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The Contribution of *Legionella anisa* to *Legionella* Contamination of Water in the Built Environment

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Abstract: *Legionella* bacteria can proliferate in poorly maintained water systems, posing risks to users. All *Legionella* species are potentially pathogenic but *Legionella pneumophila* (*L. pneumophila*) is usually the primary focus of testing. However, *Legionella anisa* (*L. anisa*) also colonises water distribution systems, is frequently found with *L. pneumophila* and could be a good indicator for increased risk of nosocomial infection. Anonymized data from three commercial *Legionella* testing laboratories afforded analysis of 565,750 water samples. Data covered July 2019 to August 2021, including the COVID-19 pandemic. Results confirmed *L. anisa* commonly colonises water distribution systems, being the most frequently identified non-*L. pneumophila* species. Proportions of *L. anisa* and *L. pneumophila* generally remained similar but increases in *L. pneumophila* during COVID-19 lockdown suggests static water supplies might favour its growth. Disinfection of hospital water systems was effective but re-colonization did occur, appearing to favour *L. pneumophila*, although *L. anisa* colony numbers also increased as a proportion of the total. While *L. pneumophila* remains the main species of concern as a risk to human health, *L. anisa's* role should not be under-estimated, either as a potential infection risk or as an indicator of the need to intervene to control *Legionella* colonization of water supplies.

Keywords: *Legionella pneumophila*; *Legionella anisa*; water systems; hospital; disinfection and recolonization; COVID-19 lockdown

1. Introduction

Mains water supplies are intended to be wholesome and safe for drinking, cooking, and bathing, but are not sterile and can contain pathogenic bacteria. If hot and cold water systems (HCWS) are not well designed or managed, then these waterborne pathogens, including *Legionella* bacteria, can proliferate to an extent that they pose risks to users of the water system [1]. Controlling the growth of legionellae in large buildings, with complex water systems can be challenging, especially if exacerbated by poor designs that make maintaining safe water temperatures problematic, or building modifications or construction materials that encourage development of biofilms and provide pockets within the water system where legionellae can thrive [1].

At least half of all known *Legionella* species have been shown to cause human disease, but all species are thought potentially to be pathogenic [2]. The major clinical manifestation of infection due to *Legionella* species is a type of pneumonia known as Legionnaires' disease (LD), although non-pneumonic legionellosis (Pontiac Fever) and extrapulmonary infection may occur [2]. *Legionella pneumophila* (*L. pneumophila*) is the most commonly detected species amongst clinical cases of legionellosis (in USA more than 90% of isolates are *L. pneumophila* and 83% *L. pneumophila* serogroup 1 (Sg1) [3]; in the EU 83% of isolates were *L. pneumophila* Sg1 in 2020 and 82% in 2020 [4]. Other *Legionella* species have been reported to cause disease but are less well researched [5,6,7]. Non- *L. pneumophila* species occasionally cause LD [6,8,9], severe pneumonia [10] and Pontiac Fever [11]. In

total, 2-7% cases of legionelloses are estimated to be caused by non- *L. pneumophila* species [12], most of these confirmed infections occurring in immunosuppressed patients [2]. Non- *L. pneumophila* species are less-commonly detected in clinical diagnoses or during environmental surveillance due to their slow culture growth and the absence of specific and rapid diagnostic/analytical tools [13].

LD outbreaks are more commonly associated with HCWS than with other system types, although this may reflect the higher number of them compared to other water systems in the built environment [14]. As part of an overall water management strategy, periodic sampling and testing for the presence of legionellae in water systems can be extremely valuable in determining whether the microbiological risk controls applied are effective [1]. For the testing of environmental samples, agar plate culture is still the most widely used standard technique for the detection and quantification of viable *Legionella* spp. [15,16], however, this technique does favour *L. pneumophila* over non-pneumophila species [17].

While *L. pneumophila* is usually the primary focus of testing, *L. anisa* is also a common coloniser of water distribution systems, although an uncommon hospital-acquired pathogen [18]. Non-pneumonic conditions attributable to *L. anisa* infections have included chronic endocarditis [19], pleural infection [20], osteomyelitis [9] and mycotic aortic aneurysm [21]. *L. anisa* has been detected in water samples from cooling towers [22,23] and has been identified as the cause of outbreaks of Pontiac Fever associated with a decorative fountain in a hotel lobby and a water feature in a restaurant [24,25], as well as the occasional cases of LD. The French national *Legionella* reference centre determined that in 2001-2002 *L. anisa* was the most frequently-identified non- *L. pneumophila* species, accounting for 13.8% of environmental water sample isolates, but only 0.8% of clinical isolates [26]. This may suggest that *L. anisa* is less pathogenic to humans than *L. pneumophila* [26,27], however, there is also likely to be significant under-reporting of clinical cases due to the diagnostic testing undertaken.

This is supported by data from the UKHSA Legionella Reference Laboratory. Examining isolates retained by the laboratory from environmental and clinical samples submitted, and thus associated with clinical infection, over a period from 2004 – 2021 from a total of 2321 environmental samples 2179 (93.9%) were *L. pneumophila* and 96 (4.14%) were *L. anisa*, while during the same period from a total of 1360 clinical samples 1327 (97.6%) were *L. pneumophila* and only one (0.07%) were *L. anisa* (V. Chalker, UKHSA, pers comm.).

Only 10 cases of Legionellosis associated with *L. anisa* were reported by national surveillance systems in New Zealand, Australia, Europe, USA and Japan, from a total of 8066 confirmed cases of legionellosis (i.e. <0.1%) [7]. In a multinational survey of community-acquired pneumonia where *Legionella* was detected in 508 respiratory tract samples, *L. anisa* only accounted for 0.2% of cases, whilst *L. pneumophila* accounted for 91.5% of cases [15].

Some *Legionella* spp may multiply only in specific protozoan hosts and the inability of *L. anisa* to multiply in human phagocytic cells may be related to the development of Pontiac Fever rather than pneumonic legionellosis in exposed individuals [28].   Further supporting this is a possible association in cooling towers between the presence of the protozoan *Acanthamoeba* and *L. anisa* [23].

To identify and trace strains causing legionellosis, it is important to correctly identify and type the *Legionella* strains in the patient, but even more in the environment with a particular focus on elucidating the potential pathogenic role of non- *L. pneumophila* species [12]. Because amoebal co-culture may be required for better recovery [29,30] this methodology is less likely to be available for clinical material [2]. All clinical sample test methods have inherent weaknesses for the identification of *Legionella*, so more than one test method is advised.   Testing from a single specimen type limits the ability to diagnose legionellosis, particularly urinary antigen tests (UAT), and in addition these do not allow the matching of patient isolate and environmental samples [31]. While UAT methods for *L. pneumophila* Sg1 are considered moderately sensitive and highly specific [32], they do not reliably detect infection by species other than *L. pneumophila* Sg1 [2].   Thus, total dependence on UAT may miss as many as 40% of cases of legionellosis [33]. The number and distribution of infections caused by non- *L. pneumophila* legionellae are thus underestimated [7] leading to under-diagnosis of both community-acquired and nosocomial *Legionella* infection [2].

By contrast, in Denmark, more than 80% of LD cases are diagnosed by polymerase chain reaction (PCR), likely increasing the detection of non-Sg1 cases [34]. Denmark has a relatively high frequency of clinical cases caused by *L. pneumophila* strains that in other countries are more commonly associated with the environment, with non- *L. pneumophila* species reportedly causing at least 4.5% of all LD cases [27]. In 2010, to increase the sensitivity of testing methods New Zealand adopted PCR as the primary diagnostic tool for all species of *Legionella* in respiratory samples [7]. In a Spanish study, a total of 210 urine samples from patients with suspected pneumonia were analysed by UAT and also by a semi-nested PCR technique. UAT detected 4 positive samples (1.9%) for *L. pneumophila* Sg1, while 15 samples tested positive by PCR (7.1%). Of the 15 positive samples, 4 were positive for *L. pneumophila*, corresponding to the UAT positive samples, 1 sample was positive for *L. anisa* and a further 10 were positive for unidentifiable *Legionella* species [6]. In a Colombian study into hospitalised HIV-associated pneumonia patients, *Legionella* co-infection was found in 17 pneumonia cases. Bronchioalveolar lavage samples from all patients were culture negative for *Legionella*, but PCR positive, six of the co-infections being with *L. anisa* and three with *L. pneumophila* [35].

While culture is still the primary method used to detect legionellae, this has been revolutionized by the application of Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-ToF MS). This is a widely accepted method for identifying bacterial species grown on agar plates, including legionellae [13,27], accurately identifying bacteria routinely isolated in microbiology laboratories with a high positive predictive value ranging in different studies from 95.4% [36] to 99.2% [37] for a range of bacteria and 99.1% specificity for *Legionella* [38].

Other detection methods, for example, Lateral Flow or Most Probable Number methods, may compare favourably with culture for the detection of *L. pneumophila*, however, do not detect non- *L. pneumophila* species, including *L. anisa* [1,39].

Multiplex PCR assays have been developed which allow simultaneous detection of *Legionella* species and *L. anisa* [5].

Regarding the value of responding to an identified problem in the built environment from legionellae other than *L. pneumophila*, UK health and safety guidance [40] suggests that their presence or growth in a water system could indicate that suitable growth conditions for *L. pneumophila* have at some point been achieved. As such, these organisms should be seen as indicators of system colonization and appropriate action taken on their detection [15,41].

While *L. pneumophila* is the species most frequently cultured from the natural environment, other disease-relevant *Legionella* species such as *L. micdadei, L. bozemanii, L. longbeachae* and *L. anisa* have yet to be extensively explored in premise plumbing systems [23], despite *L. anisa* first being isolated from environmental samples of cooling tower and potable water sources as long ago as 1980 [22]. After *L. pneumophila*, *L. anisa* is the *Legionella* species most frequently detected in the environment [12,26,27,41,42,43,44,45,46,47], including from both hot water systems and cooling tower systems [44].

L. anisa is frequently found in conjunction with *L. pneumophila* in hospital water systems [48,49,50] and could be a good indicator for increased risk of nosocomial LD [41]. It has also been suggested that presence of a more abundant *Legionella* population, including *L. anisa,* in a water system may limit the detection of *L. pneumophila*, therefore action should be taken to eradicate all *Legionella* contamination of hospital water distribution systems [2,41].

In a survey of 20 hospitals across 14 US states, nine (45%) of the hospital water systems were found to be colonized with *L. anisa* with a sample positivity rate of 57/674 (8.5%). There were no diagnosed infections due to *L. anisa*, but clinical testing of respiratory specimens was not specific to *L. anisa* [51]. *L. anisa* was detected in 29 out of 108 (27%) electronic tap water samples at John Hopkins University School of Medicine, Baltimore. This compared to 25 out of 108 (23%) testing positive for *L. pneumophila* [52]. In a Japanese study of public buildings, more than 40% of hot water distributions examined were contaminated with *L. pneumophila* and *L. anisa* [43]. *L. anisa* persisted in a Japanese hospital water system for at least the 6 years of the study and caused sporadic contamination of shower heads. Regular shower head flushing was insufficient to eradicate the problem and routine shower head replacement was adopted as a control measure [53]. *L. anisa* in biofilm in hot water systems can be difficult to remove via thermal disinfection [54] and may also show some degree of

biocidal resistance in hot water system biofilm when compared to *L. pneumophila* [46,55]. *L. anisa* concentrations in water systems can be reduced using chlorine dioxide disinfectant, however, this can be a protracted process [56].

By monitoring both *L. pneumophila* and non- *L. pneumophila* species, changes to building management can be made to address potentially different risks at different times of the year. Higher concentrations of *Legionella* spp. may occur in buildings and hot water taps during periods of lower water use [11]. Poor water circulation and temporary or permanent water stagnation allows *Legionella* to colonize water systems [57]. Consequently, building shut-downs or reduced occupancy during the Covid-19 pandemic could have increased colonization, and alerts were issued to assess this risk [58]. Interestingly, examining a subset of the UKHSA Reference Laboratory data over a later timeframe of June 2019 to July 2021, thus spanning the Covid-19 lockdown period in the UK, from a total of 393 environmental samples 359 (91.35%) were *L. pneumophila* and 31 (7.89%) were *L. anisa*, while during the same period from a total of 204 clinical samples 194 (95.1%) were *L. pneumophila* but none (0.0%) was *L. anisa* (V. Chalker, UKHSA, pers comm.).

This paper describes an analysis of routine water testing for *Legionella* species undertaken by commercial laboratories over a period from July 2019 to August 2021, therefore spanning the pandemic, and the relationship between the presence of *L. anisa* and *L. pneumophila*.

2. Materials and Methods

Through the UK trade body the Legionella Control Association, contacts were made with commercial laboratories that undertake routine water testing for *Legionella* for a range of clients. Following standard protocols [59,60] water samples from premises were regularly and routinely collected from pre-determined sampling points and delivered to the testing laboratory. Here they were treated and used to inoculate agar plates. Suspect *Legionella* colonies were identified by MALDI-ToF MS.

Three laboratories agreed to share anonymized data, each providing a database of test results with numbers of samples testing positive for *L. pneumophila*, *L. anisa* and a range of other *Legionella* species.

3. Results

Overview

As this study relied on the goodwill of the laboratories to participate, there were some differences in how they were prepared to share their data. One data set provided information on *Legionella*-positive samples only, while the other two provided information on the total number of samples tested. In two of the three data sets, the *Legionella*-positive sample data were also broken down by month, one being from July 2019 to August 2021 and the other from January 2020 to June 2021. For the third data set, the results provided were not time-resolved but were from a similar time frame. Although each data set provided a breakdown of *Legionella*-positive results into a broad range of species, for the purpose of the current analysis the focus was on *L. pneumophila* and *L. anisa*, therefore other species were grouped together as 'other'.

Legionella Data set 1

This data set provided information on *Legionella*-positive samples only. Between July 2019 and August 2021, nearly 12,000 positive *Legionella* samples were recorded. As summarized in Figure S1 (Supplementary Materials; available online), the most prevalent species identified in these samples was *L. pneumophila* (47%). The next most prevalent species identified was *L. anisa* (46%). These data are presented in Figure 1 as monthly data over the 26 month period.
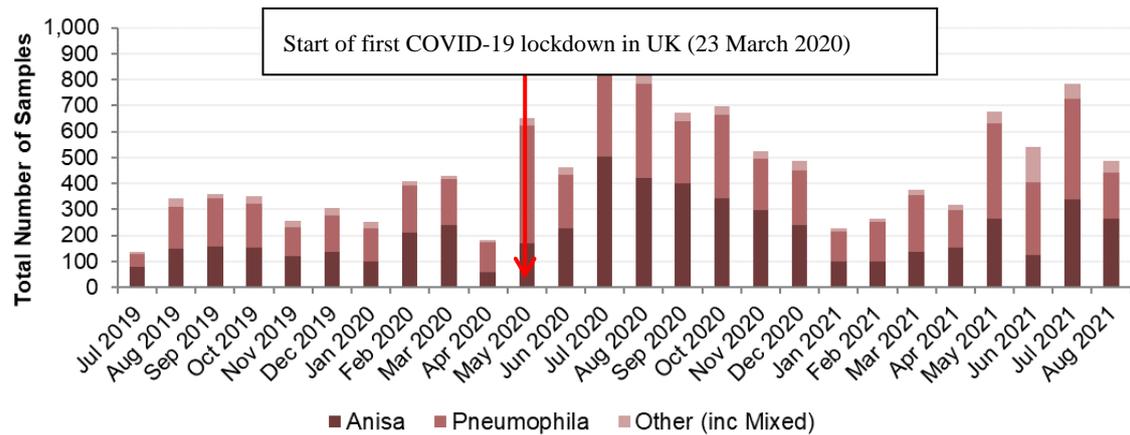
Figure 1. Data Set 1 - Total number of *Legionella*-positive samples recorded, by date and species.

Over the 26-month period the general proportion of *L. anisa* and *L. pneumophila* remained similar. However, there were some months with large differences, most noticeably April and May 2020 and June 2021.

Expressed as a percentage of the total number of *Legionella*-positive samples, those identified as *L. pneumophila* ranged from 35% to 70%, with April and May 2020 the largest percentage of the total at 63% and 70% respectively which also corresponded with a dip in the percentage of samples identified as *L. anisa* (Table S1, Supplementary Material and Figure 2 below). For *L. anisa* the overall percentage of the total was generally slightly lower than for *L. pneumophila* and ranged from 23% to 59%. The 23% value in June 2021 corresponded to a month when the percentage of 'other *Legionella* species' was greatest at 26%, seen clearly in Figure 2, which was an outlier from the proportion of 'other *Legionella* species' usually found.
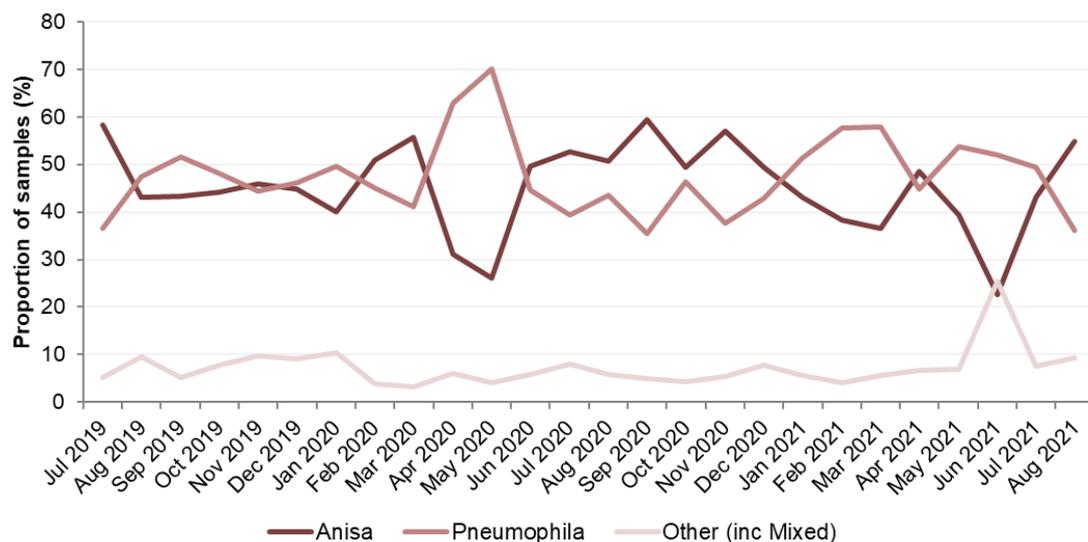

Figure 2. Data Set 1 - Proportion of *Legionella*-positive samples recorded, by date and species.

By March 2020, as the COVID-19 pandemic started to have global impact, lockdown restrictions were applied in the UK leading to reduced building occupancy. This may have influenced the number of samples taken and the number of *Legionella*-positive samples recorded. In Figure 1, an arrow indicates when the UK COVID-19 lockdown began, more specifically from 23rd March 2020. Initially the number of *Legionella*-positive samples fluctuated between April 2020 and June 2020, before increasing to its highest in July 2020. This may be due to places of employment being closed for a 3 month period, with stagnation of water supplies, before gradually being reopened. Seasonal fluctuations in positive samples is another potential influence, with higher numbers of positives in

the warmer summer/ early autumn period. This may be evident to some extent in August 2019 but more evident in July/August 2020 and July 2021, and in the post-lockdown period May to December 2020, where each month had higher numbers of *Legionella*-positive samples than in any pre-COVID lockdown months in the data set.

Overall, 23% of *Legionella*-positive samples were taken in the eight months of the data set pre-COVID and 77% in the 18 months post-COVID. However, the proportion of those samples identified as *L. anisa* did not alter (approximately 46% of *Legionella*-positive samples; Figure S2, Supplementary Material). A Chi-square test indicates that there is no significant association between *Legionella* species and Pre/Post-COVID (p=0.467).

Legionella Data set 2

This data set related to an intervention and intensive sampling exercise in a hospital between January 2020 and June 2021. It provided data on the total number of samples taken and the numbers that were *Legionella*-positive before and after a site-wide disinfection of all hot and cold water systems with hydrogen peroxide. Out of a total of 613 samples taken, 322 were positive for *Legionella* (52.5%). Overall, 167 of these were pre-cleaning samples (out of 283; 59.0%) and 155 were post-cleaning samples (out of 330; 47.0%). The most prevalent species identified in these samples was *L. pneumophila* (49%). The next most prevalent species identified was *L. anisa* (38%). Figure 3 shows the total number of *Legionella*-positive samples, by species, before and after the cleaning intervention.
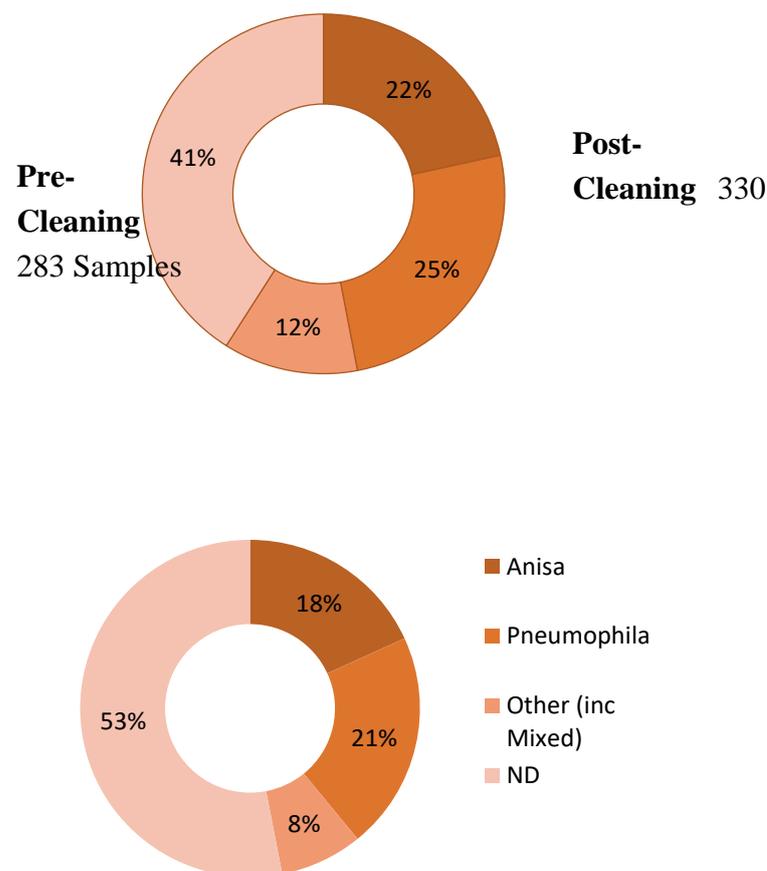

Figure 3. Data Set 2 - Total number of *Legionella*-positive samples recorded, by species for pre and post cleaning.

Figure 4 shows a monthly breakdown of the data. Although the data spans an 18-month period, the sampling was not undertaken every month, thus data is missing for April 2020, August to October 2020 and January 2021, and no pre-cleaning samples were taken for the first six months (see Table S2, Supplementary Material). Due to relatively low numbers of some sampling results, statistical analysis was not feasible.
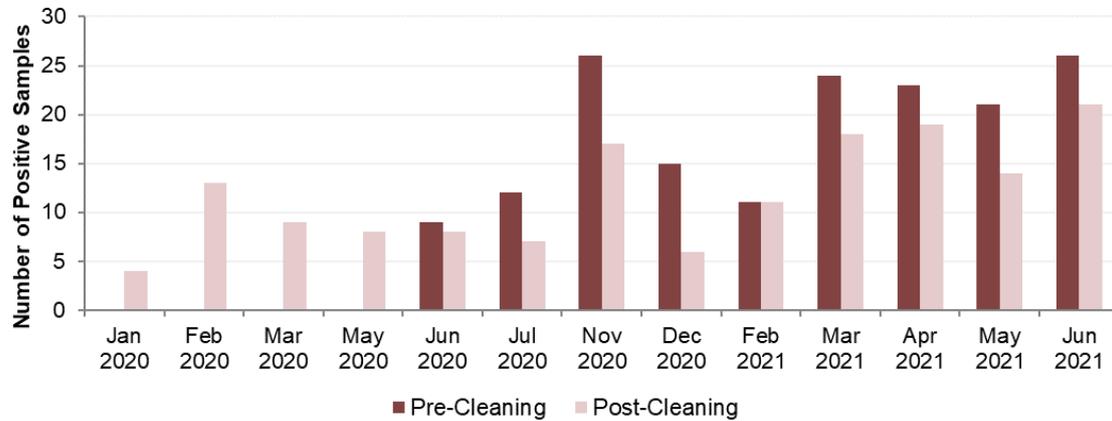

Figure 4. Data Set 2 - Total number of *Legionella*-positive samples recorded, by date for pre and post cleaning.

Only post-cleaning samples are available that span the two time-frames of pre and post COVID. Overall, 17% of *Legionella*-positive samples were taken pre-COVID and 83% post-COVID. The proportion of those samples identified as *L. anisa* roughly halved post-COVID compared to pre-COVID (from 65% of *Legionella*-positive samples down to 33%). See Figure 5.
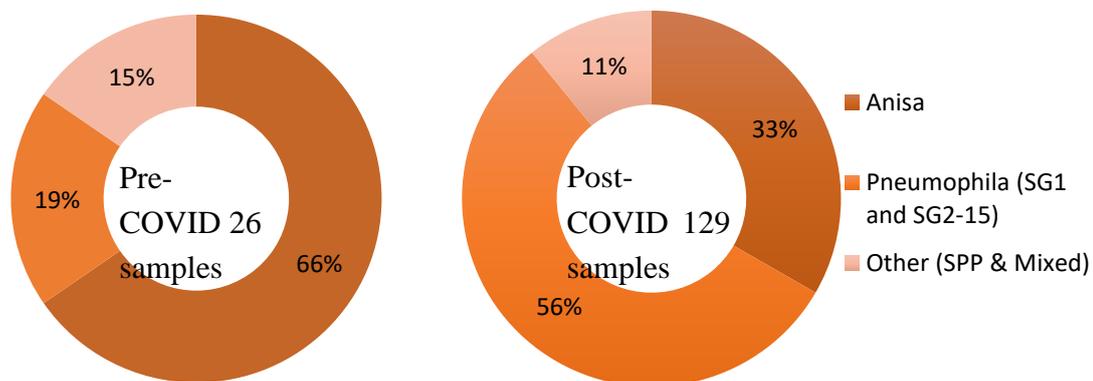

Figure 5. Data Set 2 -Number of *Legionella*-positive samples recorded before and after the COVID-19 outbreak, by species.

Table 1 presents data where there were both pre and post cleaning samples (n = 282). The breakdown between species identified in *Legionella*-positive samples pre-cleaning are presented across the rows, and for post-cleaning are presented in columns. For example, out of 61 locations, at 18 *L. anisa* remained the predominant species identified post-cleaning, six changed to *L. pneumophila* being the predominant species and three to 'other *Legionella*', with 34 locations going from *L. anisa* being isolated to None Detected. For *L. pneumophila*, at 54 out of 81 locations *L. pneumophila* remained

the predominant species identified, eight changed to *L. anisa* being the predominant species and four to 'other *Legionella*', with 15 locations going from *L. pneumophila* being isolated to None Detected. This meant that *L. anisa* went from predominating at 61 locations pre-cleaning to 38 locations post-cleaning, and *L. pneumophila* went from predominating at 81 locations pre-cleaning to 71 locations post-cleaning. Locations where no Legionellae were detected increased from 115 pre-cleaning to 161 post-cleaning.

Table 1. Data Set 2 - The effect of cleaning on the species identified in the *Legionella*-positive samples.

		Post-Cleaning Sample				Grand Total
		Anisa	Pneumophila	Other	ND	
Pre-Cleaning Sample	**Anisa**	18	6	3	34	**61**
	Pneumophila	8	54	4	15	**81**
	Other	6	6	4	9	**25**
	ND	6	5	1	103	**115**
Grand Total		**38**	**71**	**12**	**161**	**282**

As well as the number of *Legionella*-positive samples, this data set also included *Legionella*-positive colony count data. Between January 2020 and June 2021, there were over 630,000 positive *Legionella* colonies recorded. Approximately 70% of these were from pre-cleaning samples and 30% were from post-cleaning samples. The most prevalent species identified in these colonies was *L. pneumophila* (70%). The next most prevalent species identified was *L. anisa* (20%). See Table 2.

Table 2. Data Set 2 - Total number of colonies recorded, by date and species for pre and post cleaning.

Date	Pre-Cleaning Sample Colonies				Post-Cleaning Sample Colonies			
	Anisa	Pneumophila	Other	Total	Anisa	Pneumophila	Other	Total
Jan 2020					40	1,520	0	**1,560**
Feb 2020					12,920	80	1,268	**14,268**
Mar 2020					5,820	360	2,400	**8,580**
May 2020					2,920	2,120	1,460	**6,500**
Jun 2020	4,762	7,630	0	**12,392**	3,081	1,540	0	**4,621**
Jul 2020	3,180	460	6,400	**10,040**	3,340	0	180	**3,520**
Nov 2020	11,080	25,080	4,200	**40,360**	1,260	10,820	1,400	**13,480**
Dec 2020	2,520	8,280	0	**10,800**	120	3,180	0	**3,300**
Feb 2021	2,821	26,496	0	**29,317**	408	19,635	4,400	**24,443**
Mar 2021	11,673	50,127	2,120	**63,920**	8,937	37,380	0	**46,317**
Apr 2021	28,875	39,480	17,260	**85,615**	940	11,540	3,860	**16,340**
May 2021	3,400	111,440	0	**114,840**	0	20,880	200	**21,080**
Jun 2021	6,680	53,540	9,700	**69,920**	11,460	15,240	3,160	**29,860**
Total	**74,991**	**322,533**	**39,680**	**437,204**	**51,246**	**124,295**	**18,328**	**193,869**

As also seen from Table 2, pre-cleaning, *L. anisa* made up 17.2% of the total and *L. pneumophila* 73.8%. Post-cleaning, of a much lower number of colonies (193,869 compared to 437,204), *L. anisa* made up a larger proportion at 26.4%, up from 17.2% of the total, and the proportion of *L. pneumophila* was reduced to 64.1%, down from 73.8%. Out of 282 data points that had pre- and post-cleaning colony counts, counts went down in 136 (48.2%) and were unchanged from None Detected in 103 (36.5%) instances. In only 43 (5.2%) instances were colony counts found to increase post-cleaning. Most notable of these were: a shower hose that went from a pre-clean colony count of 842 CFU *L.*

pneumophila Sg1 to 4,400 CFU comprising 400 *L. pneumophila* Sg1 and 4,000 *L. anisa*; a shower hose that went from a pre-clean colony count of 779 CFU *L. pneumophila* Sg1 to 8,667 CFU *L. pneumophila* Sg1; a cold tap that went from a pre-clean colony count of 1200 CFU L. anisa to 5,000 CFU *L. pneumophila* Sg1; a shower hose that went from None Detected pre-clean to a colony count of 5,400 CFU *L. anisa*. Of the 136 data points showing a reduction in colony counts from pre- to post-cleaning, 19 showed a reduction by >5,000 CFU, most notably: a shower hose that went from a pre-clean colony count of 16,421 CFU *L. pneumophila* Sg1 to 489 CFU *L. pneumophila* Sg1; a shower hose that went from a pre-clean colony count of 12,600 CFU *L. pneumophila* Sg1 to 200 CFU *L. pneumophila* Sg1; a shower hose that went from a pre-clean colony count of 12,400 CFU *L. pneumophila* Sg1 to 100 CFU *L. pneumophila* Sg1; a shower hose that went from a pre-clean colony count of 20,000 CFU *L. pneumophila* Sg1 to 4,600 CFU *L. pneumophila* Sg1; a shower hose that went from a pre-clean colony count of 44,200 CFU *L. pneumophila* Sg1 to 4,400 CFU *L. pneumophila* Sg1; a shower hose that went from a pre-clean colony count of 20,600 CFU *L. pneumophila* Sg1 to 3,200 CFU *L. pneumophila* Sg1. Although not statistically proven, the above data indicated that highest counts were greatly reduced by the hydrogen peroxide disinfection intervention.

Most of the sample points were either showers or wash hand basins with taps incorporating thermostatic mixing valves (TMVs). Data in Table S3 (Supplementary Material) summarises how colony counts and the species isolated compares for both these sample types pre- and post-cleaning, and this is expressed graphically in Sankey diagrams in Figure 6 for shower hoses and Figure 7 for TMVs to show species changes.
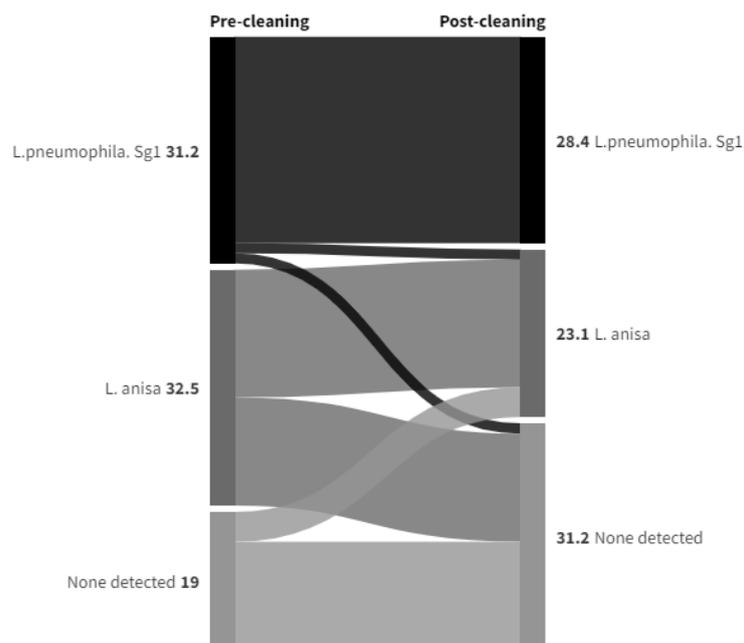

Figure 6. Data Set 2 – Changes in species isolated pre- and post-cleaning in shower hoses .

A greater proportion of TMVs than showers showed no presence of *Legionella* both pre- and post-cleaning (35.4% compared to 14.9%) and also more changed from having *L. pneumophila* Sg1 colony counts pre-clean to None Detected post-clean (8.1% compared to 1.4%). The proportion changing from *L. anisa* colony counts to None Detected was similar for both sample types. Although *Legionella* colony counts were not being eliminated, the effect of the cleaning intervention on reducing colony counts appeared to be greater with showers – *L. pneumophila* Sg1 and *L. anisa* colony counts were reduced in 23% and 12.2% of samples respectively compared to 7.4% and 1.9% respectively in TMVs. Although there were only a few instances where colony counts were increased post-cleaning compared to the same sample point pre-cleaning, there was some indication that showers were more likely to be recolonized. In 5.4% of shower samples *L. pneumophila* Sg1 remained the predominant colonizing species with counts increased, compared to 3.1% of TMVs. Also in 5.4% of shower samples *L. anisa* remained the predominant colonizing

species with counts increased, compared to 1.2% of TMVs, and 4.1% of shower samples changed from None Detected to *L. anisa* pre- to post-clean compared to 1.9% of TMVs, also suggesting the possibility that *L. anisa* had a greater propensity for recolonization.
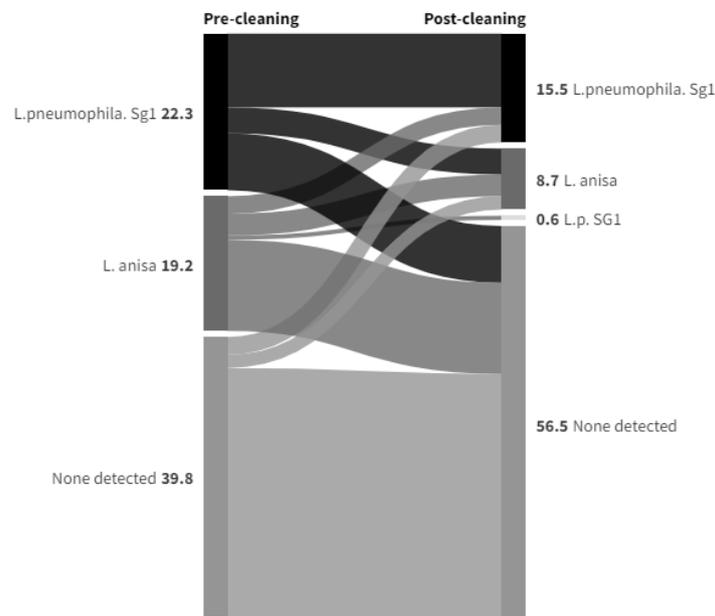

Figure 7. Data Set 2 – Changes in species isolated pre- and post-cleaning in TMVs .

Only post-cleaning samples were available that span the two time-frames both pre and post COVID. Overall 13% of *Legionella*-positive sample colonies were identified pre-COVID and 87% post-COVID. The majority of colonies identified pre-COVID were *L. anisa* (72%), however, post-COVID the majority of colonies were *L. pneumophila* (77%). See Figure 8.
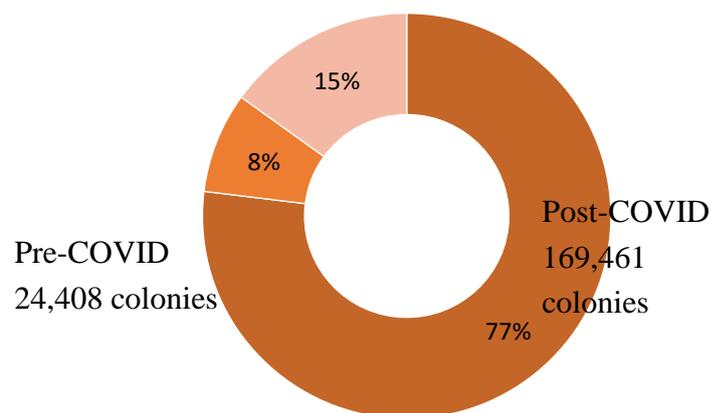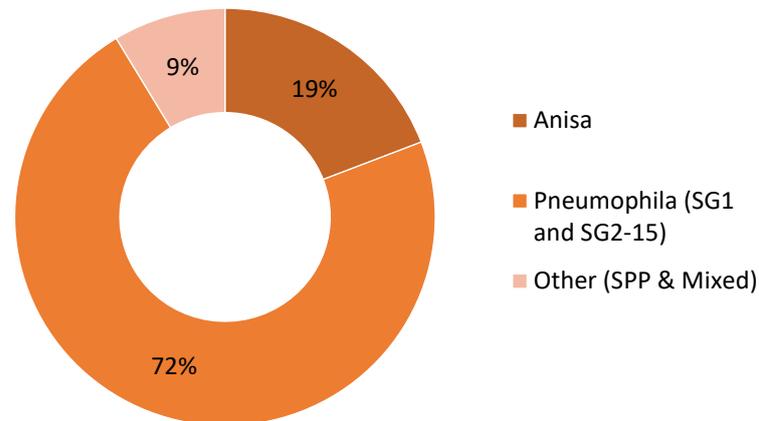
Figure 8. Data Set 2 - Number of *Legionella*-positive samples recorded before and after the COVID-19 outbreak, by species.

Legionella Data set 3

This dataset comprised 553,159 water samples in total, submitted by a range of clients for routine *Legionella* testing. Of these, 60,075 samples were identified as being positive for *Legionella* (11% of all samples). The most prevalent species identified in these samples was *L. anisa* (32,517; around 54% of all positive samples or 6% of all samples). This included 202 samples which yielded *L. anisa* as well as other species. The next most prevalent species identified was *L. pneumophila* (17,617; 30% of all positive samples or 3% of all samples). In a further 4,162 samples other *Legionella* species were identified (7% of all positive samples or <1% of all samples). This is presented in Figure 9 and Table 3.
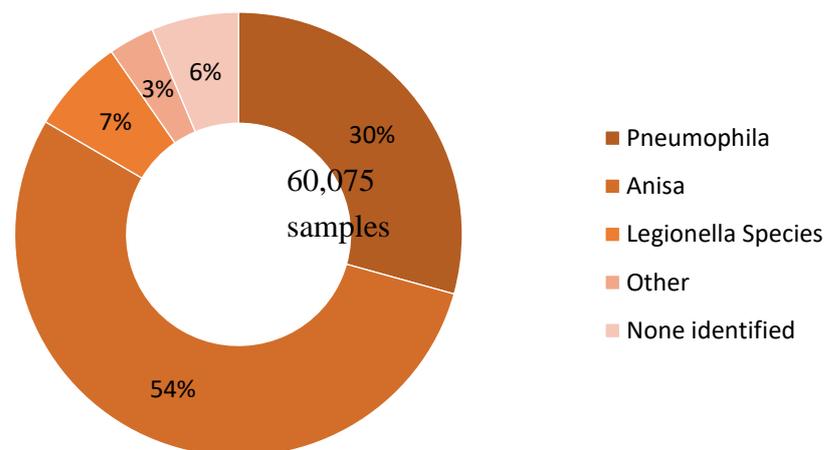

Figure 9. Data Set 3 - Total number of *Legionella*-positive samples recorded, by species.

Table 3. Data Set 3 - Total number of *Legionella*-positive samples recorded, by species.

Species Detected	Count	(Proportion)
Legionella pneumophila	17,617	(29.3%)
Legionella anisa	32,517	(54.1%)
Legionella species	4,162	(6.9%)
Other	1,992	(3.3%)
Blank (no species identified)	3,787	(6.3%)
TOTAL	**60,075**	

4. Discussion

Through unprecedented access to data made available from three *Legionella* testing laboratories, analysis was possible on a total of 565,750 water samples tested for *Legionella*. One data set also provided a species breakdown of *Legionella*-positive colony counts totaling over 630,000.

The results supported the previously reported view that *L. anisa* is a common coloniser of water distribution systems and the most frequently-identified non- *L. pneumophila* species. Identification of *L. anisa* among non- *L. pneumophila* species is probably becoming more apparent, as detection methods such as PCR and MALDI-ToF MS allow for more readily achievable speciation of bacteria routinely isolated in microbiology laboratories, and this may be reflected in the data examined.

Some differences in the proportions of *L. anisa* compared to *L. pneumophila* were found in the three databases studied. In data set 1, over a 26-month period the general proportion of *L. anisa* and *L. pneumophila* remained similar at 46% and 47% respectively. Where this differed, most noticeably in April and May 2020, the proportion of *L. pneumophila* positive samples increased to 63% and 70% respectively. This may have been a result of samples taken after a period of premises shutdown following the UK COVID-19 lockdown, suggesting static water supplies might favour growth of *L. pneumophila*. Although a smaller total number of samples were submitted for analysis in April 2020, this should not have influenced the proportions of *L. anisa* and *L. pneumophila*.

Although data set 2 spanned the period before and after COVID-19 lockdown, and indicated a large shift in proportion toward *L. pneumophila* post-COVID, the greatest influence was more likely to have been the intervention of a hospital site-wide disinfection of all hot and cold water systems with hydrogen peroxide, and subsequent re-colonization. Although sampling locations where no *Legionella* species were detected had increased from 115 pre-cleaning to 161 post-cleaning, where re-colonization was detected post-cleaning it appeared to favour *L. pneumophila*. *L. anisa* positive samples went down from 61 to 38, while *L. pneumophila* positive samples only fell from 81 to 71. However, examining *Legionella* colony counts showed that the number of *L. anisa* colonies increased as a proportion of the total from 17.2% to 26.4% post-cleaning, while the proportion of *L. pneumophila* was reduced from 73.8% to 64.1% of the total. The total colony counts had however reduced from 437,204 pre-cleaning to 193,869 post-cleaning. Showers appeared to be more likely to be colonized than TMVs and to be recolonized post-cleaning, possible more so with *L. anisa*.

Data set 3, by recording the total number of water samples tested as well as those found to be *Legionella* positive, gave an insight that positivity rate was around 10%. However, in these samples *L. anisa* predominated over *L. pneumophila*, 54.1% of the total compared to 29.3%.

5. Conclusions

The overall picture from three data sets of routine *Legionella* monitoring of water samples taken from the built environment was that, while the previous focus has tended to be on *L. pneumophila*, it is clear that *L. anisa* is also a major contributor. Samples positive for *L. anisa* were found in a similar proportion to those positive for *L. pneumophila* in one data set, and in a much greater proportion in another. The results may also support previous hypotheses (Mulder and Yu 2002, van der Mee-Marquet, et al 2005) that the presence of a more abundant *Legionella* population, including *L. anisa,* in a water system may limit the detection of *L. pneumophila*.

There was some evidence that *L. anisa* colonization of water supplies may be more sensitive to environmental influences; *L. pneumophila* may have flourished more in water supplies left static over a shutdown period, and have recolonized water supplies after disinfection, although that may differ according to sample type. There was some indication in a hospital water supply that showers may be more dynamic than TMVs in terms of the reduction of both *L. pneumophila* and *L. anisa* post-cleaning, but also in terms of recolonization. While *L. pneumophila* remains the main *Legionella* species of concern as a risk to human health, the role of *L. anisa* should not be under-estimated, either as a potential infection risk or as an indicator of the need to intervene to control *Legionella* colonization of water supplies. As the evidence from these data sets suggests *L.anisa* colonization of water systems is more prevalent in the UK than previously thought, public health officials dealing with clinical specimens should be encouraged to consider other screening methods in addition to UAT to detect other *Legionella* species.

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