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

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.



1 **Biofilms as an early indicator of *Legionella* colonisation within evaporative** 2 **cooling towers**

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10

11 **Abstract**

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

26

27 **Key words**

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



30 **Water Impact Statement**

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



41 1. Introduction

42 Engineered water systems such as potable water distribution systems, shower heads, fountains and
43 evaporative cooling systems including cooling towers are significant sources of *Legionella*
44 *pneumophila* (*L. pneumophila*) and outbreaks of Legionnaires' disease. These environments are
45 particularly conducive to producing aerosols capable of containing *L. pneumophila* (Paranjape *et al.*,
46 2020). Cooling towers pose a particular concern as they create conditions that promote both biofilm
47 (sessile) and planktonic (free-living) microbial growth, supported by temperatures between 25-35°C,
48 a near-neutral pH, exposure to sunlight, continuous aeration, steady nutrient supply and an
49 extensive surface area that facilitates biofilm development in particular (Di Gregorio *et al.*, 2017).
50 While outbreaks of Legionnaires' disease are sporadic, those associated with cooling towers tend to
51 involve multiple cases. Cooling towers are the most frequently confirmed source of *L. pneumophila*
52 aerosolisation, cited as contributing to at least 28% of all outbreaks (Di Pippo *et al.*, 2018) with
53 Hamilton *et al.* (2018) demonstrating that of 136 outbreaks identified globally, cooling towers were
54 implicated or suspected in 30% of the total number, 50% of confirmed outbreak-associated cases,
55 and 6% of outbreak-associated deaths. Aerosolised Legionellae from cooling towers can disperse
56 some distance from the source (Walser *et al.*, 2014) and possibly as far as 6 km (Nhu Nguyen *et al.*,
57 2006), thus posing a substantial potential public health and occupational health risk.

58
59 Cooling towers are a vital widespread infrastructure, serving as a cost-effective and energy-efficient
60 cooling solution for a wide range of systems and processes, including power generation,
61 petrochemical industries and factories such as plastics injection moulding. As heat exchange devices,
62 they circulate water at elevated temperatures from industrial processes over a pack material. Air
63 flows in the opposite or perpendicular direction to the water, promoting heat transfer and resulting
64 in heat loss. The cooled water is then collected in a basin, to be either recirculated within the system
65 or discarded into the wastewater system (Figure 1A and 1B). The pack material, typically made of
66 polyvinyl chloride (PVC; Figure 1C), is crucial to these systems as it provides an extensive surface
67 area, increasing the contact time between water and air, which in turn enhances the efficiency of
68 the cooling process. Biofilms (complex microbial communities encased in extracellular polymeric
69 substances) have been shown to develop on PVC in other engineered water systems (Goraj *et al.*,
70 2021; Learbuch *et al.*, 2021) and represent a significant microbial reservoir. At the system level,
71 surface-attached biofilm communities can contain several orders of magnitude more total microbial
72 cells than the planktonic (bulk-water) phase within drinking water systems (Flemming *et al.*, 2016).
73 The large surface area of the pack in a cooling tower contributes to biofilm-forming potential in
74 these systems by providing more sites for microbial attachment and growth.



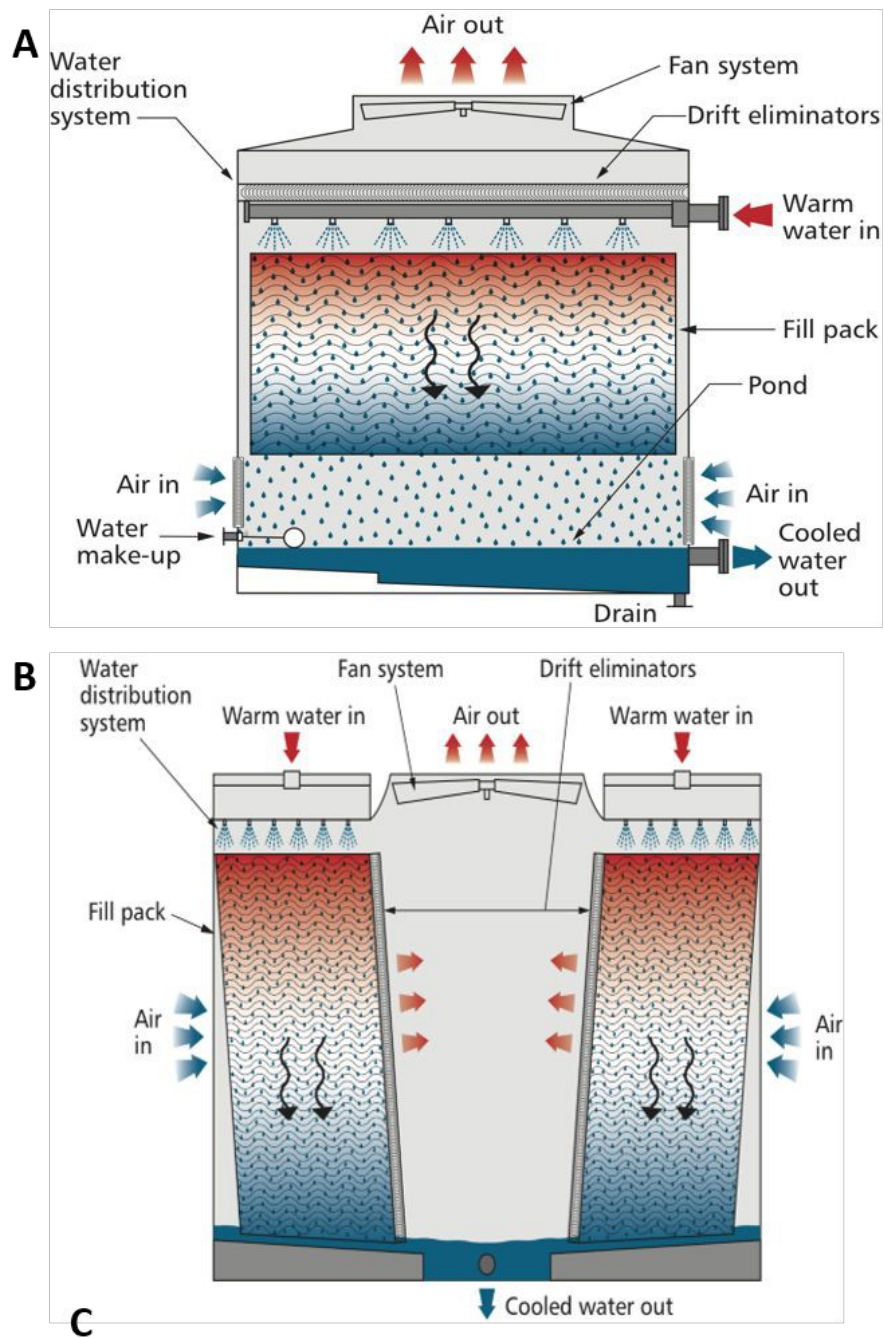


Figure 1 Cooling tower designs indicating air and water flow, temperature gradient, pack location and structure. Schematics show (A) an induced draught counter flow cooling tower and (B) an induced draught double cross flow cooling tower. (C) Examples of typical pack honeycomb or corrugated designs and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



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

85
86 To assess the effectiveness of microbial control measures, including biocide treatment, and to detect
87 any system malfunctions, routine water quality monitoring is deployed. This includes quantification
88 of viable planktonic bacterial concentrations within bulk-water using heterotrophic plate counts
89 (National Academies of Sciences, Engineering, and Medicine, 2020) and testing specifically for
90 *Legionella* through culture methods (International Organization for Standardization, 2017). In GB,
91 current action levels for *Legionella* dictate that concentrations below 100 colony forming units
92 (CFU)/litre require no immediate action, levels between 100 and 1,000 CFU/litre necessitate a
93 reassessment of the control programme and an adjustment in biocide dosage, and levels above
94 1,000 CFU/litre demand immediate remedial action (HSE, 2020). While these monitoring techniques
95 provide some level of control, *Legionella* culture methods can take between 10 and 14 days to
96 acquire results, this causes a delay in implementing any remedial action and risks that outbreaks
97 could occur in the interim (Walker and McDermott, 2021). Moreover, current monitoring practices
98 predominantly focus on sampling bulk-water for the presence of planktonic bacteria, which, while
99 informative as background snapshots of water quality, does not capture the full ecological picture.
100 As a result, sporadic outbreaks continue to occur despite compliance with current testing protocols,
101 highlighting a persistent public health risk. Therefore, there is an urgent need and growing emphasis
102 on the development and application of emerging detection techniques, such as quantitative
103 polymerase chain reaction (qPCR), to assess water samples for *Legionella* more rapidly and with
104 greater sensitivity, enabling quicker response times to prevent outbreaks. There is also a perception
105 that elevated planktonic bacterial counts could reflect system conditions that favour *Legionella*
106 proliferation, serving as a proxy for increased risk. Recent research has explored whether overall
107 microbial concentration and viability in bulk-water could serve as indicators of *Legionella* presence.
108 Campaña *et al.* (2023) and Kyritsi *et al.* (2018) both report a potential relationship between total

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

112

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

134

135 One of the most critical functions of biofilm when considering *L. pneumophila* is that biofilms attract
136 microbial grazers such as free-living amoeba because the greatest concentration of microorganisms
137 is found within the biofilm, such as the biofilms on the pack of cooling towers, providing a plentiful
138 food source (Huws *et al.*, 2005). *L. pneumophila* exploits this to replicate within the free-living
139 amoeba. Furthermore, *Legionella spp.* can obtain nutrients directly from other living microorganisms
140 and through necrotrophic feeding from decaying microbial cells, which are reported to comprise
141 nearly 50% of a biofilm (Temmerman *et al.*, 2006). The complex relationships between *Legionella*,
142 biofilms and protozoa are often overlooked, with no standardised sampling regime monitoring any



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

147
148 It was hypothesised that biofilms in cooling towers can act as ecological niches for *Legionella* and,
149 when paired with emerging detection technologies, biofilm sampling could serve as a more reliable
150 'lead indicator' of *Legionella* presence (including *L. pneumophila*) than conventional bulk-water
151 sampling. Hence, the primary aim of this study was to ascertain the prevalence of *L. pneumophila* in
152 both biofilms and bulk-water from operational cooling towers. To enable this, a novel biofilm
153 sampling technique was developed, optimised and implemented specifically for application in
154 cooling tower environments. In addition, the study sought to explore potential correlations between
155 overall microbial concentration, microbial viability, and the presence of *Legionella* in both biofilm
156 and bulk-water samples.

157 2. Materials and Methods

158 2.1 Overview of experimental design

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

170 2.2 Cooling Tower Selection and Site Details

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



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

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

183 **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-nitropropionamide (DBNPA) and Quaternary Ammonium Compound (QAC) Benzyl- C8-18-Alkyldimethyl Chloride

184 2.3 Optimising biofilm sampling within Cooling Towers

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

197
 198 To account for this potential heterogeneity and ensure the collection of reliable and representative
 199 biofilm samples from operational cooling tower pack, it was crucial to understand how biological
 200 variability between replicates could be influenced by specific environmental factors. Key among



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

206
207 To determine any potential effect of the temperature gradient from the top to the bottom of the
208 cooling tower pack, biofilm samples were collected from the top, middle, and bottom sections of the
209 pack, reflecting the typical thermal stratification observed in operating cooling towers. Biofilms were
210 collected using 100 cm² quadrats positioned approximately 90 cm inward from the outer face of the
211 pack, representing a consistent depth of sampling within the pack structure. This depth was selected
212 to balance accessibility with the need to capture conditions representative of the internal biofilm
213 environment. Temperature at each sampling point was measured three times with a digital
214 thermometer, allowing 30 seconds for the reading to stabilise.

215
216 To optimise the surface area sampled, 100 cm², 200 cm², and 300 cm² quadrats were used to collect
217 samples from a single pack location (middle) to a depth of 90 cm into the pack. The choice of a
218 minimum of 100 cm² surface area was intended to capture variability in biofilm composition,
219 enabling a focused examination, while surfaces above 300 cm², were deemed impractical for
220 handling within the pack. This rationale ensured a comprehensive yet manageable approach to
221 assessing surface area variations during biofilm sampling.



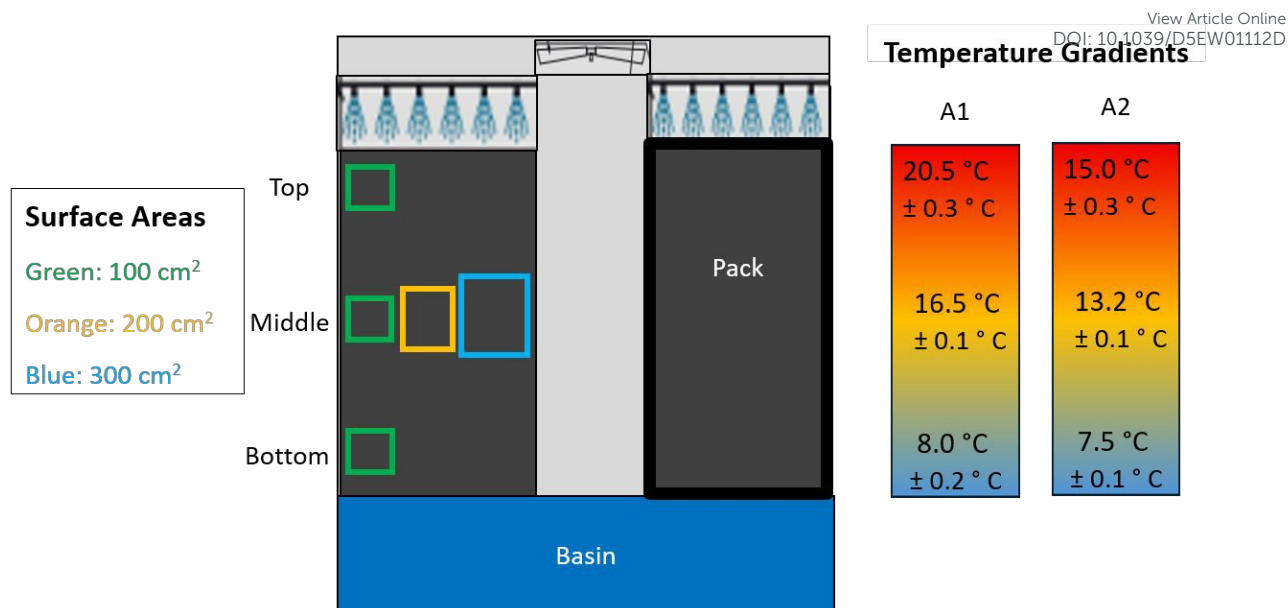


Figure 2 Schematic of cooling tower sampling locations within the pack (top, middle, bottom) indicating the corresponding surface areas that were sampled and the temperature gradient measured in each tower: A1 and A2 (mean ± standard deviation), demonstrating a decrease from the top to the bottom of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

222

223 2.4 Sampling campaign for longitudinal monitoring of cell concentration and

224 *Legionella* detection

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

233 2.5 Sample Collection Methods

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

236 2.5.1 Cooling Tower Biofilm Sampling

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



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

243
244 The selection of optimised sample areas and locations was informed by the findings from 2.3,
245 ensuring representative and repeatable results within the unique environment of an operational CT.
246 A medium-density polyethylene (MDPE) quadrat was used as a template to standardise the sampling
247 area of the pack (this was cleaned with 70% ethanol between replicates and sampling points). After
248 sampling, the swabs were transferred into falcon tubes for transport. Upon arrival at the on-site
249 laboratory, each sample tube was filled with sterile phosphate buffered saline (50 ml) and vortexed
250 at 3000 rpm for thirty seconds to resuspend the biofilm (triplicates of PBS were used as controls). To
251 achieve a thoroughly homogenised biofilm suspension, the tubes were then rotated 180 degrees
252 and vortexed again for an additional thirty seconds.

253

254 2.5.2 Cooling Tower Bulk-water Sampling

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

261

262 2.6 Sample analysis

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

265 2.6.1 Total and Intact Cell Counts

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



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

282 2.6.2 *Legionella* detection and quantification

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

289 2.6.2.1 *Legionella* genus

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

296 2.6.2.2 *L. pneumophila sg1*

297 To analyse for the presence of, and to quantify, *L. pneumophila sg1* by qPCR, the Genomadix
298 (Genomadix Bioscience, Ottawa, Canada, version 1.0.3) system was employed. This system includes
299 a portable DNA analyser, the Genomadix Cube, along with a single-use disposable concentration kit
300 and test cartridge. Briefly, 20 ml of either biofilm suspension or bulk-water samples was introduced
301 into the filter-based concentration kit. The captured intact bacteria were eluted from the filter and
302 the eluate transferred to a test cartridge which contains qPCR primers, a probe, and an internal
303 positive control, loaded into the Genomadix Cube analyser. These primers and probes are designed
304 against a highly conserved region of *L. pneumophila (mip)* gene (Wilson *et al.*, 2003). The Genomadix
305 software subsequently converts qPCR-derived copy numbers into equivalent colony-forming units
306 (CFU) for data visualisation, analysis and interpretation.



307 2.7 Data Analysis

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

314 3. Results

315 3.1 Optimising Biofilm Sampling: Temperature, surface area and cell 316 concentrations

317 In both cooling towers, biofilm TCC and ICC did not differ significantly across sampling locations
318 (Figure 3), despite the temperature gradients between the top and bottom of the pack (A1:TCC
319 $\chi^2=4.5$, $p=0.12$; ICC $\chi^2=2.38$, $p=0.31$; A2:TCC $\chi^2=1.64$, $p=0.44$; ICC $\chi^2=3.3$, $p=0.20$).

320 Sampled surface area similarly had no significant effect on biofilm TCC or ICC, in either tower (Figure
321 4; A1:TCC $\chi^2=4.62$, $p=0.10$; ICC $\chi^2=5.6$, $p=0.06$; A2:TCC $\chi^2=3.82$, $p=0.15$; ICC $\chi^2=5.6$, $p=0.06$). Although
322 temperature/location and surface area had an overlapping condition (middle, 100 cm²), the datasets
323 were collected at different time points; therefore, no shared data appears across Figures 3 and 4.

324
325 As no significant effects of temperature or surface area were observed, subsequent longitudinal
326 biofilm sampling used a standardised 100 cm² quadrat positioned in the middle of the pack.



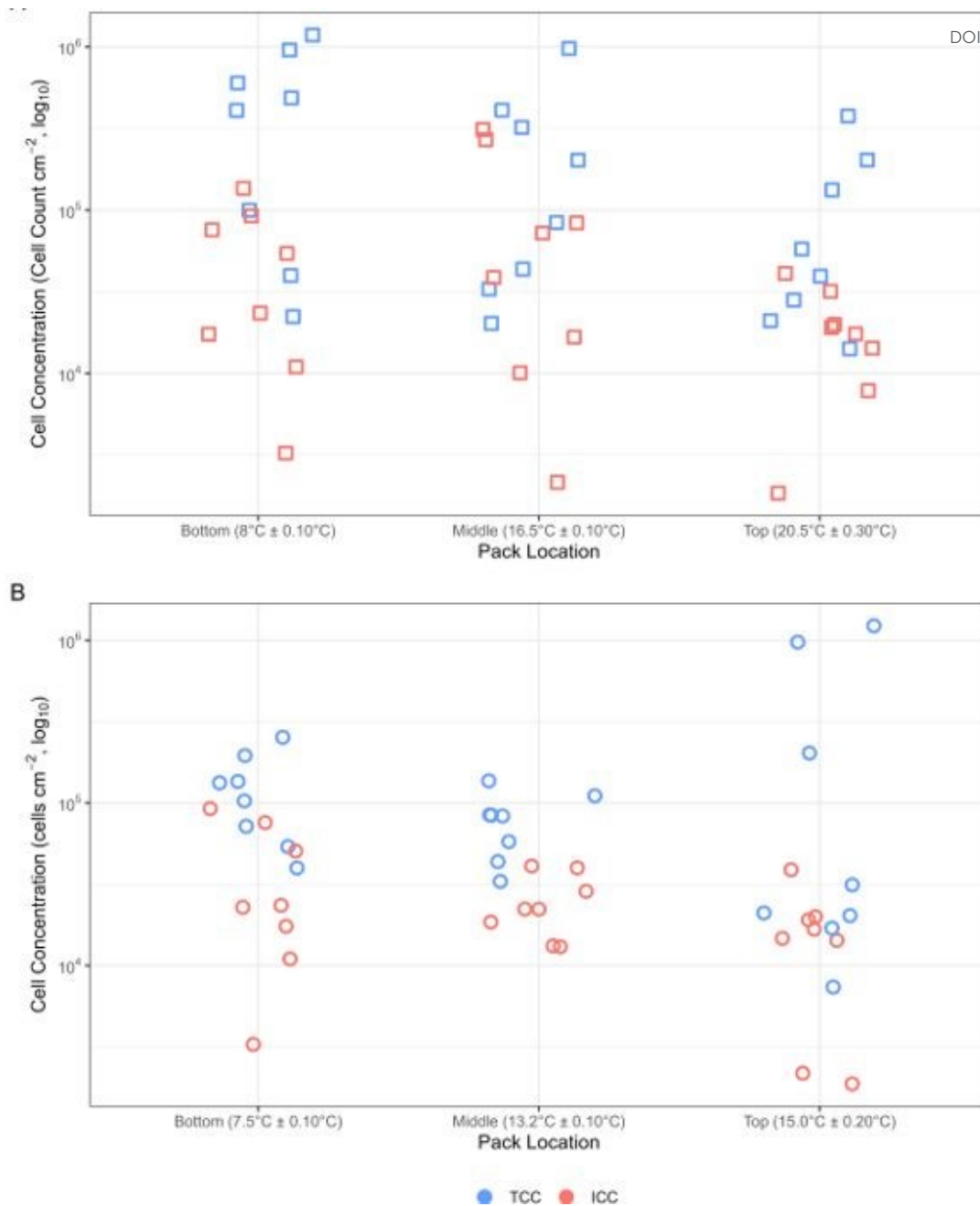


Figure 3 Impact of sample location and temperature on biofilm total cell concentration (TCC) and intact cell concentration (ICC) for cooling tower (A) A1 and (B) A2. Logged data is presented, the mean temperatures measured at each location are provided in brackets, \pm standard deviation.



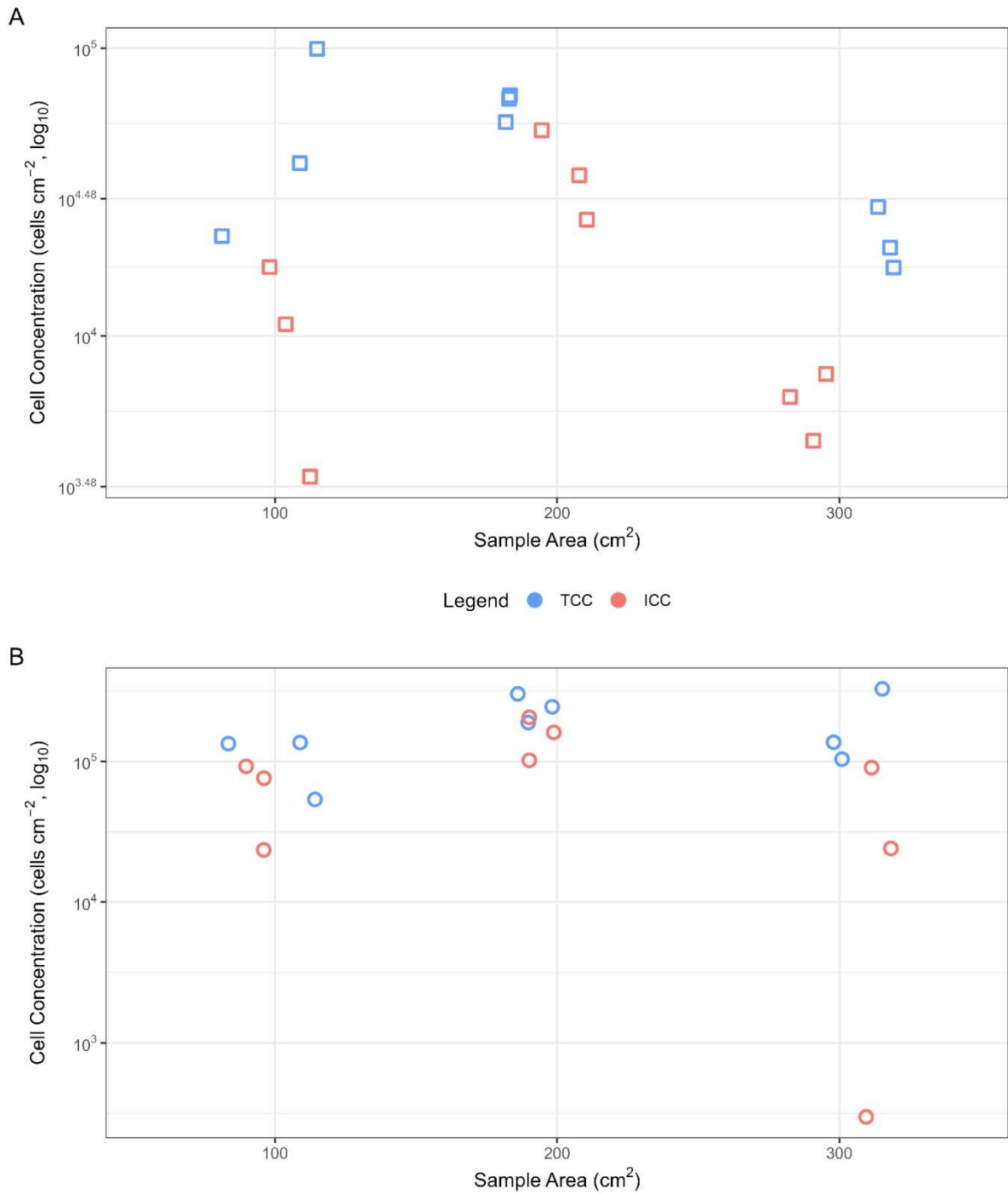


Figure 4 Impact of sampled surface area on biofilm total cell concentration (TCC) and intact cell concentration (ICC) for cooling towers (A) A1 and (B) A2. Log of the raw data is plotted.

327



328 3.2 Longitudinal assessment of microbial concentration, viability and

329 *Legionella* quantification

330 3.2.1 Microbial concentration and viability

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

334
335 Biofilm TCC and ICC varied significantly over time (Figure 5A/B) in both towers (A1: TCC $\chi^2=15.19$,
336 $p=0.03$; ICC $\chi^2=22.37$, $p < 0.01$; A2: TCC $\chi^2=17.20$, $p=0.02$; ICC $\chi^2=16.13$, $p=0.02$). ICC% also varied
337 temporally, this was only statistically significant in A1 ($\chi^2 = 21.67$, $df = 7$, $p < 0.01$), not A2 ($\chi^2 = 12.65$,
338 $df=7$, $p=0.08$). Bulk-water cell concentrations also varied temporally (Figure 5 C/D), though ICC
339 trends differed between towers, peaking in June-July in A1, with more fluctuations occurring in A2.
340 Intact-to-total cell ratios (ICC%) also varied over time in the bulk-water for both tower A1 (A1:
341 $\chi^2=14.56$, $df=7$, $p=0.04$) and A2 ($\chi^2=18.47$, $df=7$, $p=0.01$).

342
343 Comparing between towers, biofilm and bulk-water TCC and ICC were consistently higher in A2,
344 which used combined oxidising and non-oxidising biocides, than A1, which used oxidising biocides
345 only (biofilm TCC: $\chi^2=452.00$, $p < 0.01$; ICC: $\chi^2=264.00$, $p < 0.01$; bulk-water TCC $W=404$, $p=0.02$; ICC:
346 $W=387$, $p=0.04$). However, ICC% did not significantly differ between the towers, indicating
347 comparable relative viability despite varied microbial abundance.

348



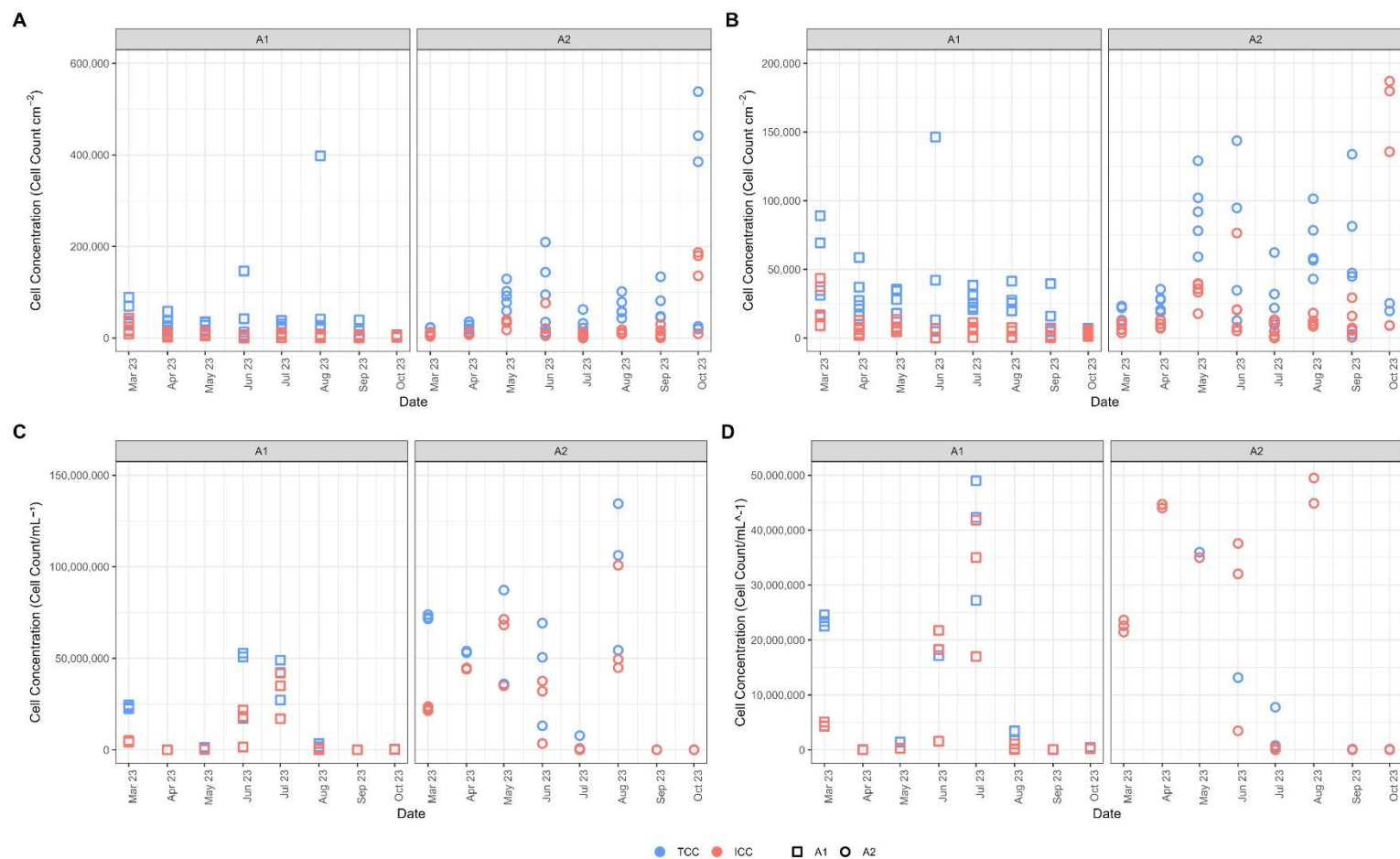


Figure 5: Total and Intact cell concentrations (TCC and ICC) for biofilm (A,B) and bulk-water (C,D) of cooling towers A1 and A2 throughout the sampling period. Raw data are shown for both towers; panels B and D replicate A and C, respectively, but on a reduced y-axis to highlight detail.



350 3.2.2 *Legionella* Detection and Quantification

351 3.2.2.1 *Legionella* genus

352 The temporal dynamics of *Legionella spp.* concentration in A1 and A2 biofilms (gene copies cm⁻²) and
353 bulk-water (gene copies L⁻¹) over the 8-month monitoring period are presented in Figure 6. Across
354 both towers, *Legionella spp.* was detected more frequently in biofilms than in bulk-water, which
355 fluctuated between undetectable and intermittent high-magnitude peaks. Biofilm and bulk-water
356 *Legionella* concentrations were not significantly correlated in either tower (Spearman's ρ : A1=0.29,
357 $p>0.05$; A2=0.38, $p>0.05$), indicating distinct dynamics.

358
359 In A1, biofilm-associated *Legionella spp.* was repeatedly detected between March and May,
360 preceding a peak in bulk-water concentrations in July; concentrations then remained low in both
361 sample types from August onwards. In A2, elevated biofilm *Legionella spp.* concentrations occurred
362 in April and June, preceding or coinciding with increases in bulk-water concentrations in June and
363 August. These findings suggest that biofilm sampling may provide earlier indication of *Legionella*
364 colonisation than bulk-water monitoring alone.

365
366 Temporal variation in *Legionella spp.* concentrations was significant in A1 biofilms ($\chi^2=16.04p=0.01$)
367 and bulk-water from both towers (A1: $\chi^2=14.08$, $p=0.03$, A2: $\chi^2=19.29$, $p<0.01$), whereas A2
368 biofilm-associated *Legionella* genus concentrations were comparatively stable over time
369 ($\chi^2=9.35,p=0.23$).



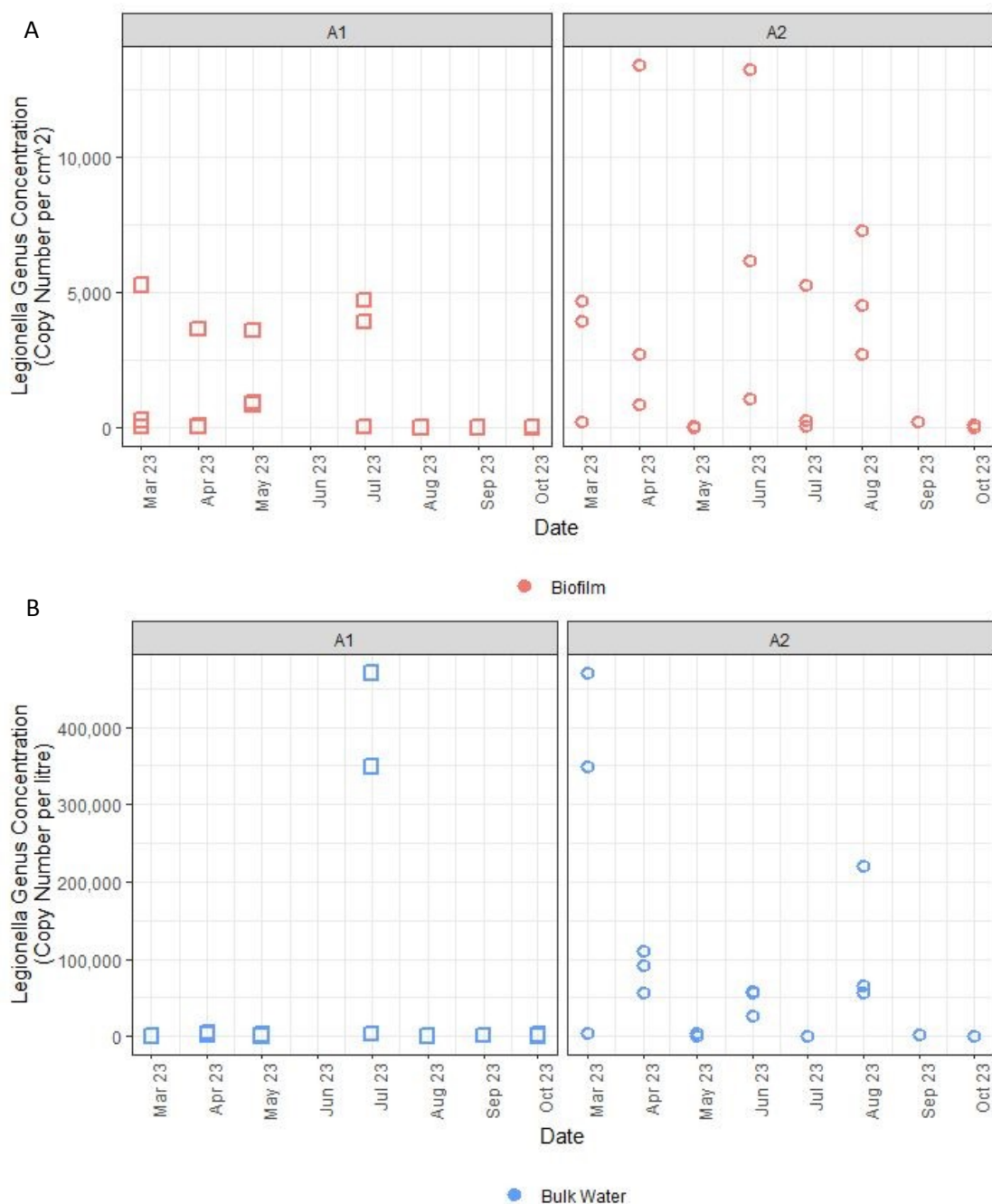


Figure 6 *Legionella spp.* concentrations in (A) biofilm and (B) bulk-water from cooling towers A1 and A2 over an 8-month sampling period. Raw data points (n=3) for both towers (A1 and A2) are plotted.

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.



375

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

386

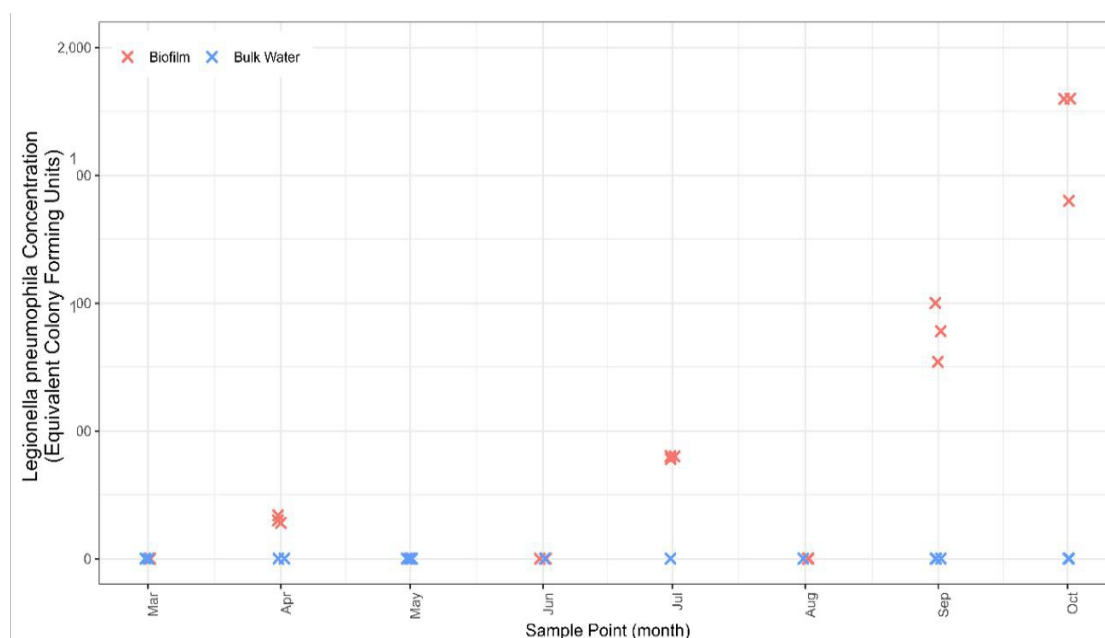


Figure 7 *Legionella pneumophila* sg1 concentrations in biofilm and bulk-water within cooling tower A2. Raw data points are presented (n=3).

387 3.2.3 Correlation between TCC or ICC and *Legionella* spp.

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

391

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



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

397
398 For the broader *Legionella* genus, correlations with TCC, ICC and ICC-to-TCC ratio were inconsistent
399 across towers and sample types. In biofilms, TCC showed no overall correlation in either tower
400 ($\rho=0.16$, $p=0.31$), while significant positive relationships with ICC ($\rho=0.57$, $p=0.006$) and ICC-to-TCC
401 ratio ($\rho=0.51$, $p=0.018$) were observed only in cooling tower A1. In bulk-water, A1 showed no
402 significant correlations, whereas A2 showed moderate positive correlations between *Legionella*
403 abundance and both TCC ($\rho=0.60$, $p<0.01$) and ICC ($\rho=0.58$, $p<0.01$). ICC-to-TCC ratio was not
404 correlated with *Legionella* abundance in either tower. Overall, these findings indicate that microbial
405 concentration and viability metrics were not reliable or consistent predictors of *Legionella*
406 abundance.

407 4. Discussion

408 4.1 Biofilm sampling optimised for operational cooling towers and integration 409 of rapid *Legionella* analysis

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

417
418 Biofilm sampling from the pack targets the dominant surface area within cooling towers and the
419 typical primary ecological niche of *Legionella*, enhancing and complementing traditional bulk-water
420 samples from the basin or make-up water, which only provide a snapshot of conditions at a single
421 time point and are prone to dilution effects due to the large water volume within operational
422 cooling towers. As *Legionella* colonisation and proliferation typically originate within biofilms, with
423 cells subsequently released into the bulk-water, biofilm sampling enables earlier detection of
424 contamination compared with conventional bulk-water monitoring and provides a more
425 representative assessment of *Legionella* persistence and distribution within cooling tower systems.

426



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

436
437 The growing need for early detection of *L. pneumophila* has driven the development of rapid
438 detection methods, which offer advantages over traditional culture techniques. These have been
439 validated on planktonic *Legionella* in bulk-water, with limited exploration in biofilms. Importantly,
440 the application of two commercial qPCR platforms - Genesig for *Legionella spp.* and Genomadix for
441 *L. pneumophila* - demonstrated that these techniques can be effectively applied to biofilms sampled
442 using the method developed herein as well as water samples from operational systems, enabling
443 more comprehensive monitoring of *Legionella*.

444
445 In both towers, elevated biofilm-associated *Legionella* signals were observed prior to, or coinciding
446 with, subsequent increases in bulk-water measurements, suggesting that biofilm monitoring may
447 provide earlier evidence of colonisation events than conventional bulk-water sampling alone.
448 Collectively, the successful application of the biofilm-focused sampling and analysis approach to
449 more rapidly detect *Legionella* in operational cooling towers lays the groundwork for future
450 research, surveillance and wider industry adoption.

451

452 4.2 Biofilms as ecological niches for *Legionella*

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



460 limited value of bulk-water monitoring alone for assessing *Legionella* presence and colonisation View Article Online
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461 status.

462
463 The results suggest that biofilms are the primary niche for *Legionella* colonisation and persistence
464 within the cooling towers , from which cells can be intermittently released into the surrounding bulk-
465 water. Previous clinical-based studies similarly shown that *Legionella* proliferation within biofilms
466 typically precedes its release into the bulk-phase (Zhao *et al.*, 2023). Biofilms likely enhance
467 *Legionella* persistence by protecting cells from stresses of the bulk-water phase and enhancing
468 nutrients (Flemming, 2020). The role of protozoa as biofilm grazers may also facilitate *Legionella*
469 survival promoting replication by acting as hosts (Declerck, 2010; Boamah *et al.*, 2017), although
470 *Legionella* do proliferate independently of protozoa (Surman *et al.*, 2002; Abdel-Nour *et al.*, 2013).

471
472 Dispersal of biofilm cells may occur through shedding of cells or biofilm aggregates into the bulk-
473 water as part of a continuous exchange between the phases, or through larger mobilisation events
474 triggered by hydraulic changes, such as increased shear stress in pipe systems (Fish *et al.*, 2020; Khu
475 *et al.*, 2023), or by biocide application, which can induce rapid biofilm sloughing in cooling water
476 systems (Di Pippo *et al.*, 2018). These processes may explain the sporadic nature of *Legionella*
477 occurrence within cooling towers, where bulk-water samples may only detect *Legionella* when it
478 coincides with transient release events. In this context, positive bulk-water detections may
479 sometimes represent snapshots of mobilisation rather than reflecting the true extent of system
480 colonisation. *Legionella* intermittently released from established biofilms into the bulk-water may
481 increase transient planktonic concentrations and provide an inoculum for colonisation of
482 downstream surfaces. This continual cycle of release, transport, and recolonisation could contribute
483 to persistent system-wide colonisation. These findings further support incorporating biofilm
484 sampling into routine surveillance, as bulk-water monitoring alone may fail to capture both the
485 extent and spatial dynamics of *Legionella* colonisation. In the current study, each cleaning event in
486 tower A2 was followed by increased *L. pneumophila* concentrations in the biofilm, suggesting
487 maintenance activities may impact *Legionella* (re)occurrence on the internal surfaces as well release
488 into the bulk-water. These dynamics could result in large numbers of *L. pneumophila* being
489 intermittently mobilised from the biofilm into bulk-water, which may then be aerosolised causing
490 sporadic Legionellosis outbreaks (Declerck, 2010). Thus, incorporating biofilm sampling into
491 *Legionella* surveillance provides a critical advancement to management frameworks that
492 complements traditional bulk-water monitoring.

493



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

500 4.3 Exclusive detection of *L. pneumophila* serogroup 1 within biofilm samples

501 This study presents the first known detection and quantification of *L. pneumophila* exclusively in
502 biofilm samples from operational cooling towers. This is a notable finding given the rarity, and
503 typically sporadic, occurrence of *L. pneumophila* in water samples from cooling towers across Europe
504 and North America. The results are consistent with previous studies of water distribution systems,
505 which reported that *L. pneumophila* predominated in biofilms (De Filippis *et al.*, 2018; Waak *et al.*,
506 2018) and that *Legionella spp.* is generally more abundant in biofilms than in bulk-water (Garner *et al.*,
507 2018). This consistency across different engineered systems further supports the robustness and
508 adaptability of the sampling method, and application across a broader range of cooling towers
509 would provide wider validation of its performance under diverse operational conditions. A valuable
510 next step would be to establish how far in advance biofilm *L. pneumophila* detection precedes
511 subsequent waterborne detection in real-world systems. While this cannot be experimentally
512 controlled or engineered in operational towers, longitudinal observations that track the transition
513 from biofilm detection to detectable levels in bulk-water would provide important evidence for the
514 predictive value of this approach, as well as insights into interpreting data to inform intervention
515 timing.

516
517 These findings provide strong support for the original hypothesis that biofilms could act as lead
518 indicators - and had monitoring been restricted to bulk-water testing, *L. pneumophila* would have
519 gone undetected, masking potential contamination of the bulk-water and the associated public
520 health risks. Application of the validated and reproducible biofilm sampling and analysis protocol
521 has demonstrated the value of incorporating biofilm sampling into surveillance frameworks as an
522 early indicator for *Legionella* presence, thus improving detection accuracy, and supporting timely
523 implementation of water safety plans.

524 4.4 No correlation between cell concentration and *Legionella* concentration

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



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

530

531 *L. pneumophila* was detected exclusively in A2 biofilms, despite similar or lower TCC and ICC values
532 compared to A1, indicating that microbial load alone is not a reliable predictor of *Legionella* risk. This
533 is in contrast to van der Kooij *et al.* (2017), in which significant, albeit weak, correlations were
534 reported between total cell count, heterotrophic plate count, and *Legionella* growth within biofilm
535 model systems. Importantly, their study used a laboratory setup that differed significantly in
536 physicochemical parameters from the operational cooling towers we examined. The use of flow
537 cytometry enabled detection of viable but non-culturable (VBNC) cells and provided a more sensitive
538 analysis in comparing cell concentrations and assessing cell viability relative to *Legionella*
539 concentrations. The difference in trends between the studies underscores the limitations of
540 extrapolating laboratory results to real-world systems.

541

542 The finding that bulk-water cell counts did not correlate with *Legionella* presence is consistent with
543 the work of Sanchis *et al.* (2023), which challenges traditional assumptions by showing that
544 heterotrophic plate count (HPC) values above 100 CFU/ml do not necessarily correlate with an
545 increased risk of *Legionella* presence. Their findings, which rely on conventional methods for
546 evaluating cooling tower bulk-water samples, suggest that only HPC values under 100 CFU/ml could
547 serve as reliable indicators for the absence or minimal concentrations of *Legionella*. Similarly, Duda
548 *et al.* (2015) underscore the limitations of using HPC as a predictive tool for *Legionella* colonisation,
549 demonstrating that such measures failed to indicate *Legionella spp.* presence in over 64% of cases
550 involving cooling tower samples. Although other studies (Campaña *et al.*, 2023; Kyritsi *et al.*, 2018)
551 have suggested possible correlations under specific conditions. These studies, while
552 methodologically diverse to this research, underscore a critical point: the predictive value of HPC for
553 *Legionella* risk assessment is highly variable and often unreliable. This highlights the nuanced and
554 complex nature of *Legionella* ecology and necessitates a move beyond relying solely on bacterial
555 abundance analysis.

556

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



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

563 4.5 Practical implications & Challenges

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

570
571 Integrating culture-based methods with rapid detection techniques such as qPCR could further
572 enhance biofilm monitoring. While culture remains the gold standard for assessing bacterial viability
573 and regulatory compliance, qPCR provides faster detection and greater sensitivity but cannot
574 distinguish viable and non-viable cells, which is a limitation of this study. However, qPCR application
575 supported rapid on-site analysis aligning with the focus of this study on earlier detection, while flow
576 cytometry (ICC), provided additional context for interpreting microbial viability and dynamics.
577 Combining these approaches (culture, flow cytometry and qPCR) could bridge the gap between
578 novel methodologies and existing water safety frameworks, facilitating wider adoption without
579 significant protocol changes. These integrated strategies could be further enhanced by incorporating
580 immunomagnetic separation (IMS) techniques, which offer the potential to concentrate *Legionella*
581 cells and improve detection sensitivity, particularly in samples with low bacterial loads.

582
583 Detection of *L. pneumophila* or *Legionella spp.* from biofilm samples would not require any change
584 to current operational responses. The same remedial measures recommended for bulk-water
585 contamination such as targeted biocide dosing, mechanical cleaning, flushing, and system
586 optimisation, should be applied. However, identifying contamination within biofilms provides earlier
587 warning, allowing operators more time to plan and implement interventions before *Legionella*
588 spreads into the bulk-water. This early insight supports a shift from reactive to proactive
589 management, reducing the risk of outbreaks and the associated operational, legal, and reputational
590 consequences. Proactive biofilm management therefore offers a cost-effective and sustainable
591 strategy to safeguard public health and ensure long-term cooling tower safety.

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592 5. Conclusions

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

601
602 Biofilm and bulk-water samples exhibited distinct microbial dynamics, with no correlation between
603 total cell counts or cell viability and the presence of *Legionella spp.*, including *L. pneumophila*. This
604 underscores that microbial load alone is insufficient to predict *Legionella* risk and highlights the need
605 for comprehensive monitoring approaches that consider microbial community characteristics,
606 biofilm–bulk-water interactions, and relevant environmental factors.

607
608 Collectively, these findings advance the understanding of *Legionella* ecology in operational cooling
609 towers and demonstrate the value of incorporating biofilm-based assessments into surveillance
610 strategies. By revealing that traditional bulk-water monitoring may underestimate abundance and
611 hence potentially risk, this study situates biofilm sampling as a critical tool for enhancing the
612 sensitivity, accuracy, and effectiveness of *Legionella* detection in environmental monitoring and
613 public health protection.



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615

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625

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627 Methodology was developed by MK, KF, JB, with input from BC, AP and DS. Fieldwork was conducted
628 by MK with support from AP. Data analysis and visualisation were undertaken by MK with input from
629 KF. All authors contributed to data interpretation. Data curation was performed by MK. The original
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631 DS. Supervision was provided by KF, JB and BC. Funding was acquired by KF, with input from JB.
632 Project administration was coordinated by KF.

633

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



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

Data Availability Statement for Kerry *et al*

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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.

