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Water Impact Statement for Kerry *et al*

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Applying a biofilm-sampling method developed for operational cooling towers, combined with rapid analyses, demonstrated *Legionella* colonises biofilms on internal surfaces. *L.pneumophila* was exclusively detected in biofilms, preceding conventional water-sample detection; the first direct evidence of biofilms being lead-indicators of colonisation in towers. Incorporating biofilm-based monitoring into management could enable earlier intervention, limiting release, aerosolisation and *Legionella*-associated occupational and public-health risks.

Biofilms as an early indicator of *Legionella* colonisation within evaporative cooling towers

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Abstract

Cooling towers are the source of over a quarter of Legionnaires' disease outbreaks. They provide ideal conditions for planktonic and biofilm-associated microbial growth, including *Legionella pneumophila*. *Legionella* monitoring typically relies on culture or, less often, rapid qPCR-based analysis of bulk-water samples only and hence may miss early colonisation. We propose that biofilms, recognised as microbial reservoirs, can be utilised as a sample type to provide early indicators in cooling towers. This study developed, optimised, and deployed a novel biofilm sampling regime across multiple cooling tower systems on a single site. Biofilm and planktonic cell concentrations, viability, and *Legionella* presence were quantified alongside cooling tower metadata. *L. pneumophila* was detected exclusively in biofilms, while *Legionella spp*. concentrations were more frequently detected in biofilms than bulk-water, with increases in biofilm-associated *Legionella spp*. often preceding elevations in bulk-water. Cell concentration and viability did not correlate with *Legionella* presence, highlighting the limits of non-specific metrics. These findings position biofilm monitoring as a more representative and reliable early-warning tool for *Legionella* detection, enabling more proactive and precise environmental health surveillance.

Key words

Legionella pneumophila, cooling towers, biofilms, qPCR, lead indicator, water infrastructure, public health

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Water Impact Statement

The first application of a biofilm sampling method developed for use in operational cooling towers, combined with rapid analysis techniques, demonstrated that *Legionella* can colonise biofilms on internal surfaces of cooling towers. Notably, *L. pneumophila* was exclusively detected in biofilms, preceding detection in conventional water samples, highlighting that biofilms can serve as lead-indicators of colonisation. Incorporating biofilm-based monitoring into routine cooling tower management offers a tool for earlier *Legionella* detection compared to conventional bulk-water monitoring. This would facilitate more timely and informed implementation of interventions before bacteria are released into bulk-water and aerosolised. Overall, this approach supports a shift from reactive to proactive management, helping to reduce occupational and public health risks from *Legionella*-associated respiratory infections.

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1. Introduction

Engineered water systems such as potable water distribution systems, shower heads, fountains and evaporative cooling systems including cooling towers are significant sources of *Legionella pneumophila* (*L. pneumophila*) and outbreaks of Legionnaires' disease. These environments are particularly conducive to producing aerosols capable of containing *L. pneumophila* (Paranjape *et al.,* 2020*).* Cooling towers pose a particular concern as they create conditions that promote both biofilm (sessile) and planktonic (free-living) microbial growth, supported by temperatures between 25-35°C, a near-neutral pH, exposure to sunlight, continuous aeration, steady nutrient supply and an extensive surface area that facilitates biofilm development in particular (Di Gregorio *et al*., 2017). While outbreaks of Legionnaires' disease are sporadic, those associated with cooling towers tend to involve multiple cases. Cooling towers are the most frequently confirmed source of *L. pneumophila* aerosolisation, cited as contributing to at least 28% of all outbreaks (Di Pippo *et al*., 2018) with Hamilton *et al*. (2018) demonstrating that of 136 outbreaks identified globally, cooling towers were implicated or suspected in 30% of the total number, 50% of confirmed outbreak-associated cases, and 6% of outbreak–associated deaths. Aerosolised Legionellae from cooling towers can disperse some distance from the source (Walser *et al*., 2014) and possibly as far as 6 km (Nhu Nguyen *et al*., 2006), thus posing a substantial potential public health and occupational health risk.

Cooling towers are a vital widespread infrastructure, serving as a cost-effective and energy-efficient cooling solution for a wide range of systems and processes, including power generation, petrochemical industries and factories such as plastics injection moulding. As heat exchange devices, they circulate water at elevated temperatures from industrial processes over a pack material. Air flows in the opposite or perpendicular direction to the water, promoting heat transfer and resulting in heat loss. The cooled water is then collected in a basin, to be either recirculated within the system or discarded into the wastewater system (Figure 1A and 1B). The pack material, typically made of polyvinyl chloride (PVC; Figure 1C), 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 efficiency of the cooling process. Biofilms (complex microbial communities encased in extracellular polymeric substances) have been shown to develop on PVC in other engineered water systems (Goraj *et al*., 2021; Learbuch *et al*., 2021) and represent a significant microbial reservoir. At the system level, surface-attached biofilm communities can contain several orders of magnitude more total microbial cells than the planktonic (bulk-water) phase within drinking water systems (Flemming *et al*., 2016). The large surface area of the pack in a cooling tower contributes to biofilm-forming potential in these systems by providing more sites for microbial attachment and growth.

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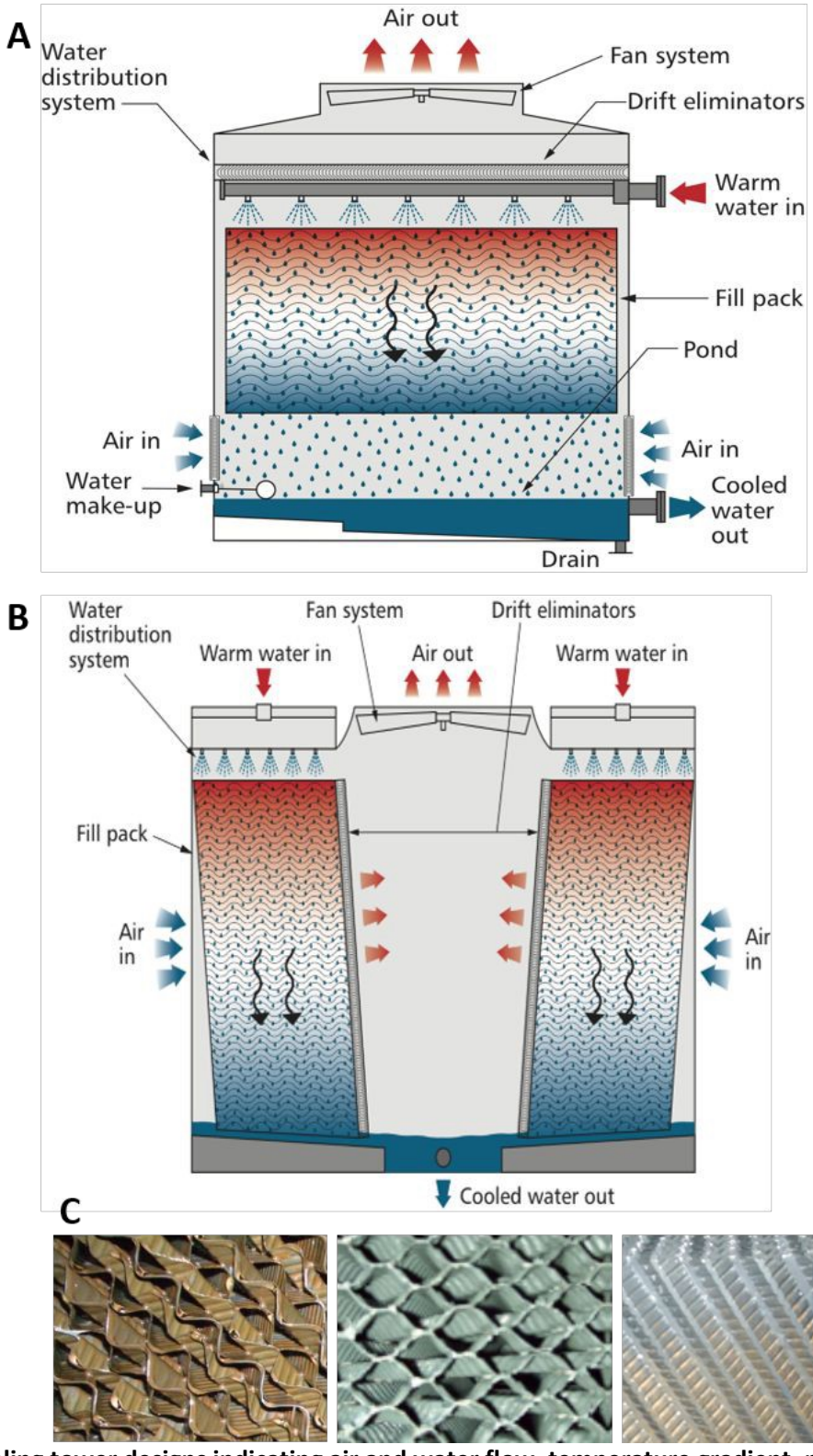

Figure 1 Cooling tower designs indicating air and water flow, temperature gradient, pack location and structure. Schematics show (A) an induced draught counter flow cooling tower and (B) an induced draught double cross flow cooling tower. (C) Examples of typical pack honeycomb or corrugated designs and surfaces. Images courtesy of Health and Safety Executive, 2024.

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Given that cooling towers are a significant source of large outbreaks of Legionnaires' disease worldwide, many governments have implemented policies to mitigate this risk. In Great Britain (GB), the control of *Legionella* within cooling towers is overseen by the Health and Safety Executive (HSE) through a regulatory framework that mandates a comprehensive water safety plan. This includes risk assessment, a water treatment programme, ongoing planktonic monitoring and maintenance, as well as meticulous record keeping (HSE, 2024). Failure to comply with these regulations can trigger enforcement by the HSE, ranging from notification of contravention letters to enforcement notices or prosecution. Most improvement notices issued in the past decade were for ineffective implementation of written control schemes (Crook *et al.*, 2020), reflecting the absence of a clear action plan to respond to *Legionella* colonisation before an outbreak occurs.

To assess the effectiveness of microbial control measures, including biocide treatment, and to detect any system malfunctions, routine water quality monitoring is deployed. This includes quantification of viable planktonic bacterial concentrations within bulk-water using heterotrophic plate counts (National Academies of Sciences, Engineering, and Medicine, 2020) and testing specifically for *Legionella* through culture methods (International Organization for Standardization, 2017). In GB, current action levels for *Legionella* dictate that concentrations below 100 colony forming units (CFU)/litre require no immediate action, levels between 100 and 1,000 CFU/litre necessitate a reassessment of the control programme and an adjustment in biocide dosage, and levels above 1,000 CFU/litre demand immediate remedial action (HSE, 2020). While these monitoring techniques provide some level of control, *Legionella* culture methods can take between 10 and 14 days to acquire results, this causes a delay in implementing any remedial action and risks that outbreaks could occur in the interim (Walker and McDermott, 2021). Moreover, current monitoring practices predominantly focus on sampling bulk-water for the presence of planktonic bacteria, which, while informative as background snapshots of water quality, does not capture the full ecological picture. As a result, sporadic outbreaks continue to occur despite compliance with current testing protocols, highlighting a persistent public health risk. Therefore, there is an urgent need and growing emphasis on the development and application of emerging detection techniques, such as quantitative polymerase chain reaction (qPCR), to assess water samples for *Legionella* more rapidly and with greater sensitivity, enabling quicker response times to prevent outbreaks. There is also a perception that elevated planktonic bacterial counts could reflect system conditions that favour *Legionella* proliferation, serving as a proxy for increased risk. Recent research has explored whether overall microbial concentration and viability in bulk-water could serve as indicators of *Legionella* presence. Campaña *et al.* (2023) and Kyritsi *et al.* (2018) both report a potential relationship between total

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microbial load and the likelihood of *Legionella* detection, proposing that under certain environmental conditions, higher viable microbial concentrations may reflect an increased Legionella risk.

A critical gap in *Legionella* monitoring is the failure to account for the ecological environment of biofilms, which are crucial for the survival and proliferation of *Legionella* in engineered water environments. It is plausible that *Legionella* may predominantly inhabit biofilms within cooling tower systems, being released only intermittently into the circulating bulk-water. Routine sampling of circulating water at a single point in time, thus stands a low chance of detecting *Legionella* that may be present within the system. This intermittent release, combined with potential dilution within large system volumes, means that bulk-water sampling alone may substantially underestimate the true extent of *Legionella* colonisation. Although the importance of biofilms is recognised, their potential, especially within cooling towers, to act as leading indicators, i.e., to provide an early warning of future problems, has not been explored. Biofilms are complex microbial communities which facilitate symbiotic interactions to exchange nutrients, while extracellular polymeric substances produced in the biofilm provides a physicochemical barrier to protect from environmental stresses and can deplete biocide concentrations (Flemming, 2020). Biofilm and planktonic communities are known to be significantly different in microbial quantity and community composition in other engineered water systems (Liu *et al*., 2020; Wang *et al*., 2023) and the favourable growth conditions in cooling towers could mean that biofilm microbial concentrations could be considerably higher than those in bulk-water. In particular, the cooling tower pack offers extensive surface area and relatively slow flow rates that enhance biofilm formation making it a plausible niche *for L. pneumophila* colonisation. However, cooling tower biofilms in general remain largely unstudied and unutilised as a sampling or monitoring source and *Legionella* colonisation of the pack within cooling tower systems specifically never being explored.

One of the most critical functions of biofilm when considering *L. pneumophila* is that biofilms attract microbial grazers such as free-living amoeba because the greatest concentration of microorganisms is found within the biofilm, such as the biofilms on the pack of cooling towers, providing a plentiful food source (Huws *et al*., 2005). *L. pneumophila* exploits this to replicate within the free-living amoeba. Furthermore, *Legionella spp.* can obtain nutrients 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). The complex relationships between *Legionella*, biofilms and protozoa are often overlooked, with no standardised sampling regime monitoring any

aspect of cooling tower biofilms (Pereira *et al*., 2021). As a result, any data collected is limited to monitoring organisms in their planktonic state. If, as is likely, any planktonic microbes recorded originate from an established biofilm, bulk-water analysis becomes a 'lagging indicator' with any actions taken due to such sampling post hoc.

It was hypothesised that biofilms in cooling towers can act as ecological niches for *Legionella* and, when paired with emerging detection technologies, biofilm sampling could serve as a more reliable 'lead indicator' of *Legionella* presence (including *L. pneumophila*) than conventional bulk-water sampling. Hence, the primary aim of this study was to ascertain the prevalence of *L. pneumophila* in both biofilms and bulk-water from operational cooling towers. To enable this, a novel biofilm sampling technique was developed, optimised and implemented specifically for application in cooling tower environments. In addition, the study sought to explore potential correlations between overall microbial concentration, microbial viability, and the presence of *Legionella* in both biofilm and bulk-water samples.

2. Materials and Methods

2.1 Overview of experimental design

To demonstrate the value of biofilm sampling, it was critical that live, operational systems were studied under real-world conditions, where natural variation and the lack of experimental control necessitate a longitudinal approach. A longitudinal field study was conducted sampling biofilm and bulk-water across two operational cooling towers on a single site to capture seasonal variations and routine cleaning events, thereby maximising opportunities to detect *L. pneumophila*, which had been historically rare at the site. This design enabled evaluation of biofilms as potential leading indicators for the presence of *Legionella spp.*, including *L. pneumophila*, and facilitated examination of correlations between total and intact cell concentrations (TCC and ICC) in biofilm and bulk-water with the presence of *Legionella.* A biofilm sampling regime was developed and optimised for use in operational cooling towers and implemented alongside bulk- water sampling incorporating cell concentration and viability analysis (via flow cytometry) and *Legionella* detection (via qPCR)

2.2 Cooling Tower Selection and Site Details

To explore biofilm and bulk-water under different operational conditions, two towers (A1 and A2) were selected for biofilm sampling method optimisation and the longitudinal study based on a shared pack design but contrasting operational biocide regimes (Table 1). These towers were both

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supplied with coarsely filtered water from nearby natural sources and used in several on-site operations.

The field site was a nuclear-waste processing, storage and decommissioning facility employing multiple cooling towers for process heat rejection purposes. Sampling at this facility presented unique logistical constraints: samples could not be removed from the site until completion of a mandatory quarantine period. This prevented adherence to ISO 11731, which requires *Legionella* culture analysis to be undertaken within 2 d of collection. To overcome this, on-site laboratory capabilities were established for *Legionella* detection via qPCR, while on-site flow cytometry was used for determination of TCC and ICC, enabling rapid analysis following collection.

Table 1 Cooling Tower technical description, monitoring and control strategies

Cooling Tower	Engineered Structure	Oxidising/ Non-Oxidising Biocide	Biocide Regime
A1	Induced Cross Flow	Oxidising	Sodium hypochlorite coupled with ultrasonic cavitation physical treatment
A2	Induced Cross Flow	Combination of oxidising and non-oxidising	Weekly rotations of non-oxidising biocides 2,2-dibromo-3-nitrilopropionamide (DBNPA) and Quaternary Ammonium Compound (QAC) Benzyl- C8-18-Alkyldimethyl Chloride

2.3 Optimising biofilm sampling within Cooling Towers

The cooling tower pack was identified as the most appropriate location for biofilm sampling, primarily due to its extensive surface area, on which biofilm development is most likely to occur and from which aerosolisation of *Legionella* would most plausibly originate (Pereira *et al*., 2021), thus posing the greatest risk of transmission. If the hypothesis that biofilms act as an ecological niche for *Legionella* is correct, then representative samples from this crucial zone within the cooling towers are the most appropriate and important to study, as this area is likely to contribute most to *Legionella* aerosolisation. Additionally, characteristics inherent to the pack, such as favourable temperature range, nutrient availability and low flow rates, further promote biofilm growth (Flemming, 2020). The accessibility of the pack area across different cooling towers also provided a practical advantage, facilitating consistent biofilm sampling and comparison between systems. However, spatial variability within the pack may influence biofilm distribution, a factor that should be considered when interpreting results.

To account for this potential heterogeneity and ensure the collection of reliable and representative biofilm samples from operational cooling tower pack, it was crucial to understand how biological variability between replicates could be influenced by specific environmental factors. Key among

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these factors is (i) temperature, as a gradient occurs from the top to the bottom of the pack, and (ii) surface area, as a larger surface area could present more ecological niches and gradients of nutrients, oxygen and biocide. Therefore, a preliminary sampling regime was implemented to assess systematically the impact of both temperature and surface area on biofilm cell concentration and viability (Figure 2).

To determine any potential effect of the temperature gradient from the top to the bottom of the cooling tower pack, biofilm samples were collected from the top, middle, and bottom sections of the pack, reflecting the typical thermal stratification observed in operating cooling towers. Biofilms were collected using 100 cm^2 quadrats positioned approximately 90 cm inward from the outer face of the pack, representing a consistent depth of sampling within the pack structure. This depth was selected to balance accessibility with the need to capture conditions representative of the internal biofilm environment. Temperature at each sampling point was measured three times with a digital thermometer, allowing 30 seconds for the reading to stabilise.

To optimise the surface area sampled, 100 cm^2, 200 cm^2, and 300 cm^2 quadrats were used to collect samples from a single pack location (middle) to a depth of 90 cm into the pack. The choice of a minimum of 100 cm^2 surface area was intended to capture variability in biofilm composition, enabling a focused examination, while surfaces above 300 cm^2, were deemed impractical for handling within the pack. This rationale ensured a comprehensive yet manageable approach to assessing surface area variations during biofilm sampling.

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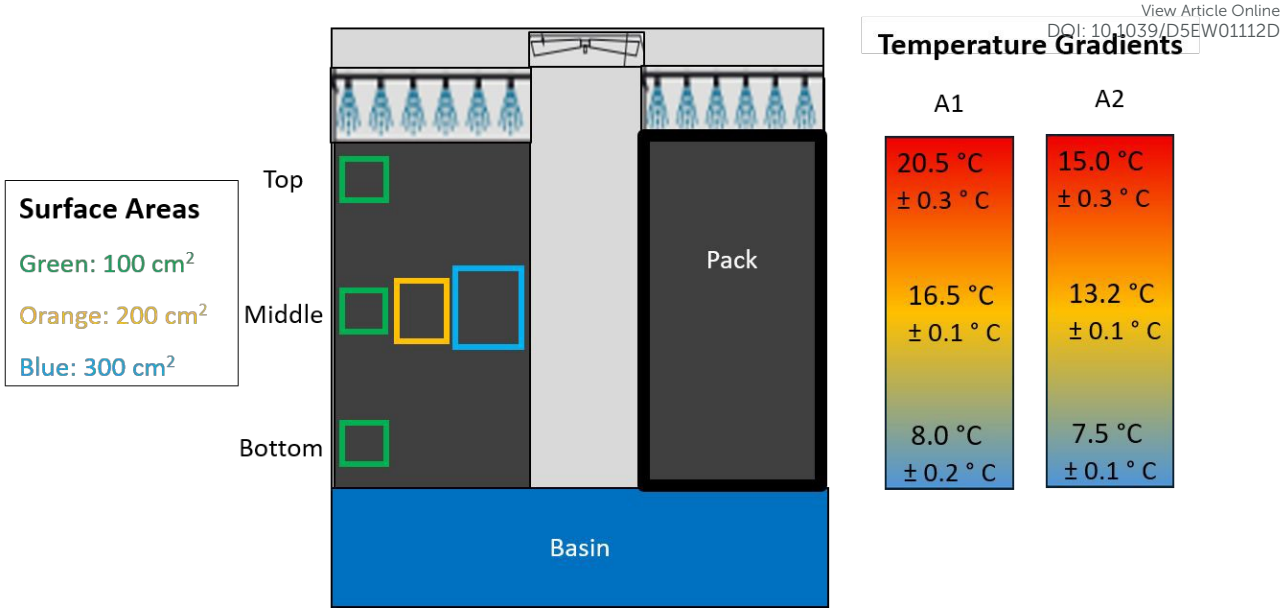

Figure 2 Schematic of cooling tower sampling locations within the pack (top, middle, bottom) indicating the corresponding surface areas that were sampled and the temperature gradient measured in each tower: A1 and A2 (mean ± standard deviation), demonstrating a decrease from the top to the bottom of the pack.

2.4 Sampling campaign for longitudinal monitoring of cell concentration and *Legionella* detection

Sampling was conducted at eight time points between March and October, providing a valuable opportunity to assess the influence of both seasonal variation and operational interventions on microbial dynamics. During each sampling time point, biofilm samples (n=5) and bulk-water samples (n=3) were collected from each cooling tower for analysis of total and intact cell concentrations (TCC and ICC) and *Legionella* detection and quantification. Routine cooling tower cleaning interventions were carried out by the site maintenance team in accordance with the written controls scheme. These interventions were recorded alongside sampling activities to provide operational context for data interpretation.

2.5 Sample Collection Methods

The biofilm removal and bulk-water collection methods were the same across the optimisation and longitudinal components of the research.

2.5.1 Cooling Tower Biofilm Sampling

Biofilm samples (n=5) were collected from each cooling tower pack using sterile cotton swabs (Thermo Scientific Sterilin). This biofilm removal method was specifically chosen to align with established practices for biofilm collection in other engineered water systems, such as drinking

water distribution and plumbing systems, where swabbing has been widely used and validated (De Sotto *et al*., 2020; Liu *et al*., 2020). Employing this method enhances comparability across studies by ensuring consistency in sampling protocols.

The selection of optimised sample areas and locations was informed by the findings from 2.3, ensuring representative and repeatable results within the unique environment of an operational CT. A medium-density polyethylene (MDPE) quadrat was used as a template to standardise the sampling area of the pack (this was cleaned with 70% ethanol between replicates and sampling points). After sampling, the swabs were transferred into falcon tubes for transport. Upon arrival at the on-site laboratory, each sample tube was filled with sterile phosphate buffered saline (50 ml) and vortexed at 3000 rmp for thirty seconds to resuspend the biofilm (triplicates of PBS were used as controls). To achieve a thoroughly homogenised biofilm suspension, the tubes were then rotated 180 degrees and vortexed again for an additional thirty seconds.

2.5.2 Cooling Tower Bulk-water Sampling

Bulk-water samples (n=3) were obtained from each cooling tower following the ISO 11731 protocol. In brief, one litre volumes of water were collected in triplicate, either from the cooling tower basin or from a designated sampling port when the basin was inaccessible, into sterile polypropylene bottles containing 0.01% sodium thiosulfate to neutralise any residual chlorinated biocide present. These bulk-water samples were taken directly to the laboratory, thus no transport conditions as per ISO 11731 were necessary and they required no preparation prior to analysis.

2.6 Sample analysis

Biofilm and bulk-water samples were subdivided into aliquots for analysis to determine TCC and ICC and the presence, absence and quantification of *Legionella spp*. and *L. pneumophila*.

2.6.1 Total and Intact Cell Counts

TCC and ICC counts of cooling tower biofilm and bulk-water samples were obtained via flow cytometry using the staining and gating protocol detailed in Fish *et al,* (2020), adapted from (Gatza *et al*., 2013). Briefly, 0.5 ml sample aliquots were stained with either SYBR Green (Life Sciences, California, USA) to quantify total cells, or a mixture of SYBR Green and Propidium Iodide (Life Sciences, California, USA) to quantify intact cells. After vortexing, the stained samples were incubated for 10 minutes at 37°C in the dark and analysed on a BD Accuri C6 flow cytometer (50 µl, medium flow rate). Negative controls included sterile distilled deionised water that were unstained (n=3), stained with SYBR Green (n=3) and stained with the SYBR Green and Propidium Iodide

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combination (n=3). The standard C6 analysis template was modified as per Fish *et al*. (2020) to include singlet–doublet discrimination, providing a quantitative assessment of sample homogenisation (for the current study, samples for which >90% of the data were singlets were classed as homogenised). Cell counts (cells μl^{-1}) were obtained directly from the C6 software and subsequently converted to concentrations depending on sample type: for planktonic samples, counts were multiplied by 1000 µl to give cells ml^{-1}; for biofilms, counts were multiplied by the suspension volume (50 ml) and divided by the surface area from which the biofilm was removed to give cells mm^{-2}.

2.6.2 *Legionella* detection and quantification

All study samples (biofilm, bulk-water and negative controls), were subjected to DNA extraction using the DNeasy PowerWater Kit (Qiagen), which is widely used for high-quality microbial DNA recovery from engineered and environmental water matrices (Liu *et al*., 2020). In accordance with the protocol, each sample received 15 µl of an endogenous control reaction mix. The extracted DNA was then analysed for detection and quantification of the *Legionella* genus and the specific pathogen *L. pneumophila* sg1.

2.6.2.1 Legionella genus

Legionella spp. was quantified using the Genesig qPCR platform (Primer Design Ltd, Southampton, UK) following the manufacturer's standard protocol, with each 15 µL reaction containing qPCR master mix, *Legionella*-specific primers and probe, internal extraction control primers and probe, and RNase/DNase-free water. For biofilm samples, qPCR outputs were converted to gene copies cm^{-2} by accounting for the resuspension volume and sampled surface area, whereas bulk-water samples were expressed as gene copies L^{-1}.

2.6.2.2 L. pneumophila sg1

To analyse for the presence of, and to quantify, *L. pneumophila* sg1 by qPCR, the Genomadix (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 filter-based concentration kit. The captured intact bacteria were eluted from the filter and the eluate transferred to a test cartridge which contains qPCR primers, a probe, and an internal positive control, loaded into the Genomadix Cube analyser. These primers and probes are designed against a highly conserved region of *L. pneumophila* (*mip*) gene (Wilson *et al*., 2003). The Genomadix software subsequently converts qPCR-derived copy numbers into equivalent colony-forming units (CFU) for data visualisation, analysis and interpretation.

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2.7 Data Analysis

All data visualisation and statistical analysis was conducted using R v4.3.2 (R Core Team, 2025) within the R Studio integrated environment (v2023.09.1+494; Posit Software, 2023) using base R and the package ggplot2 (Wickham, 2016). Raw data points are plotted unless otherwise stated. As data was non-normally distributed non-parametric tests (Wilcoxon or Kruskal Wallis) were conducted to test for statistical significance between variables. Spearman's rank correlation was used to assess the relationship between cell counts and *Legionella spp.* or *L. pneumophila* concentrations.

3. Results

3.1 Optimising Biofilm Sampling: Temperature, surface area and cell concentrations

In both cooling towers, biofilm TCC and ICC did not differ significantly across sampling locations (Figure 3), despite the temperature gradients between the top and bottom of the pack(A1:TCC χ^2=4.5, p=0.12; ICC χ^2=2.38, p=0.31; A2:TCC χ^2=1.64, p=0.44; ICC χ^2=3.3, p=0.20). Sampled surface area similarly had no significant effect on biofilm TCC or ICC, in either tower (Figure 4; A1:TCC χ^2=4.62, p=0.10; ICC χ^2=5.6, p=0.06; A2:TCC χ^2=3.82, p=0.15; ICC χ^2=5.6, p=0.06). Although temperature/location and surface area had an overlapping condition (middle, 100 cm^2), the datasets were collected at different time points; therefore, no shared data appears across Figures 3 and 4.

As no significant effects of temperature or surface area were observed, subsequent longitudinal biofilm sampling used a standardised 100 cm^2 quadrat positioned in the middle of the pack.

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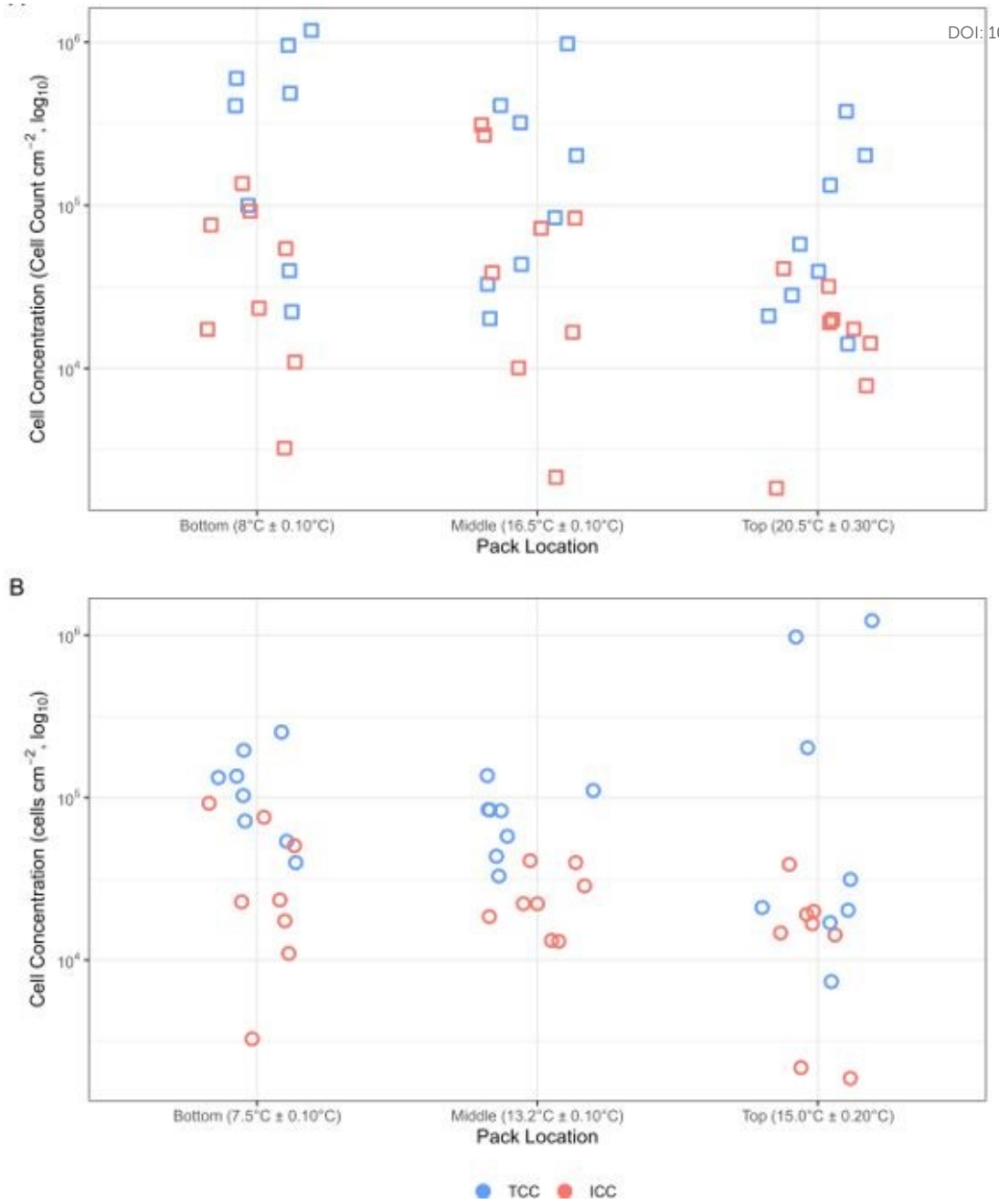

Figure 3 Impact of sample location and temperature on biofilm total cell concentration (TCC) and intact cell concentration (ICC) for cooling tower (A) A1 and (B) A2. Logged data is presented, the mean temperatures measured at each location are provided in brackets, ± standard deviation.

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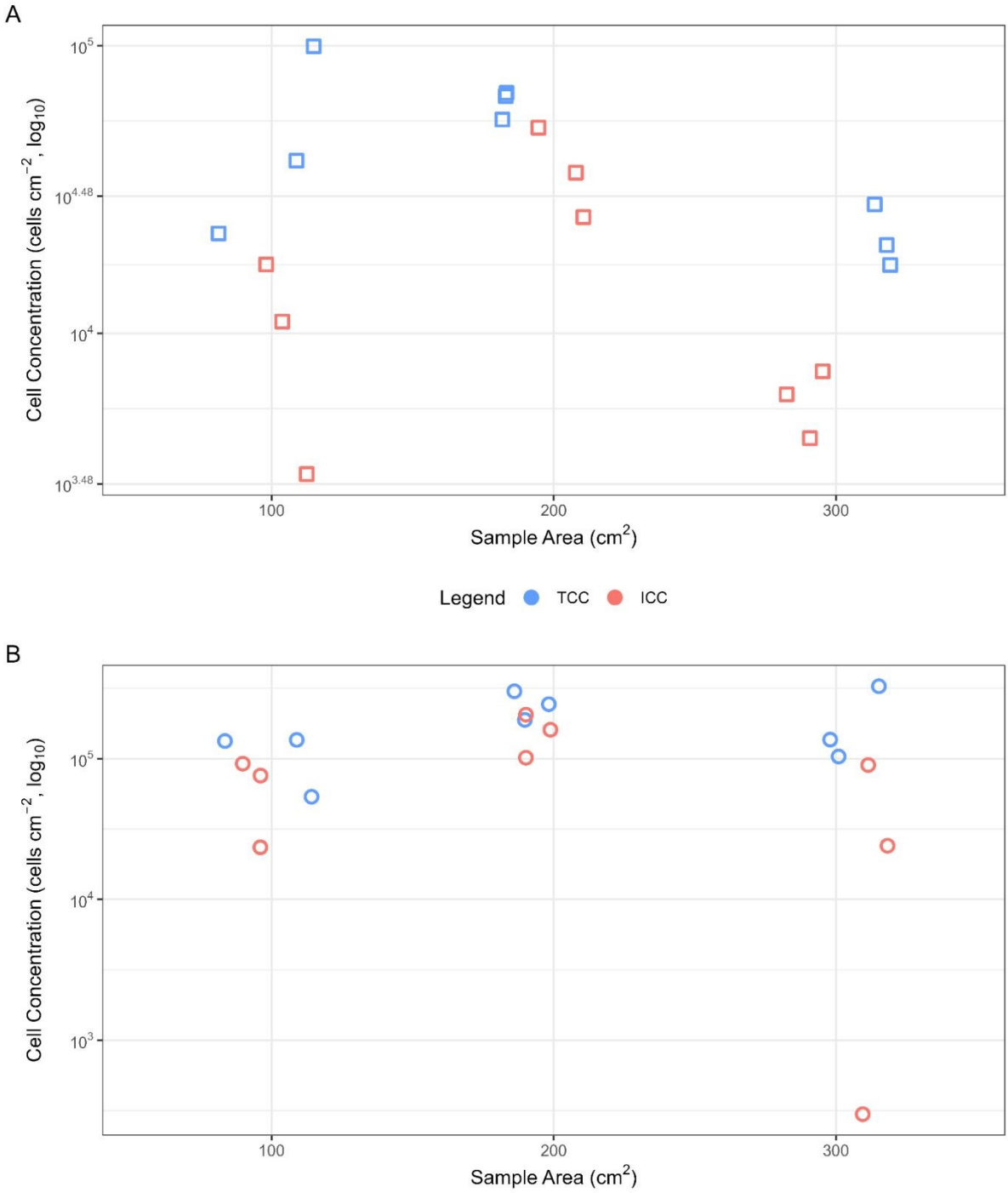

Figure 4 Impact of sampled surface area on biofilm total cell concentration (TCC) and intact cell concentration (ICC) for cooling towers (A) A1 and (B) A2. Log of the raw data is plotted.

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3.2 Longitudinal assessment of microbial concentration, viability and *Legionella* quantification

3.2.1 Microbial concentration and viability

TCC and viability (as assessed by ICC) differed significantly between biofilms and bulk-water throughout the sampling period for each tower ($p < 0.01$), indicating that the two sample types were consistently distinct (Figure 5).

Biofilm TCC and ICC varied significantly over time (Figure 5A/B) in both towers (A1: TCC χ^2=15.19, p=0.03; ICC χ^2=22.37, p< 0.01; A2: TCC χ^2=17.20, p=0.02;ICC χ^2=16.13, p=0.02). ICC% also varied temporally, this was only statistically significant in A1 (χ^2 = 21.67, df = 7, p <0.01), not A2 (χ^2 = 12.65, df=7, p=0.08). Bulk-water cell concentrations also varied temporally (Figure 5 C/D), though ICC trends differed between towers, peaking in June-July in A1, with more fluctuations occurring in A2. Intact-to-total cell ratios (ICC%) also varied over time in the bulk-water for both tower A1 (A1: χ^2=14.56, df=7, p=0.04) and A2 (χ^2=18.47, df=7, p=0.01).

Comparing between towers, biofilm and bulk-water TCC and ICC were consistently higher in A2, which used combined oxidising and non-oxidising biocides, than A1, which used oxidising biocides only (biofilm TCC: χ^2=452.00, p<0.01; ICC: χ^2=264.00, p<0.01; bulk-water TCC W=404, p=0.02; ICC: W=387, p=0.04). However, ICC% did not significantly differ between the towers, indicating comparable relative viability despite varied microbial abundance.

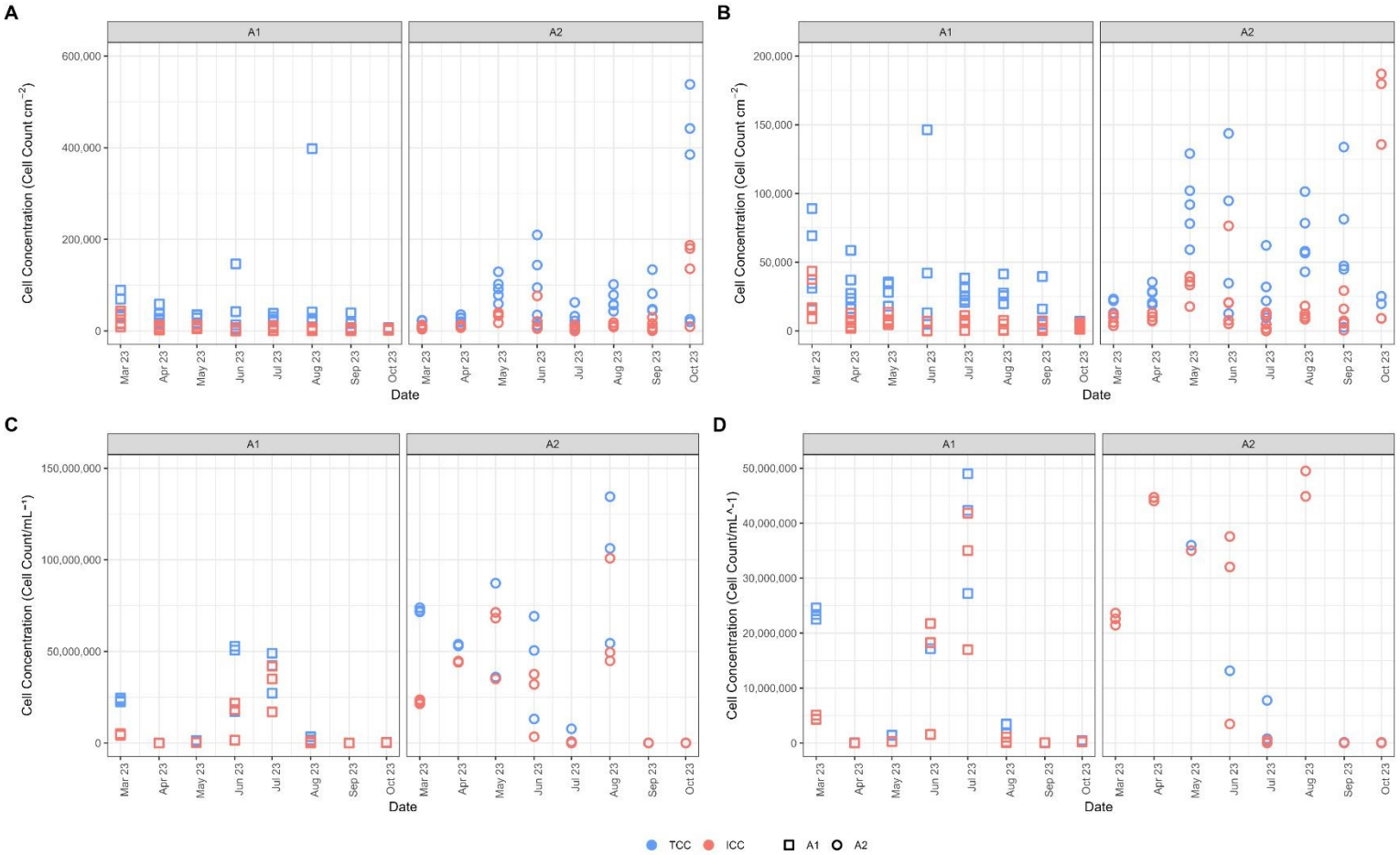

Figure 5: Total and Intact cell concentrations (TCC and ICC) for biofilm (A,B) and bulk-water (C,D) of cooling towers A1 and A2 throughout the sampling period. Raw data are shown for both towers; panels B and D replicate A and C, respectively, but on a reduced y-axis to highlight detail.

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3.2.2 *Legionella* Detection and Quantification

3.2.2.1 *Legionella* genus

The temporal dynamics of *Legionella spp*. concentration in A1 and A2 biofilms (gene copies cm^{-2}) and bulk-water (gene copies L^{-1}) over the 8-month monitoring period are presented in Figure 6. Across both towers, *Legionella* spp. was detected more frequently in biofilms than in bulk-water, which fluctuated between undetectable and intermittent high-magnitude peaks. Biofilm and bulk-water *Legionella* concentrations were not significantly correlated in either tower (Spearman's ρ: A1=0.29, p>0.05; A2=0.38, p>0.05), indicating distinct dynamics.

In A1, biofilm-associated *Legionella* spp. was repeatedly detected between March and May, preceding a peak in bulk-water concentrations in July; concentrations then remained low in both sample types from August onwards. In A2, elevated biofilm *Legionella spp*. concentrations occurred in April and June, preceding or coinciding with increases in bulk-water concentrations in June and August. These findings suggest that biofilm sampling may provide earlier indication of *Legionella* colonisation than bulk-water monitoring alone.

Temporal variation in *Legionella spp.* concentrations was significant in A1 biofilms (χ^2=16.04p=0.01) and bulk-water from both towers (A1: χ^2=14.08, , p=0.03, A2: χ^2=19.29, p<0.01) , whereas A2 biofilm-associated *Legionella* genus concentrations were comparatively stable over time (χ^2=9.35,p=0.23).

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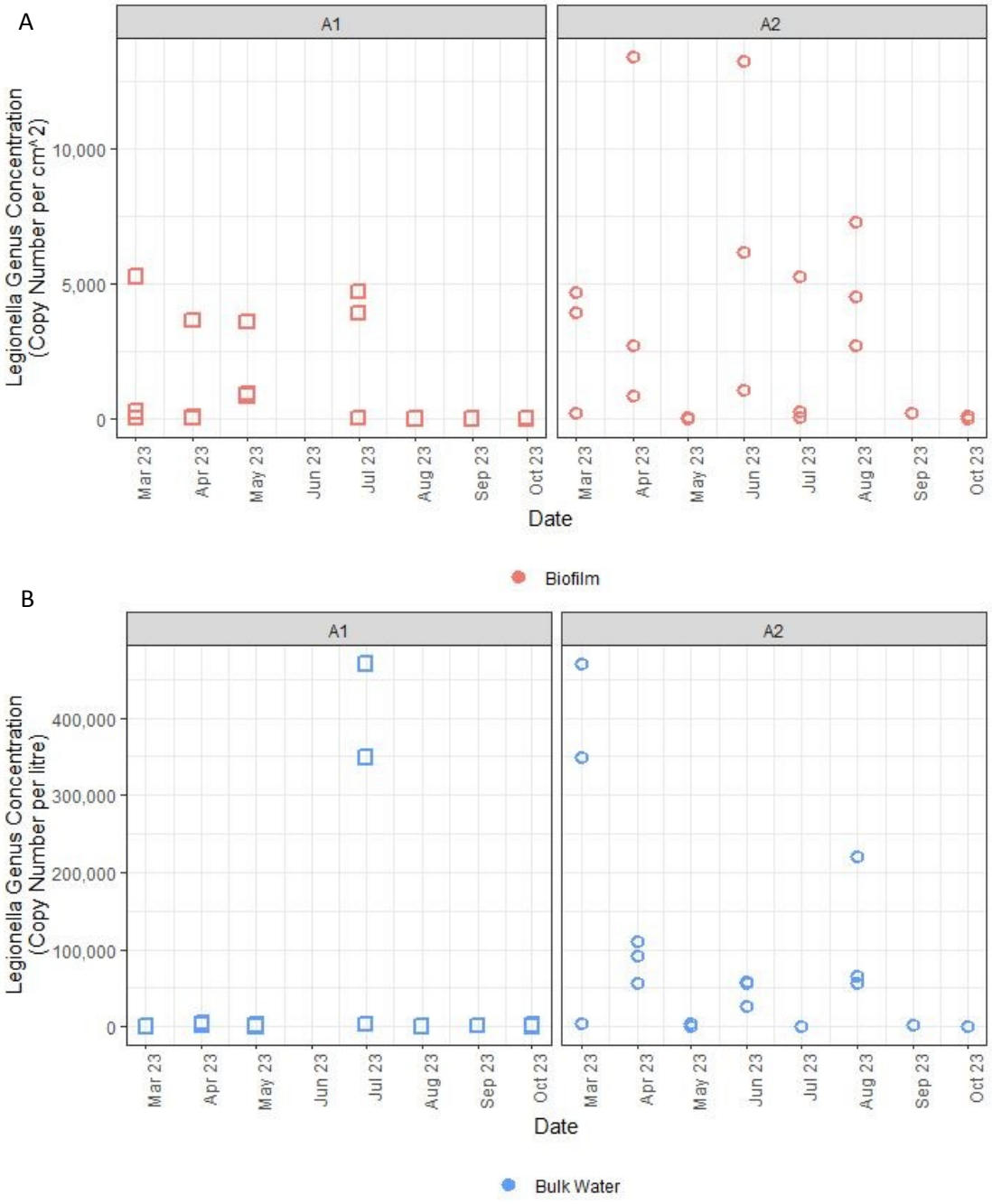

Figure *6 Legionella spp.* concentrations in (A) biofilm and (B) bulk-water from cooling towers A1 and A2 over an 8-month sampling period. Raw data points (n=3) for both towers (A1 and A2) are plotted.

370

371 ### 3.2.2.2 *L. pneumophila sg1 Detection and Quantification*

372 *L. pneumophila* sg1 was exclusively detected in biofilms from cooling tower A2, with no detection in
373 the bulk-water during the sample period (Figure 7). Crucially, this proves the hypothesis that biofilms
374 could be a lead indicator of *L. pneumophila* presence within operational cooling tower systems.

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A progressive escalation in *L. pneumophila* sg1 equivalent CFUs was observed in biofilms from A2, although it remained undetected in tower A2 bulk-water, and both sample types from A1 throughout the sampling period. *L. pneumophila* sg1 abundance increased by factors of 2.59 from April to July, 2.23 from July to September, and 1.88 from September to October, peaking at the end of the sampling period. Cleans conducted in tower A2 during June and August were followed by a period where no *L. pneumophila* sg1 was detected in biofilms or bulk-water, before it re-emerged in A2 biofilm, at a higher abundance then prior to the clean. These findings indicate that *L. pneumophila* persistence was associated primarily with biofilm rather than bulk-water, reinforcing the role of biofilms as a key habitat for this organism and a potential source of recontamination within cooling towers.

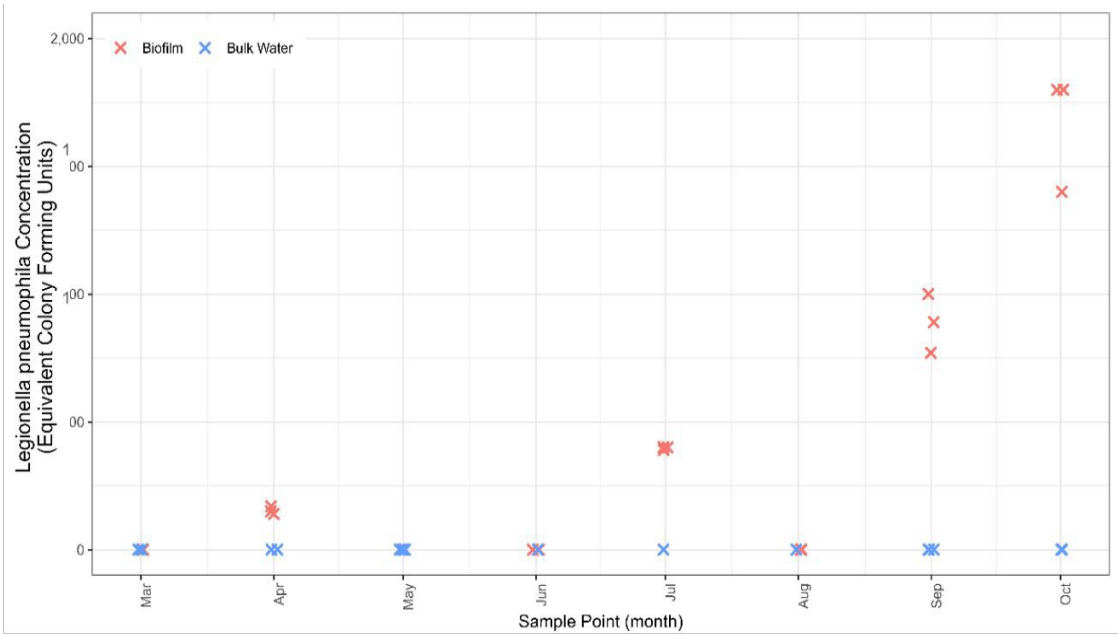

Figure 7 *Legionella pneumophila* sg1 concentrations in biofilm and bulk-water within cooling tower A2. Raw data points are presented (n=3).

3.2.3 Correlation between TCC or ICC and *Legionella spp.*

To assess whether microbial concentration could be used to indicate *Legionella* trends, correlations between TCC or ICC were tested with respect to *L. pneumophila* (where it was detected) and the broader *Legionella* genus.

Biofilm cell concentrations (TCC and ICC) and the presence or abundance of *L. pneumophila* within cooling tower A2 did not have any significant correlation (Spearman's ρ: TCC=0.42, p = 0.06; ICC=0.32, p=0.11). Similarly, ICC-to-TCC ratio (%) showed no significant relationship with *L.*

pneumophila abundance (ρ=-0.29, p=0.35). As *L. pneumophila* was detected only in biofilm samples no correlations with bulk-water cell concentrations were possible.

For the broader *Legionella* genus, correlations with TCC, ICC and ICC-to-TCC ratio were inconsistent across towers and sample types. In biofilms, TCC showed no overall correlation in either tower (ρ=0.16, p=0.31), while significant positive relationships with ICC (ρ=0.57, p=0.006) and ICC-to-TCC ratio (ρ=0.51, p=0.018) were observed only in cooling tower A1. In bulk-water, A1 showed no significant correlations, whereas A2 showed moderate positive correlations between *Legionella* abundance and both TCC (ρ=0.60, p<0.01) and ICC (ρ=0.58, p<0.01). ICC-to-TCC ratio was not correlated with *Legionella* abundance in either tower. Overall, these findings indicate that microbial concentration and viability metrics were not reliable or consistent predictors of *Legionella* abundance.

4. Discussion

4.1 Biofilm sampling optimised for operational cooling towers and integration of rapid *Legionella* analysis

This study successfully developed and validated a novel biofilm sampling technique for operational cooling towers, which was applied across two cooling tower systems. To our knowledge, this represents the first standardised method designed specifically for cooling tower biofilms. The method overcomes logistical challenges and accounts for environmental variability, such as temperature gradients and surface-area variability. Importantly, the sampling method remains practical and transferable across systems as it does not require additional equipment, operational changes, or strict timing constraints.

Biofilm sampling from the pack targets the dominant surface area within cooling towers and the typical primary ecological niche of *Legionella,* enhancing and complementing traditional bulk-water samples from the basin or make-up water, which only provide a snapshot of conditions at a single time point and are prone to dilution effects due to the large water volume within operational cooling towers. As *Legionella* colonisation and proliferation typically originate within biofilms, with cells subsequently released into the bulk-water, biofilm sampling enables earlier detection of contamination compared with conventional bulk-water monitoring and provides a more representative assessment of *Legionella* persistence and distribution within cooling tower systems.

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Cotton swabs were chosen for biofilm collection because they enabled immediate, non-disruptive sampling without the need for pre-installed devices or shutdowns, this also aligned with established protocols such as showerhead sampling (CDC, 2019). This contrasts with methods such as coupons which require prior installation, flow cells or advanced biosensors, which can alter biofilm composition, require controlled conditions and remain costly (Azeredo *et al*., 2017). The robustness of the method is demonstrated by the successful collection and analysis of hundreds of samples across diverse towers. However, a clear system for recording sampling points is essential to ensure that previously sampled sites are not reused, which could result in unrepresentative younger biofilm samples.

The growing need for early detection of *L. pneumophila* has driven the development of rapid detection methods, which offer advantages over traditional culture techniques. These have been validated on planktonic *Legionella* in bulk-water, with limited exploration in biofilms. Importantly, the application of two commercial qPCR platforms - Genesig for *Legionella spp.* and Genomadix for *L. pneumophila* - demonstrated that these techniques can be effectively applied to biofilms sampled using the method developed herein as well as water samples from operational systems, enabling more comprehensive monitoring of *Legionella*.

In both towers, elevated biofilm-associated *Legionella* signals were observed prior to, or coinciding with, subsequent increases in bulk-water measurements, suggesting that biofilm monitoring may provide earlier evidence of colonisation events than conventional bulk-water sampling alone. Collectively, the successful application of the biofilm-focussed sampling and analysis approach to more rapidly detect *Legionella* in operational cooling towers lays the groundwork for future research, surveillance and wider industry adoption.

4.2 Biofilms as ecological niches for Legionella

Across both cooling towers, biofilm samples showed more frequent and consistent *Legionella* detection than corresponding bulk-water samples. Biofilm and bulk-water *Legionella* genus concentrations did not correlate suggesting distinct dynamics between the two ecological niches within cooling tower systems. . While similar patterns have been observed in other engineered water systems, (De Filippis *et al*., 2018; Margot *et al*., 2024; Van der Kooij *et al*., 2005), this study presents the first demonstration of this trend within the specific context of operational cooling towers, which have unique microbial dynamics and water quality parameters. This highlights the

limited value of bulk-water monitoring alone for assessing *Legionella* presence and colonisation status.

The results suggest that biofilms are the primary niche for *Legionella* colonisation and persistence within the cooling towers , from which cells can be intermittently released into the surrounding bulk-water. Previous clinical-based studies similarly shown that *Legionella* proliferation within biofilms typically precedes its release into the bulk-phase (Zhao *et al*., 2023). Biofilms likely enhance *Legionella* persistence by protecting cells from stresses of the bulk-water phase and enhancing nutrients (Flemming, 2020). The role of protozoa as biofilm grazers may also facilitate *Legionella* survival promoting replication by acting as hosts (Declerck, 2010; Boamah *et al*., 2017), although *Legionella* do proliferate independently of protozoa (Surman *et al*., 2002; Abdel-Nour *et al*., 2013).

Dispersal of biofilm cells may occur through shedding of cells or biofilm aggregates into the bulk-water as part of a continuous exchange between the phases, or through larger mobilisation events triggered by hydraulic changes, such as increased shear stress in pipe systems (Fish *et al*, 2020; Khu *et al*., 2023), or by biocide application, which can induce rapid biofilm sloughing in cooling water systems (Di Pippo *et al*., 2018). These processes may explain the sporadic nature of *Legionella* occurrence within cooling towers, where bulk-water samples may only detect *Legionella* when it coincides with transient release events. In this context, positive bulk-water detections may sometimes represent snapshots of mobilisation rather than reflecting the true extent of system colonisation. *Legionella* intermittently released from established biofilms into the bulk-water may increase transient planktonic concentrations and provide an inoculum for colonisation of downstream surfaces. This continual cycle of release, transport, and recolonisation could contribute to persistent system-wide colonisation. These findings further support incorporating biofilm sampling into routine surveillance, as bulk-water monitoring alone may fail to capture both the extent and spatial dynamics of *Legionella* colonisation. In the current study, each cleaning event in tower A2 was followed by increased *L. pneumophila* concentrations in the biofilm, suggesting maintenance activities may impact *Legionella* (re)occurrence on the internal surfaces as well release into the bulk-water. These dynamics could result in large numbers of *L. pneumophila* being intermittently mobilised from the biofilm into bulk-water, which may then be aerosolised causing sporadic Legionellosis outbreaks (Declerck, 2010). Thus, incorporating biofilm sampling into *Legionella* surveillance provides a critical advancement to management frameworks that complements traditional bulk-water monitoring.

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Since the current standard is to take bulk-water samples only, by the time mobilisation from the biofilm occurs and planktonic *Legionella* are detected, the concentrations could already be high and have been aerosolised before remedial actions can take place. Biofilm sampling therefore not only provides an earlier indicator of contamination, it also improves detection sensitivity and provides critical insight into the microbial ecology underpinning *Legionella* persistence in engineered water systems.

4.3 Exclusive detection of *L. pneumophila* serogroup 1 within biofilm samples

This study presents the first known detection and quantification of *L. pneumophila* exclusively in biofilm samples from operational cooling towers. This is a notable finding given the rarity, and typically sporadic, occurrence of *L. pneumophila* in water samples from cooling towers across Europe and North America. The results are consistent with previous studies of water distribution systems, which reported that *L. pneumophila* predominated in biofilms (De Filippis *et al*., 2018; Waak *et al*., 2018) and that *Legionella spp*. is generally more abundant in biofilms than in bulk-water (Garner *et al*., 2018). This consistency across different engineered systems further supports the robustness and adaptability of the sampling method, and application across a broader range of cooling towers would provide wider validation of its performance under diverse operational conditions. A valuable next step would be to establish how far in advance biofilm L*. pneumophila* detection precedes subsequent waterborne detection in real-world systems. While this cannot be experimentally controlled or engineered in operational towers, longitudinal observations that track the transition from biofilm detection to detectable levels in bulk-water would provide important evidence for the predictive value of this approach, as well as insights into interpreting data to inform intervention timing.

These findings provide strong support for the original hypothesis that biofilms could act as lead indicators - and had monitoring been restricted to bulk-water testing, *L. pneumophila* would have gone undetected, masking potential contamination of the bulk-water and the associated public health risks. Application of the validated and reproducible biofilm sampling and analysis protocol has demonstrated the value of incorporating biofilm sampling into surveillance frameworks as an early indicator for *Legionella* presence, thus improving detection accuracy, and supporting timely implementation of water safety plans.

4.4 No correlation between cell concentration and *Legionella* concentration

There was no correlation between TCC, ICC and the presence of *Legionella spp.* including *L. pneumophila* in either biofilm or bulk-water samples. These findings challenge a conventional

expectation that higher bacterial counts create a conducive environment for *Legionella* growth, an assumption in existing literature (Springston & Yocavitch, 2017) and prevalent in "expert judgement" and practice.

L. pneumophila was detected exclusively in A2 biofilms, despite similar or lower TCC and ICC values compared to A1, indicating that microbial load alone is not a reliable predictor of *Legionella* risk. This is in contrast to van der Kooij *et al*. (2017), in which significant, albeit weak, correlations were reported between total cell count, heterotrophic plate count, and *Legionella* growth within biofilm model systems. 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 enabled detection of viable but non-culturable (VBNC) cells and provided a more sensitive analysis in comparing cell concentrations and assessing cell viability relative to *Legionella* concentrations. The difference in trends between the studies underscores the limitations of extrapolating laboratory results to real-world systems.

The finding that bulk-water cell counts did not correlate with *Legionella* presence is consistent with the work of Sanchis *et al*. (2023), which 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. Although other studies (Campaña *et al*., 2023; Kyritsi *et al*., 2018) have suggested possible correlations under specific conditions. 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. This highlights the nuanced and complex nature of *Legionella* ecology and necessitates a move beyond relying solely on bacterial abundance analysis.

Taken in combination these results emphasise the need for comprehensive monitoring strategies that incorporate biofilm sampling, microbial community profiling, physicochemical factors, and advanced detection tools to better assess and manage *Legionella* risk in operational cooling towers. To truly understand *Legionella* dynamics, identifying bacterial compositions or physiochemical

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factors that either promote or inhibit *Legionella* growth in biofilm samples is crucial to transition to more proactive management strategies.

4.5 Practical implications & Challenges

The exclusive detection of *L. pneumophila* in biofilm samples highlights that colonisation can occur well before contamination becomes detectable in the bulk-water phase. Current UK Health and Safety Executive (HSE) guidance focuses solely on bulk-water sampling, without defined action levels for biofilms. These findings underscore the importance of incorporating biofilm sampling into routine surveillance to provide earlier warning of colonisation events and enhance the timeliness and effectiveness of water safety interventions.

Integrating culture-based methods with rapid detection techniques such as qPCR could further enhance biofilm monitoring. While culture remains the gold standard for assessing bacterial viability and regulatory compliance, qPCR provides faster detection and greater sensitivity but cannot distinguish viable and non-viable cells, which is a limitation of this study. However, qPCR application supported rapid on-site analysis aligning with the focus of this study on earlier detection, while flow cytometry (ICC), provided additional context for interpreting microbial viability and dynamics. Combining these approaches (culture, flow cytometry and qPCR) could bridge the gap between novel methodologies and existing water safety frameworks, facilitating wider adoption without significant protocol changes. These integrated strategies could be further enhanced by incorporating immunomagnetic separation (IMS) techniques, which offer the potential to concentrate *Legionella* cells and improve detection sensitivity, particularly in samples with low bacterial loads.

Detection of *L. pneumophila* or *Legionella spp*. from biofilm samples would not require any change to current operational responses. The same remedial measures recommended for bulk-water contamination such as targeted biocide dosing, mechanical cleaning, flushing, and system optimisation, should be applied. However, identifying contamination within biofilms provides earlier warning, allowing operators more time to plan and implement interventions before *Legionella* spreads into the bulk-water. This early insight supports a shift from reactive to proactive management, reducing the risk of outbreaks and the associated operational, legal, and reputational consequences. Proactive biofilm management therefore offers a cost-effective and sustainable strategy to safeguard public health and ensure long-term cooling tower safety.

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5. Conclusions

This study successfully developed and implemented a novel biofilm sampling technique for operational cooling towers, overcoming logistical challenges and providing a reproducible method for biofilm collection and analysis. Using this approach, *L. pneumophila* was detected exclusively in biofilm samples, highlighting biofilms as critical lead indicators for early and reliable *Legionella* detection. Additionally, *Legionella spp*. was detected more persistently in biofilm samples then in bulk-water samples, with elevated biofilm concentrations preceding (or coinciding with) peaks in bulk-water detections , emphasising the ecological importance of biofilms in supporting bacterial persistence.

Biofilm and bulk-water samples exhibited distinct microbial dynamics, with no correlation between total cell counts or cell viability and the presence of *Legionella spp.*, including *L. pneumophila*. This underscores that microbial load alone is insufficient to predict *Legionella* risk and highlights the need for comprehensive monitoring approaches that consider microbial community characteristics, biofilm–bulk-water interactions, and relevant environmental factors.

Collectively, these findings advance the understanding of *Legionella* ecology in operational cooling towers and demonstrate the value of incorporating biofilm-based assessments into surveillance strategies. By revealing that traditional bulk-water monitoring may underestimate abundance and hence potentially risk, this study situates biofilm sampling as a critical tool for enhancing the sensitivity, accuracy, and effectiveness of *Legionella* detection in environmental monitoring and public health protection.

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Data Availability: The datasets containing the numerical values used to generate the figures in this article will be deposited in the University of Sheffield's Online Research Data (ORDA) repository and made openly available upon publication. A DOI and direct access link will be provided in the final version of the manuscript.

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Data Availability Statement for Kerry *et al*

The datasets containing the numerical values used to generate the figures in this article will be deposited in the University of Sheffield's Online Research Data (ORDA) repository and made openly available upon publication. A DOI and direct access link will be provided in the final version of the manuscript.