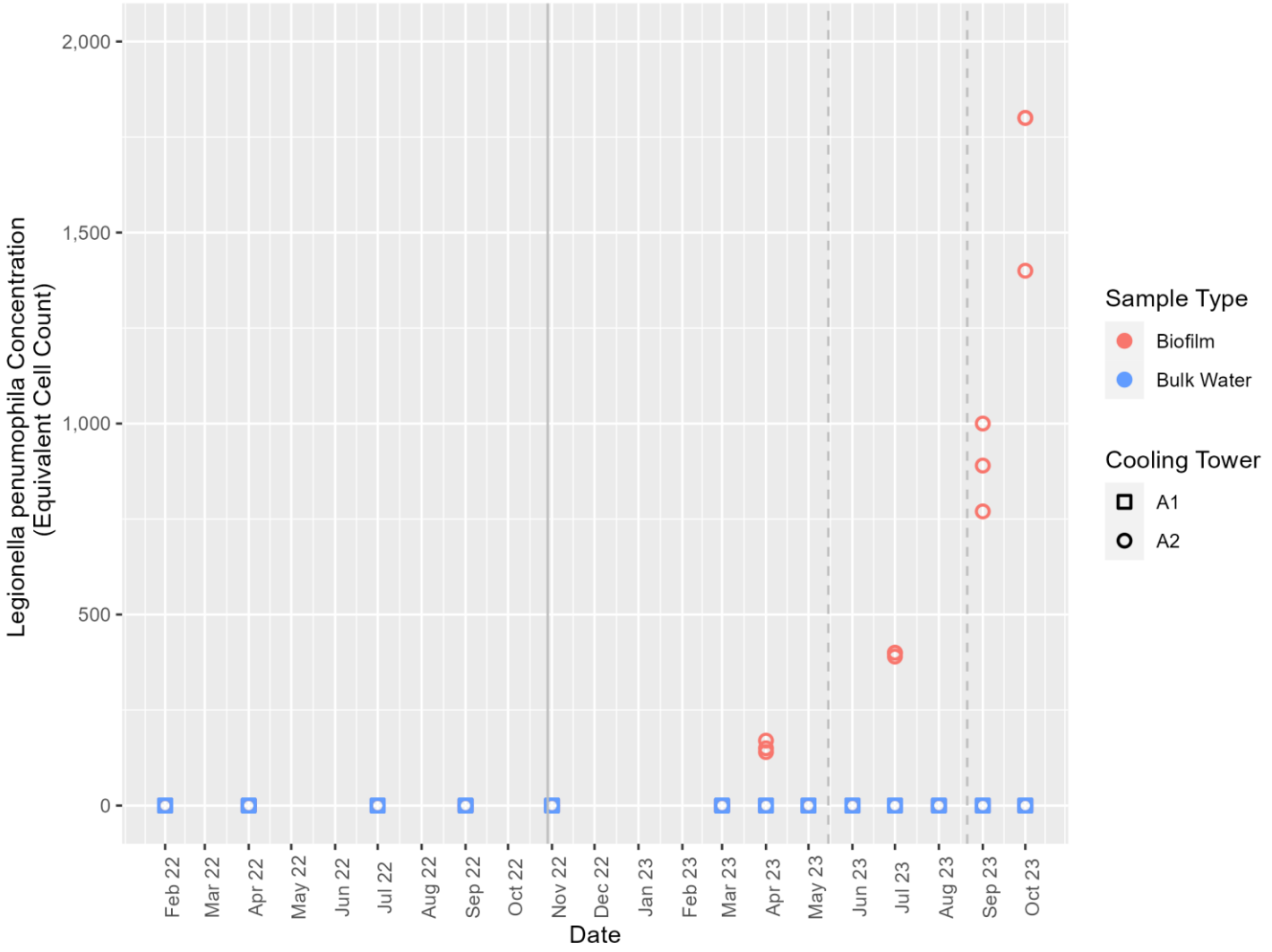

***Figure 11**: Comparative analysis of L. pneumophila presence in biofilm (n=3 for each date, totalling 39 samples) and bulk water samples (n=3 for each date, totalling 39 samples) from cooling towers A1 and A2 across 2022 and 2023, quantified as equivalent colony forming units (CFU) using the Genomadix platform. The solid line indicates changes associated with the implementation of a new pack in cooling tower A2, while the dotted line represents periods following the cleaning of cooling tower A2.*

5.2.2 *Legionella* Genus Detection and Quantification

This section examines *Legionella* spp. concentrations in biofilm and bulk water samples collected from cooling towers A1 and A2 and analysed using the Genesig platform. Focusing on the relationships between these sample types, the influence of biocide regimes, and the effects of seasonal variation and cleaning regimens. Figure 12 illustrates *Legionella* concentrations over the sampling period, with cleans and operational changes marked by vertical dotted and solid black lines, respectively.

5.2.2.1 Comparison of *Legionella* spp. Detection and Quantification across Sample Types and Influence of Biocide regimes

A significant observation in this study is the consistent higher *Legionella* spp. concentrations in biofilm samples compared to bulk water across both operational cooling towers (Wilcoxon Rank-Sum Test U test: A1: $W=392$, $p < 0.05$; A2: $W=549$, $p < 0.05$). Despite this, no discernible correlation was observed between biofilm and bulk water concentrations (Spearman Correlation coefficient, A1: $\rho=0.029$, $p > 0.05$; A2: $\rho=0.375$, $p > 0.05$). Scatter plot accompanied by linear regression (Appendix), reinforces the lack of correlation with low coefficients of determination ($R2 < 0.05$). Comparison of the two cooling towers revealed no significant differences in *Legionella* spp. concentrations in either biofilm or bulk water samples, despite differences in biocide regimes (Wilcoxon Rank-Sum Test U test: biofilm, $W = 356$, $p > 0.05$; bulk water, $W = 314$, $p > 0.05$)

5.2.2.2 Temporal and Cleaning Effects on *Legionella* spp. Detection and Quantification

Fluctuations in Legionella spp. concentrations were evident, with both cooling towers exhibiting sustained periods of elevated biofilm concentrations in March and July 2023. Cooling tower A1 also displayed elevated levels in April and May 2023. As shown in Figure 12, these periods of elevated concentrations included individual samples with lower values, highlighting variability even within months generally characterized by higher concentrations.

Kruskal-Wallis tests confirmed significant monthly differences for both sample types across the study period (A1 biofilm: $\chi^2 = 25.684$, $p < 0.05$; A2 biofilm: $\chi^2 = 42.089$, $p < 0.05$; A1 bulk water: $\chi^2 = 26.235$, $p < 0.05$; A2 bulk water: $\chi^2 = 117.31$, $p < 0.05$). Post-hoc Wilcoxon rank-sum tests with Bonferroni correction identified specific monthly differences while controlling for multiple comparisons. These tests indicated that Legionella concentrations in cooling tower A1 remained relatively stable over time, despite variability in individual samples, as no significant differences were detected between months. In contrast, cooling tower A2 demonstrated greater variability, particularly in May 2023, where concentrations significantly decreased compared to adjacent months. However, this variability fell within the broader range of fluctuations observed during other periods, such as between March and April 2023, suggesting that the changes in May were not uniquely large but rather part of ongoing concentration dynamic

Finally, the impact of cleaning regimens was assessed, focusing on cooling tower A2 due to the lack of comparable cleaning events in A1. The May–June 2023 cleaning event led to a significant increase in *Legionella* spp. concentrations in biofilm samples (Wilcoxon rank-sum test, $W=245$, $p < 0.05$), with concentrations rising from an average of 43,863 copies/litre in May to 684,670 copies/litre in June, representing a 1,460% increase.

Similarly, bulk water concentrations increased significantly during this period ($W=0$, $p < 0.05$), rising from an average of 1,200 copies/litre in May to 46,727 copies/litre in June, a 3,794% increase. In contrast to the substantial increase observed during May-June, the August–September cleaning event was associated with a significant 98.49% reduction in bulk water concentrations ($W=441$, $p < 0.05$), but no significant change in biofilm concentrations ($W=245$, $p > 0.05$).

In summary, biofilm samples consistently showed higher *Legionella* spp. concentrations than bulk water, with no significant correlation between the two. While cooling tower design and biocide regimes had limited impact, temporal variations and cleaning regimens played a critical role, particularly in cooling tower A2, where cleaning events had contrasting effects on *Legionella* spp. dynamics.

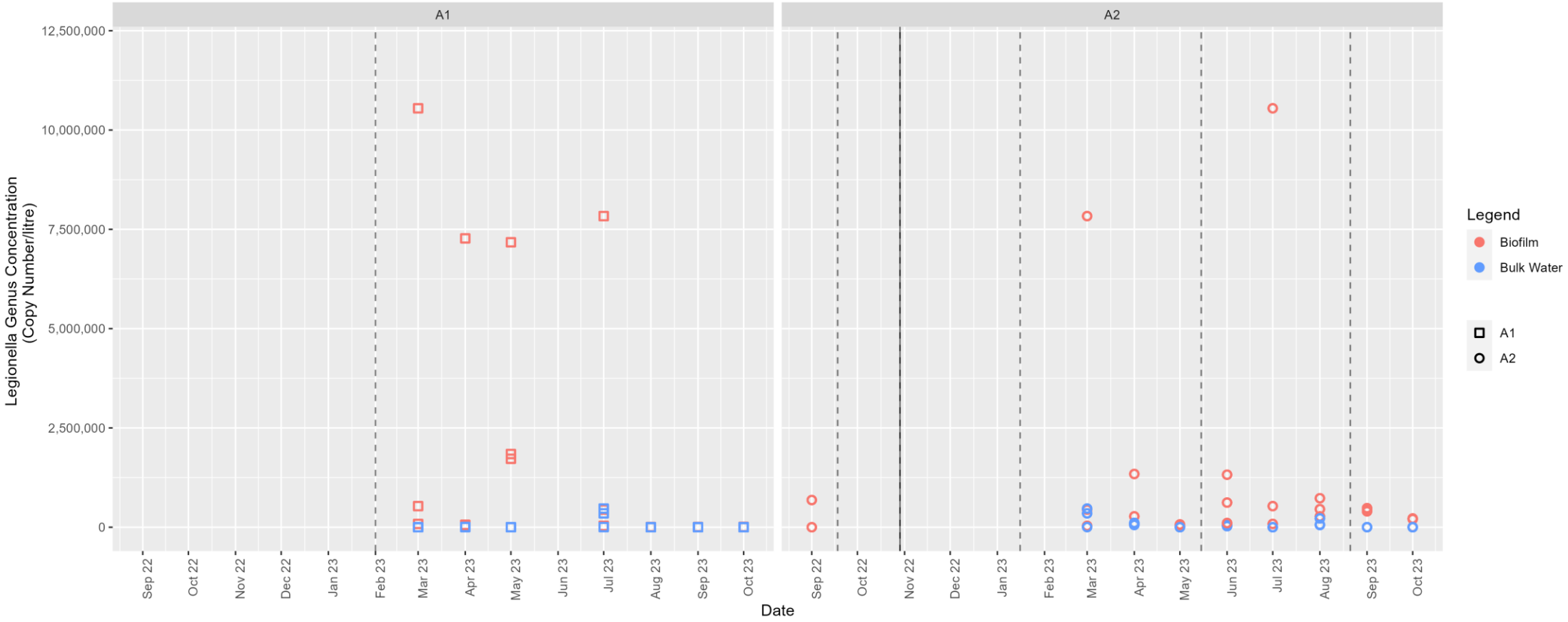

Figure 12: *Highlights Legionella genus concentrations across sampling dates for biofilm and bulk water samples within cooling tower A1 and A2*

5.2.3 Microbial Concentration and Viability

This section examines the microbial dynamics of cell concentration (TCC) and cell viability (ICC) in four cooling towers over the sampling period (Figure 13) where cleans are indicated by dotted vertical labelled and operational changes are represented by solid black lines. The study compares biofilm and bulk water TCC and ICC to assess potential correlations. It evaluates the influence of TCC and ICC on the presence and quantification of *Legionella* spp., including a specific analysis of *L. pneumophila*, which was detected in only one tower. Additionally, it explores the effects of cooling tower design, biocide regimes, seasonal changes, and cleaning schedules on biofilm and bulk water TCC and ICC throughout the field study. The ICC-to-TCC percentage highlighted differences in microbial health and was instrumental in establishing a standard for comparing microbial concentrations across cooling towers with larger microbial loads.

5.2.3.1 Comparison of Cell Concentration and Viability between Biofilm and Bulk Water

Biofilm and bulk water samples from four cooling towers across two years demonstrated inconsistent correlations in TCC and ICC counts, indicating they are independent samples. Biofilm and bulk water TCC count exhibited a combination of statistically significant and non-significant correlations, with significant results showing both positive and negative relationships. For example, cooling tower B2 exhibited a strong significant positive correlation for TCC counts (Spearman Rank Coefficient, $\rho = 0.885$, $p < 0.05$), while cooling towers A1, A2, and B1 showed no significant correlations. Furthermore, TCC correlations varied across seasons for individual cooling towers. For instance, cooling tower A2 demonstrated a strong significant negative correlation in autumn 2022 (Spearman Rank Coefficient, $\rho = -0.94$, $p = 0.017$) but shifted to a weak insignificant positive correlation in winter 2022 (Spearman Rank Coefficient, $\rho = 0.50$, $p = 1.00$).

Similarly, ICC counts between biofilm and bulk water showed no consistent or significant correlations across the four cooling towers with a mix of significant/ insignificant positive and negative correlations. For example, Cooling tower A1 had a strong significant negative correlation in spring 2022 (Spearman Rank Coefficient, $\rho = -1.00$, $p = 0.333$) but a strong positive correlation in summer 2022 ($\rho = 1.00$, $p = 0.333$). Finally, ICC to TCC % between biofilm and bulk water was not correlated (Spearman Rank Coefficient, $\rho = 0.029$, $p = 0.75$).

5.2.3.2 Relationship between Biofilm Cell Concentration, Cell Viability and *Legionella* Concentration

Biofilm cell concentration (TCC and ICC) and the presence or abundance of *L. pneumophila* within cooling tower A2 showed no significant correlation (Spearman's rank correlation coefficients, TCC: $\rho = 0.42$, $p > 0.05$; ICC: $\rho = 0.32$, $p > 0.05$). There was also no correlation with ICC to TCC % and *L. pneumophila* presence or abundance (Spearman's rank correlation coefficient, $\rho = -0.29$, $p = 0.35$).

Biofilm cell concentration (TCC) did not correlate with the presence of *Legionella* genus (Spearman's rank correlation coefficient, $\rho = 0.16$, $p = 0.31$) across all seasons for both cooling towers A1 and A2. In contrast, cell viability (ICC) and ICC to TCC % showed a statistically significant correlation with *Legionella* genus in cooling tower A1 (Spearman's rank correlation coefficient, ICC: $\rho = 0.57$, $p = 0.006$; ICC to TCC %: $\rho = 0.51$, $p = 0.0175$), but no significant correlation was

observed in cooling tower A2. Seasonal variation had no impact on the correlation between TCC, ICC, and ICC to TCC % and *Legionella* genus.

5.2.3.3 Comparison of Biofilm Cell Concentration and Viability across Cooling Towers

Cooling towers biocide regime and engineering design jointly had a significant influence on TCC and ICC across the cooling towers (Kruskal-Wallis test, TCC: $\chi^2 = 452$, $p < 0.001$; ICC: $\chi^2 = 264$, $p < 0.001$). Post- Hoc tests revealed significantly different TCC and ICC across all cooling towers pairs, except cooling tower A1 and B1. Cooling towers using a combination of oxidising and non-oxidising biocides (A2 and B2) exhibited higher microbial counts than those using only oxidising biocides (A1 and B1), with B2 showing the highest counts overall. Cooling tower design influenced microbial concentration and viability within one pair (A2 & B2). Cooling towers also showed a significant influence on ICC to TCC % across the cooling towers (Kruskal-Wallis test, $\chi^2 = 14.29$, $p < 0.01$). Subsequent post- hoc test showed that biofilm samples from cooling tower A2 significantly differed from those in B1 and B2, but not cooling tower A1.

5.2.3.4 Seasonal Effects on Biofilm Cell Concentration and Viability across Cooling Towers

Seasonal variations significantly influenced TCC and ICC counts across all cooling towers (Kruskal-Wallis rank sum, $p < 0.05$), but these effects varied by year and between TCC and ICC counts. In 2022, cooling towers A1 and A2 exhibited significant increases in TCC and ICC during the transition from winter to spring (February to April, $p < 0.01$), followed by declines in summer. In contrast, cooling tower B1 showed reductions in TCC and ICC during autumn (September to November), whereas B2 exhibited elevated microbial counts from July to September, followed by a decline in autumn.

In 2023, seasonal influences were evident, but patterns differed between towers. Cooling tower A1 remained stable throughout the year, with no significant monthly changes apart from a general decline from March to October ($p < 0.01$). A2 repeated its pattern of spring peaks in TCC and ICC ($p < 0.05$) and showed additional peaks in October during autumn. Cooling tower B1 exhibited significant fluctuations, with declines from March to June followed by increases in July, suggesting a lagged response to seasonal transitions. B2 displayed synchronised TCC and ICC peaks in spring (March to May), followed by sharp reductions at the onset of summer (May to June).

ICC to TCC percentage does not exhibit seasonal effects across the cooling towers. For Cooling towers A1, A2 and B2 there was no significant differences between the seasons in the years 2022 and 2023. However, cooling tower B1 ICC to TCC % displayed a significant increase in 2023 rising progressively from Spring to Summer to Autumn.

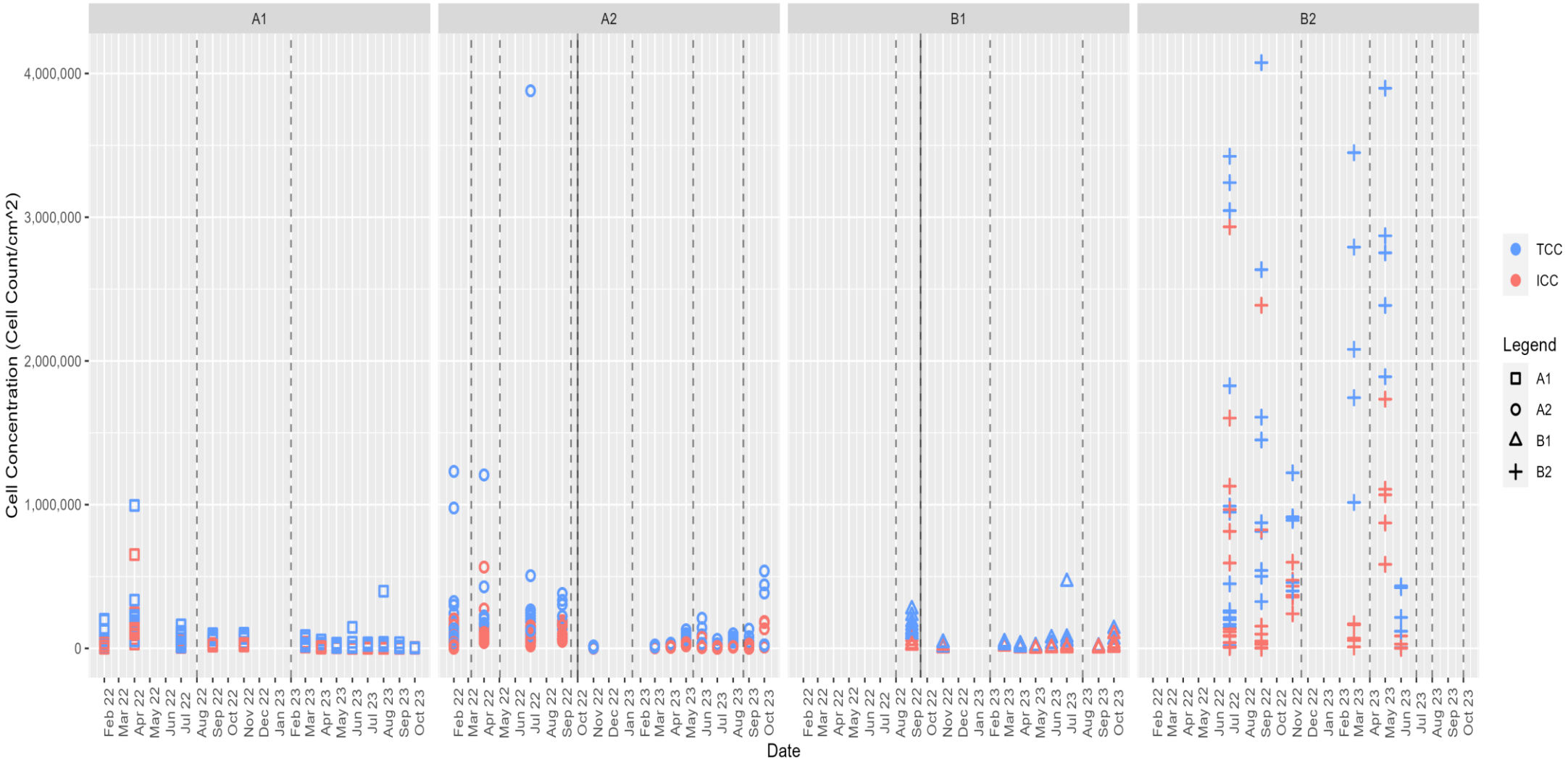

Figure 13: *Cell concentration in biofilms across sampling time. Each shape represents a different cooling tower. Dashed lines represent when the cooling tower was cleaned when solid black lines represent an operational cooling tower change. Each cooling tower is also facetted to allow for comparison.*

5.2.3.5 Cleaning Effects on Biofilm Cell Concentration and Viability

Cleaning effects on biofilm TCC and ICC vary significantly across events and cooling towers, with operational changes playing a crucial role in enhancing cleaning outcomes. In cooling tower A1, a cleaning event showed no significant effect on either TCC or ICC. In contrast, cooling tower A2 revealed that most cleaning events had no significant impact on these metrics. However, a March 2022 cleaning paradoxically increased ICC by 128% (Wilcoxon rank sum, $p < 0.01$) without significantly affecting TCC. Conversely, a combined cleaning and pack replacement in October 2022 achieved a significant reduction in both TCC and ICC by 96% ($p < 0.01$), as depicted in **Figure 14**. Cooling tower B1 demonstrated variable responses. An August 2023 cleaning reduced TCC by 92% ($p<0.05$) but left ICC significantly unchanged. Notably, an operational change in September 2022 significantly decreased TCC by 82% and ICC by 68% (TCC: $p <0.01$; ICC: $p<0.01$) show in **Figure 15**. Cooling tower B2 showed a paradoxical result in April 2023, with TCC increasing ($p <0.05$) after cleaning while ICC remained unchanged after cleaning.

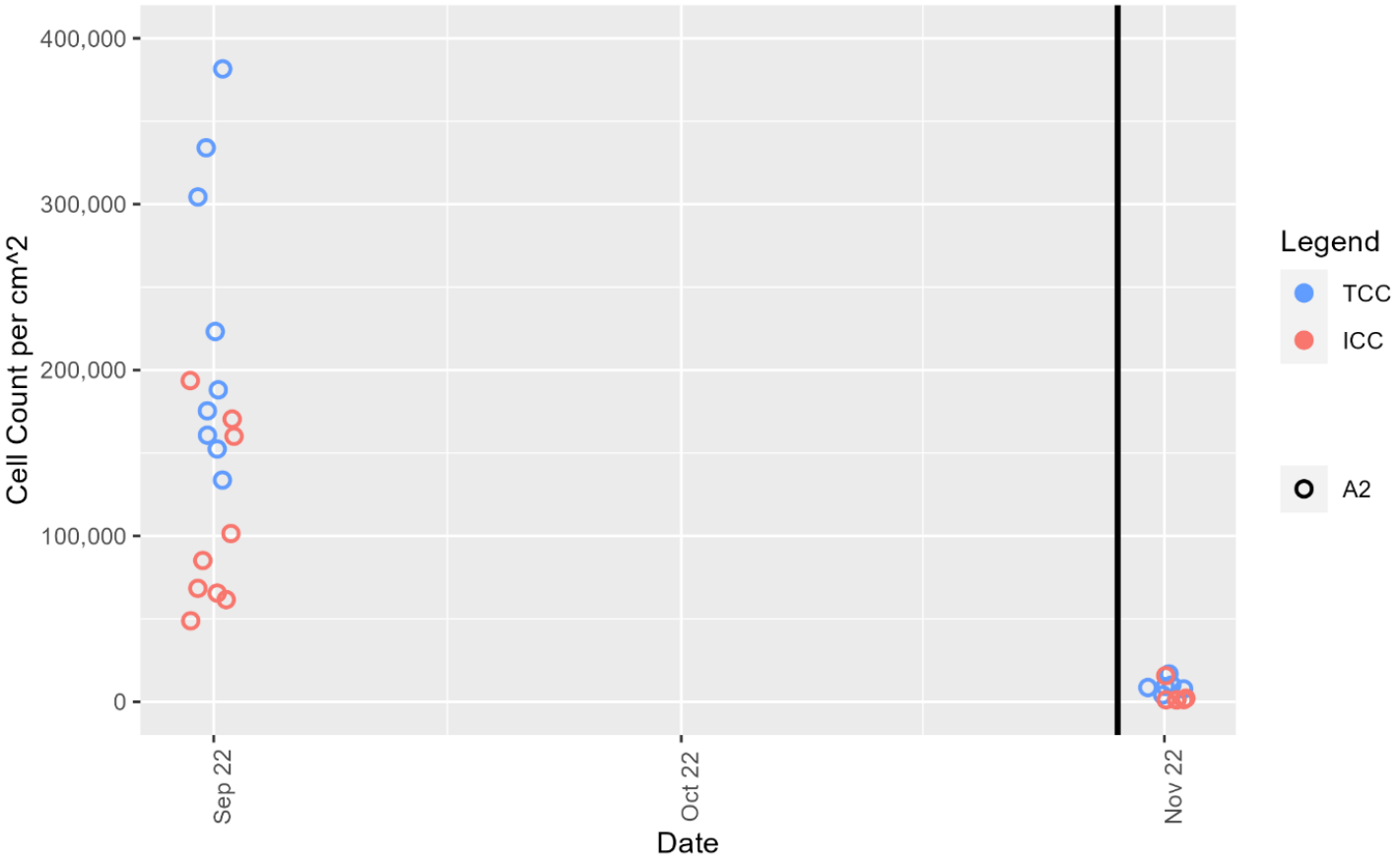

***Figure 14**: Biofilm cell concentration before and after a pack replacement and cleaning event within cooling tower A2, which took place in Late October 2022. The vertical black line represents the timing of this event.*

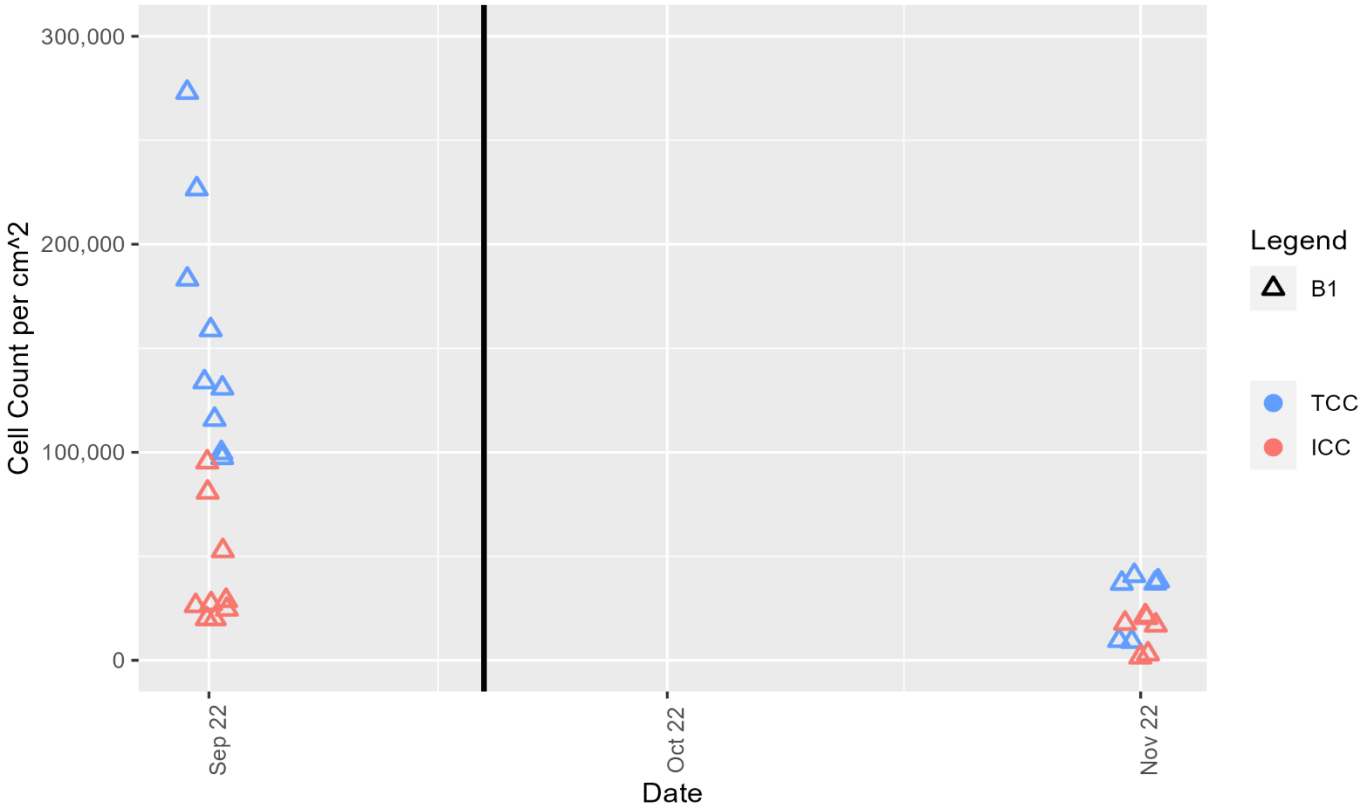

Figure 15: *Biofilm cell concentration before and after the installation of an automatic dosing system and cleaning event within cooling tower B1, which took place in Mid September 2022. The vertical black line represents the timing of this event.*

5.2.3.6 Relationship between Bulk Water Cell Concentration, Cell Viability and *Legionella* Concentration

Bulk water concentration (TCC), viability (ICC), and ICC to TCC % showed inconsistent correlations with the presence of *Legionella* genus (copy number per litre), limiting their reliability as indicators for *Legionella* detection across cooling towers and seasons. Cooling tower A1 showed no significant correlation, whereas cooling tower A2 exhibited a moderate positive correlation (Spearman rank coefficient: TCC, $\rho = 0.60$, $p < 0.01$; ICC, $\rho = 0.58$, $p < 0.01$). Seasonal effects were generally not significant for TCC correlations with *Legionella* genus in either cooling tower. However, significant seasonal correlations were observed for ICC during summer in cooling towers B180 ($\rho = 0.78$, $p = 0.017$) and B1866 ($\rho = 0.89$, $p = 0.033$). ICC to TCC % also showed no significant correlation across cooling towers or seasons.

5.2.3.7 Comparison of Bulk Water Cell Concentration and Viability across Cooling Towers

Similar to biofilm samples, cooling towers biocide regime and engineering design jointly had a significant influence on TCC and ICC across the cooling towers (Kruskal-Wallis test, TCC: $\chi^2 = 39$, $p < 0.01$; ICC: $\chi^2 = 40$, $p < 0.01$). Post- Hoc tests revealed significantly different TCC and ICC across all cooling towers pairs, except cooling tower A1 and B1. Cooling towers using a combination of oxidising and non-oxidising biocides (A2 and B2) exhibited higher microbial counts than those using only oxidising biocides (A1 and B1), with B2 showing the highest counts overall. Cooling tower design influenced microbial concentration and viability within one pair (A2 & B2). Cooling towers also showed a significant influence on ICC to TCC % across the cooling towers (Kruskal-Wallis test, $\chi^2 = 9.1$, $p < 0.05$). Subsequent post- hoc test showed that biofilm samples from cooling tower A2 significantly differed from those in A1 and B1 but not cooling tower B2. Illustration of this finding shown in **Figure 16**.

5.2.3.8 Seasonal Effects on Bulk Water Cell Concentration and Viability across Cooling Towers

Consistent with the findings in biofilm samples, seasonal variations significantly influenced bulk water TCC and ICC across all cooling towers except B1 (Kruskal-Wallis rank sum, $p < 0.05$). However, when analysing the ICC to TCC percentage, most cooling towers did not exhibit notable seasonal effects. The only exceptions were cooling tower A1, which showed seasonal differences between summer and autumn 2022, and cooling tower B1, with differences observed between summer and autumn 2023. Post hoc tests examining the influence of season on TCC and ICC revealed that cooling tower A1 showed relatively consistent TCC and ICC during 2022–2023, with significant variations observed in spring and autumn 2022 and between spring and summer 2023. Cooling tower A2 also exhibited seasonal differences in 2023, with TCC varying between spring and autumn, as well as between summer and autumn, and ICC showing differences between summer and autumn. Finally, cooling tower B2 displayed a significant decrease in TCC between spring and autumn 2023, and ICC reductions between spring, early summer, and autumn 2023.

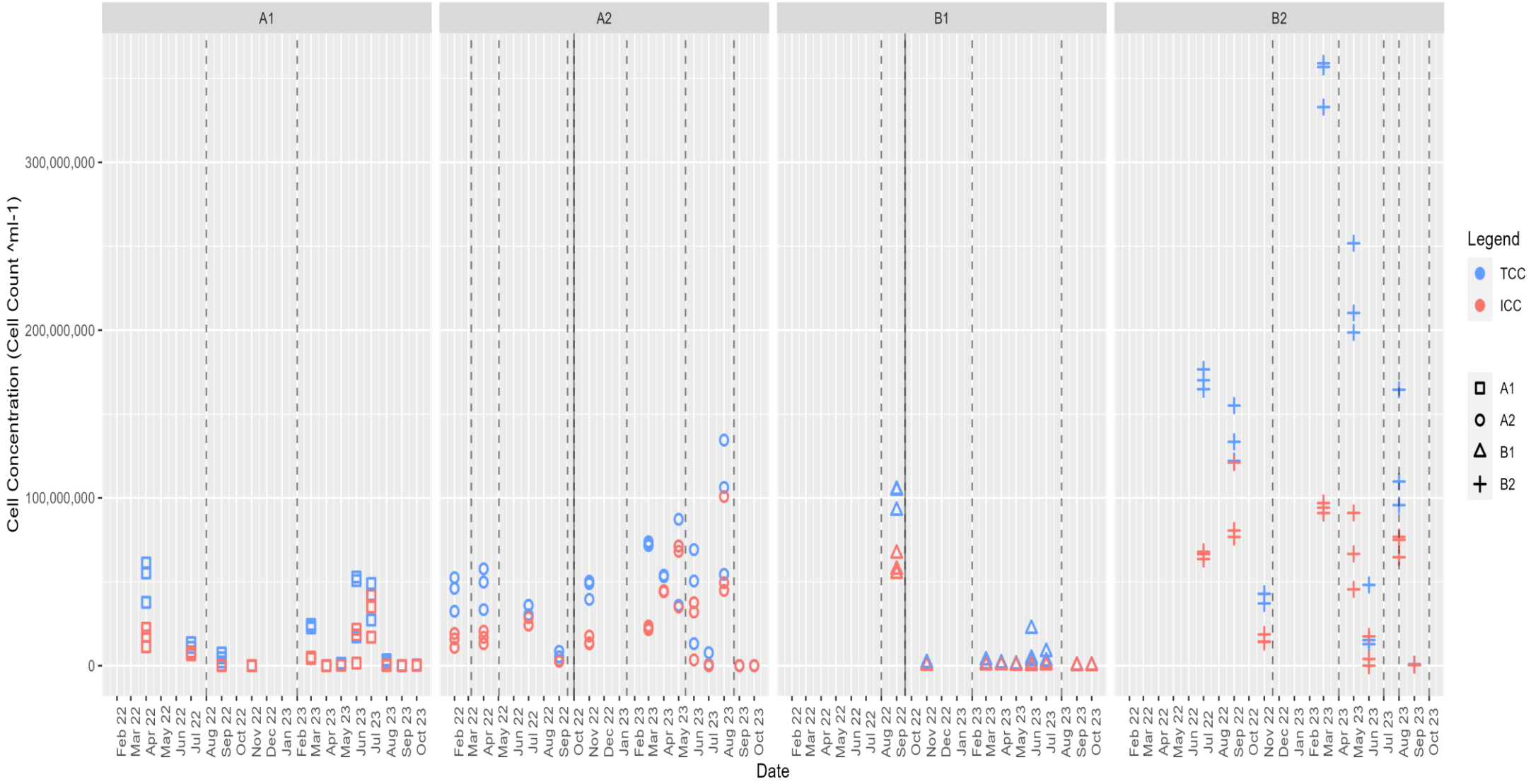

***Figure 16**: Cell concentration in bulk water samples across sampling time. Each shape represents a different cooling tower. Dashed lines represent when the cooling tower was cleaned when solid black lines represent an operational cooling tower change. Each cooling tower is also facetted to allow for comparison.*

5.2.3.9 Cleaning Effects on Bulk Water Cell Concentration and Viability

Similar to biofilm samples cleans had mixed results across cleaning dates and cooling towers. For cooling tower A1, there was no significant difference in TCC before and after the clean that occurred in August 2022, however there was a significant decrease in ICC counts ($\chi2= 3.86$, $p < 0.05$). For cooling tower A2, there was no significant difference in TCC and ICC before and after the clean in March 2022. Interestingly, after a clean in September 2022 and a pack replacement in October, cooling tower A2 exhibited a significant drop in TCC and ICC ($\chi2 = 3.86$, $p < 0.05$) shown in **Figure 17**. In 2023, cooling tower A2 had two cleans, one in May and one in August, where there was no significant difference in both TCC and ICC counts. Cooling tower B1 did show a significant decrease in TCC and ICC in November, following the installation of an automatic dosing pump in September 2022 shown in **Figure 18**. Furthermore, total and intact cell counts significantly decreased from July to September after the clean in August 2023. Cooling Tower B2 showed no significant difference in TCC but did show a significance decrease in ICC before and after the cleaning in April 2023.

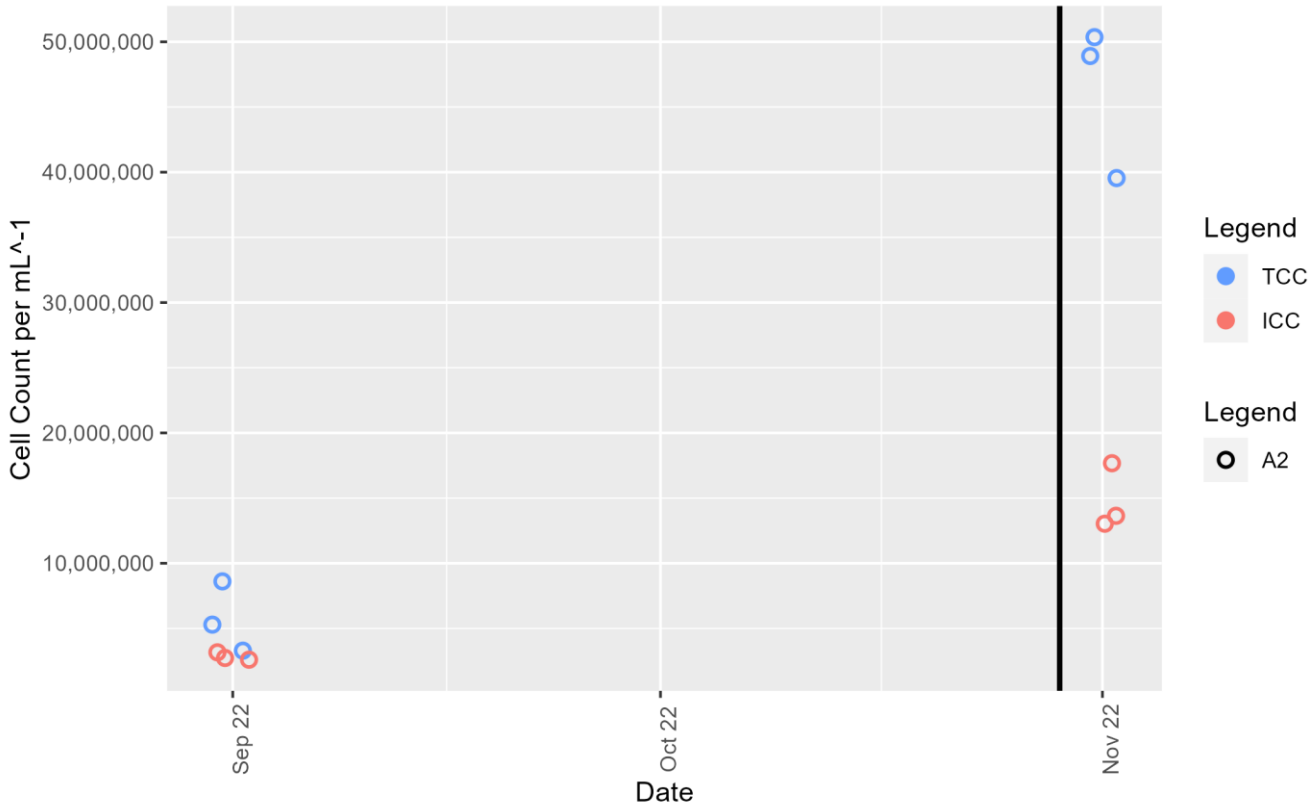

***Figure 17**: Bulk Water cell concentration before and after a pack replacement and cleaning event wihtin cooling tower A2, which took place in Late October 2022. The vertical black line represents the timing of this event.*

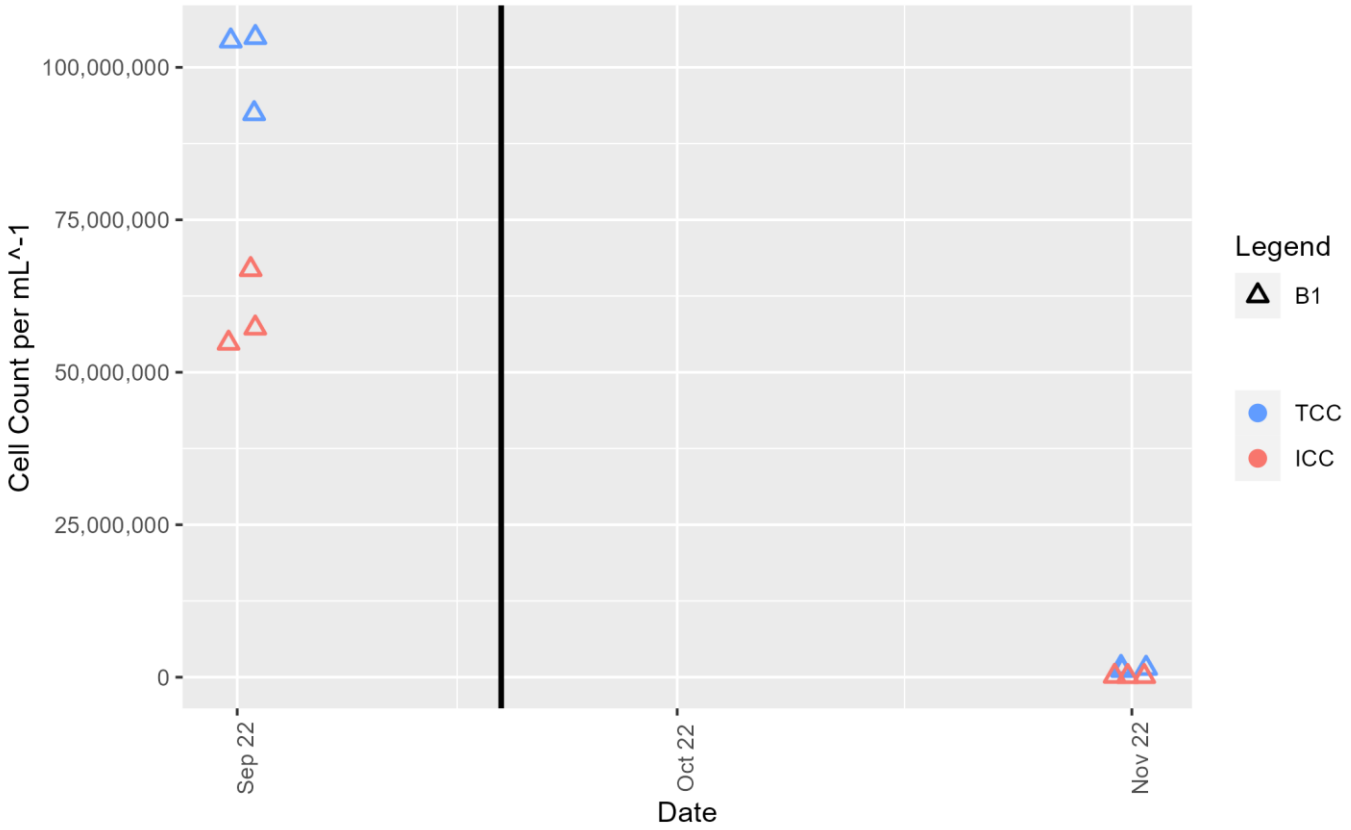

Figure 18: *Bulk Water cell concentration before and after the installation of an automatic dosing system and cleaning event wihtin cooling tower B1, which took place in Mid September 2022. The vertical black line represents the timing of this event.*

5.3 Bacterial Community Structure

To explore differences in bacterial community structure, two key parameters were examined: bacterial richness and bacterial evenness. Richness refers to the number of bacterial species present in the community, while evenness describes how evenly these species are distributed within the community. The 'Observed' metric was used to measure richness and 'Pielou's Evenness' was used to assess evenness and can be seen in Figure 19, 20 and 21.

5.3.1 Bacterial Richness and Evenness between Biofilms and Bulk Water Samples

Biofilms exhibited significantly higher bacterial richness (Observed) compared to bulk water samples for cooling towers A1 and B1 (Wilcoxon test: A1, $W=583$, $p < 0.05$; B1, $W=279$, $p < 0.01$). However, no significant differences were observed for cooling towers A2 and B2. As illustrated in Figure 19, biofilm samples consistently demonstrated a higher median richness than bulk water samples across all cooling towers. Notably, cooling towers A2 and B2 displayed a larger interquartile range, indicating a broader variability in bacterial species richness. This broader variability, coupled with data averaging over two years, likely masked any statistical significance for cooling towers A2 and B2.

In terms of community evenness (Pielou's Evenness), biofilm and bulk water samples were generally similar in bacterial species distribution, with only cooling tower A1 showing a significant difference (Wilcoxon test: A1, $W=263$, $p < 0.05$). This result suggests that biofilms in cooling tower A1 may be dominated by a few species, whereas the bulk water supports a more balanced bacterial community. Figure 19 shows that bulk water samples had a higher median evenness for cooling towers A1, B1, and B2; however, the broad interquartile ranges for B1 and B2 prevented the detection of significant differences in evenness.

While cooling towers A1 and B1 demonstrated significantly higher bacterial richness within biofilm compared to bulk water, this was not consistently reflected in bacterial evenness. In cooling tower A1, biofilms showed lower species evenness, suggesting that while more bacterial species are present, their distribution is less uniform. In contrast, cooling tower B1 did not show any significant difference in evenness between biofilm and bulk water samples, indicating similar species distribution patterns despite differences in richness. Additionally, cooling tower B2 had a higher median bacterial richness in biofilms but a lower evenness, though these results were not statistically significant. This implies that, despite biofilms in B2 having more bacterial species, these species are unevenly distributed. Caution is advised in interpreting these findings, particularly for B2, due to the variability in data.

5.3.2 Relationship Between Bacterial Richness and Evenness when *L. pneumophila* was present.

Bacterial richness and evenness did not significantly differ significantly one month before and after the detection of *L. pneumophila* during any event. *L. pneumophila* was detected in April, July, September, and October for cooling tower A2, yet bacterial species richness and community structure remained stable throughout, indicating no measurable impact on microbial diversity.

5.3.3 Bacterial Richness and Evenness Among Cooling Towers

The bacterial richness of biofilms and bulk water (**Figure 19**) in cooling towers showed similar median values, with comparable interquartile ranges for cooling towers A1 and A2, as well as B1 and B2, respectively. The results of a Kruskal-Wallis test confirmed these findings. Evenness across each cooling tower has similar median with lots of interquartile overlap showing no significant difference, this was confirmed by the Kruskal-Wallis test.

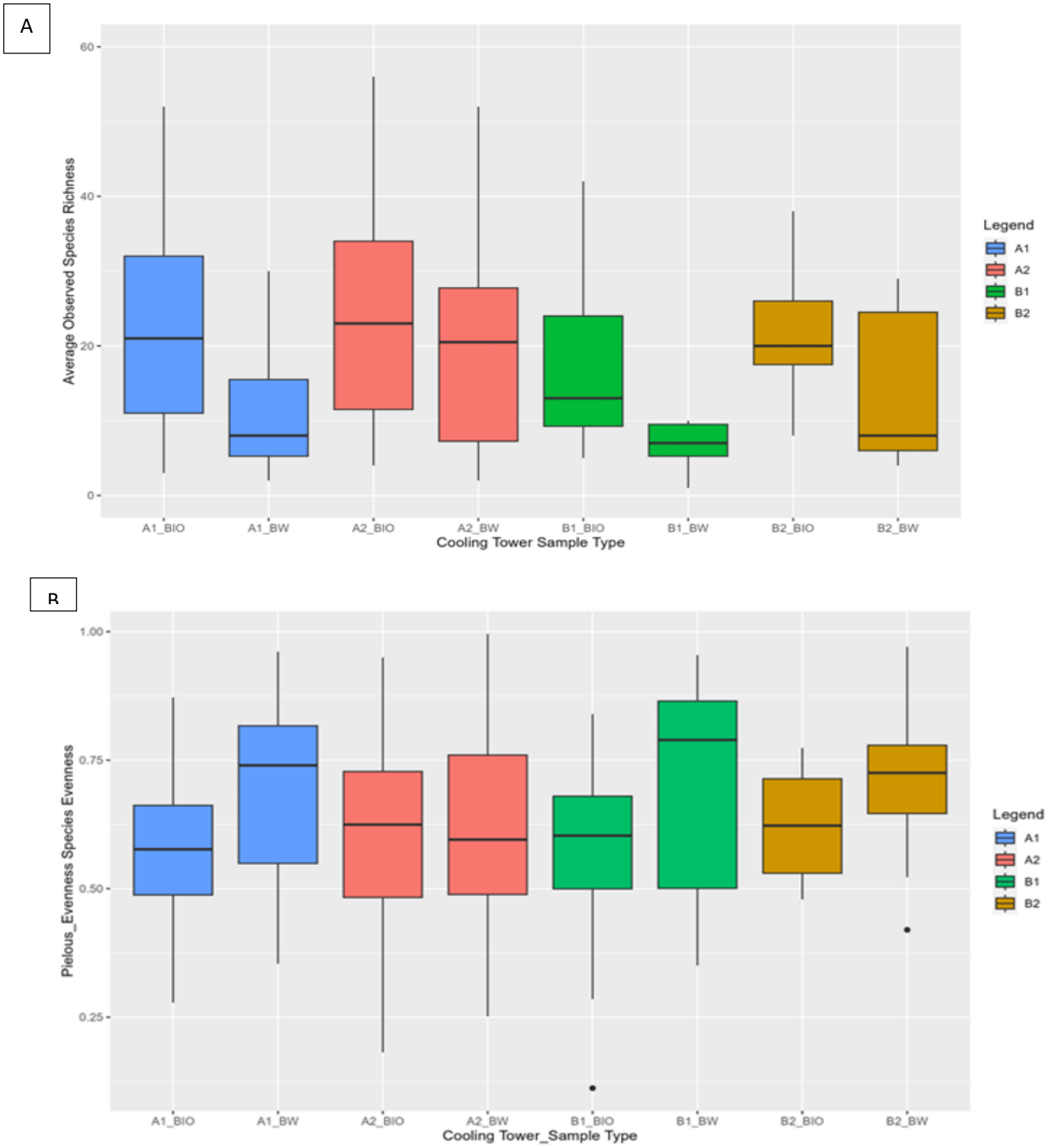

Figure 19*: Trends in Alpha diversity in cooling tower biofilm and bulk water samples.* The x-axis labels combine the cooling tower ID (e.g., "A1") with the sample type ("Biofilm" or "Bulk Water"). *Panel (A) depicts species richness, as measured by the Observed Metric, highlighting the number of distinct ASVs identified in both biofilm and bulk water samples from cooling towers. Panel (B) illustrates Pielou's Evenness, evaluating the uniformity of species distribution within the samples. Each colour represents a different cooling tower.*

5.3.4 Temporal Variation Between Bacterial Richness and Evenness and Cleans

When comparing the richness and evenness across the sampling period (Figure 20 & 21) for both biofilm and bulk water across the cooling towers, no significant differences were observed, evidenced by substantial overlap between interquartile ranges. The Kruskal-Wallis test and post-hoc Dunn test further confirmed the absence of significant differences between sampling dates. Additionally, this analysis revealed subsequent cleanings did not significantly impact the richness and evenness of the biofilms and bulk water across any cooling tower.

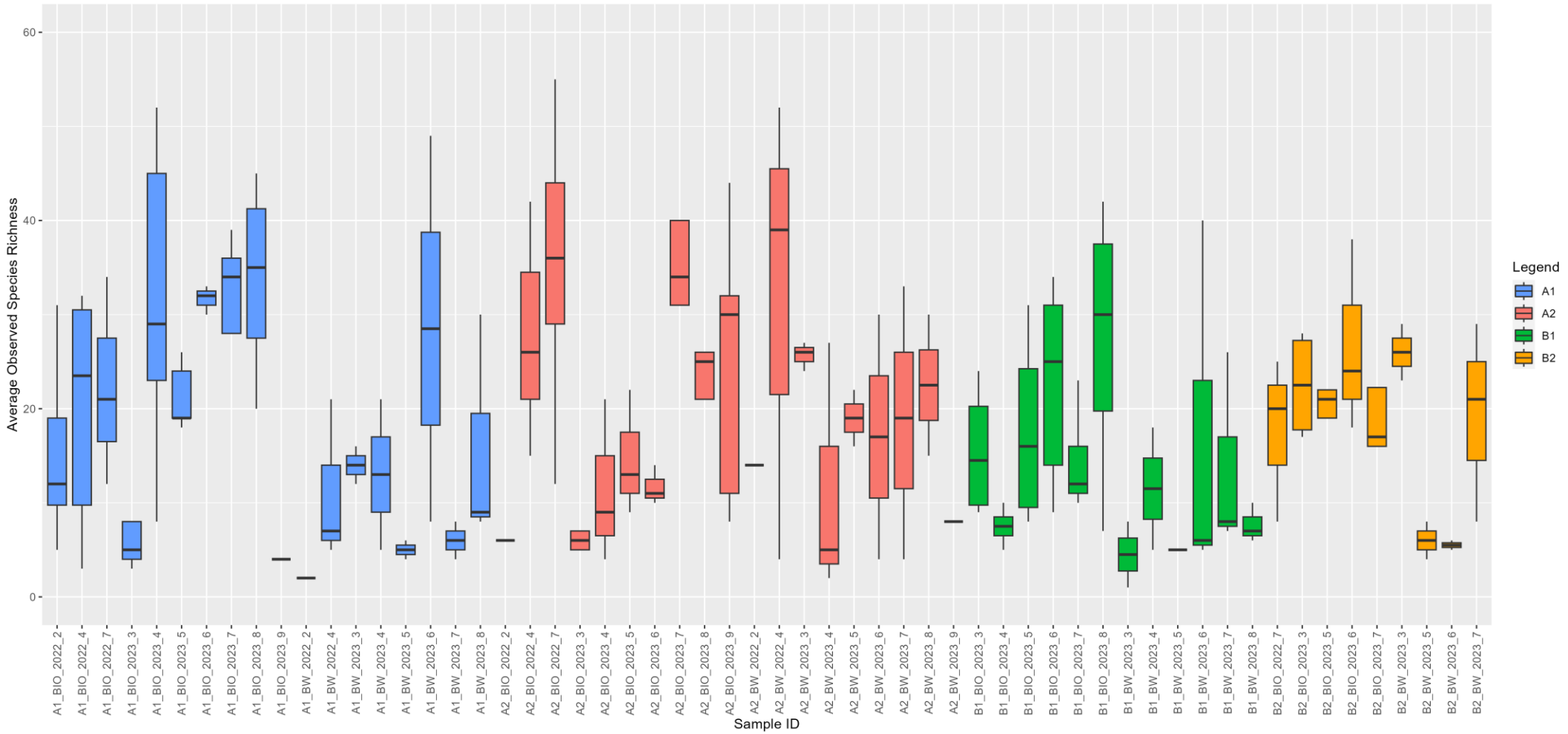

Figure 20: *Comparative Analysis of Species Richness in Cooling Tower Samples Across Sample Dates. Richness was measured using the Observed Metric, representing the number of distinct species identified in biofilm (BIO) and bulk water (BW) samples from different cooling towers. The x-axis labels (Sample ID) represent a combination of cooling tower ID, sample type (BIO = Biofilm, BW = Bulk Water) followed by year and month, allowing for direct comparisons across time points*

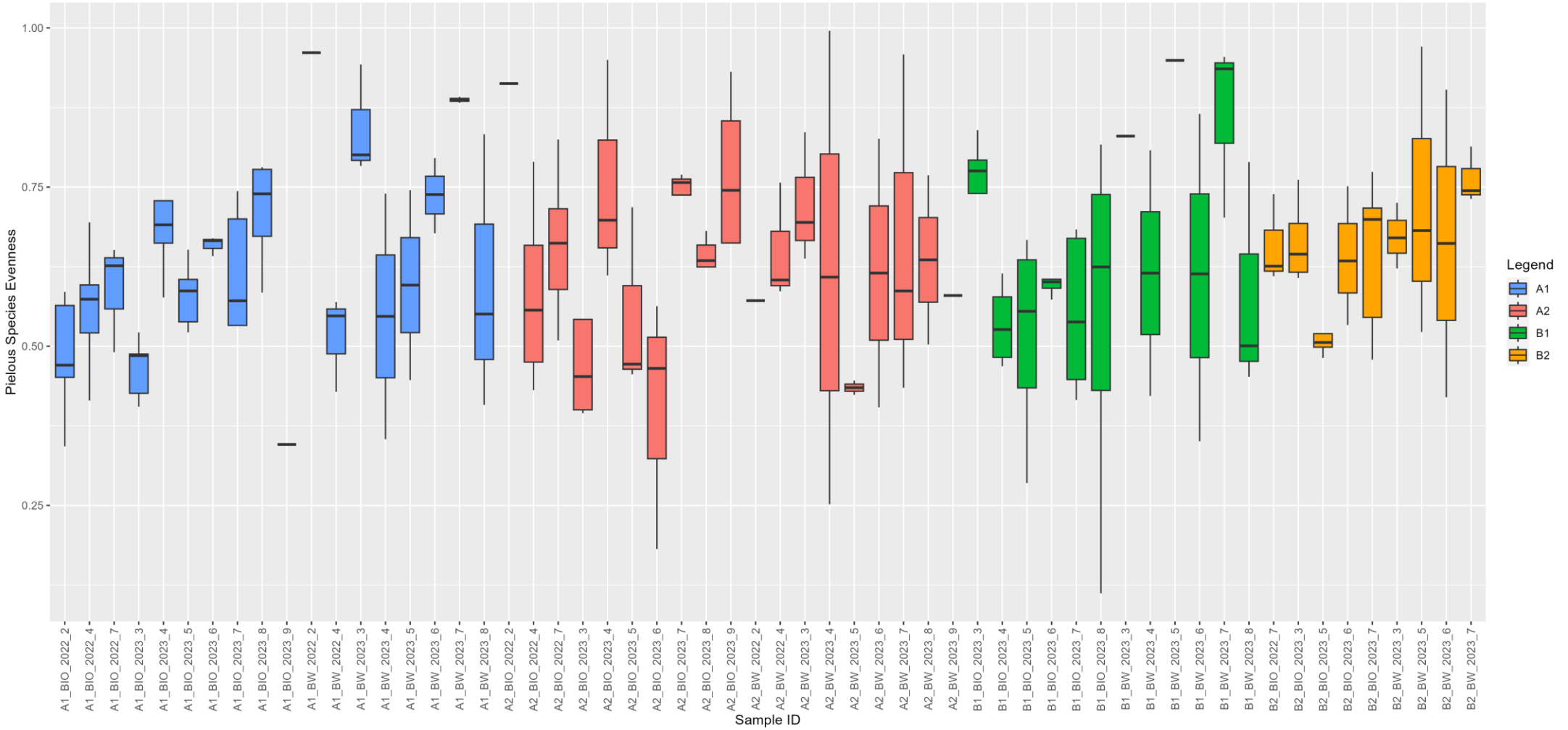

***Figure 21**: Comparative Analysis of Evenness in Cooling Tower Samples across each sample date. Evenness was measured by the Pielous Evenness Metric, highlighting the uniformity of species distribution in both biofilm and bulk water samples from cooling towers. The x-axis labels (Sample ID) represent a combination of cooling tower ID, sample type (BIO = Biofilm, BW = Bulk Water) followed by year and month, allowing for direct comparisons across time points*

5.4 Bacterial Community Composition

To investigate microbial community composition changes across sample types, cooling towers, seasons, cleaning events, and *L. pneumophila* detection, beta diversity analysis was performed using the Bray dissimilarity metric.

5.4.1 Microbial Diversity between Biofilm and Bulk Water Samples

Biofilm samples consistently exhibited significant differences in microbial communities compared to bulk water samples across all cooling towers when measured with the Bray dissimilarity metric (Permutational Multivariate Analysis of Variance test, $p < 0.001$), highlighting distinct microbial communities in these two sample types. Visualisation using hierarchical clustering analysis (Figure 22) also highlighted this finding for cooling tower A1 with clear clusters between biofilm and bulk water and even some distinct clustering between biofilm samples indicating variability within biofilm microbial communities. The same trend was observed with cooling tower A2, B1 and B2 shown in appendix.

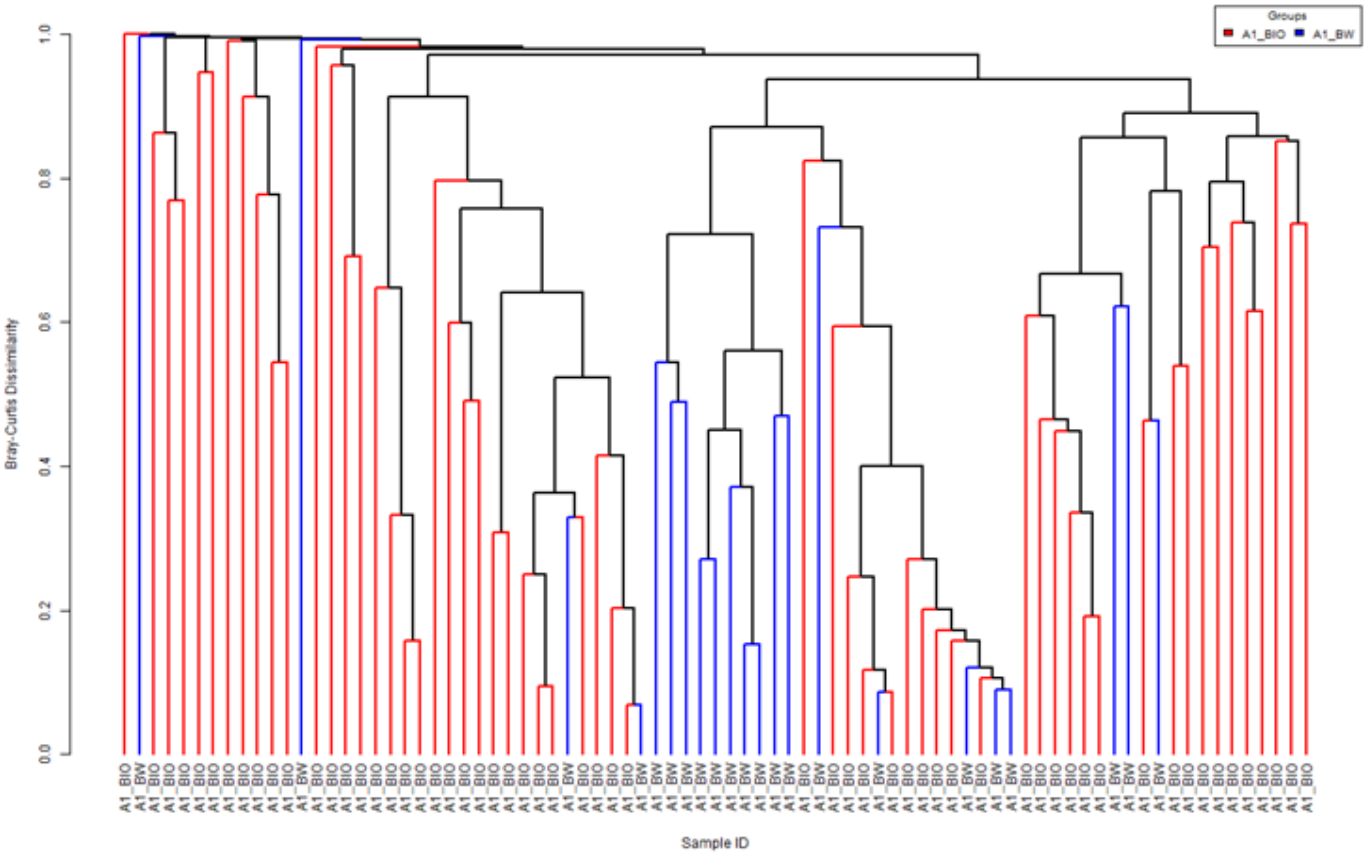

***Figure 22**: Hierarchical clustering analysis of microbial communities to compare between biofilm and bulk water samples for cooling tower A1 using the Bray Curtis dissimilarity matrix. Red lines represent biofilm samples and blue lines represent bulk water samples. Black lines represent direct link to the biofilm and bulk water samples. The height of the branches reflects the degree of dissimilarity between samples, with greater heights indicating more distinct microbial communities. The x-axis labels (Sample ID) represent a combination of cooling tower ID and sample type (BIO = Biofilm, BW = Bulk Water).*

5.4.2 Microbial Diversity between Cooling Towers

Cooling towers exhibited distinct bacterial communities within biofilm and bulk water samples (Permutational Multivariate Analysis of Variance, $p<0.01$). Pairwise analysis revealed varying results for both biofilm and bulk water samples. For instance, in biofilm samples, cooling towers A1 and A2, which are structurally similar but have different biocide regimes, did not show distinct bacterial communities. In contrast, cooling towers B1 and B2, which also share a similar structure but differ in biocide regimes, exhibited significant differences in their bacterial communities (pairwise PERMANOVA with Benjamini-Hochberg (BH) corrections, $p<0.01$) shown in **Figure 23**. However, cooling towers A1 and B1, as well as A2 and B2, which differ in engineering structure but have similar biocide regimes, showed significant differences in their bacterial communities shown in (appendix). This suggests that engineering structure may have a stronger influence on biofilm bacterial composition than biocide regimes.

Pairwise analysis for bulk water samples also revealed varying results with cooling tower A2 consistently displayed a statistically significant distinct bacterial community when compared to A1, B1 and B2 (Permutational Multivariate Analysis of Variance, $p < 0.01$), while the other cooling towers did not exhibit such differences.

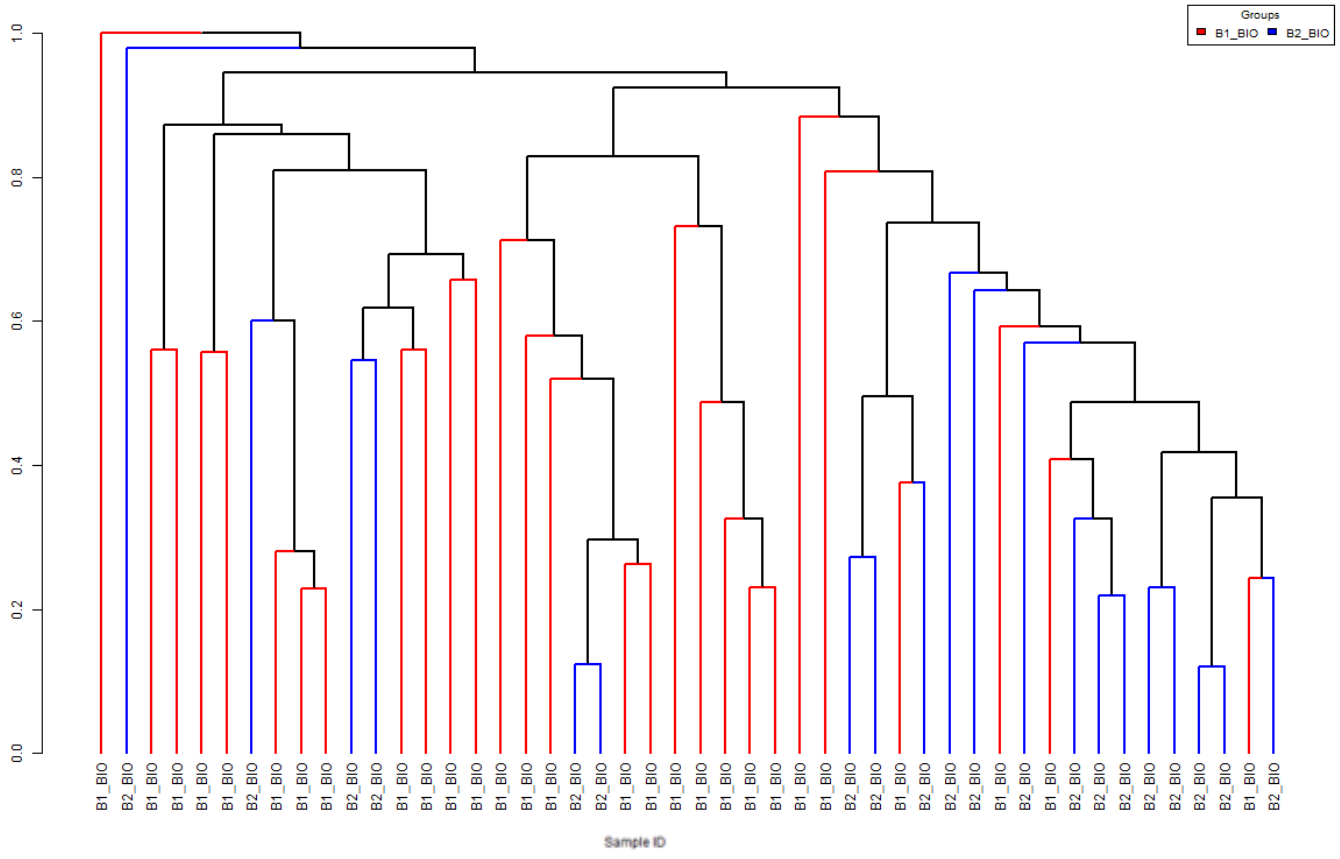

***Figure 23**: Hierarchical clustering analysis of microbial communities to compare cooling tower B1 and B2 biofilm samples Using the Bray Curtis dissimilarity matrix. Red lines represent cooling tower biofilm samples when blue lines represent cooling tower bulk water samples. The height of the branches reflects the degree of dissimilarity between samples, with greater heights indicating more distinct microbial communities. The x-axis labels (Sample ID) represent a combination of cooling tower ID and sample type (BIO = Biofilm, BW = Bulk Water).*

5.4.3 Seasonal Variation in Microbial Community Composition

Seasons had a mixed effect on the biofilm microbial community composition across cooling towers. For cooling tower A1, in the year 2022, the bacterial community composition remained stable across all seasons. However, in the year 2023, the bacterial community composition in spring (March) was significantly different compared to late spring and summer (April, May, June, July, and August) (PERMANOVA, $p < 0.01$). Additionally, in 2023, early spring (April) was significantly different compared to mid and late spring (May and June). Similarly, for cooling tower A2, in the year 2022, the biofilm bacterial community composition remained stable across all seasons. In the year 2023, the bacterial community in spring (March) differed compared to summer (June, July, and August) (PERMANOVA, $p < 0.01$). Furthermore, early autumn (September) significantly differed from summer (June and August). Cooling towers B1 and B2, on the other hand, exhibited stable biofilm bacterial communities throughout 2022 and 2023, with no significant seasonal variation. Bulk water samples showed no significant changes in bacterial composition across seasons for all cooling towers. Both biofilm and bulk water trends are further illustrated in Figure V (Appendix I).

5.4.4 Microbial Community Between Cleans

When examining beta diversity one month before and after a clean (where data allowed), it was clear that cleans did significantly change the microbial community for cooling tower A2 when the clean happened In May 2022. However, it should be noted that results from April had to be compared to July instead of May due to lack of sampling date. Furthermore, the clean that occurred in May and August 2023 did cause a significant shift in microbial commu1nity as confirmed by a PERMANOVA test ($P < 0.05$). For cooling tower B1 the clean in April 2023 did not cause a significant shift between March and May 2023. For cooling tower B2, the clean in August 2023 also caused a significant difference between July and September 2023 ($p < 0.05$). In comparison, bulk water samples were not significantly different across any cleans.

5.4.5 Microbial Community between *L. pneumophila* Detection

A Permutational Multivariate Analysis of Variance revealed a significant difference in the biofilm microbial community only between August and September in cooling tower A2 (Figure 24). When examining whether there was a significant difference between the biofilm microbial communities one month before and one month after the presence of *L. pneumophila,* considerable overlap was observed between the clusters in March and April, April and May, June and July, and July and August. This indicates no considerable difference in the microbial community when *L. pneumophila* was detected in April and July. However, the cluster observed in August was different from that in September, when *L. pneumophila* was present.

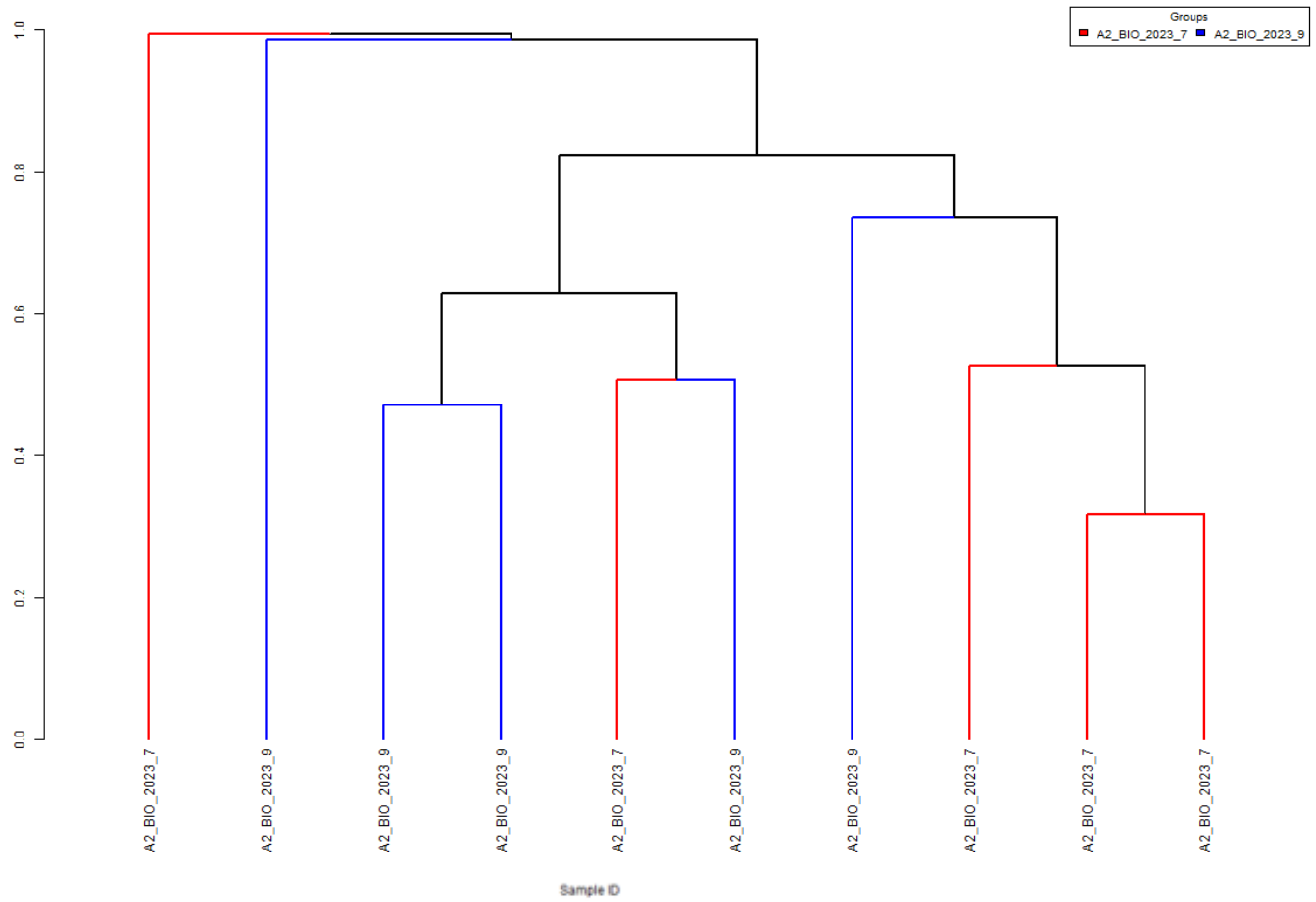

***Figure 24**: Hierarchical clustering analysis of microbial communities to compare cooling tower A2 and A2 biofilm samples Using the Bray Curtis dissimilarity matrix. Red lines represent cooling tower biofilm samples when blue lines represent cooling tower bulk water samples. The height of the branches reflects the degree of dissimilarity between samples, with greater heights indicating more distinct microbial communities. The x-axis labels (Sample ID) represent a combination of cooling tower ID, sample type (BIO = Biofilm, BW = Bulk Water) followed by year and month, allowing for direct comparisons across time points*

5.5 Bacterial Community

5.5.1 Bacterial Community Composition

Figure 25 and Figure 26 show most abundant bacterial phylum, class, family and genus present within each cooling tower over a two-year period, encompassing both biofilm and bulk water samples.

5.5.2 Characterisation of Biofilm and Bulk Water Bacterial Community within Cooling Towers

The characterisation of the biofilm and bulk water community within various cooling towers reveals a striking consistency in bacterial composition, with both matrices predominantly featuring Proteobacteria, followed by *Bacteroidota* and Firmicutes. Biofilm samples additionally exhibit small abundances of *Cyanobacteria* and Acidobacteria. At the class level, Alpha, Gamma, and Delta Proteobacteria are abundant in both biofilms and bulk water, while Bacteroidia, Bacilli, *Cyanobacteria*, and other rare taxa are uniquely characterised in biofilms. Families such as Sphingomonadaceae, Pseudomonadaceae, Bdellovibrionaceae, and *Xanthobacteraceae* are the most prevalent across both environments, with differences mainly in the relative abundance of specific families.

Further examination across different cooling towers highlights a consistent predominance of *Proteobacteria*. Cooling towers A1 and A2 show higher abundances of *Bacteroidota* and *Cyanobacteria*, whereas towers B1 and B2 are richer in Firmicutes and Acidobacteriota. Notably, cooling tower A1 features a higher abundance of Beijerinckiaceae, and both A1 and B1 show higher levels of Caulobacteraceae. Cooling tower A2 is particularly distinguished by significantly larger abundances of Clade III, Hypomicrobiaceae, Spirosomaceae, and Azospirillaceae. An analysis at the genera level across all samples did not yield additional insights, reaffirming that the key distinctions occur at the phylum and family levels.

5.5.3 Characterisation of Biofilm Bacterial Community when *L. pneumophila* was Present

To determine if community composition changed when *L. pneumophila* was detected by Genomadix (April, July, September and October) within cooling tower A2 biofilm samples, the phyla, classes, families, and genera present one month before, during, and one month after the detection of *L. pneumophila* were analysed (refer to Figures 27, 28 and 29). In April 2023, when *L. pneumophila* was detected, a unique phylum of *Cyanobacteria* was identified, which was absent in both March and May. During this month, there was a distinctive presence of *Cyanobacteria* classes, accompanied by a notably high relative abundance of Alpha-*Proteobacteria* compared to the following month, whereas the previous month showed no presence of Alpha-*Proteobacteria*. An examination of the family-level composition revealed that Sphingomonadaceae, and *Xanthobacteraceae* exhibited much higher abundances than in the months before and after, alongside a unique presence of Rhizobiales and Solimonadaceae. Further analysis of the genera present indicated that Novosphingobium, Sphingomonas, Afipia, and Phreatobacter had a significantly higher relative abundance than in the previous and subsequent months, with Nevskia, Methylobacterium, and Skermanella uniquely present during the month of April when *L. pneumophila* was detected.

In July 2023, Cyanobacteria remained uniquely present, supplemented by additional uniquely present phyla such as Bacteroidota, Firmicutes, and Acidobacteriota. The classes observed during this month, which correlated with the presence of *L. pneumophila,* included Cyanobacteria, Bacteroidia, Delta *proteobacteria*, Bacilli, and other rare taxa.

July 2023 also saw a higher abundance of the families *Xanthobacteraceae* and Hyphomicrobiaceae, along with a unique presence of Clade_III, Rhodobacteraceae, Bdellovibrionaceae, Spirosomaceae, and Beijerinckiaceae. The genera landscape in July 2023 was uniquely dominated by rare taxa, as well as Hypomicrobium, Clade_III, *Sphingopyxis* , Blastomonas, Bdellovibro, Lentibacillus, and Methylobacterium.

September 2023 comparisons revealed a small, unique presence of Firmicutes. A deeper analysis of the classes showed that Delta*Proteobacteria* was particularly unique this month, accompanied by small abundances of unique classes such as Bacilli and Bacteroidia. The family structure included a unique presence of Hypomicrobiaceae, Moraxellaceae, Bdellovibrionaceae, rare taxa, Bacillaceae, and Solimonadaaceae, with a higher relative abundance of Pseudomonadaceae compared to the previous month. The genera present in September 2023 when *L. pneumophila* was detected were *Pseudomonas*, Hypomicrobium, Acinetobacter, Bdellovibro, *Sphingopyxis* , along with rare taxa, Blastomonas, and Lentibacillus.

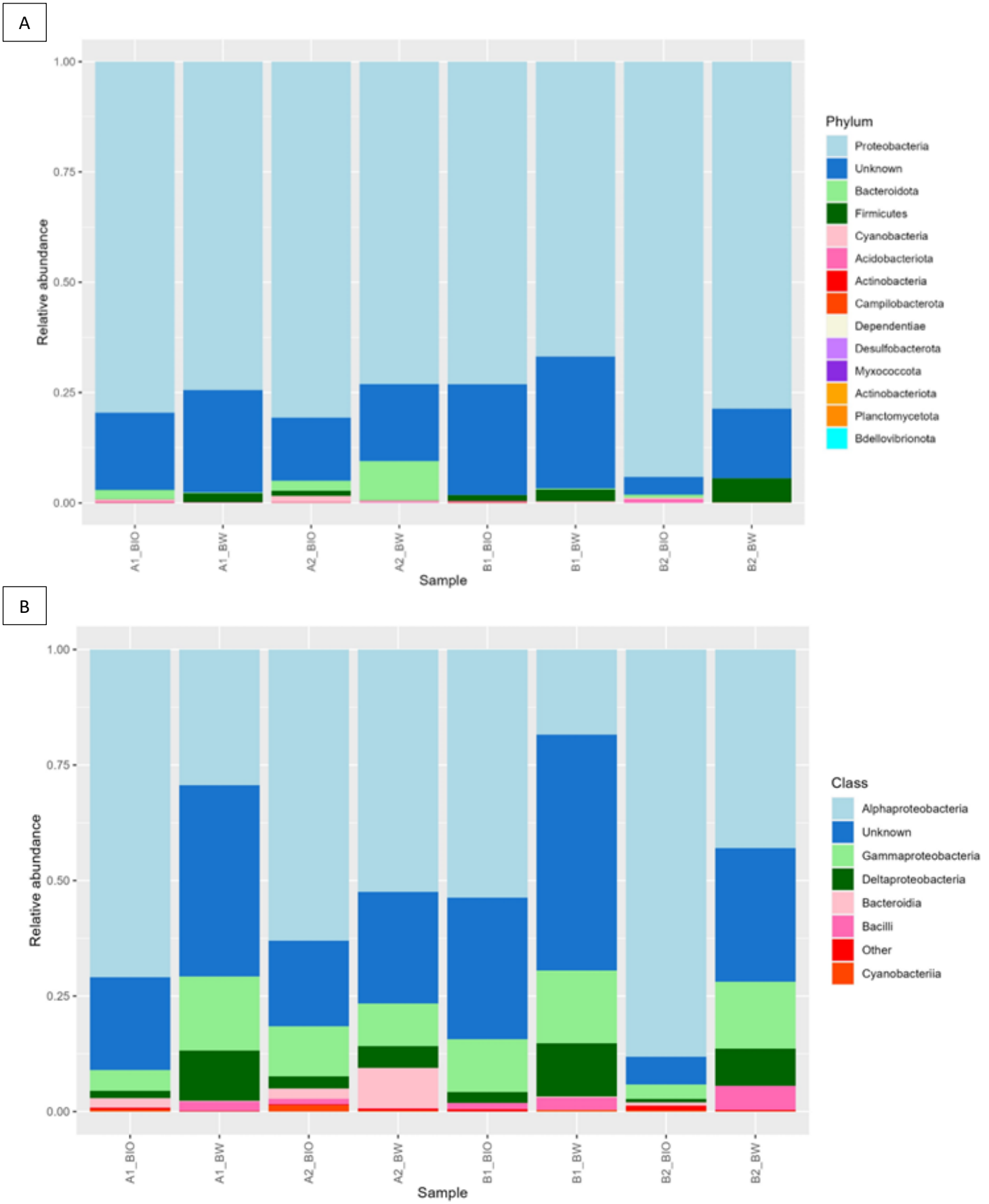

***Figure 25**: shows relative abundant bacterial phylum and class present within each cooling tower over a two-year period, encompassing both biofilm and bulk water samples. Panel A represents Phylum, Panel B represents Class. Phylum and class were filtered using a detection threshold of 0.01% (taxa must constitute at least 0.1 % of total sequences) and prevalence of 5% (taxa must be present in at least 5 % of samples).*

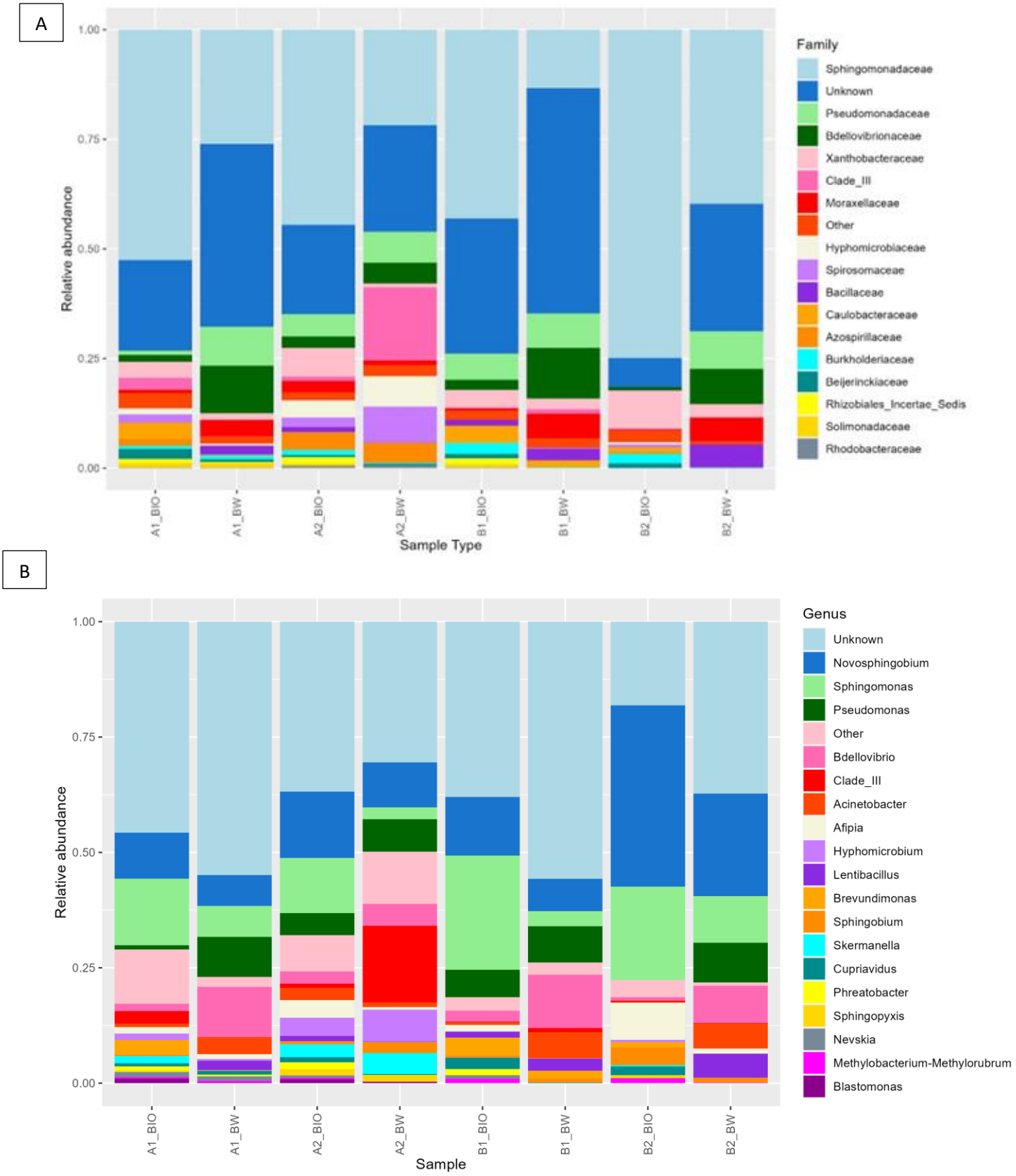

***Figure 26**: shows relative abundant bacterial phylum and class present within each cooling tower over a two-year period, encompassing both biofilm and bulk water samples. Panel A represents Phylum, Panel B represents Class. Phylum and class were filtered using a detection threshold of 0.01% (taxa must constitute at least 0.1 % of total sequences) and prevalence of 5% (taxa must be present in at least 5 % of samples).*

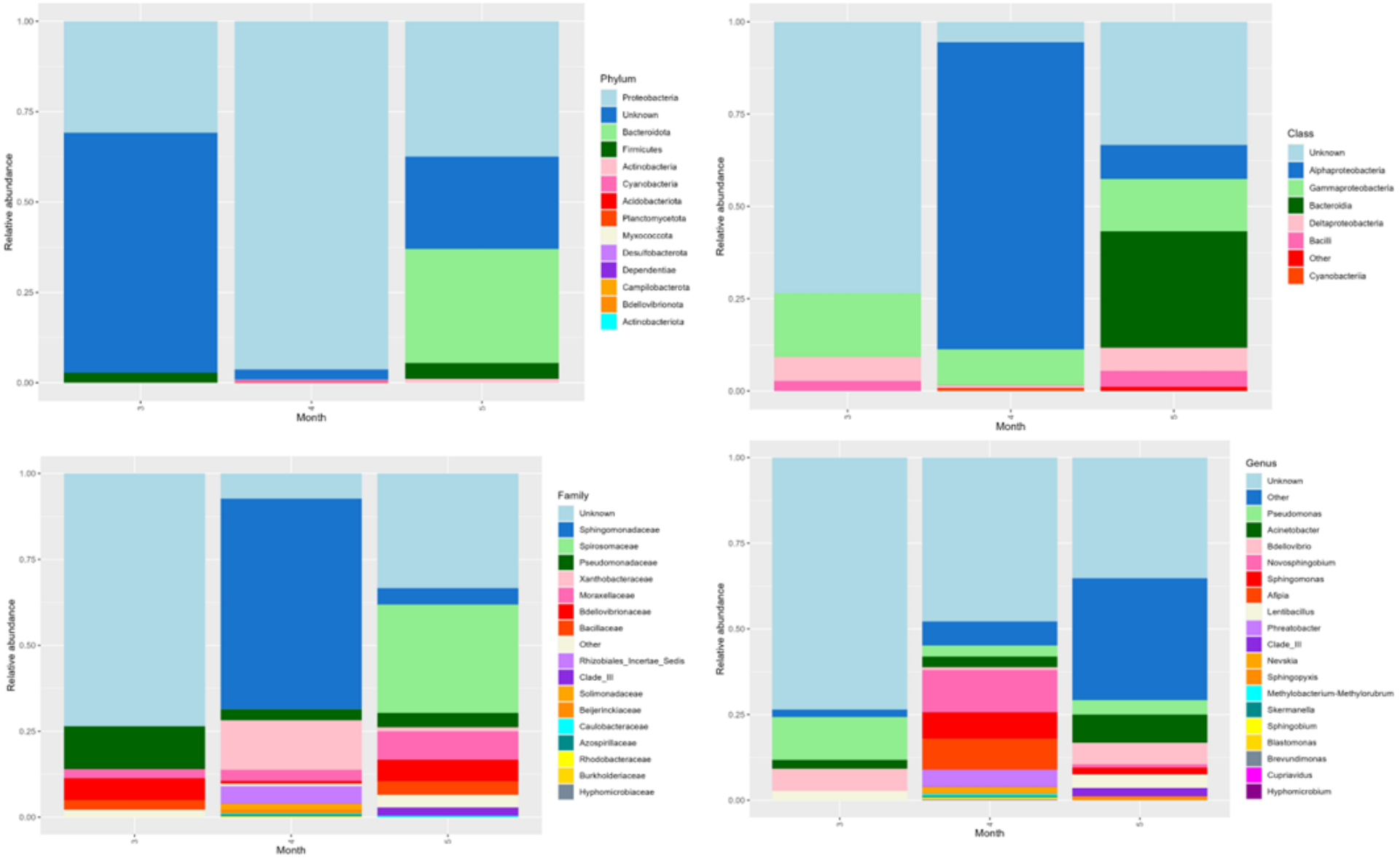

Figure 27: *Shows relative abundant bacterial phylum, class, Family and Genus within the months March, April and May for cooling tower A2. Phylum, Class, Family and Genus were filtered using a detection threshold of 0.01% (taxa must constitute at least 0.1 % of total sequences) and prevalence of 5% (taxa must be present in at least 5 % of samples).*

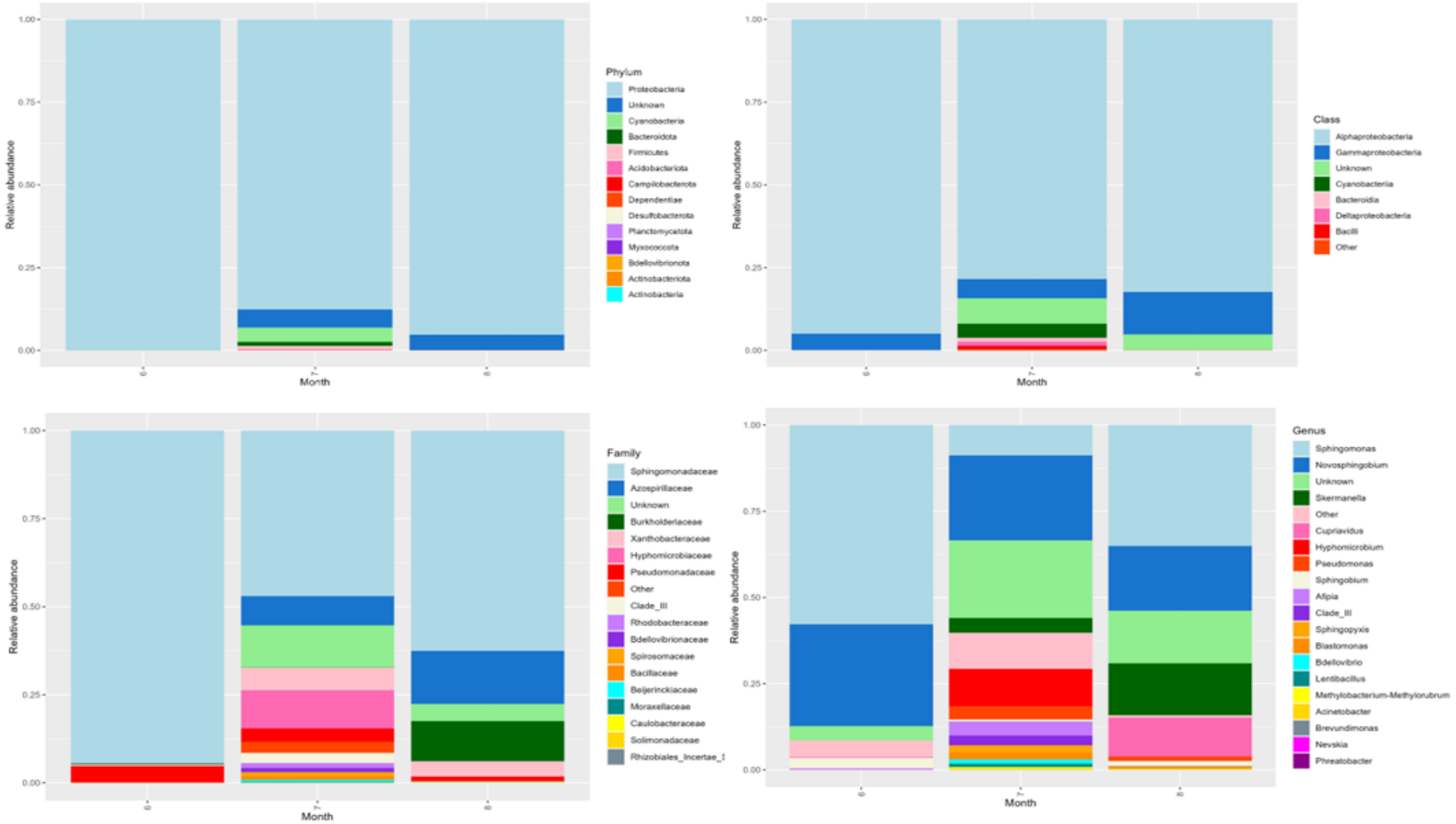

***Figure 28**: Shows relative abundant bacterial Phylum, Class, Family and Genus within the months June, July and August for cooling tower A2. Phylum, Class, Family and Genus were filtered using a detection threshold of 0.01% (taxa must constitute at least 0.1 % of total sequences) and prevalence of 5% (taxa must be present in at least 5 % of samples).*

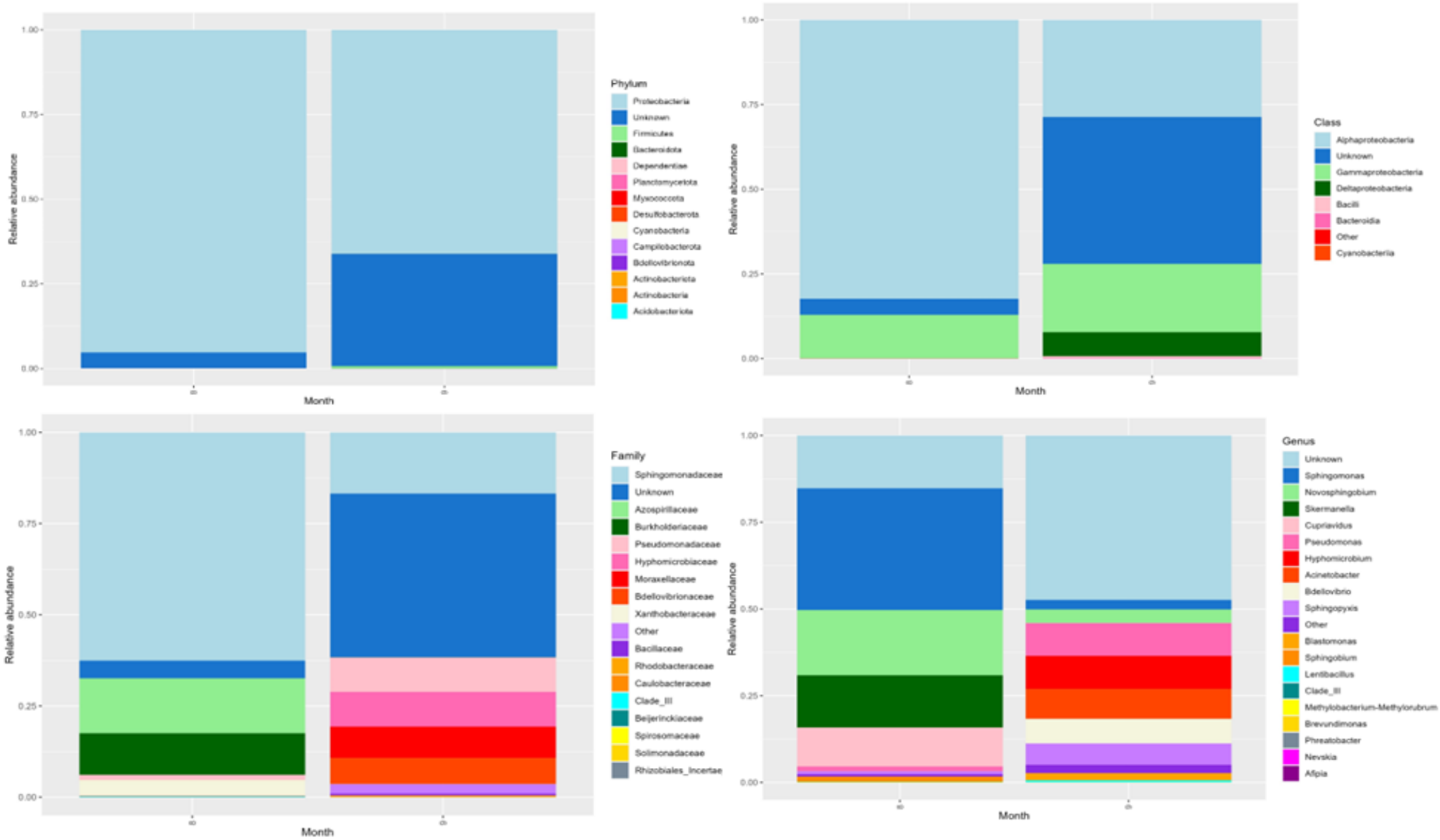

***Figure 29**: Shows relative abundant bacterial Phylum, Class, Family and Genus within the months August and September for cooling tower A2. Phylum, Class, Family and Genus were filtered using a detection threshold of 0.01% (taxa must constitute at least 0.1 % of total sequences) and prevalence of 5% (taxa must be present in at least 5 % of samples).*

5.6 Physicochemical Parameters

The physicochemical parameters measured across the four cooling towers over a two- year period provide insights into variation in calcium hardness, alkalinity, chloride conductivity, water temperature, pack temperature and Iron content. A summary of the mean, minimum and maximum values for each parameter is provided in Table 5.

***Table 5**: Mean water quality parameters across cooling towers. Values in bracket represent min and max value*

Cooling Tower	Hardness (ppm)	Alkalinity (ppm)	Chloride (ppm)	pH	Conductivity (µS/cm)	Iron (mg/L)	Temperature (°C)	Temperature Pack (°C)
A1	18.83 (8.0 - 46.0)	27.07 (15.0 - 50.0)	30.65 (10.0 - 83.0)	6.84 (6.3 - 7.5)	259.12 (39.0 - 615.0)	0.11 (0.1 - 0.5)	17.11 (12.3 - 25.3)	15.98 (9.8 - 22.2)
A2	43.33 (28.0 - 100.0)	29.59 (20.0 - 78.0)	39.24 (18.0 - 175.0)	6.9 (6.5 - 7.7)	172.29 (79.0 - 837.0)	0.07 (0.0 - 1.13)	17.37 (8.7 - 26.8)	14.63 (8.2 - 19.5)
B1	13.67 (9.0 - 20.0)	20.64 (15.0 - 25.0)	19.52 (5.8 - 45.0)	6.69 (6.1 - 7.2)	254.17 (74.0 - 563.0)	0.1 (0.1 - 0.1)	14.08 (6.6 - 21.6)	12.62 (8.1 - 16.9)
B2	21.3 (12.0 - 45.0)	21.57 (12.0 - 35.0)	75.97 (48.0 - 115.0)	6.61 (6.3 - 7.0)	273.22 (127.0 - 456.0)	4.33 (0.9 - 194.0)	13.96 (8.7 - 22.9)	16.15 (10.3 - 22.8)

5.6.1 Calcium Hardness Variability and *L. pneumophila* Correlation in Cooling Towers

Figure 30 displays that calcium hardness was recorded the highest in cooling tower A2, exhibiting dramatic fluctuations and pronounced peaks and troughs throughout the timeline. In contrast, the other cooling towers (A1, B1, and B2) generally maintained more stable calcium levels, hovering around 25 ppm, with A1 and B2 experiencing occasional peaks. Notably, there was evaluations in calcium hardness in cooling tower A2 coincided with *L. pneumophila* qPCR detection near the end of the months of April but a drop in calcium hardness coincided in July 2023.

5.6.2 Alkalinity Variability and *L. pneumophila* Correlation in Cooling Towers

Figure 31 illustrates that cooling towers A1 and A2 have a relatively higher average alkalinity, around 30 ppm, compared to cooling towers B1 and B2, which maintain an alkalinity around 20 ppm. Furthermore, towers A1 and A2 exhibit greater variability in alkalinity, including some extreme peaks. Notably, there were no significant changes in alkalinity levels in cooling tower A2 when *L. pneumophila* was detected.

5.6.3 Chloride Variability and *L. pneumophila* Correlation in Cooling Towers

Figure 32 shows that cooling tower B2 had the highest chloride levels throughout the sample timeline, followed by cooling tower A1 and A2 with the lowest being found in cooling tower B1. Throughout the timeline cooling tower A2 had the largest spikes, however, there was no peaks or drops when *L. pneumophila* occurred in April and July.

5.6.4 Conductivity Variability and *L. pneumophila* Correlation in Cooling Towers

Figure 33 illustrates that each cooling tower typically maintains conductivity levels between 200 and 400 microSiemens per cm. However, all towers except for B2 experience peaks that exceed 400 microSiemens per cm at various times throughout the observation period. Notably, there were no significant changes in conductivity one month before, during, or after the detection of *L. pneumophila* in any of the towers.

5.6.5 Water Temperature Variability and *L. pneumophila* Correlation in Cooling Towers

Figure 34 shows that cooling towers A1 and A2 reach higher peak temperatures compared to towers B1 and B2. Despite this, considerable variance and overlap exist among the temperatures across all towers. Specifically for tower A2, an increase in water temperature was noted in April and July 2023, coinciding with the detection of *L. pneumophila.* However, these temperature increases followed previous drops and remained within the typical range for this tower.

5.6.6 Pack Temperature Variability and *L. pneumophila* Correlation in Cooling Towers

The data illustrated in Figure 35 shows seasonal pack temperature variations within four cooling towers, with measurements taken from the middle of the pack. The data indicates an increase from March to July, with temperature peaks observed in April and July. These peaks correlate with the detection of L. pneumophila in the same months. Despite a temperature decline in August and September, the bacterium was detected again in September

5.6.7 Iron Variability and *L. pneumophila* Correlation in Cooling Towers

Iron levels were found to be same throughout the entire testing period at 0 ppm. Therefore, no change was found throughout the testing period and cooling towers.

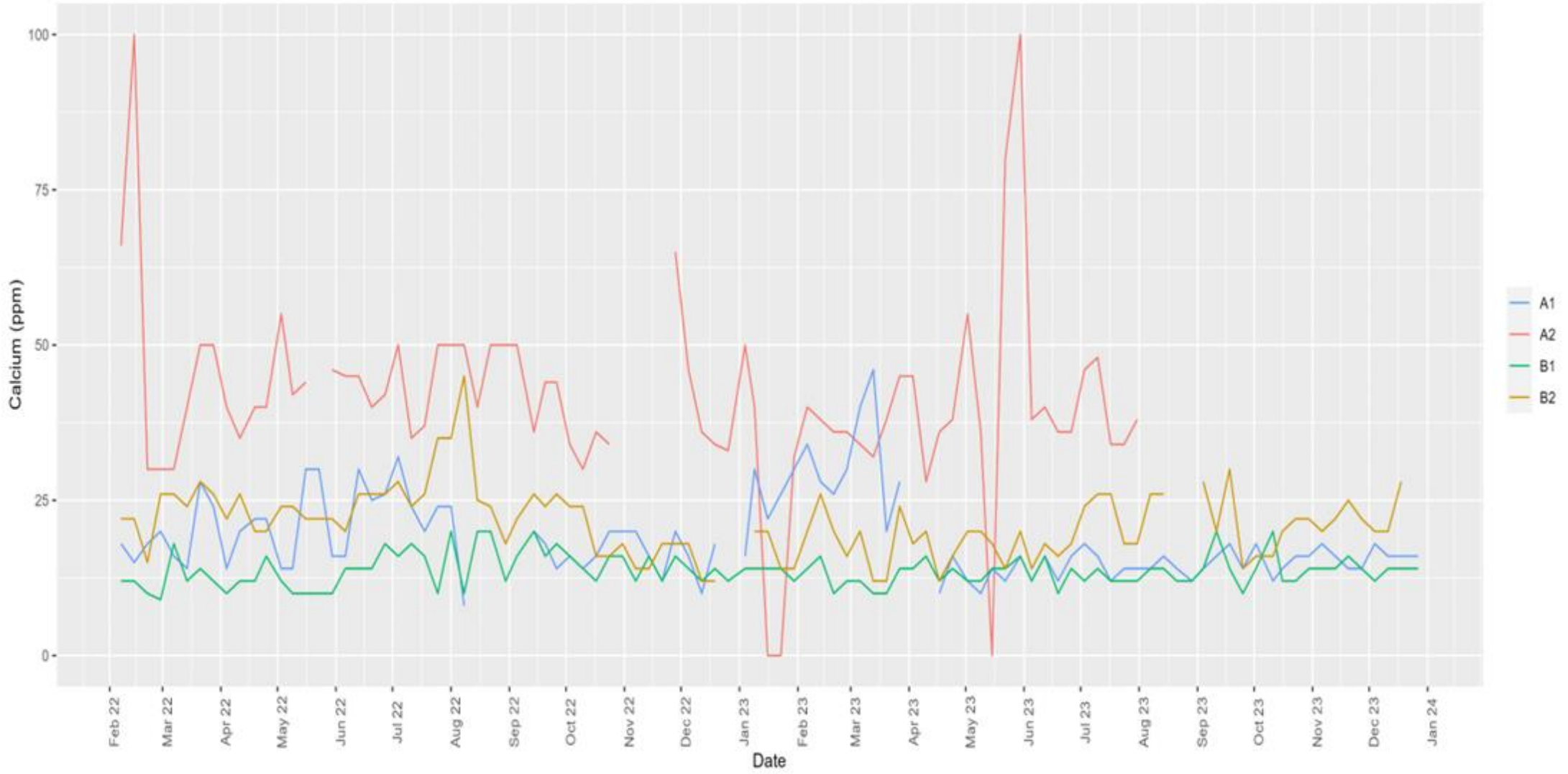

Figure 30: *Time series plot showing fluctuation in calcium levels (ppm) recoded by four different cooling towers (A1, A2, B1, B2) from February 2022 to June 2023. Each Cooling tower is represented by a distinct colour to illustrate trends and variations across the specific period.*

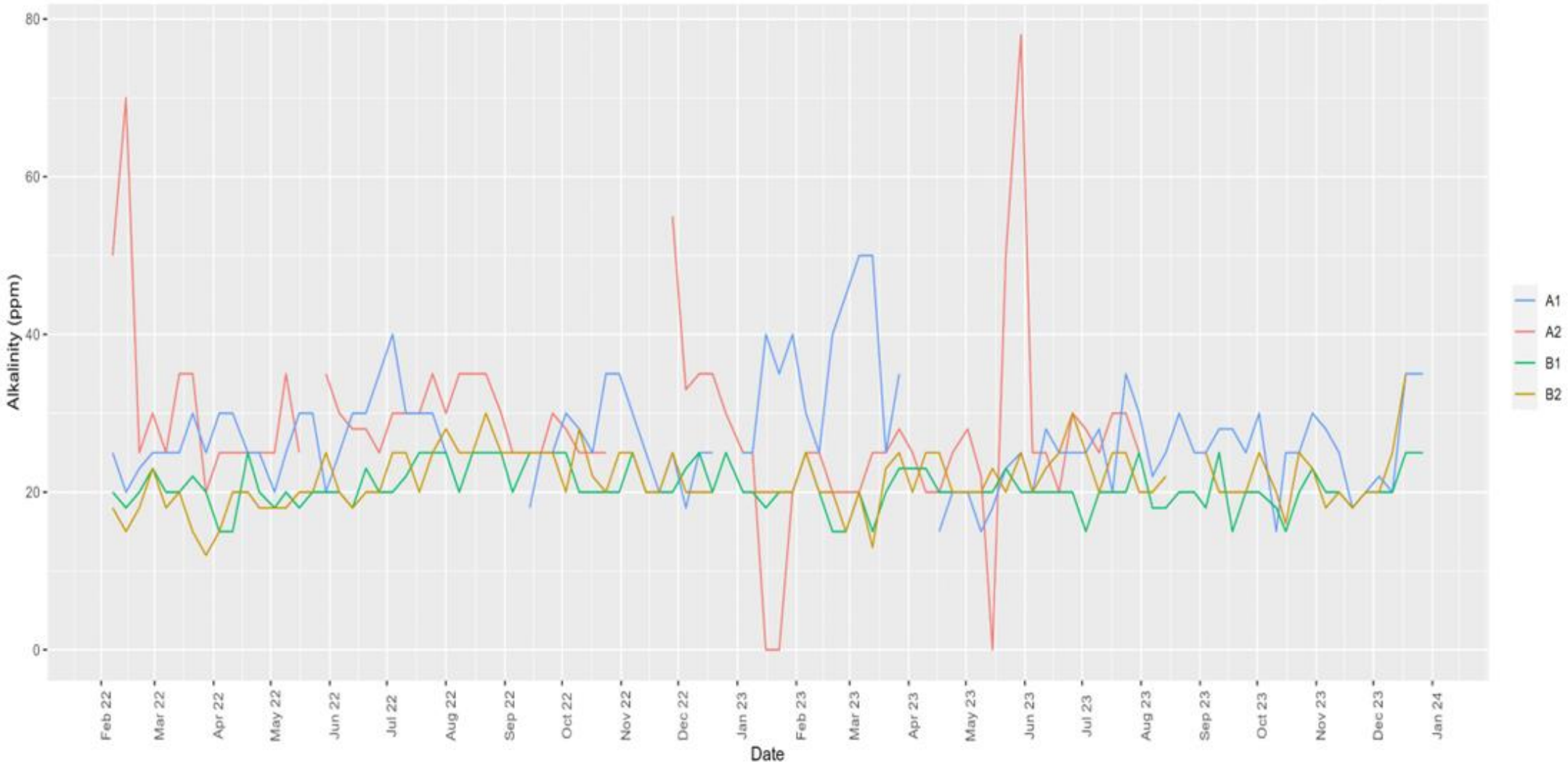

***Figure 31**: Time series plot showing fluctuation in Alkalinity (ppm) recoded by four different cooling towers (A1, A2, B1, B2) from February 2022 to June 2023. Each Cooling tower is represented by a distinct colour to illustrate trends and variations across the specific period.*

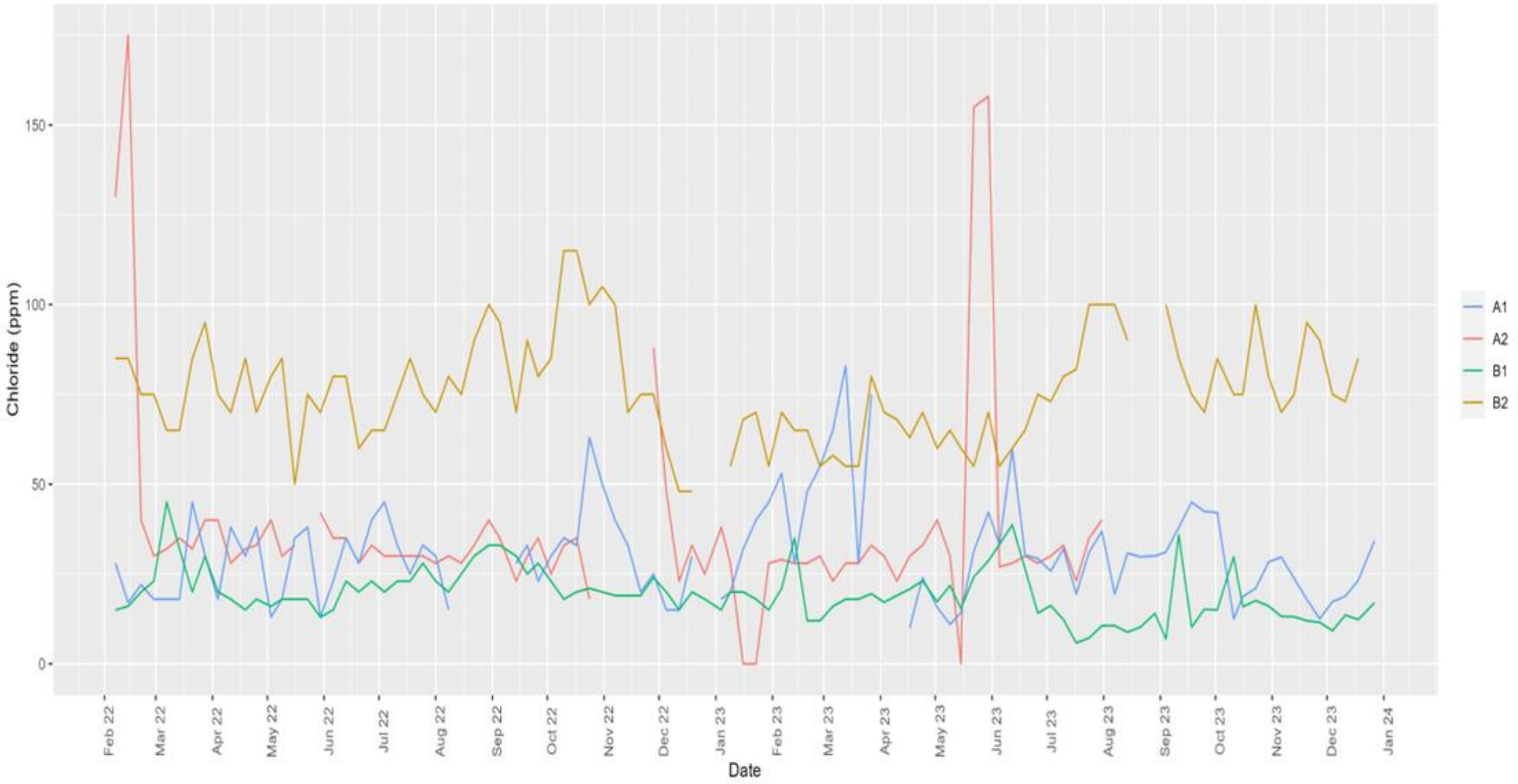

***Figure 32**: Time series plot showing fluctuation in chloride (ppm) recoded by four different cooling towers (A1, A2, B1, B2) from February 2022 to June 2023. Each Cooling tower is represented by a distinct colour to illustrate trends and variations across the specific period.*

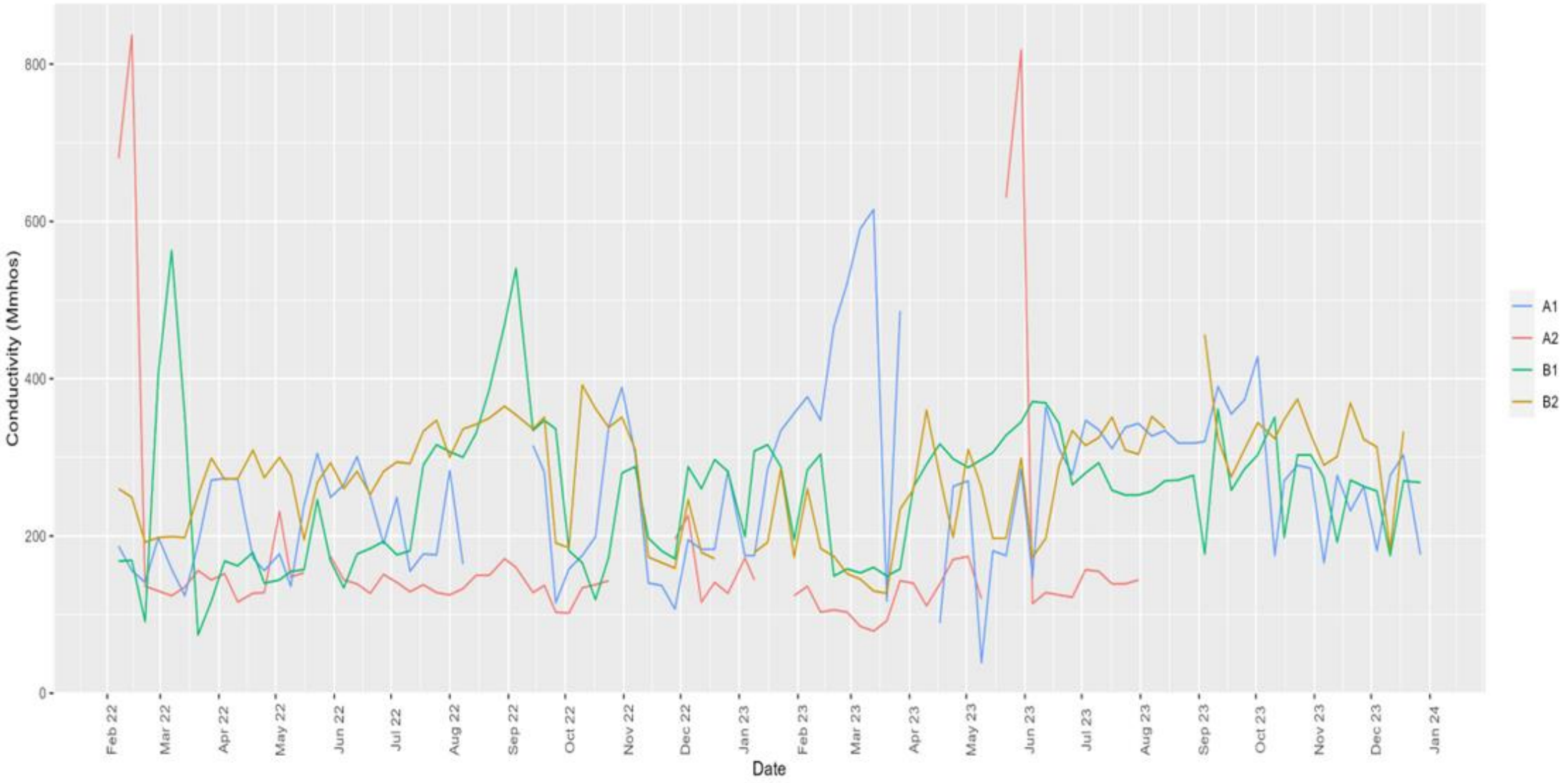

***Figure 33**: Time series plot showing fluctuation in conductivity (Mmhos) recoded by four different cooling towers (A1, A2, B1, B2) from February 2022 to June 2023. Each Cooling tower is represented by a distinct colour to illustrate trends and variations across the specific period.*

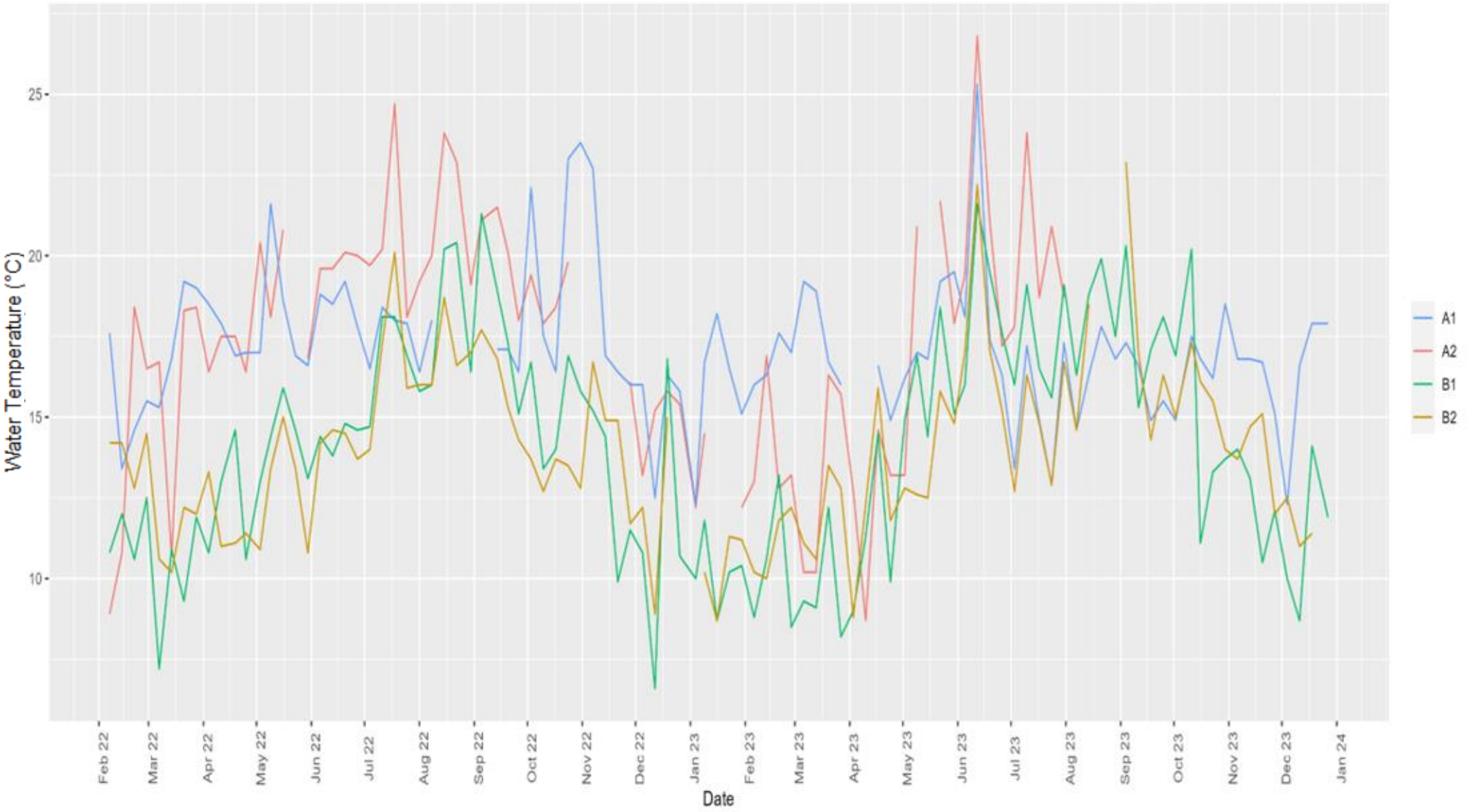

***Figure 34**: Time series plot showing fluctuation in water temperature (°C) recoded by four different cooling towers (A1, A2, B1, B2) from February 2022 to June 2023. Each Cooling tower is represented by a distinct colour to illustrate trends and variations across the specific period.*

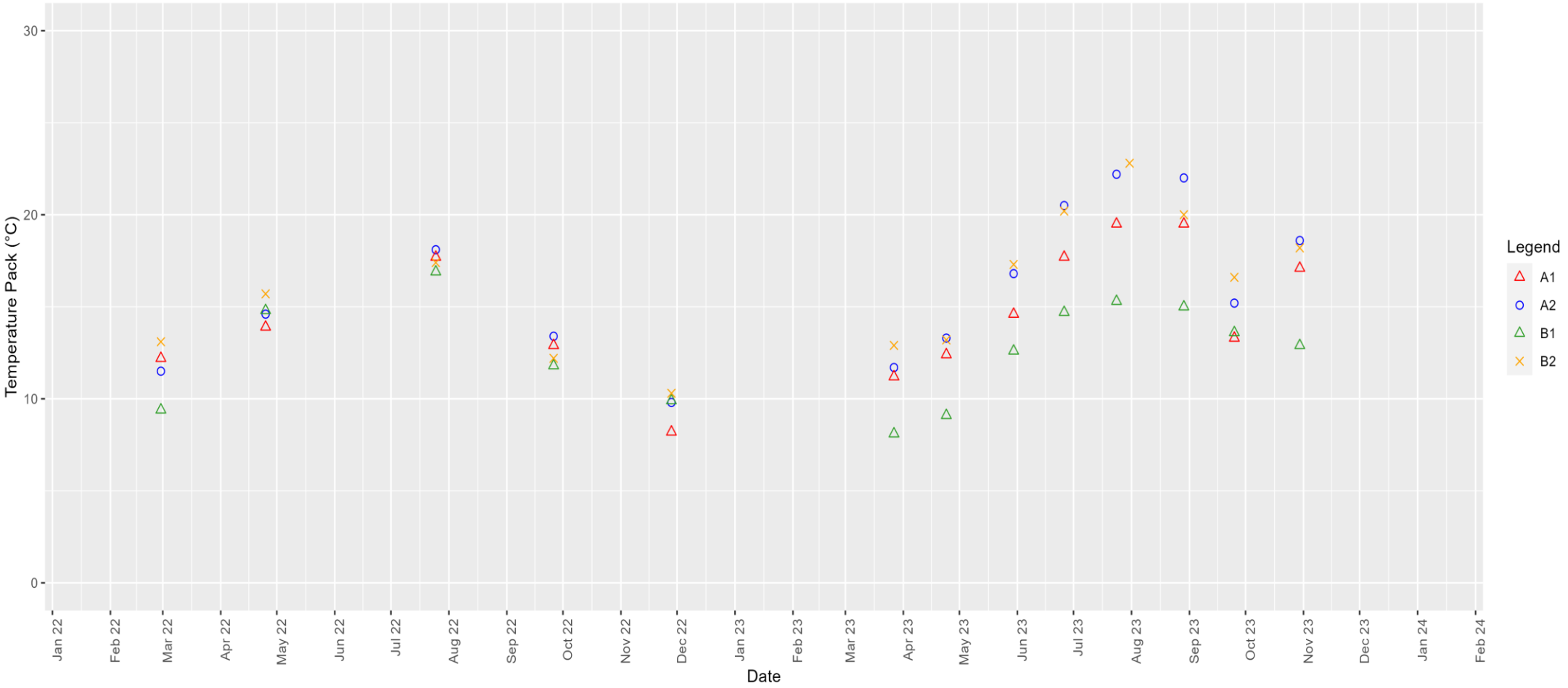

***Figure 35**: Time series plot showing fluctuation in pack temperature (°C) recoded by four different cooling towers (A1, A2, B1, B2) from February 2022 to June 2023. Each Cooling tower is represented by a distinct colour to illustrate trends and variations across the specific period.*

Chapter 6 Discussion

The study conducted at the field site leveraged a unique research environment, characterised by its diverse array of cooling towers, each distinguished by its own engineered structure, specific biocide regimes, and distinct Physicochemical parameters. In this study, 'bulk water' samples refer to planktonic samples collected from the cooling tower basin following the ISO 11731 protocol. These towers provided a robust platform for the development, optimisation, and application of a tailored biofilm sampling strategy. The accessibility of these cooling towers enabled an extensive and methodical sampling routine over an 18-month period. This routine not only differentiated between biofilm and bulk water samples but also considered variations in engineered structures, biocide regimes, seasonal changes, and maintenance cycles. The presence of an onsite laboratory was also pivotal, allowing for the immediate analysis of both biofilm samples and bulk water. This capability enabled near real-time assessment of microbial concentrations, viability and the presence of *Legionella* spp., including *L. pneumophila.* Additionally, samples could be DNA extracted on site and stored immediately for downstream microbial community analysis. This unique combination of sophisticated on-site facilities, diverse sampling strategies, and comprehensive real-time data analysis enabled a thorough investigation of this research aims and objectives.

6.1 Biofilms as Lead Indicators

6.1.1 Exclusive discovery of *L. pneumophila* in Biofilms

In this study, the first known detection and quantification of *L. pneumophila* within biofilm samples extracted from operational cooling towers is presented. This discovery is particularly significant given the rarity of *L. pneumophila* occurrences in cooling towers within Europe and the USA, where its detection is generally considered a sporadic event. The exclusive identification of *L. pneumophila* within biofilm samples not only showcases the potential of biofilms as critical indicators for monitoring efforts but also underscores the limitations of conventional monitoring strategies, which are confined to planktonic samples and would have overlooked such detections within the same study period. The findings emphasise the need for a more comprehensive monitoring policy, practice, and even regulations, which should integrate the assessment of biofilm samples alongside traditional planktonic samples complementing each other. Integrating biofilm assessments offers a forward-looking approach by enhancing the sensitivity and comprehensiveness of monitoring efforts, significantly improving the capabilities for early detection and timely implementation of a water safety plan. These are essential in mitigating the risk of *L. pneumophila* outbreaks and maintaining public health.

6.1.2 Biofilms as Ecological Niches for *Legionella* spp.

For the first time, this study has identified *Legionella* spp. in higher concentrations within cooling tower biofilm samples compared to cooling tower bulk water samples, further corroborating the pivotal role of biofilms as an ecological niche and their potential as lead indicators. Additionally, within operational cooling towers, a lack of correlation was observed between biofilm and bulk water *Legionella spp.* concentrations, indicating that these represent discrete sample types with distinct microbial dynamics. While such findings are not novel in other engineered water systems, as demonstrated in previous studies (van der Kooij et al., 2005; De Filippis et al., 2017;

Margot et al., 2024), this study presents the first demonstration of this phenomenon within the specific context of operational cooling towers. These studies have shown that biofilms in drinking water distribution networks and building plumbing systems often harbour higher concentrations of Legionella spp. compared to bulk water. The importance of biofilms is widely recognised for the survival of *Legionella spp.* in diverse environments, such as freshwater systems (Declerck, 2010), water distribution systems (De Filippis, 2018; Waak, 2018), shower hoses (Cavallaro, 2023), and in vitro systems (Taylor, 2013; Stewart Muthye and Cianciotto, 2012). This is further highlighted in various guidelines (ASHRAE, 2019; WHO, 2007; HSE, 2014). These findings are attributed to the protective characteristics of biofilms, such as nutrient availability and resistance to shear forces, which support *Legionella spp.* growth (Costerton, 1995; Flemming, 2020; Pereira, Silva, and Melo, 2021).

This study builds on these observations by providing novel insights into *Legionella* concentrations in biofilms compared to bulk water, and whether these two sample types are correlated, within the specific context of operational cooling towers. Cooling towers exhibit unique microbial dynamics and water quality parameters compared to other engineered systems (Tsao et al., 2019), yet their biofilms have not been systematically studied until now. This research gap can be attributed to four main challenges: (1) the assumption that cooling tower biofilms behave similarly to those in other engineered water systems without systematic evidence; (2) the absence of standardised biofilm sampling methods; (3) the logistical difficulties of obtaining biofilm samples compared to more accessible bulk water samples; and (4) ongoing debates regarding the ability of *Legionella spp.* to persist and proliferate without protozoa, which has led to an underestimation of the role of biofilms (Barbosa, 2024). This study reveals that routine planktonic sampling methods (bulk water samples) remains a critical component of Legionella surveillance and public health. However, they do not capture the distinct bacterial concentration and communities including *Legionella* spp., that reside within biofilms. These findings underscore the need for improved cooling tower management strategies that incorporate biofilm sampling. As demonstrated in this study, biofilm samples can be readily and reliably collected across the cooling tower pack using a developed and optimised biofilm sampling regime. This highlights their pivotal role in the *Legionella* life cycle and establishes biofilms as a viable and valuable lead indicator of *Legionella spp.* contamination, warranting their integration into traditional monitoring practices.

A further consideration is the role of protozoa, such as amoebae, which are known to graze on bacterial populations within biofilms, fostering environments where *Legionella spp.* can persist and replicate intracellularly (Declerck, 2010). They also contribute to nutrient cycling by feeding on bacteria and recycling organic matter, which further supports *Legionella* proliferation (Nisar et al., 2020). While protozoa are recognized as important hosts that enhance *Legionella* survival and replication, they were not included in this study's investigation of biofilms within cooling towers for several reasons. Protozoa-*Legionella* interactions are well-documented in bulk water systems as they can serve as intracellular hosts for *Legionella*, protecting the bacteria from environmental stressors, disinfectants, and even predatory bacteria (Boamah et al., 2017), rendering this aspect less novel for the scope of the present research. Despite the relationship between biofilms and protozoa, this study was specifically designed to explore biofilms as independent ecological niches and lead indicators of *Legionella spp.* presence, focusing on their unique role in cooling

tower environments. Moreover, previous research suggests that *Legionella spp.* can proliferate within biofilms independently of protozoa (Surman et al., 2002; Abdel-Nour et al. 2013), supporting this study's approach to investigating biofilms without this added variable. Finally, practical and logistical considerations also influenced this decision. Incorporating protozoa into the analysis would have required additional sampling protocols, which could complicate the longitudinal assessment of *Legionella* dynamics in biofilms versus bulk water. While protozoa undoubtedly play a significant role in *Legionella* ecology, the scope and design of this study prioritised biofilms as self-contained ecosystems. Future studies might delve into protozoa-*Legionella* interactions within biofilms, but this research has established a critical foundation for understanding biofilms as lead indicators of *Legionella spp.* in cooling tower environments.

6.1.3 Novel and Optimised Sample Technique to Determine and Quantify *L. pneumophila* and spp.

This study successfully demonstrates that biofilm samples can be collected and analysed from various operational cooling towers, including two distinct cooling tower structures (induced crossflow and induced draft flow) and two biocide regimes, using the novel and optimised sampling technique developed in this research. To the best of our knowledge, this is the first study to design and validate a biofilm sampling method that can be consistently applied across operational cooling towers, addressing a critical gap in Legionella surveillance practices. This method overcomes challenges such as the lack of standardisation and logistical complexities in biofilm research within cooling towers. The robustness of the novel method is evidenced by the successful collection and analysis of hundreds of biofilm samples, reinforcing its practicality and reliability. This study's biofilm sampling approach specifically targets the cooling tower pack, where biofilms are most likely to thrive and develop, and where Legionella aerosolisation is most likely to occur. Furthermore, cooling tower pack is also located just upstream of the standardised bulk water sampling locations, such as the basin or makeup water, making it a logical and complementary site for sampling biofilms.

The biofilm sampling strategy also incorporates key environmental considerations, such as temperature gradients across the cooling tower pack and variations in biofilm surface area, whilst ensuring it remains practical for routine application. By offering a clear, reproducible protocol that does not require additional equipment, operational changes, or specific location and time constraints, this approach ensures practicality and accessibility for routine application. Thereby providing a strong foundation for establishing standardised biofilm sampling practices in operational cooling towers, improving the consistency of Legionella surveillance and supporting better public health outcomes.

Another key methodological decision was the use of swabs for biofilm sampling, chosen for their practicality, flexibility and already established in routine guidelines for showerheads (CDC, 2019). Swabs enabled immediate sampling from cooling tower pack surfaces without requiring pre-installed devices or operational shutdowns, minimising disruption. In contrast, methods such as coupons or biofilm monitoring devices (e.g. Robbins devices, rotating disc reactor or flow cells) (Azeredo et al., 2017) introduce foreign substrates that can affect biofilm development, bacterial composition or

require controlled laboratory conditions that do not replicate the complex biology and physiochemical conditions within operational cooling towers. Similarly, while advanced methods such as impedimetric biosensors show promise, they remain expensive and insufficiently validated for use in cooling tower environments (Islam et al., 2020). This study's swab-based approach represents an innovative, practical solution that bridge the gap between laboratory-based techniques and real-world application, enhancing routine surveillance practices and promoting reliable Legionella detection.

Despite limited research in this area, the results of this study closely align with those of Waak et al. (2018) and De Filippis et al. (2018), who reported that L. pneumophila was exclusively present in biofilm samples within water distribution systems. Additionally, Garner et al. (2018) observed that Legionella spp. were more abundant in biofilms than in bulk water, further validating the critical role of biofilms in water distribution systems. The corroboration of these findings, despite the use of different engineering structures in this study, further proves the reliability and adaptability of the novel biofilm sampling routine developed here. This consistency across varied systems highlights the robustness of the method and its potential for broader application. By providing a validated and reproducible method for biofilm sampling in cooling towers, this study contributes to filling a critical gap in the literature, promoting future research, and emphasizing the importance of biofilm sampling for the detection and quantification of *L. pneumophila*. Ultimately, this advancement enhances monitoring efforts, supports early detection, and facilitates the timely implementation of water safety plans to protect public health.

6.1.4 Integrating Biofilm Sampling with Rapid Detection: Advancing Beyond Bulk Water Monitoring

An increasing awareness of the need for early detection of *L. pneumophila* has sparked significant research into emerging detection techniques. These rapid detection methods exhibit distinct advantages over traditional culturing methods, as discussed in Section 2.3. Most existing techniques, however, have been developed and tested on planktonic *Legionella* in bulk water samples, leaving their applicability to biofilms relatively unexplored. This study sought to bridge this gap by exploring how biofilm sampling could be integrated with rapid detection techniques to enhance *Legionella* monitoring practices. Specifically, it evaluated the performance of two commercial quantitative polymerase chain reaction (qPCR) methods—Genomadix and Genesig—for detecting *L. pneumophila* and *Legionella spp.*, respectively, in both biofilm and bulk water samples.

The results highlight that rapid detection methods, such as qPCR, can be successfully applied to both biofilm and bulk water samples for detecting *Legionella spp.* The detection of *Legionella spp.* in bulk water samples aligns with prior studies on other engineered water systems (e.g., Wullings and van der Kooij, 2010; Waak et al., 2018; Buse et al., 2020), which demonstrated the effectiveness of qPCR for identifying planktonic *Legionella*. However, this study revealed several advantages of using biofilm as the sample type. *L. pneumophila* was detected exclusively in biofilm samples, and *Legionella spp.* was consistently found in higher concentrations in biofilms compared to bulk water. These results provide a more accurate representation of *Legionella* levels within cooling tower systems, underscoring the limitations of protocols that focus solely on planktonic samples.

Incorporating biofilm sampling into routine monitoring practices is essential for obtaining a more comprehensive understanding of *Legionella* risks. This integration offers a critical step toward improving rapid detection methods and enhancing environmental monitoring frameworks.

Beyond qPCR, this study evaluated other emerging detection methods, including lateral flow assays, immunomagnetic separation, and an additional commercial qPCR kit. However, these methods failed to reliably or accurately detect *L. pneumophila* when tested against known positive *L. pneumophila* lenticule discs (NCTC 12821). Variability in their performance likely stemmed from limited test availability, sparse methodological data (e.g., for lateral flow assays), and test-specific constraints, such as the specificity of immunomagnetic separation for wild strains as indicated by the manufacturer. Differences in detection efficiency compared to studies like the Nephros PluraPath (2024) further highlight the urgent need for standardized methodologies, particularly in stock preparation and concentration protocols.

While these emerging detection methods were not applied to biofilm samples in this study, they may perform better when adapted for biofilm contexts. The higher concentrations of *Legionella spp.* observed in biofilms compared to bulk water suggest that biofilm sampling could align better with the sensitivities of these methods than planktonic testing. Moreover, the exclusive detection of *L. pneumophila* in biofilm samples during this study reinforces the importance of prioritising biofilm sampling in routine monitoring practices to more reliably detect *Legionella* and better protect public health over more emergent detection methods.

6.2 No Correlation between Cell Concentration and *Legionella* spp.

In examining the correlation between cell concentration (TCC), cell viability (ICC), and the presence of *Legionella* spp., including *L. pneumophila,* the findings challenge the conventional expectation that higher bacterial counts create a conducive environment for *Legionella* growth, an assumption often uncritically accepted in the existing literature (Springston, 2017). This study also explored, for the first time, the correlation between the ICC to TCC ratio and the presence of *Legionella*, adding a new dimension to if cell counts can correlate to *Legionella*. A noteworthy aspect of this study was the inclusion of both biofilm and bulk water samples, with biofilms being a particularly critical focus due to their previously underexplored role in *Legionella* ecology. When comparing cell counts before and after *L. pneumophila* was detected, in most instances, there were no significant differences in cell counts associated with the detection of *L. pneumophila.* Intriguingly, in scenarios where differences were observed, cell counts were lower at the time of *L. pneumophila* detection, contradicting the idea that a higher bacterial load is a prerequisite for *Legionella* proliferation.

A particularly intriguing outcome of this investigation was the isolated detection of *L. pneumophila* in one cooling tower (A2), despite cooling tower B2 having significantly higher TCC and ICC counts. Additionally, it was observed that *Legionella* spp. concentrations were similar between cooling towers A1 and A2, even though A2 exhibited significantly higher cell counts. These findings imply that the presence and concentration of *L. pneumophila,* as well as other

Legionella species, may be influenced by factors beyond simple microbial concentrations and viability, casting doubts on the reliability of cell counts as a standalone indicator for assessing *Legionella* risk.

In this study, the ratio of cell viability to cell concentration was analysed to determine its relationship with *Legionella spp.* concentrations. Observations showed that despite similar *Legionella spp.* concentrations in cooling towers A1 and A2, *L. pneumophila* was detected exclusively in cooling tower A2. Both A1 and A2 exhibited a similar, low intact to total cell ratio, indicating a predominance of non-viable cells within their microbial communities. Conversely, cooling towers B1 and B2, which had much higher intact to total cell ratios, showed no presence of *Legionella*. These findings suggest that the ratios of viable and non-viable cells cannot reliably predict the presence of *Legionella*, and the viability of the surrounding microbial community may not be a crucial factor for the survival and growth of *Legionella*. The lack of correlation between the cell viability to cell concentration ratio and *Legionella spp.* currently cannot be compared to existing research due to a lack of studies. The inference that *Legionella* does not require viable microbial communities has been evidenced for the first time and aligns with the findings conducted by Temmerman et al. (2006), who found that *Legionella* can exhibit necrotrophic growth by utilising nutrients from heat-killed microbial cells in tap water.

The observed detachment between cell concentration, cell viability and *Legionella* detection underscores the complexity of *Legionella* ecology, suggesting that its proliferation within water systems cannot be predicted by bacterial load alone. Consequently, this calls for a more comprehensive approach that considers not just the microbial load but also the composition of the bacterial community and key physicochemical parameters to fully understand the conditions that favour *Legionella* growth and persistence within both biofilms and bulk water.

6.2.1 Comparative Analysis with Biofilm Studies

The lack of correlation between cell counts and *Legionella* within biofilm samples is a distinctive feature of this investigation, particularly given the scarcity of literature directly addressing this relationship, yet it is prevalent in 'expert judgement' and practice. This finding stands in contrast to a study by van der Kooij et al. (2017), which reported significant, albeit weak, correlations between total cell count, heterotrophic plate count, and *Legionella* growth within biofilms. Importantly, their study used a laboratory setup that differed significantly in physicochemical parameters from the operational cooling towers we examined. The use of flow cytometry in these real-world settings enabled a more detailed and comprehensive analysis, allowing for higher sensitivity in comparing cell concentrations and assessing cell viability relative to *Legionella* concentrations. This methodology has enhanced our understanding of how cell concentrations and viability correlate within both biofilms and bulk water with *Legionella* presence within operational cooling towers.

6.2.2 Comparative Analysis with Bulk Water Studies

This study also found that bulk water cell counts also did not correlate with *Legionella* presence that again, contradicting the expectation that higher bacterial counts create a conducive environment for *Legionella* growth. This finding actually aligns with some studies, for instance, the work of Sanchis, Inza & Figueras (2023) challenges traditional assumptions by showing that heterotrophic plate count (HPC) values above 100 CFU/ml do not necessarily

correlate with an increased risk of *Legionella* presence. Their findings, which rely on conventional methods for evaluating cooling tower bulk water samples, suggest that only HPC values under 100 CFU/ml could serve as reliable indicators for the absence or minimal concentrations of *Legionella*. Similarly, Duda et al. (2015) underscore the limitations of using HPC as a predictive tool for *Legionella* colonisation, demonstrating that such measures failed to indicate *Legionella* spp. presence in over 64% of cases involving cooling tower samples. These studies, while methodologically diverse to this research, underscore a critical point: the predictive value of HPC for *Legionella* risk assessment is highly variable and often unreliable. Conversely, research by Campaña et al. (2023) and Kyritsi et al. (2018) suggested a potential relationship between overall microbial concentration in bulk water samples and *Legionella* presence, positing that under certain environmental conditions, microbial load could indeed reflect an increased *Legionella* risk. The discrepancy between these findings and those presented here, as well as those reported by Duda et al (2015) & Sanchis, Inza, & Figueras (2023) highlights the nuanced and complex nature of *Legionella* ecology. This complexity necessitates a move beyond simple numerical analyses. To truly grasp *Legionella* dynamics, identifying bacterial compositions or physicochemical factors that either promote or inhibit *Legionella* growth is crucial for developing more effective management strategies.

This study's use of flow cytometry represents a significant methodological advancement, allowing for the detection of viable but non-culturable (VBNC) cells, which are often overlooked by traditional plate counts. The ability to identify these cells is paramount, as they may play a crucial role in *Legionella* survival, proliferation, and pathogenicity and less than 1% of bacteria found in environments are culturable (Wang, 2010). Thus, the approach developed and presented here not only provides a more accurate depiction of the microbial load but also showcases the importance of utilising advanced methodologies to understand the complexity of microbial interactions within cooling towers. The sensitivity and comprehensiveness of these methods reveal the limitations in relying solely on conventional microbial load measurements for *Legionella* risk assessment. Given these considerations, alignment between the research findings presented here and those of Sanchis, Inza, & Figueras (2023), despite their use of traditional methods, further emphasizes the need for a multifaceted approach to *Legionella* risk assessment. This approach should not only consider cell counts but also the specific characteristics of the microbial community and other factors beyond mere microbial abundance.

Considering the limitations of relying solely on cell counts for *Legionella* detection, it is important to recognise the need for a more comprehensive strategy for monitoring *Legionella*. Although these counts are still valuable for assessing biocide efficacy, cleanings and malfunctions that may lead to bacterial growth in cooling towers, they should be complemented with a broader range of indicators. These other indicators should focus on microbial community characteristics, physicochemical parameters, and interactions between biofilms and bulk water on the detection of *Legionella* spp. especially pneumophila. Gradually introducing these elements can enhance the predictive models for *Legionella* presence and provide a strategic advantage in customizing monitoring and control measures for water systems.

6.3 Biofilm Community & Structure

This work has shown that sampling biofilm using a specific detection method can yield new insight and advance indication of *L. pneumophila,* while also showing that cell numbers are not enough. Biofilms are complex multispecies environments, and literature has suggested that biofilms communities play a pivotal role in the survival and proliferation of *Legionella* within cooling towers (Abu Khweek, 2018; Di Pippo, et al., 2018; Pereira, Silva & Melo., 2021). These communities have not been studied or quantified, particularly from the pack of an operational cooling tower, with little information about the microbial composition and structure present. Another crucial aspect of biofilms is the substantial amounts of EPS that might influence the survival and detectability of *Legionella* and although not the focus of this study, the importance of EPS warrants further investigation, as it likely plays a critical role in the persistence and detection of *Legionella* within these environments.

The selection of the pack, constructed from PVC, for use in cooling tower systems was strategic, primarily due to its massive surface area and identification as a potential hotspot for the aerosolisation of *Legionella*. The large surface area provided by the pack is instrumental in facilitated microbial attachment and nutrient accumulation. Moreover, its structural design moderates water flow, creating an optimal environment for biofilms to establish and mature over time. Furthermore, PVC has been demonstrated to exhibit the highest species richness among various material used within water distribution systems (Li et al., 2021), a factor correlated with *Legionella* spp., in cooling towers (Llewellyn et al., 2017) and inherently promotes biofilm formation (Li et al., 2021).

In this study examination of microbial community compositions associated with *L. pneumophila* presence within biofilms, pivotal insights emerge. Notably, the detection of *Legionella* coincides with the presence of phyla Cyanobacteria and Firmicutes, alongside families such as Sphingomonadaceae, Moraxellaceae, and Xanthobacteraceae, known for their ability to utilise organic compounds. Additionally, families like Hypomicrobiaceae and Rhodobacteraceae, involved in nitrogen fixation, were consistently identified when *L. pneumophila* was detected. Intriguingly, instances where *L. pneumophila* was detected also showed the presence of the family Bdellovibrionaceae, suggesting a predatory mechanism that could indirectly benefit *Legionella* by reducing competition. These observations indicate that *L. pneumophila* within biofilms may thrive via two primary ecological mechanisms: First, through ecological niches rich in bacterial communities that enhance the availability of organic compounds or nitrogen; second, through ecological dynamics that suppress competitive bacterial populations. Crucially, the variation in bacterial families and genera observed each time *L. pneumophila* was detected underscores a significant insight: the specific species present may be less critical than the functional capabilities of the overall bacterial community. This finding aligns with the work of Llewellyn et al. (2017), who observed a correlation between *Legionella* presence and higher species richness in bulk water samples. Such parallels suggest that the resilience and proliferation of *Legionella* in biofilm environments are intricately linked to the functional diversity and ecological roles of the surrounding microbial community, rather than the dominance or absence of any single taxon.

This study's subsequent analysis of bacterial communities within biofilms revealed significant differences compared to bulk water samples, highlighting the unique presence of the phyla *Bacteroidota*, Cyanobacteria, and Acidobacteriota,

which are exclusively found in biofilms. There was also a higher abundance of specific families such as Xanthobacteraceae, Clade III, Hypomicrobiaceae, and Burkholderiaceae, along with other rare taxa. This contrasts with the findings in bulk water samples, where these specific phyla and families are typically less dominant or absent. Furthermore, a core community predominantly containing the phylum *Proteobacteria*, notably Alpha*proteobacteria*, alongside the consistent presence of the genera Sphingomonas and Novosphingobium, was detected. Comparisons with findings from Di Gregorio et al. (2017) and Wang et al. (2014) also show a similar core community present within biofilms. This commonality underscores a fundamental characteristic of biofilm microbial communities across various cooling tower systems, suggesting potential ecological roles these genera play in biofilm stability and function. Moreover, both referenced studies and our current findings observe a long tail of taxa, characterised by a plethora of bacterial species present in small abundances, indicative of a vast, intricate microbial ecosystem. Simultaneously, our analysis has revealed a notable increase in the abundance of Delta*proteobacteria* and Gamma*proteobacteria*, particularly within the families Bdellovibrionaceae and Pseudomonadaceae, presenting a unique microbial diversity in this study's samples. This distinct composition contrasts with the communities documented in previous studies, suggesting that the specific sampling location on the pack used in this research, as opposed to the basin sampled in prior studies, could contribute to observed variances in microbial community structure. These environmental variations might influence microbial selection pressures, nutrient gradients, and physical conditions, leading to the nuanced composition of these communities we have identified.

The divergence between microbial populations in biofilms and bulk water underscores the limitations of traditional water sampling methods. Upon closer examination, it becomes clear that biofilm microbial communities exhibit a core set of taxa, primarily within the Proteobacteria phylum, consistently observed across various studies and environmental conditions. This core community represents a foundational aspect of biofilm ecosystems, suggesting essential ecological roles in stability and function. However, it is the nuanced composition of low-abundance and rare taxa within biofilms that adds a significant layer of complexity. These taxa, though present in smaller numbers, can profoundly influence microbial interactions and ecological dynamics. They may provide additional organic compounds and nitrogen or even play a role in reducing competitive bacterial populations, potentially affecting conditions conducive to *Legionella* proliferation. This finding underscores the importance of prioritising biofilm sampling in water monitoring strategies. By focusing on biofilms, we gain a more accurate understanding of microbial communities, which can either promote or inhibit the presence of *Legionella* species, which are not present in bulk water samples. Adopting a holistic view of microbial ecology in water systems is crucial for the effective monitoring and management of *Legionella*, highlighting the necessity of considering the broader functional capabilities of microbial communities in shaping conditions conducive to *Legionella* presence.

Whilst this study primarily focuses on bacterial community dynamics within biofilms, it is important to acknowledge the role of other microbial components, such as amoebae and protozoa, which further influence biofilm dynamics. These organisms serve a dual role: acting as a protective niche for the persistence and intracellular replication of Legionella (as highlighted in Section 2.1.2) and influencing biofilm dynamics through selective grazing, predation, and

nutrient recycling (Nisar et al.,2020). These activities contribute to the formation of microenvironments within the biofilm's extracellular polymeric substances (EPS) and drive trophic cascades within the microbial ecosystem (Flemming and Wingender, 2010). This inherently complex dynamic can cause shifts in bacterial communities, influencing the presence or absence of *Legionella* spp.

This selective predation of fast-growing, dominant bacteria can provide ecological opportunities for slower-growing or less competitive species to thrive, such as *Legionella*. Additionally, selective feeding may influence bacterial diversity, or, conversely, inhibit it (Jürgens & Matz, 2002). If the remaining bacterial community promotes the production of EPS, *Legionella* has a greater opportunity to obtain nutrients, either from lysed bacteria or nutrients trapped within the EPS matrix. Selective feeding can also increase bacterial species richness, which has been correlated with the presence of *Legionella* (Llewellyn et al. 2017). Furthermore, selective feeding may allow rare taxa to persist, which, in this study, were found to co-occur when *L. pneumophila* was present. Selective feeding could also drive the dominance of bacteria that positively correlate with *Legionella* or suppress bacteria that negatively correlate with it. For example, *Acinetobacter spp.* positively correlate with *Legionella* by coexisting in biofilms and creating favourable conditions for its persistence (Stewart, Muthye and Cianciotto, 2012), whereas *P. aeruginosa* negatively correlates with *Legionella* (Paranjape et al., 2020b) due to its production of antimicrobial compounds like rhamnolipids and its competitive exclusion in biofilms.

Therefore, investigating protozoan-biofilm interactions would complement the findings of this study; however, this was not conducted, as justified in Section 6.1.2. In the context of protozoan community analysis, broader data collection and 18S rRNA analysis would have been required, presenting practical challenges in terms of time, resources, and interpretative complexity. These considerations further reinforce the rationale for narrowing the scope of this study to bacterial interactions in its aims and objectives.

6.4 Bulk Water Bacterial Community and Structure

In this research, we have placed a significant emphasis on the analysis of biofilms within cooling towers due to their critical role in the survival and proliferation of *Legionella*. However, it is important to consider the bacterial communities present in bulk water. While distinct from biofilms, this complementary insight allows us to capture a full spectrum of microbial interactions, from transient populations that may seed biofilms.

Throughout this study, the major phylum within bulk water was Proteobacteria, followed by Firmicutes. At the family level, Sphingomonadaceae, Pseudomonadaceae, Bdellovibrionaceae, Xanthobacteraceae, Moraxellaceae, and Bacillaceae were consistently present, underlining a shared microbial structure within the bulk water. Genus-level analysis further aligned with these findings, with *Novosphingobium*, *Sphingomonas*, *Pseudomonas*, *Bdellovibrio*, *Acinetobacter*, and *Lentibacillus* being prevalent across samples. Interestingly, cooling tower A2's major phylum was still Proteobacteria, but it had no Firmicutes present, instead featuring Bacteroidota and small amounts of Cyanobacteria. This trend continued at the family level, as uniquely, cooling tower A2 had a much higher abundance of Clade_III, Hypomicrobiaceae, Spirosomaceae, and Azospirillaceae. Again, this trend was evident at the genus level,

as cooling tower A2 uniquely had much higher abundances of Clade_III, Hyphomicrobium, Flectobacillus, and Skermanella.

To draw comparisons with this study, which explores both biofilm and bulk water microbial communities within the UK, examining the research conducted by Llewellyn et al. (2017), who investigated planktonic microbial communities within cooling towers across nine regions in the United States, and Paranjape et al., (2020b), who focused on select locations in Canada, reveals a remarkable consistency in core planktonic community composition. This underscores the profound selective pressures exerted by the biofilm environments within these structures. This consistency is particularly evident in the predominant abundance of *Proteobacteria*, a finding that aligns with Llewellyn et al. (2017) and Paranjape et al (2020b). The presence of Bacteroidetes and Cyanobacteria as the second and third most abundant phyla, respectively, echoed this pattern but only in one of this study cooling towers, specifically cooling tower A2. Interestingly, while Proteobacteria emerge as a common denominator across all investigated cooling towers, the presence of Bacteroidetes and Cyanobacteria as the second and third most abundant phyla, respectively, introduces a layer of diversity. This pattern, particularly observed in cooling tower A2, suggests a nuanced microbial ecosystem that, while consistent at its core, exhibits variations. Such a distinction is particularly notable when compared to the broader patterns observed by Llewellyn et al. (2017), where the phyla Planctomycetes and Verrucomicrobia were also present, but absent in data reported here. The comparative analysis further extends to the family and genera taxa with (Llewellyn, 2017), where similarities and differences were found; specifically, a high abundance *Pseudomonas* was a small abundance of *Methylobacterium* was similar but *Limnobacter*, *Legionella* and *Cupriviadus* was completely absent in this study. In summary, this shows that although there is a core microbial community present, many Physicochemical parameters influence the bacterial composition and structure especially at the genus level.

One striking difference in this research was the complete absence of *Legionella* in bulk water within the 16S rRNA gene analysis, despite the presence of a similar core microbial community at the phylum, family, and genus levels. Notably, *Pseudomonas* emerged as the third most prevalent genus, which may partly explain the absence of *Legionella* spp. This observation aligns with findings by Paranjape et al. (2020b), which demonstrated that *P. aeruginosa* had a higher abundance in *Legionella* spp. absent samples, potentially showing that *P. aeruginosa* inhibiting *Legionella* spp. growth. The discrepancy in *Legionella* presence could be attributed to several factors, including differences in the methods used for 16S rRNA gene analysis, variations in the abundance of lower taxa, differences in physicochemical parameters, or a combination of these factors. The differentiation in 16S analysis methods might affect the detectability of specific taxa, including *Legionella*, by either enhancing or limiting the resolution at which these organisms can be identified. Similarly, the variations in lower abundance taxa and physicochemical parameters might create environmental niches that either discourage or are inhospitable to *Legionella*'s growth in bulk water, despite their presence in biofilm samples. The notable presence of *Pseudomonas*, particularly, suggests a competitive microbial interaction that could further elucidate the environmental conditions unfavourable to *Legionella* proliferation in cooling towers.

6.5 Cooling Tower Engineered Structure and Biocide Regime influence on Bacterial Community Composition, Structure and Microbial Load

This study's findings demonstrate that a combination of engineered structures and biocide regimes can impact both biofilm and bulk water sample microbial communities. Due to the novelty of this finding, there is no current literature comparing the effects of oxidising and non-oxidising biocides and engineered structures in operational cooling towers on the microbiome within biofilms and bulk water samples. One study (I.S.M. Pinel, et al., 2020) that used a full-scale system has compared the differences between disinfected (chlorine) samples in the basin and non-disinfected samples in the feed water. It found that the most abundant phyla were Proteobacteria and Actinobacteria, with the orders SAR11 Clade and Frankiales. However, these abundances dramatically dropped in the cooling tower water, with the most abundant orders being Caulobacterales, Obscuribacterales, followed by Rhizobiales, Sphingomonadales, and Beta*proteobacteria*les, which although found in the feed water, was much lower abundance. The cooling tower showed less diversity than the feed water. This finding is similar to the findings of this study, showing that disinfection practices create a strong core community but still differ in low-abundance taxa. Despite this, the change in low-abundance taxa is significant, as evidenced when *L. pneumophila* was present, implying a big impact despite their low abundance.

This study illuminates a critical aspect of cooling tower management: the substantial impact of routine cleans and operational changes on cell concentration, viability, *Legionella spp.* concentration, and microbial community composition within operational cooling towers. Routine cleaning of the four cooling towers in this study included high-pressure washing, scrubbing, and chemical treatment with a bio-dispersant and oxidising biocide.

Results showed that *L. pneumophila* within biofilms was consistently undetectable for at least a month following cleaning. However, cleaning methods failed to consistently reduce *Legionella spp.* concentrations in biofilms and bulk water, with one event even increasing their concentration in both. Similarly, reductions in cell concentration and viability were inconsistent, with some cleaning events showing no significant impact or even increases. These outcomes highlight the resilience of biofilms, largely due to their extracellular polymeric substance (EPS) matrix, which acts as a protective barrier against disinfectants like chlorine. Additionally, free-living amoebae that harbour bacteria may exist in cyst form within biofilms or bulk water, further reducing the efficacy of chemical treatments. In bulk water, cleaning inconsistencies may also result from the release of cells from disrupted biofilms, replenishing planktonic populations.

Combining cleaning with operational changes, such as continuous dosing pumps or pack replacements, demonstrated more reliable reductions in *Legionella spp.* concentrations, cell concentration, and viability. However, operational changes may also have unintended effects, as observed in this study, where *L. pneumophila* detection coincided with a pack replacement. This suggests that microbial community shifts caused by such changes could create conditions for favourable pathogen proliferation.

Interestingly, *L. pneumophila* was consistently undetectable after cleaning, even when cell concentration and viability remained unaffected. This indicates that cleaning practices may disrupt the microbial community in ways that prevent *L. pneumophila* from surviving or proliferating. The observed variability between towers, such as the resilience of biofilms in cooling tower B1, highlights the complexity of microbial dynamics and suggests that some communities are more resistant to disruption due to lower microbial concentration and viability.

Cleaning had little effect on bulk water bacterial richness and evenness, likely because these communities are less diverse and may consist of biofilm species mobilised into the water or adapted to biocide presence. This aligns with previous research (Llewellyn et al., 2017), which identified a consistent core microbial community across large geographic areas due to strong selective pressures within cooling towers.

Emerging research into probiotic approaches offers a promising yet challenging direction in cooling tower maintenance. Probiotic treatments aim to introduce beneficial microorganisms that outcompete opportunistic pathogens like *Legionella* (Cavallaro et al., 2022). However, maintaining stable populations of beneficial microbes in dynamic, high-turnover environments such as cooling towers is challenging due to factors like nutrient fluctuations, water flow, and biocide use. Additionally, understanding how probiotics interact with existing microbial communities is critical to avoiding unintended ecological consequences, such as fostering niches for other pathogens.

This study highlights the limitations of current cleaning practices, which often fail to achieve consistent microbial control. By integrating operational changes and gaining better insights into the complex interactions within microbial communities, future efforts can improve the effectiveness and reliability of pathogen suppression in these systems. Advancing our understanding of microbial dynamics will enable the development of optimized water treatment strategies that reduce the risks associated with *Legionella* and other opportunistic pathogens.

6.6 Physicochemical Parameters on Bacterial Community Composition, Structure and Microbial Load

This study is the first to investigate the relationship between pack temperature and the presence of *Legionella* species, including *L. pneumophila,* in cooling tower systems using biofilm samples. Our analysis found no significant correlation between pack temperature and the prevalence of *Legionella* spp. This result may be attributed to the protective role of biofilms, particularly the extracellular polymeric substances (EPS), which could shield bacterial communities from thermal variations in cooling tower packs. Given the current gap in literature on this interaction, further experimental work is needed to understand the potential of EPS in protecting these bacteria from temperature-induced stress. These novel findings highlight the necessity for additional research to validate our observations and to consider their implications for alternative *Legionella* monitoring strategies.

Moreover, the study evaluated the influence of various physicochemical parameters, including water temperature, calcium hardness, alkalinity, chloride, conductivity, and iron content on microbial concentrations, microbial viability, and the presence of *Legionella* spp. in bulk water samples. Contrary to certain guidelines (ASHRAE, 2018; CDC, 2017) that suggest these parameters can predict *Legionella* risk, our findings revealed no correlations. This challenges the

effectiveness of using water quality parameters as predictors for *Legionella* presence, echoing the findings of Pierre et al. (2019). However, contrasting results from Campaña et al. (2023) indicate that parameters such as chloride, pH, and water hardness may be linked to higher *Legionella* risks in cooling towers. This discrepancy underscores the need for a more nuanced approach to *Legionella* management, informed by a broader array of empirical data, rather than a reliance on a uniform set of water quality parameters.

6.7 Practical Implications & Challenges

An identified key area for methodological enhancement in the current research focuses on integrating culture-based methods alongside qPCR for biofilm analysis. Despite the limitations of culture techniques, as discussed in Section 2.2, they are considered the 'Gold Standard' due to their ability to assess the viability of *Legionella* spp. and to leverage historical data. Incorporating this dual approach could have replicated the lead indicator effect observed with qPCR, facilitating a quicker adaptation of biofilm sampling into existing water safety and monitoring frameworks due to the culture's compatibility with established risk levels and thresholds. This integration promises a more immediate, practical application for water safety organisations, sidestepping the need for significant alterations to current protocols or the adoption of costlier, nascent detection methods. However, the distinctive operational conditions at the Field site restricted our full exploration of this combined methodological approach.

Immunomagnetic separation, in combination with this study's novel and optimised biofilm sampling, could have potentially provided an earlier detection of *L. pneumophila*, similar to that achieved by qPCR with biofilms found in this study. This approach suggested a promising avenue for enhancing the sensitivity and timeliness of detection methods. However, before extensive longitudinal analysis could be conducted, the field site discontinued this method due to reliability issues. These difficulties were thought to be caused by the on-site lab's filtering process, which adhered to the ISO 11731 protocol. Notably, our biofilm samples did not require this filtering process, which could have mitigated some of these challenges. The premature removal of this technique underscores the operational challenges that can arise when implementing new methods in practical settings, reflecting the delicate balance between innovative approaches and the robustness required for field applications.

This study navigated procedural constraints, particularly in terms of sample handling and processing. Stringent site regulations necessitated the exclusive use of a specific DNA extraction method for off-site sample analysis. This limitation may have been associated with a higher proportion of unidentified samples in our 16S rRNA gene analysis. Further examination suggested that the DNA yield and quality, potentially influenced by this specific extraction method, might have contributed to this outcome. However, it's essential to recognize that such challenges are not unique to our investigation. The study by Cavallaro et al. (2024) similarly highlighted the influence of DNA extraction techniques on the quantification of *Legionella* spp. and the delineation of bacterial community profiles. Despite these procedural nuances, we remain confident in the robustness of our analytical approach and the insights derived from this study data. Notably, this study findings reveal a core bacterial community within bulk water that is consistent with prior literature. This consistency not only underscores the value of our study in contributing to a deeper

understanding of microbial communities in biofilm and water samples but also reinforces the credibility of our approach and the relevance of our findings to the field.

Given these findings, it is crucial to consider how cooling tower operators should respond to *Legionella* spp. and *L. pneumophila* in biofilms. Biofilm-associated *Legionella* contamination often precedes its detection in bulk water (Zhao, Sun and Liu, 2023), posing a significant public health risk even when bulk water samples remain below action levels. This study confirms that risk, as *L. pneumophila* was detected exclusively in biofilms. Over time, biofilm-residing bacteria may be released into the water column due to mechanical disturbances, flow dynamics, or biocide action, increasing the likelihood of aerosolisation in cooling towers. Since *Legionella* exposure occurs via inhalation of contaminated aerosols, biofilm contamination must be considered in risk management strategies.

Despite this, UK Health and Safety Executive (HSE) guidelines currently focus only on bulk water sampling, with no specific action levels for biofilm samples. However, biofilms provide a protective environment for *Legionella*, allowing it to persist at much higher concentrations than in bulk water, as found in this study and previous literature. The bacteria benefit from trapped nutrients, microbial interactions, and protozoan hosts, which support their survival and multiplication before release into the system. These factors highlight the need for a proactive monitoring approach that includes biofilm sampling alongside standard bulk water testing. As this study relied exclusively on qPCR methods, which detect both live and dead bacteria, additional culture-based testing is necessary before biofilm contamination can be assessed using the same action levels as bulk water (Figure 1). However, using bulk water thresholds as an initial reference point could help operators interpret biofilm results in a familiar context.

If *Legionella* spp. and/or *L. pneumophila* is detected in biofilms, operators should apply the same remedial actions outlined in HSE guidelines for bulk water contamination. This includes reinforcing existing water treatment protocols, increasing biocide dosing if necessary, and conducting mechanical cleaning, descaling, and system flushing to remove biofilms. In addition to existing water treatment protocols, this study findings recommends the use of an automatic dosing pump to apply a consistent dosage of oxidising biocides. This approach limits biofilm regrowth and has the most significant impact on lowering microbial concentration and microbial viability, reducing fluctuations that may allow *Legionella* to persist and adapt. Additionally, operators should reassess their water management plan (WMP) to identify potential weaknesses that may contribute to biofilm persistence and modify control measures accordingly.

Although applying bulk water action levels to biofilms may seem excessive, given their typically higher *Legionella* concentrations, the long-term benefits outweigh the costs. Early biofilm detection allows for pre-emptive interventions before *Legionella* spreads into bulk water, reducing the likelihood of widespread contamination. A *Legionella* outbreak can lead to legal liabilities, regulatory penalties, reputational damage, and facility shutdowns—all far more costly than proactive biofilm management.

6.8 Future Research

Future studies should focus on validating biofilm sampling techniques across diverse operational cooling tower environments to ensure regulatory adoption. While this study highlights the value of biofilm sampling for early and more accurate *Legionella* detection, further research is required to establish standardised compliance thresholds comparable to those for bulk water monitoring. This includes incorporating culture-based detection alongside molecular methods to align with existing regulatory frameworks.

Beyond regulatory validation, future research should explore the integration of biofilm sampling into routine monitoring programs, ensuring that it is both practical and cost-effective for industry adoption. This study has demonstrated the feasibility of biofilm sampling across multiple cooling tower designs and operational conditions, showing that it can be a practical and reliable monitoring tool. However, future research should expand on this further by evaluating its long-term reproducibility and cost-effectiveness across an even wider range of cooling tower systems, climate conditions, and biocide regimes. Large-scale, multi-site studies will be essential to refine standardisation efforts and confirm the robustness of biofilm sampling in diverse operational contexts, ensuring its seamless integration into industry practices. Additionally, advances in automated or real-time biofilm monitoring technologies—such as biosensors or in-line sampling devices—could further improve the feasibility of biofilm-based surveillance, reducing reliance on intermittent manual sampling.

While this study employed qPCR as a rapid detection method for *Legionella* in biofilm samples, future research should investigate the compatibility of biofilm sampling with other emerging rapid detection technologies, such as immunomagnetic separation. Evaluating the effectiveness of these techniques in conjunction with biofilm sampling could further enhance detection sensitivity, improve early warning capabilities, and provide real-time monitoring solutions, ultimately strengthening Legionella risk assessment and outbreak prevention strategies.

By developing clear action levels for biofilm samples and optimizing their incorporation into existing monitoring frameworks, future research can help establish biofilm-based monitoring as a standardized, proactive approach for *Legionella* risk assessment, ultimately improving water safety and public health protection.

Building upon the foundational research of this thesis, future studies should consider investigating the role of protozoa within biofilms in cooling tower systems and their interactions with *Legionella*. Protozoa have been recognised for their ability to harbour and potentially augment the proliferation of *Legionella*. Future research should aim to identify and quantify the types of protozoa present in biofilms, explore specific interactions between protozoa and *Legionella*, and examine how environmental factors, tower design, and maintenance practices influence these dynamics. Additionally, integrating protozoan detection into routine biofilm sampling methods could enhance *Legionella* monitoring protocols, potentially leading to the development of predictive models based on protozoan and bacterial community dynamics. This expanded focus could significantly improve the efficacy of monitoring and managing *Legionella* in cooling towers, providing a more comprehensive understanding of microbial interactions within biofilms and enabling more effective prevention strategies against *Legionella* outbreaks.

Chapter 7: Conclusions

This thesis has successfully developed and implemented a novel and optimised technique for collecting and analysing biofilm samples from operational cooling towers, effectively addressing the challenges of standardisation and logistical complexity. This method offers a robust approach for future research on biofilms within cooling towers. Additionally, this study finds that, even with the use of emerging detection methods, *L. pneumophila has* been detected exclusively in biofilm samples, not in bulk water. This underscores the importance of biofilm samples as lead indicators and marks a critical advancement in our ability to detect and quantify this pathogen with timely precision and accuracy. Given this exclusive detection within biofilms - alongside findings that biofilms contain significantly higher concentrations of *Legionella* spp. than bulk water- it becomes imperative to revise water monitoring practices to incorporate biofilm sampling. By integrating biofilm-based monitoring, we can significantly improve early detection and risk assessment enhance the safety and efficacy of water management practices, ultimately reducing the risk of *Legionella* outbreaks and protecting public health.

Biofilms and bulk water samples show that cell concentration and cell viability counts do not correlate with the presence of *Legionella* spp., including pneumophila. This finding necessitates the need for a multifaceted approach to *Legionella* detection and monitoring. Such an approach should extend beyond mere cell counts to include the examination of microbial community characteristics and the interaction between biofilms and bulk water. By adopting such comprehensive strategies, we can gain a more accurate and holistic understanding of *Legionella* dynamics, significantly enhancing our ability to monitor, detect and manage *Legionella* effectively proactively with better lead times.

Biofilms create a unique ecological niche, distinctly different from that of bulk water samples, especially in fostering *L. pneumophila* proliferation. Notably, the presence of specific bacterial families Sphingomonadaceae*,* Moraxellaceae*,* Xanthobacteraceae*,* Hypomicrobiaceae*,* Rhodobacteraceae*,* and Bdellovibrionaceae—was uniquely identified at low abundance in conjunction with *L. pneumophila.* These findings highlight the intricate microbial dynamics within biofilms that may provide additional organic compounds, nitrogen or even play a role in reducing competitive populations. These findings highlight the intricate microbial dynamics within biofilms that support *Legionella* growth, offering crucial insights into the broader capabilities of biofilm microbial communities in fostering conditions conducive to *L. pneumophila* presence.

This study significantly advances the understanding of microbial dynamics in cooling towers, revealing that engineered structures, biocide regimes, and routine cleaning practices distinctly shape biofilm and bulk water microbial communities. The most significant impact on microbial communities for both biofilm and bulk water was biocide dosing, with the engineered structure being less influential and cleaning having a limited impact. Moreover, physicochemical parameters showed no correlation to *Legionella* presence and quantification. Thus, reinforcing the need for a broader, integrated approach to *Legionella* monitoring, informed by a wider array of empirical data using

both sessile and planktonic samples, with Physicochemical parameters being insufficient alone to monitor *Legionella* due to its complexity within cooling tower.

Future research should focus on validating biofilm sampling techniques across diverse operational cooling tower environments to ensure regulatory adoption. Integrating biofilm sampling with both culture-based and molecular detection methods will allow for the development of clear compliance thresholds and regulatory action levels. Establishing standardised protocols for biofilm analysis will enhance the reliability of *Legionella* surveillance and improve risk mitigation strategies.

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Chapter 9: Appendix

Appendix (I): Dendrogram Plots

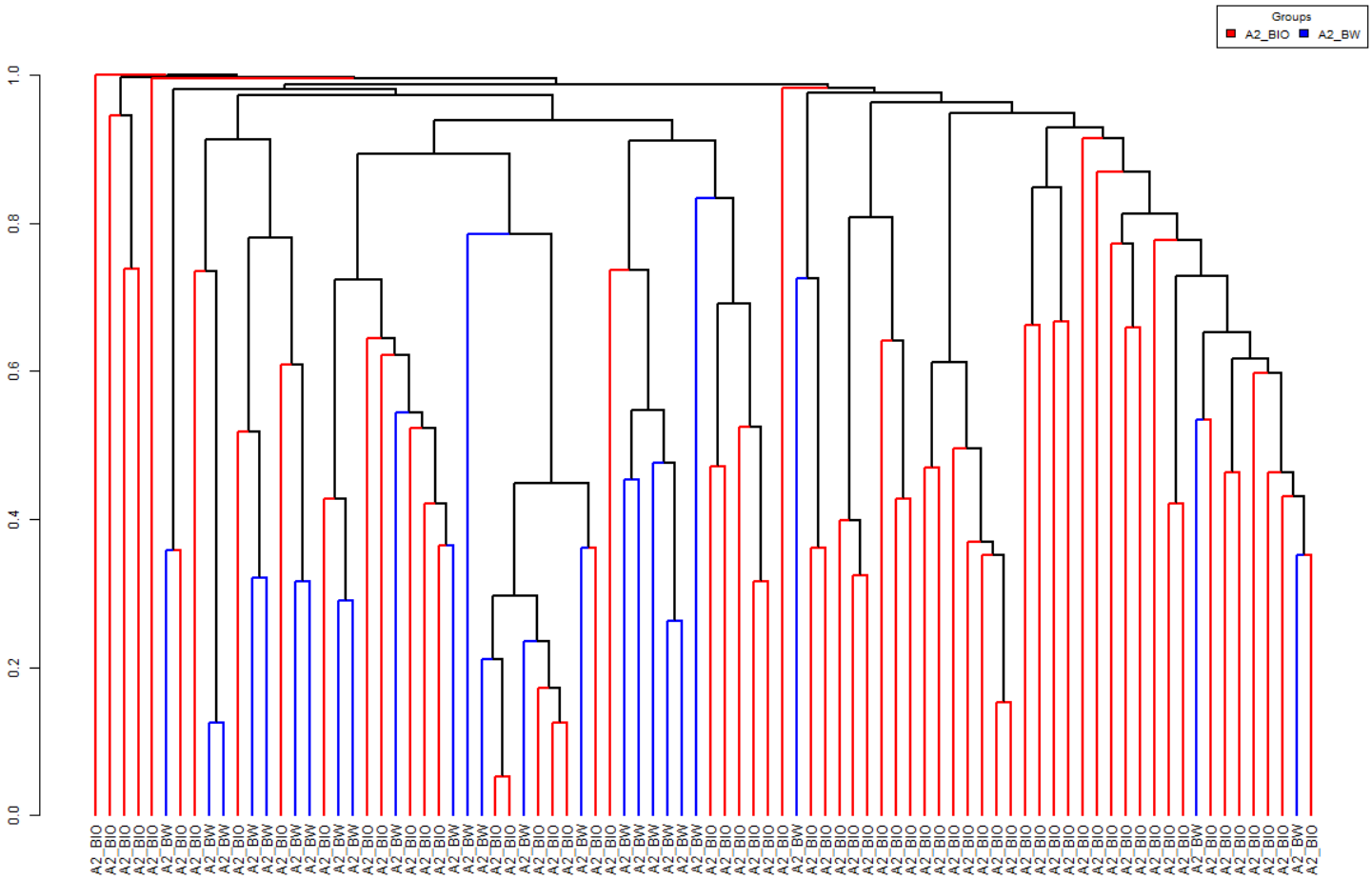


Figure I: *Hierarchical clustering analysis of microbial communities to compare between biofilm and bulk water samples for cooling tower A2 using the Bray Curtis dissimilarity matrix. Red lines represent biofilm samples and blue lines represent bulk water samples. Black lines represent direct link to the biofilm and bulk water samples.*

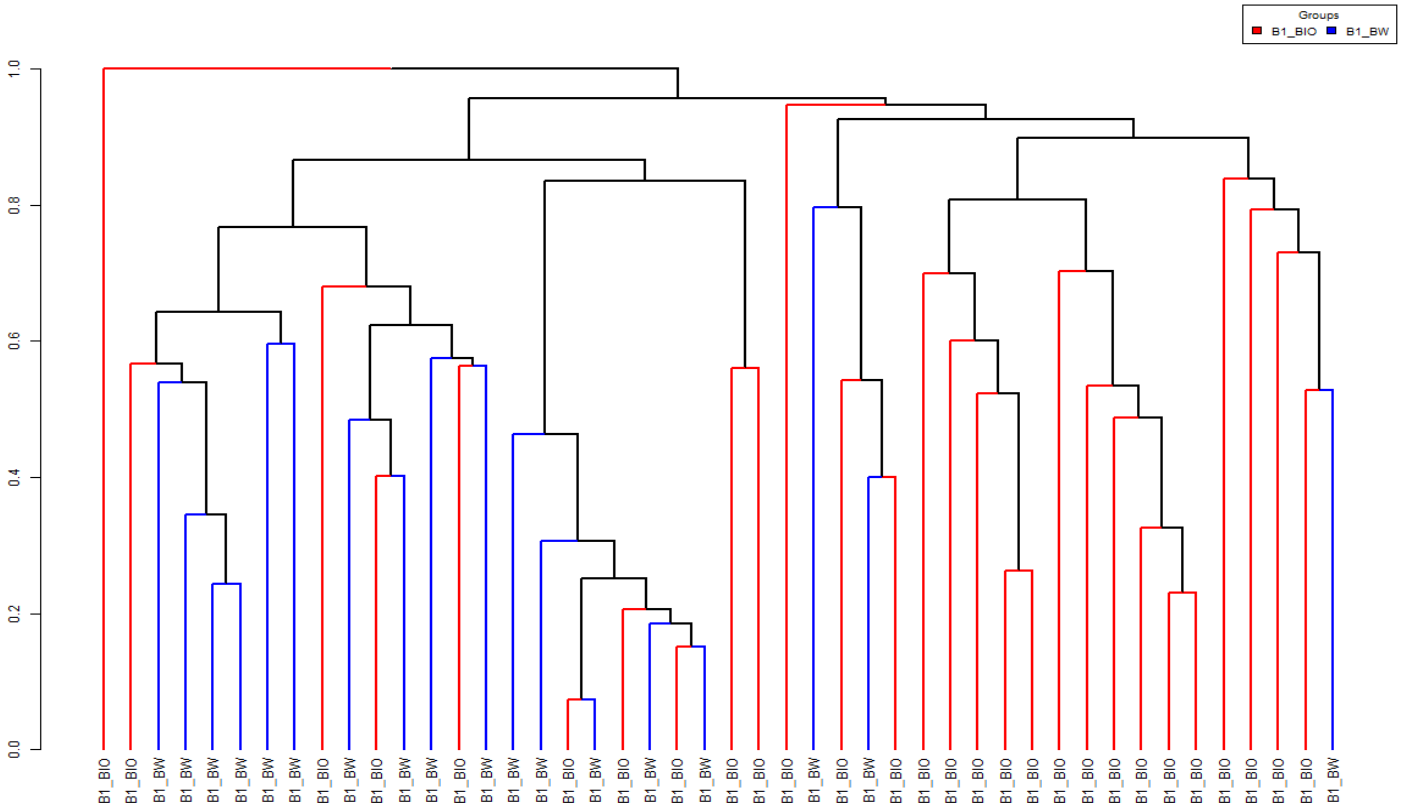


Figure II: *Hierarchical clustering analysis of microbial communities to compare between biofilm and bulk water samples for cooling tower B1 using the Bray Curtis dissimilarity matrix. Red lines represent biofilm samples and blue lines represent bulk water samples. Black lines represent direct link to the biofilm and bulk water samples.*

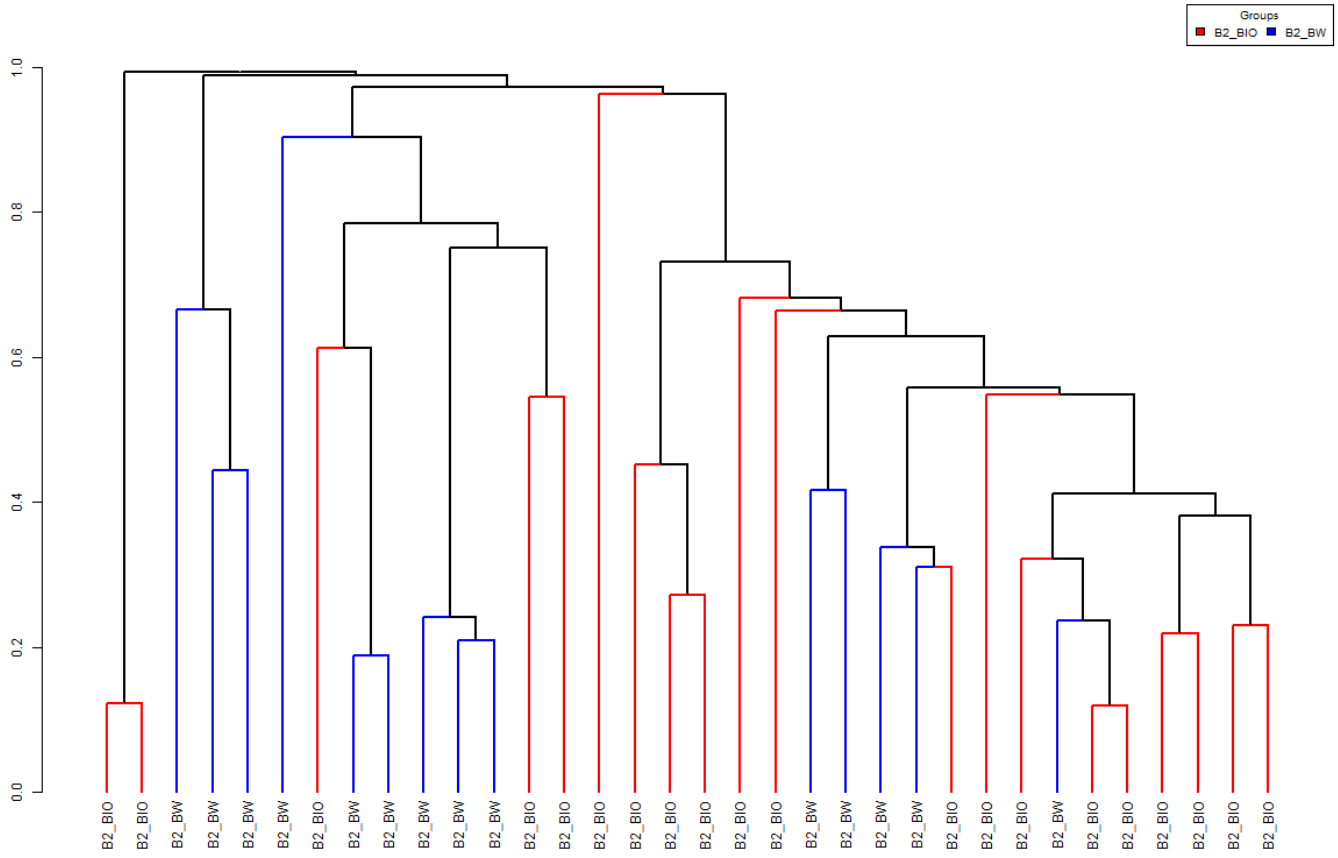


Figure III: *Hierarchical clustering analysis of microbial communities to compare between biofilm and bulk water samples for cooling tower B2 using the Bray Curtis dissimilarity matrix. Red lines represent biofilm samples and blue lines represent bulk water samples. Black lines represent direct link to the biofilm and bulk water samples.*

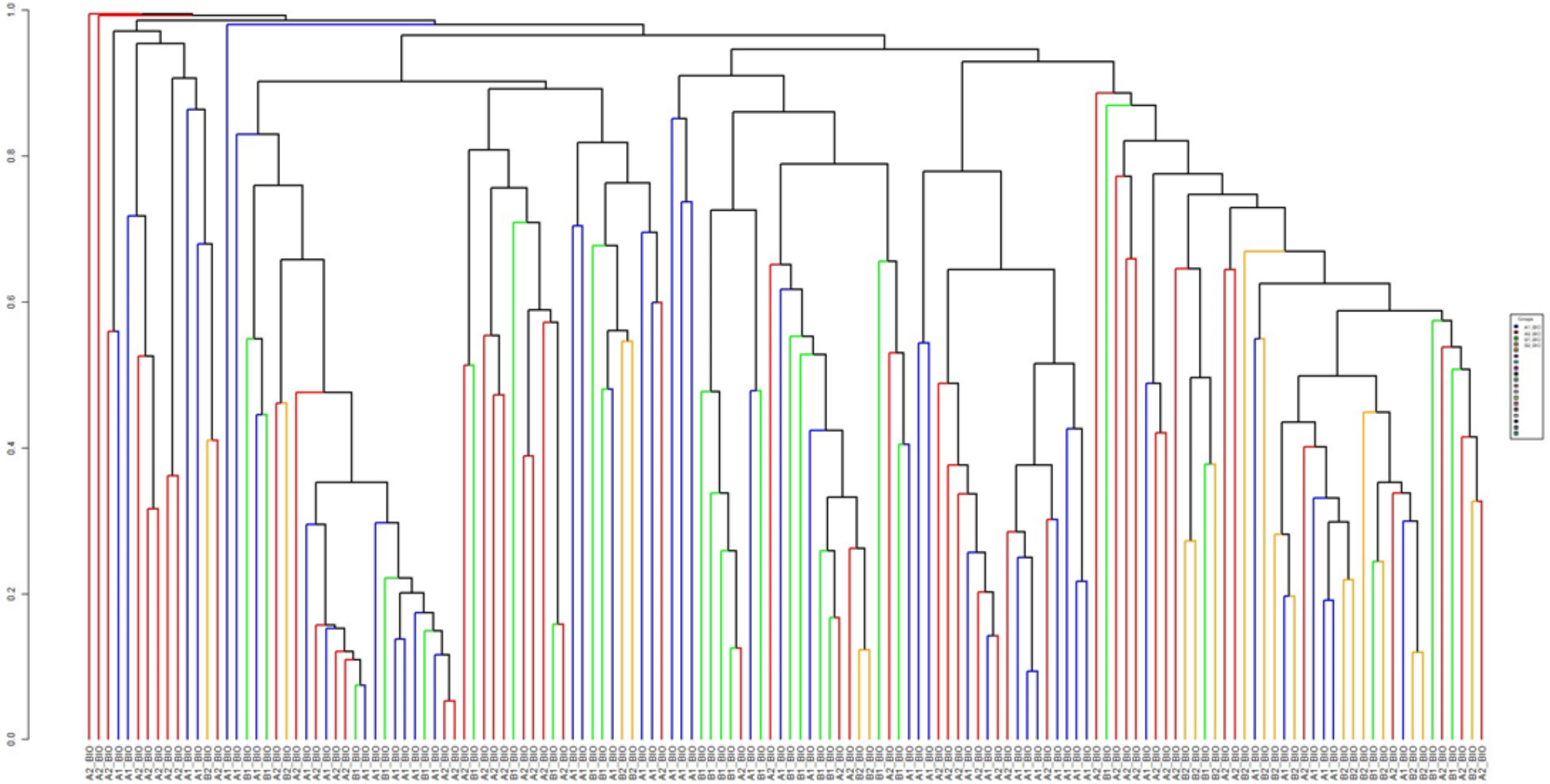


Figure IV: *Hierarchical clustering analysis of microbial communities to compare the biofilm microbial communities between four cooling towers using the Bray Crutis dissimilarity matrix. Blue represents cooling tower A1, red represent cooling tower A2, green represent cooling tower B1 and orange represent cooling tower B2.*

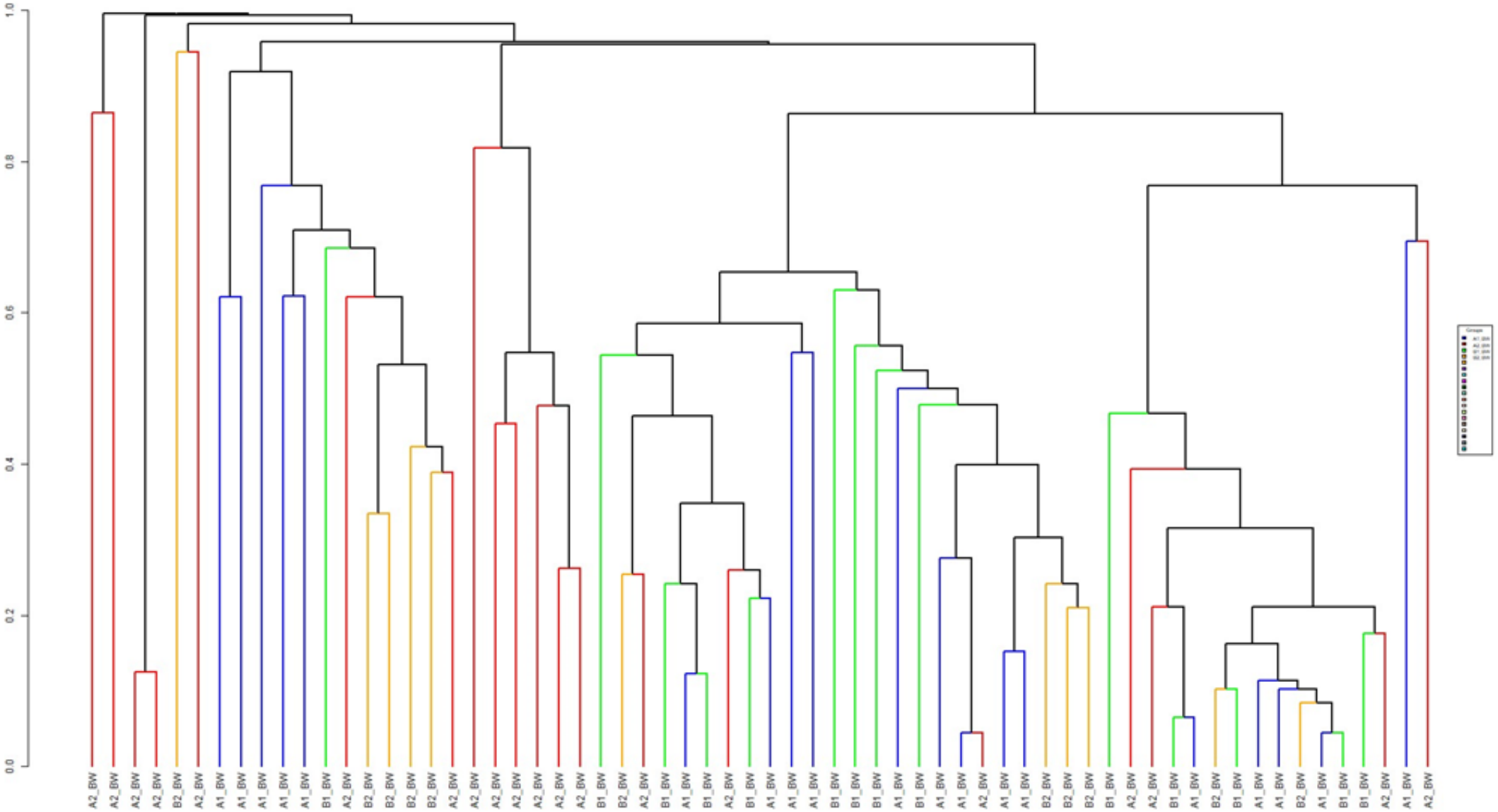
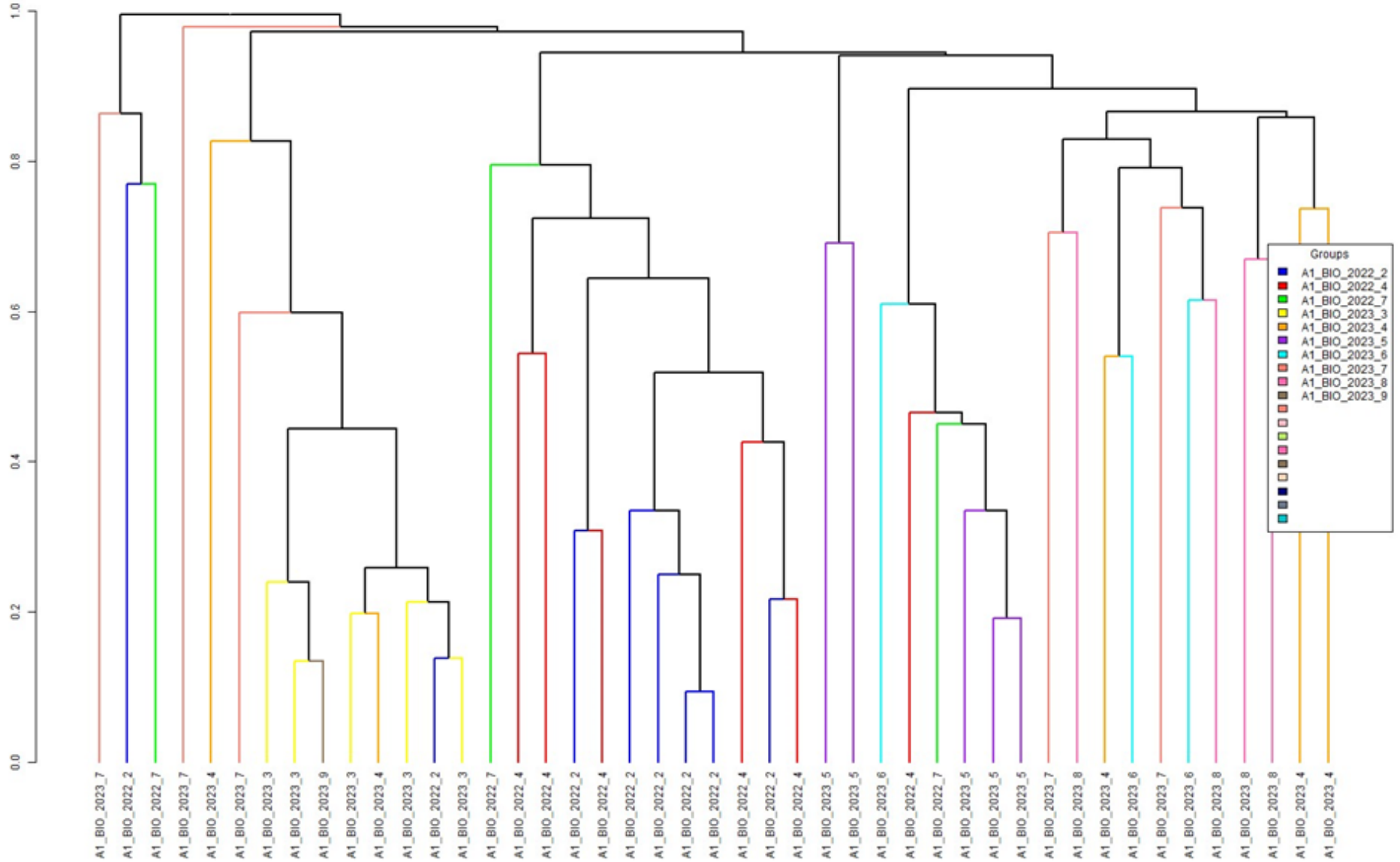
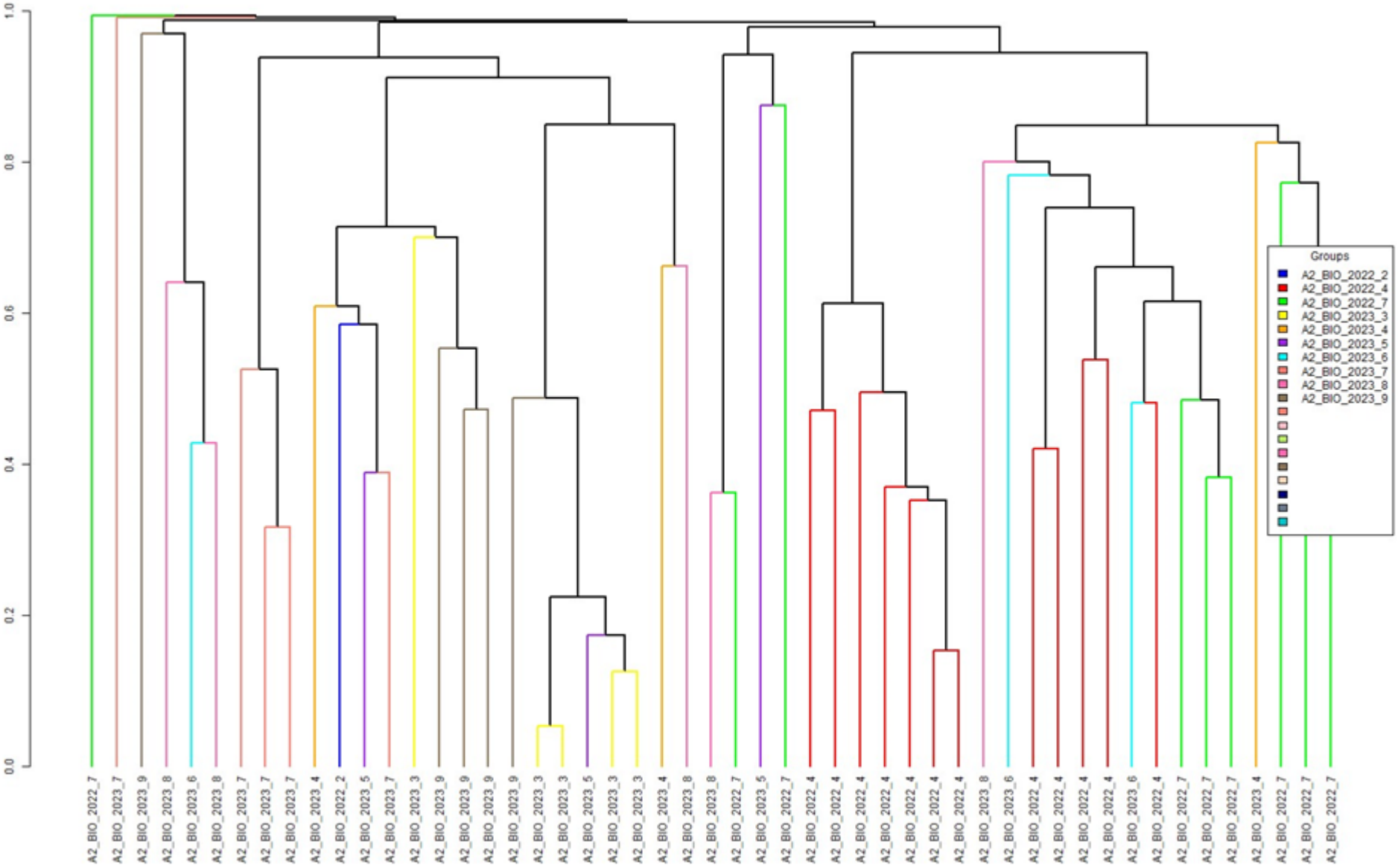


***Figure V**: Hierarchical clustering analysis of microbial communities to compare the bulk water microbial communities between four cooling towers using the Bray Crutis dissimilarity matrix. Blue represents cooling tower A1, red represent cooling tower A2, green represent cooling tower B1 and orange represent cooling tower B2.*

A


1.0
0.8
0.6
0.4
0.2
0.0
Groups
A1_BIO_2022_2
A1_BIO_2022_4
A1_BIO_2022_7
A1_BIO_2023_3
A1_BIO_2023_4
A1_BIO_2023_5
A1_BIO_2023_6
A1_BIO_2023_7
A1_BIO_2023_8
A1_BIO_2023_9
A1_BIO_2023_7
A1_BIO_2022_2
A1_BIO_2022_7
A1_BIO_2023_7
A1_BIO_2023_4
A1_BIO_2023_7
A1_BIO_2023_3
A1_BIO_2023_3
A1_BIO_2023_9
A1_BIO_2023_3
A1_BIO_2023_4
A1_BIO_2023_3
A1_BIO_2022_2
A1_BIO_2023_3
A1_BIO_2022_7
A1_BIO_2022_4
A1_BIO_2022_4
A1_BIO_2022_2
A1_BIO_2022_4
A1_BIO_2022_2
A1_BIO_2022_2
A1_BIO_2022_2
A1_BIO_2022_2
A1_BIO_2022_4
A1_BIO_2022_2
A1_BIO_2022_4
A1_BIO_2023_5
A1_BIO_2023_5
A1_BIO_2023_6
A1_BIO_2022_4
A1_BIO_2022_7
A1_BIO_2023_5
A1_BIO_2023_5
A1_BIO_2023_5
A1_BIO_2023_7
A1_BIO_2023_8
A1_BIO_2023_4
A1_BIO_2023_6
A1_BIO_2023_7
A1_BIO_2023_6
A1_BIO_2023_8
A1_BIO_2023_8
A1_BIO_2023_8
A1_BIO_2023_4
A1_BIO_2023_4

B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0.6
0.4
0.2
0.0
Groups
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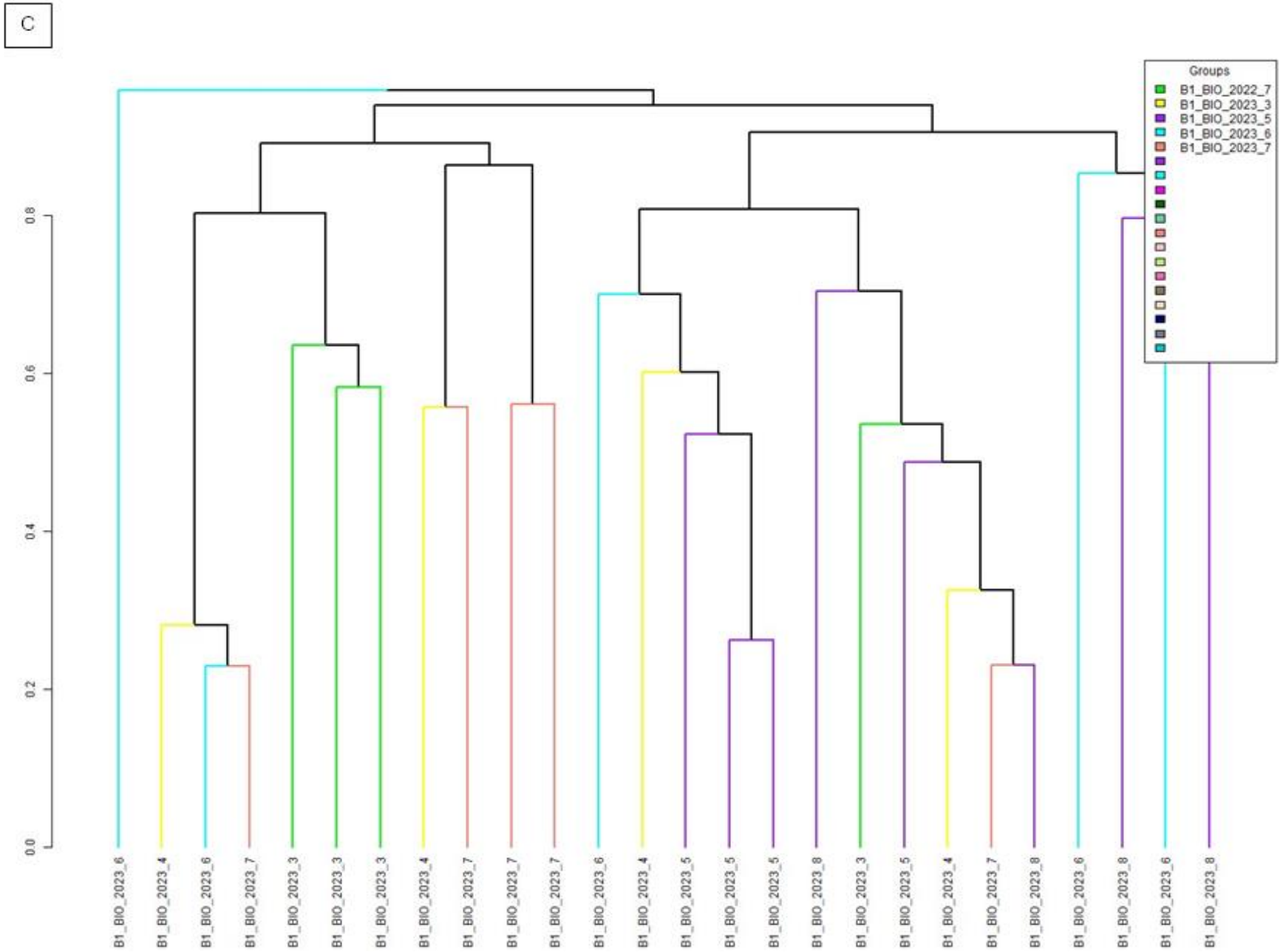
A2_BIO_2022_4
A2_BIO_2022_7
A2_BIO_2023_3
A2_BIO_2023_4
A2_BIO_2023_5
A2_BIO_2023_6
A2_BIO_2023_7
A2_BIO_2023_8
A2_BIO_2023_9
A2_BIO_2022_7
A2_BIO_2023_7
A2_BIO_2023_9
A2_BIO_2023_8
A2_BIO_2023_6
A2_BIO_2023_8
A2_BIO_2023_7
A2_BIO_2023_7
A2_BIO_2023_7
A2_BIO_2023_4
A2_BIO_2022_2
A2_BIO_2023_5
A2_BIO_2023_7
A2_BIO_2023_3
A2_BIO_2023_9
A2_BIO_2023_9
A2_BIO_2023_9
A2_BIO_2023_9
A2_BIO_2023_3
A2_BIO_2023_3
A2_BIO_2023_5
A2_BIO_2023_3
A2_BIO_2023_3
A2_BIO_2023_4
A2_BIO_2023_8
A2_BIO_2023_8
A2_BIO_2022_7
A2_BIO_2023_5
A2_BIO_2022_7
A2_BIO_2022_4
A2_BIO_2022_4
A2_BIO_2022_4
A2_BIO_2022_4
A2_BIO_2022_4
A2_BIO_2022_4
A2_BIO_2022_4
A2_BIO_2023_8
A2_BIO_2023_6
A2_BIO_2022_4
A2_BIO_2022_4
A2_BIO_2022_4
A2_BIO_2022_4
A2_BIO_2023_6
A2_BIO_2022_4
A2_BIO_2022_7
A2_BIO_2022_7
A2_BIO_2022_7
A2_BIO_2023_4
A2_BIO_2022_7
A2_BIO_2022_7
A2_BIO_2022_7


C
Groups
B1_BIO_2022_7
B1_BIO_2023_3
B1_BIO_2023_5
B1_BIO_2023_6
B1_BIO_2023_7
0.0
0.2
0.4
0.6
0.8
B1_BIO_2023_6
B1_BIO_2023_4
B1_BIO_2023_6
B1_BIO_2023_7
B1_BIO_2023_3
B1_BIO_2023_3
B1_BIO_2023_3
B1_BIO_2023_4
B1_BIO_2023_7
B1_BIO_2023_7
B1_BIO_2023_7
B1_BIO_2023_6
B1_BIO_2023_4
B1_BIO_2023_5
B1_BIO_2023_5
B1_BIO_2023_5
B1_BIO_2023_8
B1_BIO_2023_3
B1_BIO_2023_5
B1_BIO_2023_4
B1_BIO_2023_7
B1_BIO_2023_8
B1_BIO_2023_6
B1_BIO_2023_8
B1_BIO_2023_6
B1_BIO_2023_8

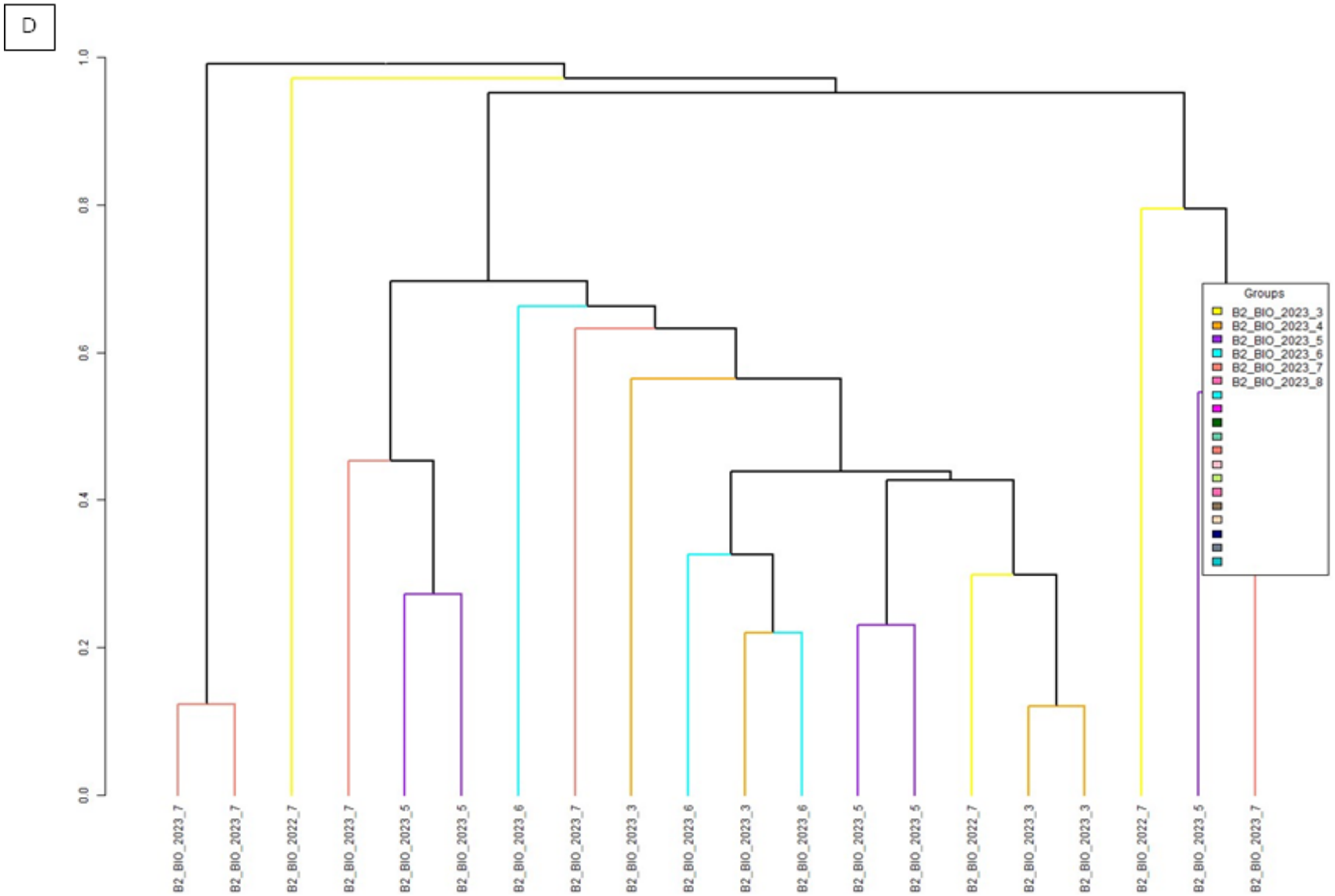
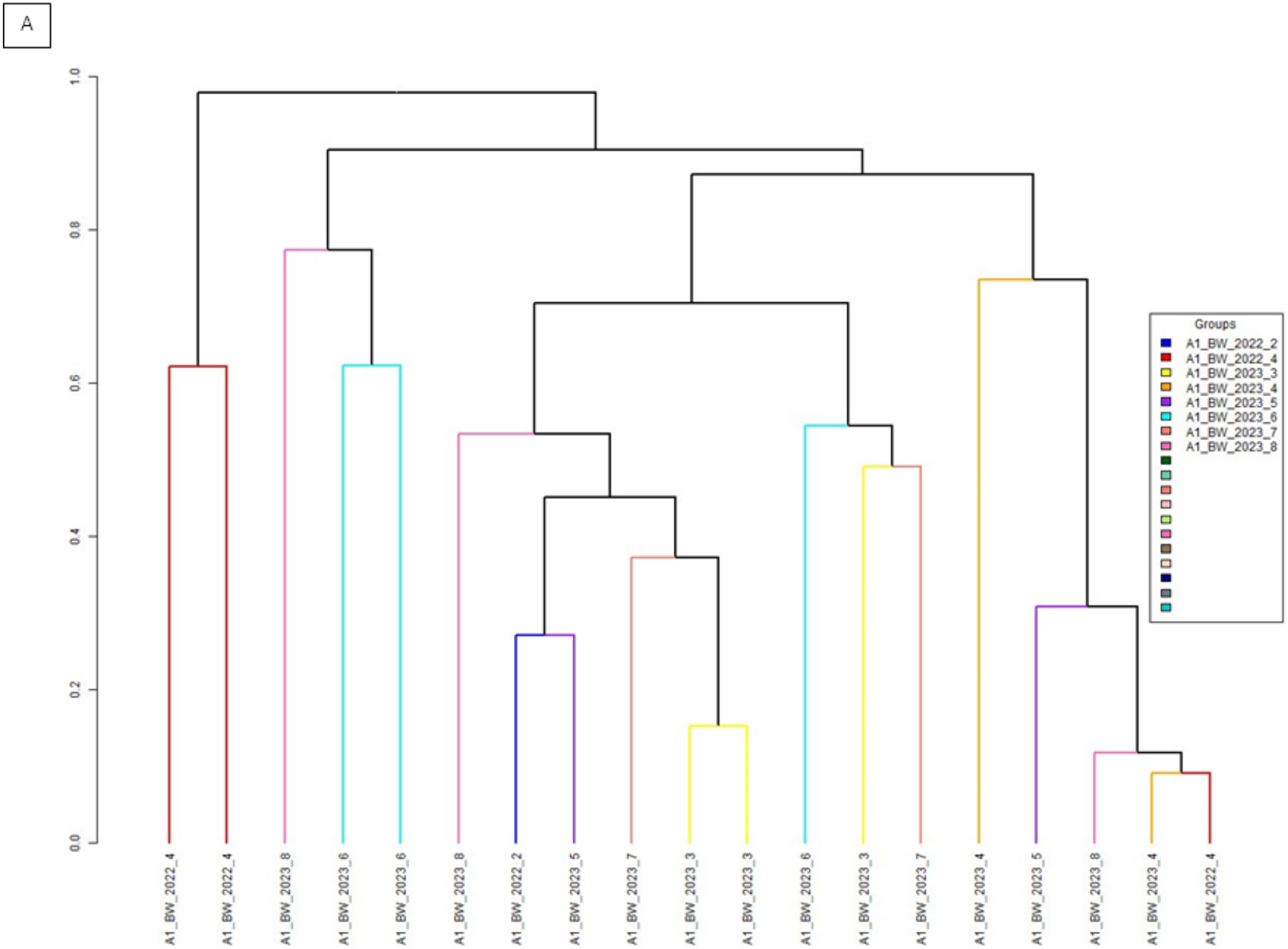
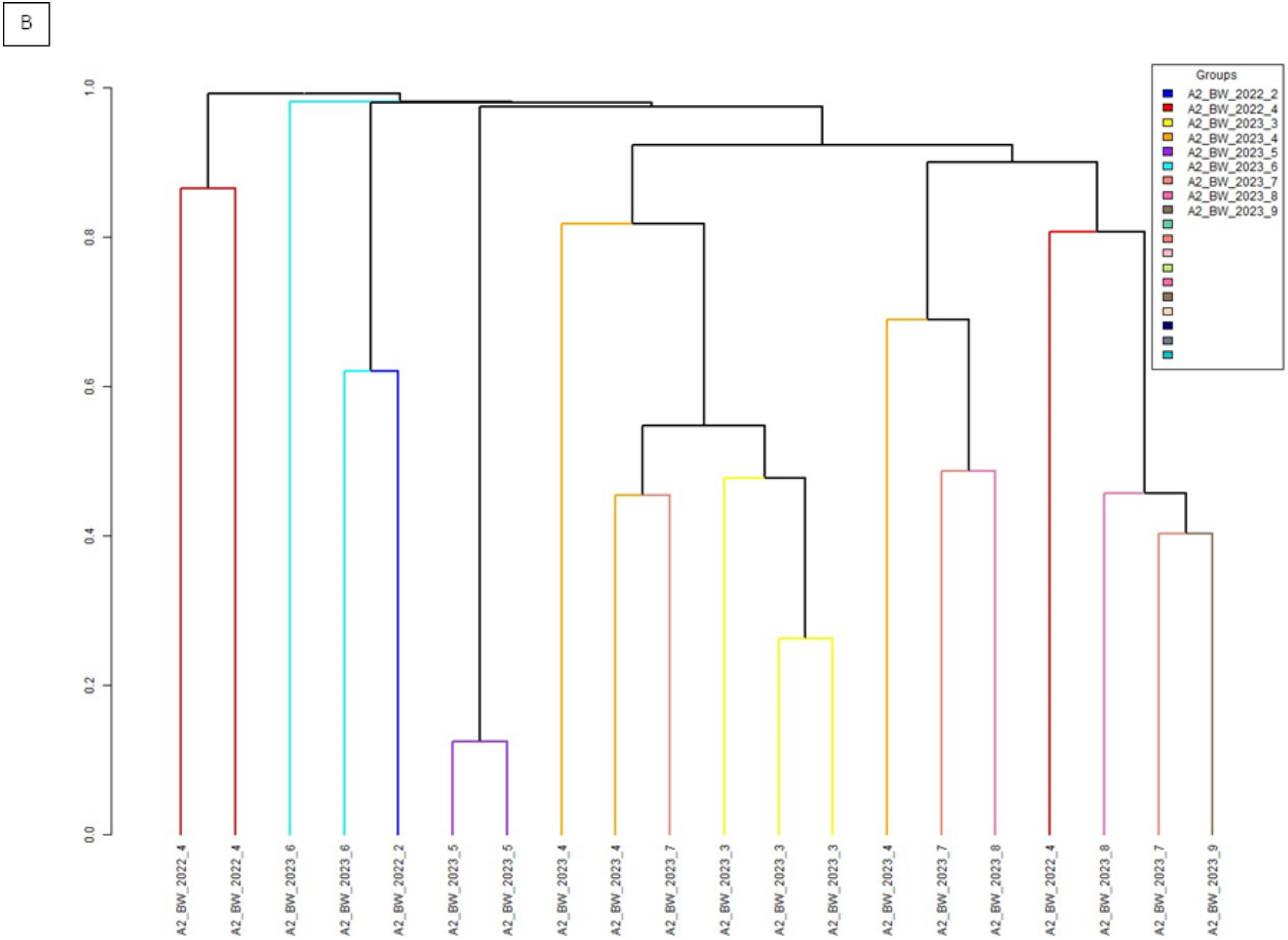


Figure VI: *depicts the hierarchical clustering of microbial communities across various sampling dates for biofilm samples for each Cooling Tower, utilizing the Bray dissimilarity matrix. Each colour represents a unique sampling date, illustrating the diversity and similarity of microbial compositions over time. The black lines indicate the absence of a significant link or similarity between the microbial communities of the compared samples. Panel A, B, C, D represent cooling tower A1, A2, B1, B2 respectively.*


A
1.0
0.8
0.6
0.4
0.2
0.0
Groups
A1_BW_2022_2
A1_BW_2022_4
A1_BW_2023_3
A1_BW_2023_4
A1_BW_2023_5
A1_BW_2023_6
A1_BW_2023_7
A1_BW_2023_8
A1_BW_2022_4
A1_BW_2022_4
A1_BW_2023_8
A1_BW_2023_6
A1_BW_2023_6
A1_BW_2023_8
A1_BW_2022_2
A1_BW_2023_5
A1_BW_2023_7
A1_BW_2023_3
A1_BW_2023_3
A1_BW_2023_6
A1_BW_2023_3
A1_BW_2023_7
A1_BW_2023_4
A1_BW_2023_5
A1_BW_2023_8
A1_BW_2023_4
A1_BW_2022_4

B


Groups
A2_BW_2022_2
A2_BW_2022_4
A2_BW_2023_3
A2_BW_2023_4
A2_BW_2023_5
A2_BW_2023_6
A2_BW_2023_7
A2_BW_2023_8
A2_BW_2023_9
1.0
0.8
0.6
0.4
0.2
0.0
A2_BW_2022_4
A2_BW_2022_4
A2_BW_2023_6
A2_BW_2023_6
A2_BW_2022_2
A2_BW_2023_5
A2_BW_2023_5
A2_BW_2023_4
A2_BW_2023_4
A2_BW_2023_7
A2_BW_2023_3
A2_BW_2023_3
A2_BW_2023_3
A2_BW_2023_4
A2_BW_2023_7
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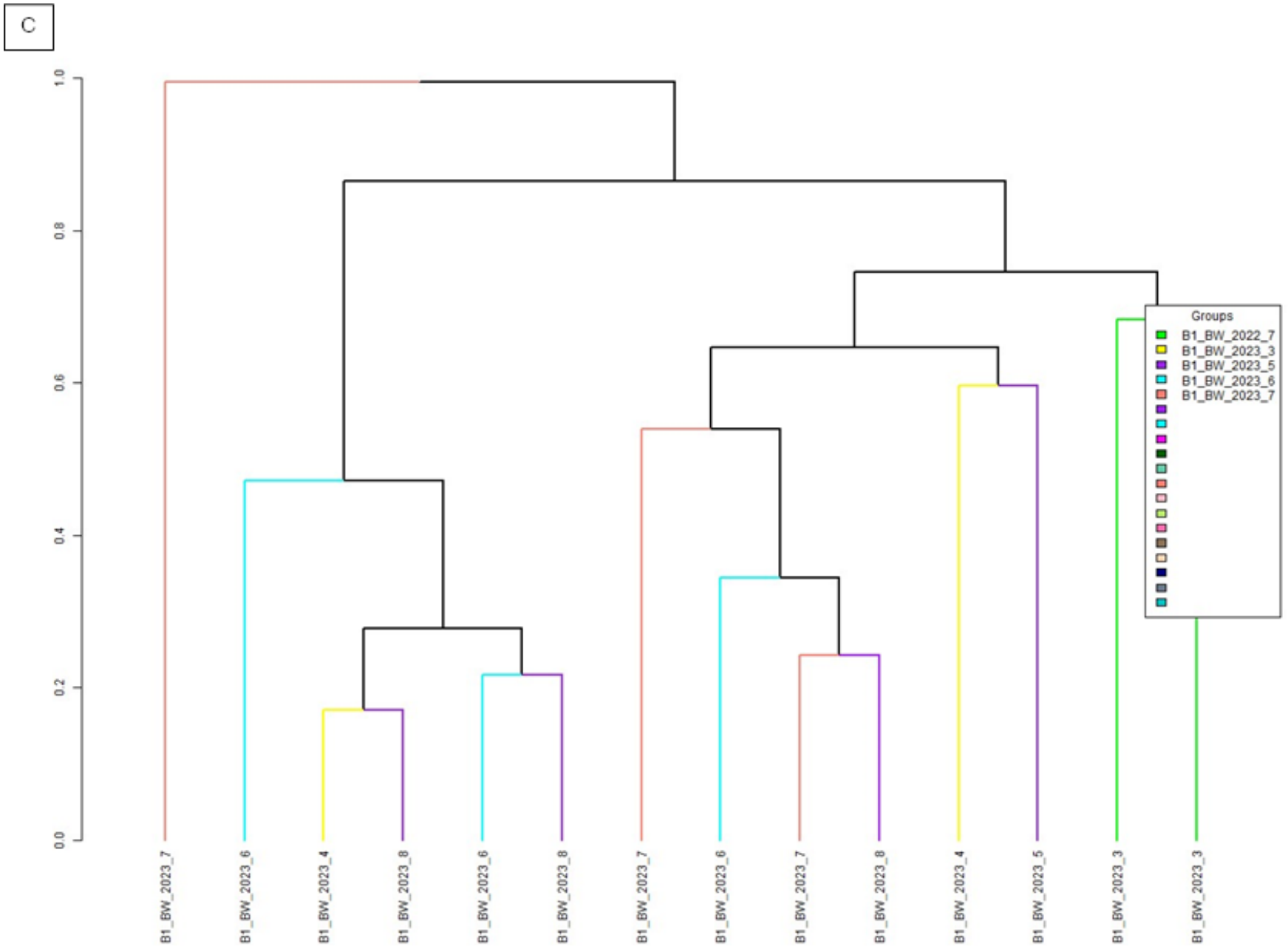
A2_BW_2023_7
A2_BW_2023_9


C
1.0
0.8
0.6
0.4
0.2
0.0
Groups
B1_BW_2022_7
B1_BW_2023_3
B1_BW_2023_5
B1_BW_2023_6
B1_BW_2023_7
B1_BW_2023_7
B1_BW_2023_6
B1_BW_2023_4
B1_BW_2023_8
B1_BW_2023_6
B1_BW_2023_8
B1_BW_2023_7
B1_BW_2023_6
B1_BW_2023_7
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B1_BW_2023_4
B1_BW_2023_5
B1_BW_2023_3
B1_BW_2023_3

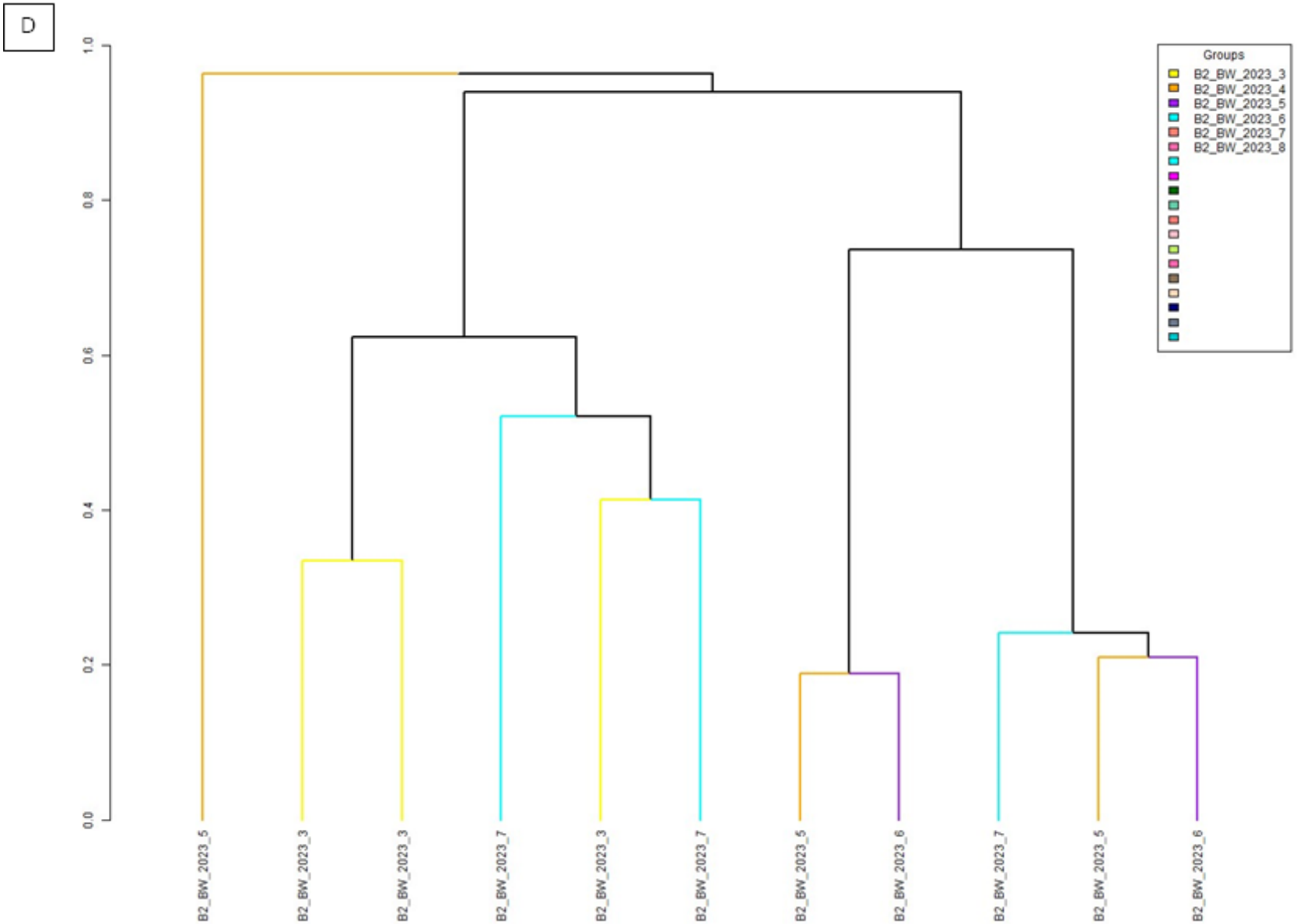

***Figure VII**: depicts the hierarchical clustering of microbial communities across various sampling dates for Bulk Water samples for each cooling tower, utilizing the Bray dissimilarity matrix. Each colour represents a unique sampling date, illustrating the diversity and similarity of microbial compositions over time. The black lines indicate the absence of a significant link or similarity between the microbial communities of the compared samples. Panel A, B, C, D represent cooling tower A1, A2, B1, B2 respectively.*

Appendix (II): Multidimensional Scaling Plot

To assess the variance in bacterial communities between biofilm and bulk water across each cooling tower, multidimensional scaling (MDS) plots were generated **Figure (VIII), Figure (IX) and Figure (X)**.

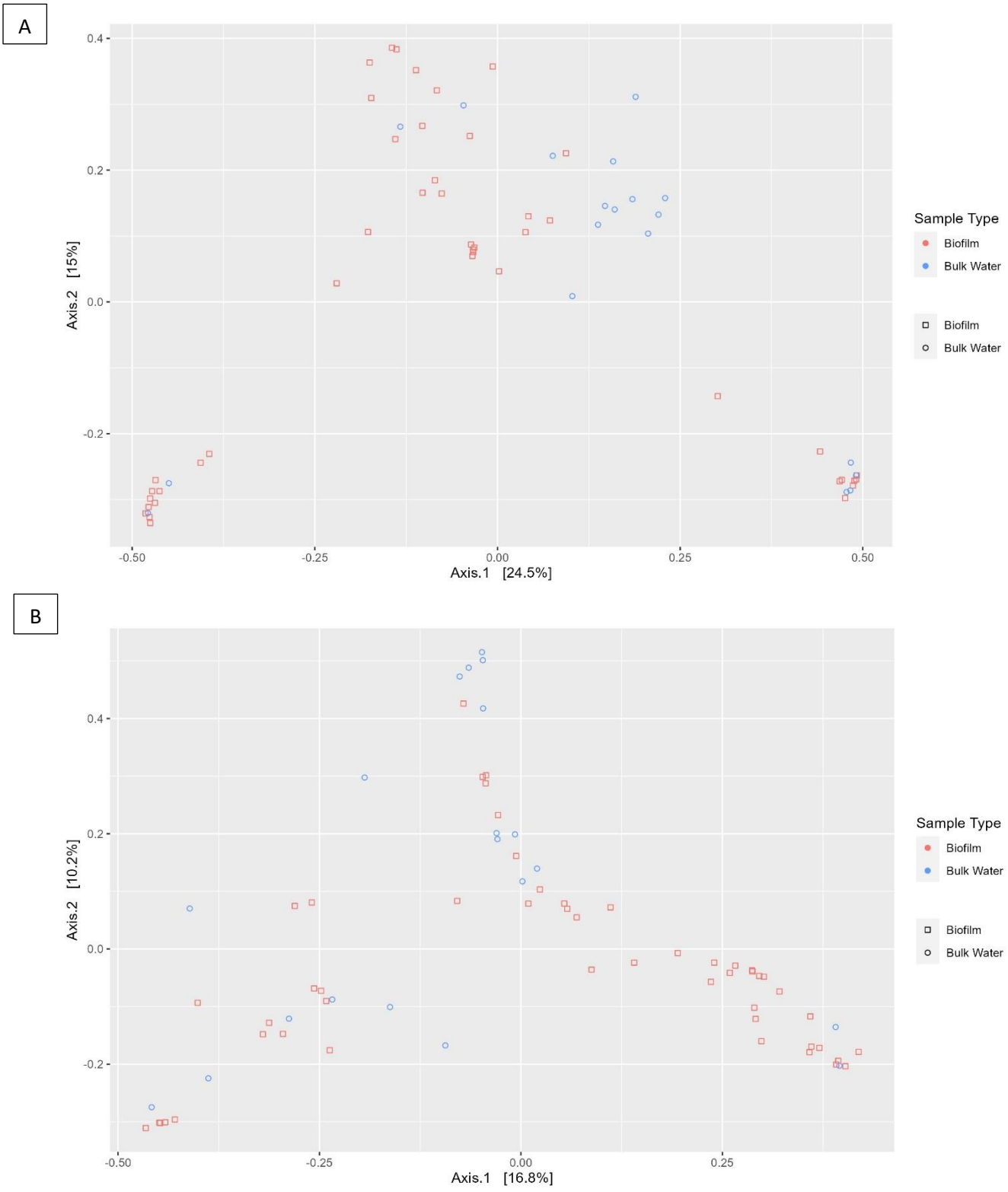

Figure (VIII): *Multidimensional Scaling plot to compare the microbial communities between biofilm and bulk water samples for cooling tower A1 (A) and A2 (B) using the Bray Curtis dissimilarity matrix.*

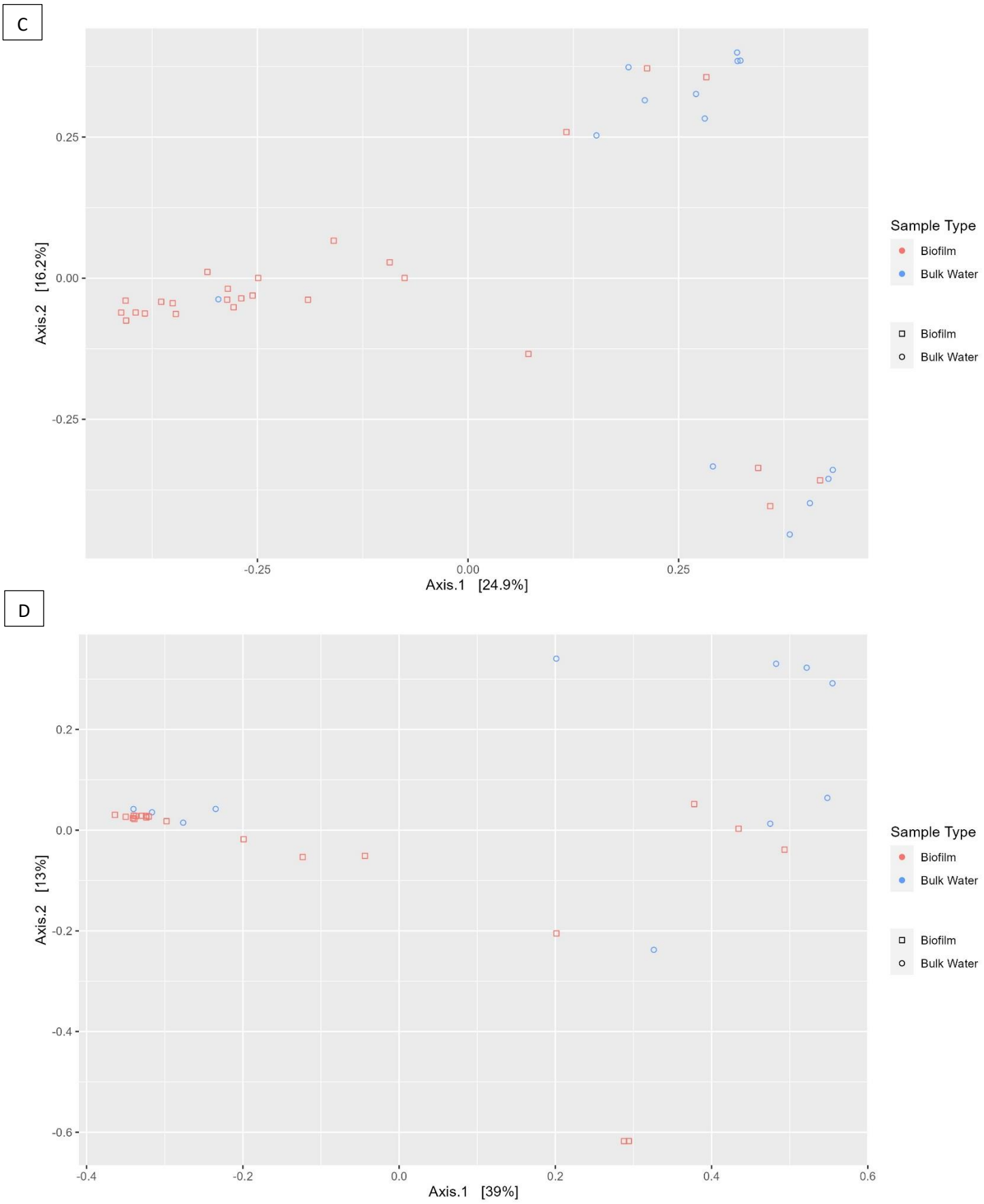

Figure (IX): *Multidimensional Scaling plot to compare the microbial communities between biofilm and bulk water samples for cooling tower B1 (C) and B2 (D) using the Bray Curtis dissimilarity matrix.*

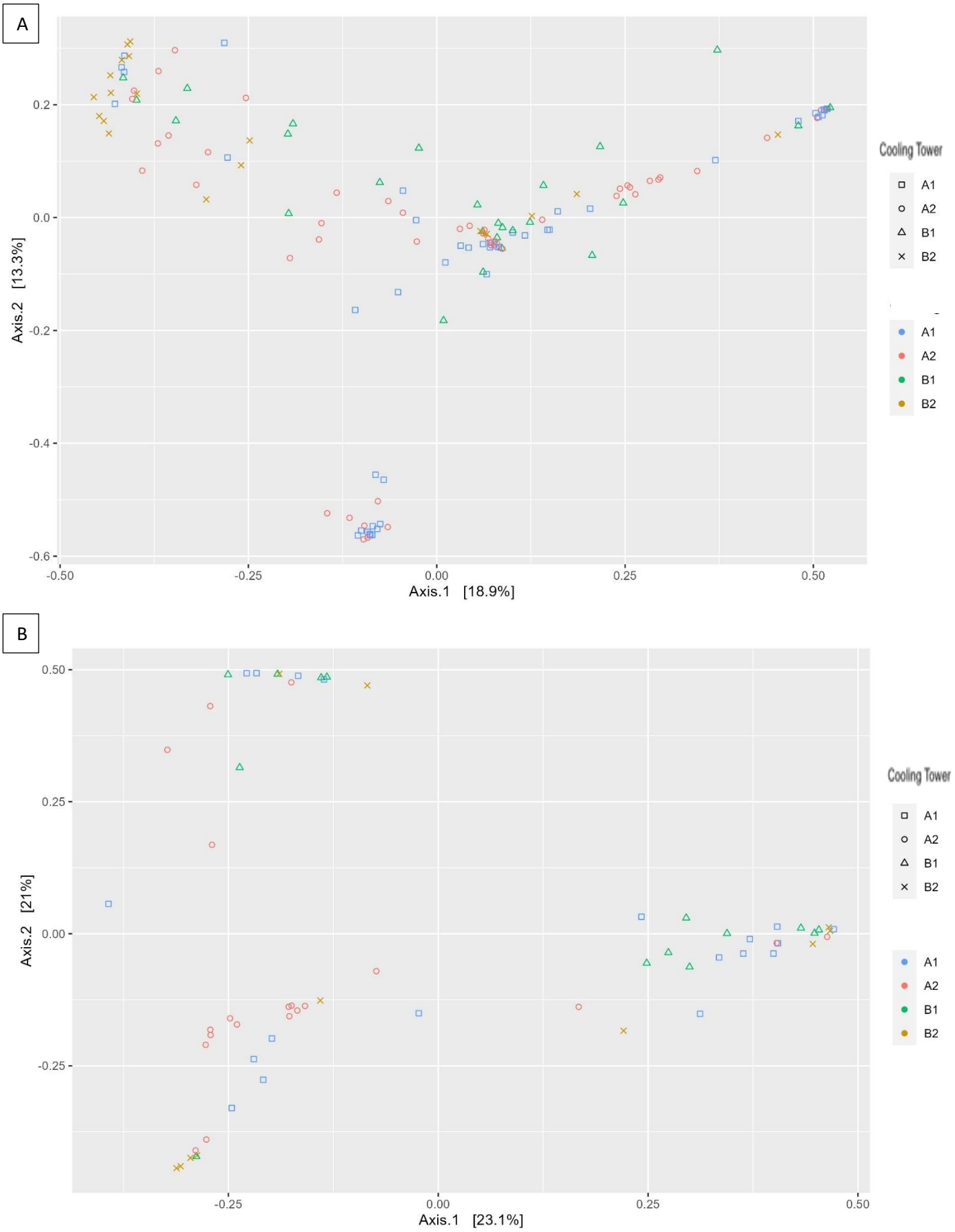

Figure (X): *Multidimensional Scaling plot to compare the microbial communities between cooling tower for biofilms (A) and bulk water (B) using the Bray Curtis dissimilarity matrix.*

Appendix (III): Taxa Tables

Taxa Tables are presented to facilitate the visualisation of taxa within the stack barplots. Taxa tables displaying families are shown in Table (I), while those presenting genera are depicted in Table (II).

Table (I): *Family Taxa Table*

Family Number	Taxa Name	Family Number	Taxa Name
1	Acidobacteriaceae_(Subgroup_1)	43	Beijerinckiaceae
2	Solibacteraceae	44	Devosiaceae
3	Nov-24	45	Hyphomicrobiaceae
4	Blastocatellaceae	46	Kaistiaceae
5	Actinomycetaceae	47	Labraceae
6	Mycobacteriaceae	48	*Methylobacteriaceae*
7	Pseudonocardiaceae	49	Methylocystaceae
8	Cytophagaceae	50	Rhizobiaceae
9	Spirosomaceae	51	Rhizobiales_Incertae_Sedis
10	Flavobacteriaceae	52	Xanthobacteraceae
11	Weeksellaceae	53	Rhodobacteraceae
12	Sphingobacteriaceae	54	Magnetospirillaceae
13	Kapabacteriales	55	Rhodospirillaceae
14	Bacteriovoracaceae	56	Clade_III
15	Sulfurimonadaceae	57	*Sphingomonadaceae*
16	Microcystaceae	58	Burkholderiaceae
17	Nostocaceae	59	Comamonadaceae
18	Xenococcaceae	60	Bdellovibrionaceae
19	Leptolyngbyaceae	61	Aeromonadaceae
20	Cyanobiaceae	62	Alteromonadaceae
21	Obscuribacteraceae	63	Pseudoalteromonadaceae
22	Babeliaceae	64	Shewanellaceae
23	Vermiphilaceae	65	Nitrosomonadaceae
24	Desulfomicrobiaceae	66	Oxalobacteraceae
25	Bacillaceae	67	Rhodocyclaceae
26	Lactobacillaceae	68	Cellvibrionaceae
27	Christensenellaceae	69	Porticoccaceae
28	Clostridiaceae	70	Diplorickettsiaceae
29	Haliangiaceae	71	Enterobacteriaceae
30	Nannocystaceae	72	Erwiniaceae
31	Brocadiaceae	73	Morganellaceae
32	Acetobacteraceae	74	Yersiniaceae
33	Azospirillaceae	75	Unknown_Family
34	Inquilinaceae	76	*Legionella*ceae
35	Caulobacteraceae	77	Methylomonadaceae
36	Hyphomonadaceae	78	Pasteurellaceae
37	Dongiaceae	79	Moraxellaceae
38	Elsteraceae	80	Pseudomonadaceae
39	Uncultured	81	Solimonadaceae
40	Micropepsaceae	82	Vibrionaceae
41	Paracaedibacteraceae	83	Rhodanobacteraceae
42	Reyranellaceae	84	*Xanthomonadaceae*

Table (II): *Genus Taxa Table*

Genus Number	Taxa Name	Genus Number	Taxa Name	Genus Number	Taxa Name	Genus Number	Taxa Name
1	Acidicapsa	43	Rhodovarius	85	Flavimaricola	127	Cellvibrio
2	Edaphobacter	44	Roseomonas	86	Gemmobacter	128	C1-B045
3	Granulicella	45	uncultured	87	Jannaschia	129	Aquicella
4	Terriglobus	46	Azospirillum	88	Paracoccus	130	Candidatus Hamiltonella
5	Candidatus_Solibacter	47	Nitrospirillum	89	Pseudorhodobacter	131	Citrobacter
6	Nov-24	48	Niveispirillum	90	Rhodobacteraceae	132	Enterobacter
7	Tellurimicrobium	49	Skermanella	91	Magnetospirillum	133	Klebsiella
8	Actinomyces	50	Inquilinus	92	Clade_III	134	Providencia
9	Mycobacterium	51	*Brevundimonas*	93	Altererythrobacter	135	Pantoea
10	Pseudonocardia	52	Caulobacter	94	Blastomonas	136	Serratia
11	Rhodocytophaga	53	Phenylobacterium	95	Novosphingobium	137	Candidatus_Ovatusbacter
12	Arcicella	54	Hirschia	96	Polymorphobacter	138	*Legionella*
13	Flectobacillus	55	Dongia	97	Porphyrobacter	139	Methylobacter
14	Pseudarcicella	56	Aliidongia	98	Rhizorhapis	140	Aggregatibacter
15	Spirosoma	57	Reyranella	99	Sphingobium	141	Acinetobacter
16	*Flavobacterium*	58	alphaI_cluster	100	Sphingomonas	142	Alkanindiges
17	Chryseobacterium	59	Beijerinckiaceae	101	*Sphingopyxis*	143	Enhydrobacter
18	Mucilaginibacter	60	Bosea	102	Sphingorhabdus	144	Azotobacter
19	Pedobacter	61	FukuN57	103	Cupriavidus	145	*Pseudomonas*
20	Kapabacteriales	62	Methylobacterium	104	Rhodoferax	146	Nevskia
21	Bacteriovorax	63	Methylocella	105	Variovorax	147	Photobacterium
22	Sulfuricurvum	64	Methylocystis	106	Bdellovibrio	148	Dyella
23	Chalicogloea_CCALA_975	65	Devosia	107	Aeromonas	149	Luteibacter
24	Scytonema_VB-61278	66	Hyphomicrobium	108	Rheinheimera	150	Stutzerimonas
25	Pleurocapsa_PCC-7319	67	Pedomicrobium	109	Pseudoalteromonas	151	Thermomonas
26	LB3-76	68	Kaistia	110	Shewanella	152	Xanthomonas
27	Prochlorococcus_MIT9313	69	Labrys	111	Burkholderia-	127	Cellvibrio
28	Candidatus_Obscuribacter	70	Methylobacterium	112	Limnobacter	128	C1-B045
29	Babeliaceae	71	Aliihoeflea	113	Pandoraea	129	Aquicella
30	Vermiphilaceae	72	Allorhizobium	114	Polynucleobacter	130	Candidatus Hamiltonella
31	Desulfomicrobium	73	Aureimonas	115	Acidovorax	131	Citrobacter
32	Bacillus	74	Phyllobacterium	116	Aquabacterium	132	Enterobacter
33	Lentibacillus	75	Rhizobium	117	Curvibacter	133	Klebsiella
34	Spirobacillus	76	Phreatobacter	118	Polaromonas	134	Providencia
35	Lactobacillus	77	Afipia	119	MND1	135	Pantoea
36	Christensenellaceae	78	Angulomicrobium	120	Collimonas	136	Serratia
37	Haliangium	79	Azorhizobium	121	Duganella	137	Candidatus_Ovatusbacter
38	Nannocystis	80	Bradyrhizobium	122	Janthinobacterium		
39	Candidatus_Brocadia	81	Pseudorhodoplanes	123	Massilia		
40	Acetobacteraceae	82	Pseudoxanthobacter	124	Undibacterium		
41	Acidiphilium	83	Rhodo*Pseudomonas*	125	Ferribacterium		
42	Acidocella	84	Tardiphaga	126	Zoogloea		

Appendix (IV): Cleaning Protocol within Cooling Towers

Cooling tower cleans are performed following the approved code of practice (HSE L8) every 6 months, with a flow chart shown in Figure (XI). Briefly, before cleaning the system, water should be disinfected using an oxidizing biocide with a suitable bio-dispersant, while the system fans are off, maintaining a residual 5 mg/l as free chlorine for a minimum of 5 hours. Then the system should be de-chlorinated, drained, and cleaned throughout, including the pack and drift eliminators. This cleaning process can involve physical removal using a 'wet vac', a strong oxidizing agent, acid clean, and solvent dispersant, depending on the fouling present. Upon completing the cleaning operation, the system should be refilled and disinfected again in the same manner as before.

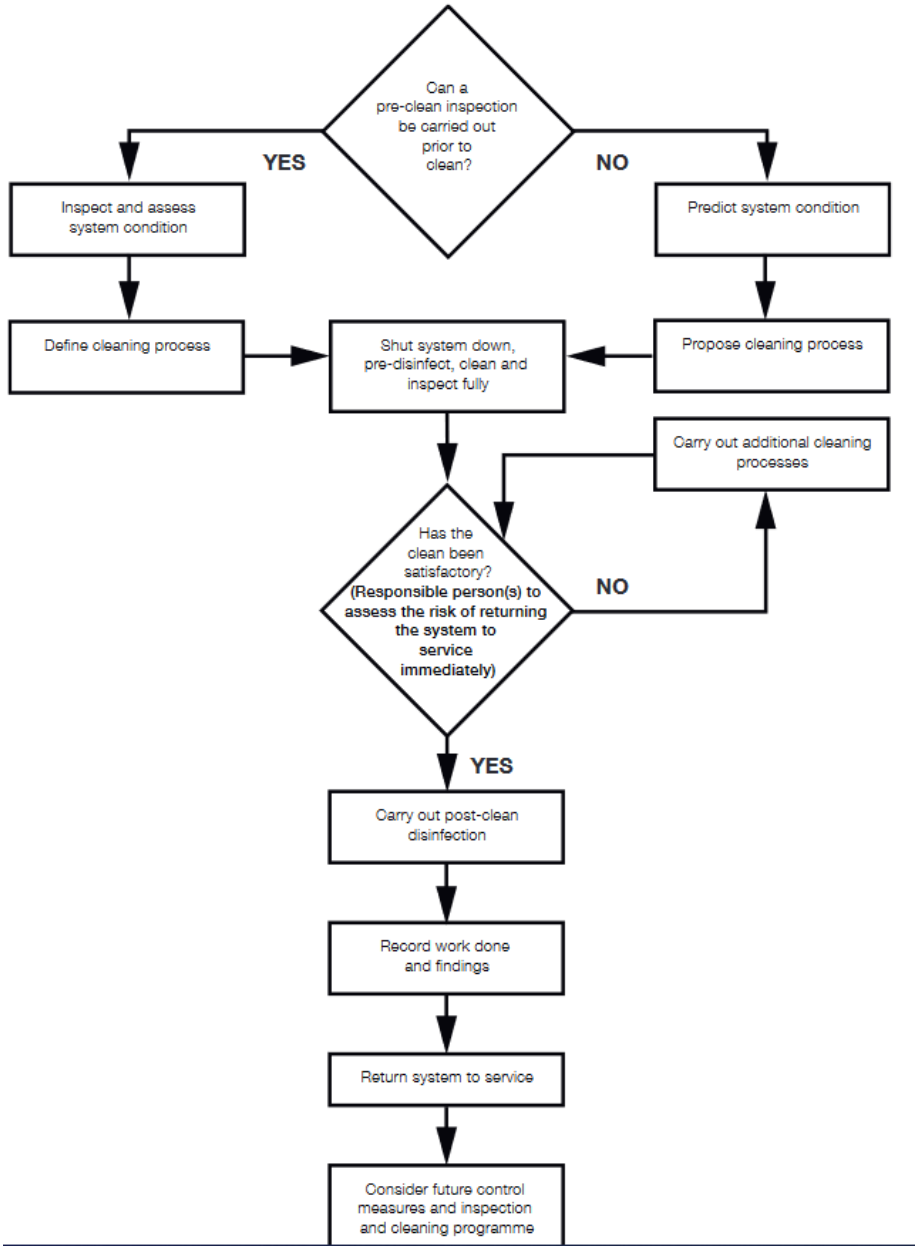

***Figure (XI)**: Flowchart of inspection and cleaning decision-making process (Health and Safety Executive, 2020.*