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INFLUENCE OF SAMPLING SEASON AND SAMPLING PROTOCOL ON DETECTION OF *LEGIONELLA PNEUMOPHILA* CONTAMINATION IN HOT WATER

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Legionella pneumophila *is an environmental pathogen of engineered water systems that can cause different forms of legionellosis — from mild fever to potentially lethal pneumonia. Low concentrations of legionellae in natural habitats can increase markedly in engineered hot water systems where water temperatures are below 55 °C. In the current study, we aimed to investigate the influence of sampling season, hot water temperature and sampling protocol on occurrence of* L. pneumophila*. A total of 120 hot water samples from 20 apartment buildings were collected in two sampling periods – winter 2014 (n = 60) and summer 2015 (n = 60). Significantly higher occurrence of* L. pneumophila *was observed in summer 2015. Significant differences in temperature for negative and positive samples were not observed, which can be explained by low water temperatures at the point of water consumption. Temperature above 55 °C was observed only once, for all other sampling events it ranged from 14 °C to 53 °C.*

Key words: Legionella *control, hot water, water temperature, sampling strategy.*

INTRODUCTION

L. pneumophila is an opportunistic environmental (Saprozoic) pathogen of engineered water systems (Ashbolt, 2015; Falkinham, 2015), that can reach human lungs via inhalation of contaminated aerosols (Anonymous, 2007) or aspiration of water containing the bacteria (Fields, 2002). Clinical manifestations of the legionellosis vary from mild fever (Pontiac's fever) to potentially lethal pneumonia (Legionnaire's disease) (Stout *et al*., 1992). In Latvia, as of 2011, when the number of legionellosis cases increased (Rozentale, 2011), the incidence of Legionnaire's disease has been recorded as about 1.7 cases per 100 000 each year (Anonymous, 2013), with an overall incidence of 1.1 per 100 000 inhabitants in the EU (Anonymous, 2013). Limitations in diagnostics and reporting are the main reasons underlying lack of knowledge on the true incidence of Legionnaire's disease and Pontiac fever (Phin, 2014). Building water systems are now recognised as the primary source of legionellosis (McCoy, 2015). Very low concentrations of legionellae in natural habitats can increase markedly in engineered hot water systems where water temperatures are below 55 °C (Mathys *et al*., 2008). Most cases of legionellosis can be traced to man-made aquatic environments where the water temperature is higher than ambient temperature (Diederen, 2008), however, documentation of the source for the spread of the etiologic agent causing legionellosis can be a problem; thus microbiological conditions of the water may change before epidemiologic data have been collected and analysed (Barbaree, 1987). A crucial role in facilitating preventive action against *L. pneumophila* contamination is played by building management, ensuring disinfection of the water system in buildings and maintenance of the appropriate circulation temperature.

In the current study, we aimed to investigate the influence of sampling season, hot water temperature and sampling protocol on detection of *L. pneumophila*.

MATERIALS AND METHODS

Sampling. A total of 120 hot water samples were taken from randomly selected 20 multi storey apartment buildings with a centralised hot water supply system in different administrative districts in Rīga. The sampling plan for the study was developed, considering the results of previous studies on occurrence of *Legionella* in Latvia, which showed that hot water samples are contaminated more fre-

quently than cold water samples, and that showerheads are sampling points with the highest frequency of *Legionella* positive results (Valciņa, 2013). Sampling was performed in two periods — first in winter 2014 (n = 60), and repeated sampling was performed during summer 2015 (n = 60). In each sampling period, three samples were taken from showerheads in each apartment — in the evening of the working day (from 5:00 PM to 9:00 PM, during the period of active water use, before previous flushing), in the morning of the working day (from 4:00 AM to 6:30 AM, after overnight stagnation, before previous flushing), and in the morning after flushing for at least 10 minutes). All samples were collected in sterile bottles and temperature of water was measured during each sampling event. Measurements were carried out with calibrated thermometers (calibration performed by accredited laboratory), in accordance with the manufacturer's methodology.

Microbiological analysis. Isolation and identification of *L. pneumophila* was performed according to standard ISO 11731. A total one litre of water sample was filtrated and concentrated using membrane filtration with a 0.45 μm pore-size polyamide filter (Millipore, USA). The filter membranes were cut into pieces, resuspended in 5 ml sterile distilled water, shaken for two minutes (Vortex Genie) and kept in a room temperature for 10 minutes. Heat treatment and acid treatment were used to reduce the growth of other bacteria. A total three 0.1 ml untreated, heat treated and acid treated aliquots of the sample were spread on Buffered Charcoal Yeast extract medium (GVPC, Oxoid, UK). The plates were incubated at 36 °C in a humidified environment for 10 days, and examined every day, beginning on the day 3. At least three characteristic colonies from each GVPC plate were selected for subculture onto plates Buffered Charcoal Extract agar medium with L-cysteine (BCYE, OXOID, UK) and Buffered Charcoal Extract agar medium without L-cysteine (BCYE-Cys, OXOID, UK) and incubated for at least 48 h at 36 °C. Colonies grown on BCYE were subsequently identified by latex agglutination test (*Legionella* Rapid Latex Test Kit, BIOLIFE Italiana S.r.l., ITALY), which allows separate identification of *L.pneumophila* Serogroup 1, Serogroup 2-15 and 10 non pneumophila *Legionella* species. Colonies from all plates were counted, confirmed and estimated number of *Legionella* were expressed as CFU/litre *Legionella* species and serogroup. Microbiological analyses were carried out in Laboratory of Medical Microbiology (Institute of Food Safety, Animal Health and Environment "BIOR").

Statistical analysis. All data were analysed using IBM SPSS Statistics 22. Analysis of variance (one-way ANOVA) was performed to determine possible significant differences between parameters.

RESULTS

In total, *L. pneumophila* was observed in 18 of 20 (90%) buildings during the study. During the first sampling period in winter 2014, *L. pneumophila* was observed in 9 of 20 (45.0%) apartment buildings while during the repeated sampling in summer 2015, *L. pneumophila* was found in 14 of 20 (70.0%) buildings. Overall 65 of 120 (54.2%) samples were *L. pneumophila* positive (Table 1).

Significantly higher ($p < 0.05$) *L. pneumophila* occurrence was observed in samples taken in summer 2015, when 41 of 60 samples (68.3%) were contaminated with *L. pneumophila*, while in the first period of sampling occurrence of *L. pneumophila* was 40.0% (24 of 60 samples positive). In total, in five buildings *L. pneumophila* was observed in both sampling periods, and in four of them all samples were *L. pneumophila* positive, with levels of colonisation ranging from 5×10^1 CFU/L to 6.7×10^3 CFU/L (Fig. 1). All samples were negative in both sampling periods only in two buildings, while in 13 buildings *L. pneumophila* was ob-

Table 1

AVERAGE *L. PNEUMOPHILA* COLONISATION AND WATER TEMPERATURE IN WINTER AND SUMMER SEASON

L. pneumophila, CFU/L	Winter 2014		Summer 2015	
	No. samples (%)	average T, °C	No. samples (%)	average T, °C
Not detected	36 (60%)	35.8	19 (31%)	42.9
1×10^3	8 (13%)	46.9	22 (37%)	41.4
$1 \times 10^3 \div 2.9 \times 10^3$	15 (25%)	40.8	9 (15%)	38.1
3×10^3	1 (2%)	22.0	10 (17%)	33.5
Total	60 (100%)	38.2	60 (100%)	40.1

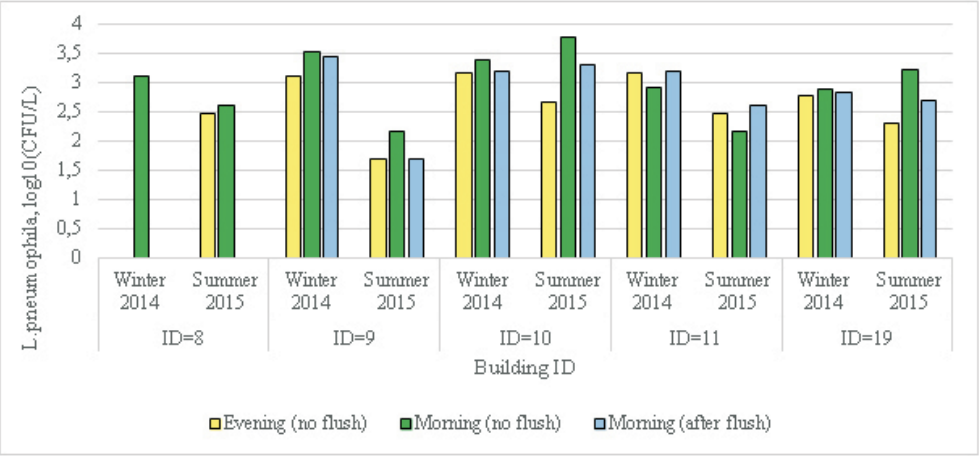

Fig. 1. Comparison of *L. pneumophila* colonisation levels in winter and summer season.

served in one sampling period. Among nine positive buildings in winter 2014, in seven levels of contamination exceeded 1 × 10³ CFU/L (max 3.5 × 10³ CFU/L). In summer 2015, in eight of 14 positive buildings level of contamination exceeded 1 × 10³ CFU/L (max 9 × 10³ CFU/L). However, no statistically significant differences in the level of *L. pneumophila* colonisation between seasons were observed.

In most cases, *L. pneumophila* was observed in all samples from the same building during one sampling period. However, in two buildings only samples taken in the morning were positive. In all positive buildings, samples taken in the morning had higher levels of colonisation than samples taken in the evening. For samples taken in the morning, on average two times higher level of *L. pneumophila* colonisation was observed, although the difference was not statistically significant (p = 0.07).

Higher temperature of hot water was observed during the second sampling period in summer 2015, when average temperature in the evening was 39.2 ± 2.8 °C (min 15.1 °C, max 69.8 °C). In the morning after overnight stagnation average temperature of hot water was 34.2 ± 2.2 °C (min 14.7 °C, max 51.0 °C) and increased up to an average of 46.7 ± 0.9 °C (min 36.7 °C, max 53.4 °C) after flushing for at least 10 minutes. During the first period of sampling, average temperatures of hot water were 0.8–2.9 °C lower — 38.4 ± 2.0 °C (min 18.9 °C, max 50.0 °C) in the evening, 31.3 ± 2.5 °C (min 14.2 °C, max 50.0 °C) in the morning and 45.2 ± 1.3 °C (min 25.2 °C, max 52.0 °C) in the morning after flushing. Overall, the average temperature decrease after overnight stagnation of water was –7.1 °C (max 30.0 °C) in winter 2014 and 5.0 °C (max 34.2 °C) in summer 2015.

An overall significant effect of water temperature on *L. pneumophila* colonisation was observed ($p < 0.05$), but statistically significant differences in water temperature for *L. pneumophila* negative samples and samples with colonization less than 1 × 10³ CFU/L ($p > 0.05$) and more than 1 × 10³ CFU/L ($p > 0.05$) were not detected (Fig. 2).

DISCUSSION

During this study, *L. pneumophila* was found in 18 of 20 apartment buildings (90%), which is significantly higher than in other European countries; occurrence of *L. pneumophila* in water distribution systems varied from 23% in Italy (Borella *et al*., 2004), 26% in Germany (Zietz *et al*., 2001) to 30% in Finland (Zacheus *et al*., 1994). The levels were also higher than in our previous study, where *L. pneumophila* was found in 53% of apartment buildings (Valciņa, 2013). The results of this study may be explained by the sampling strategy, where each apartment building was inspected twice during the study, and sampling was performed in two different seasons, i.e., winter and summer. Our results showed significantly higher ($p < 0.05$) occurrence of *L. pneumophila* in summer. This is in accordance with the results of other studies, which found a peak in *L. pneumophila* contamination during the summer (Blanky, 2015), and have supported the opinion that conditions in water supply systems are not constant. As a result, the presence and the quantity of contaminants may vary (Barbaree, 1987; Ditommaso, 2010). Consequently, in order to obtain reliable results about prevalence of *L. pneumophila* in buildings, sampling plans have to cover different seasons.

High *L. pneumophila* occurrence can be caused by multiple factors, such as insufficient control of *Legionella* load, lack of appropriate disinfection strategies and inappropriate water circulation temperature (Anonymous, 2007; Den Boer 2006; Kruse, 2016; Volker, 2016). The high frequency of *L. pneumophila* contamination in apartment buildings showed that regular preventive actions and controls are an important part of prevention against legionellosis. Regular monitoring of *Legionella* is not carried out, since the Latvian legal requirements for monitoring of drinking water quality do not demand determination of the presence of *Legionella* and risk assessment plans, which is recommended by the World Health Organisation (Anonymous, 2007), but not incorporated in Latvian legislation yet. Likewise, the lack of scientifically developed strategies for disinfection of building's

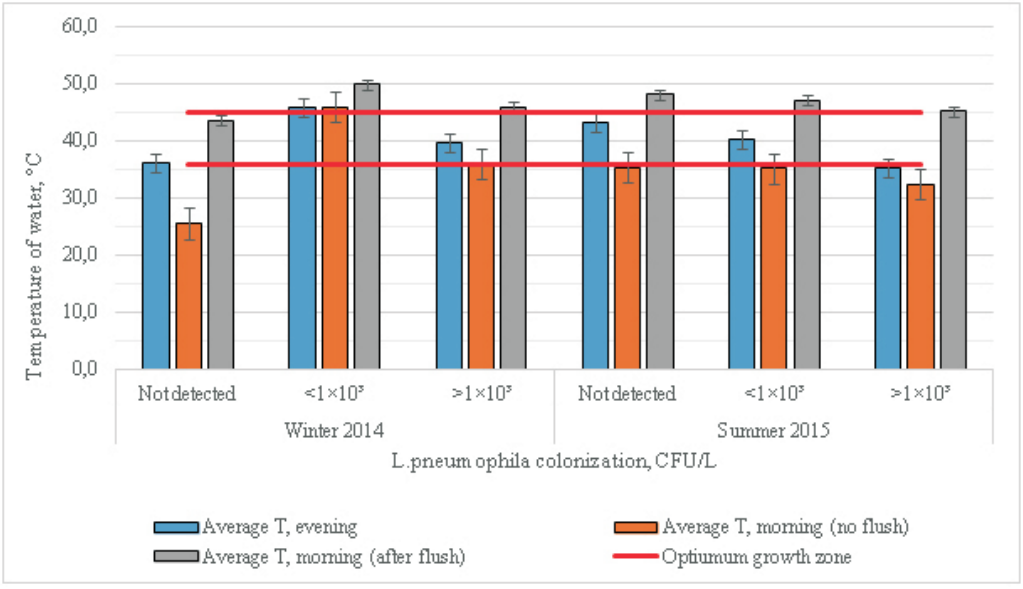

Fig. 2. Average water temperature for *L. pneumophila* negative and positive samples.

internal water supply systems reduces the efficiency of measures for *Legionella* eradication from building water supply systems.

Appropriate sampling procedures are essential for collecting representative water samples for *L. pneumophila* testing. Despite rigorous standards for regulatory purposes, there is often a lack of detail about sampling methodologies (Douterelo, 2014). The sampling method should be chosen depending on the purpose of sampling, such as post-outbreak investigation or preventive measurement. Sampling may be performed immediately after tap switching, or after at least one minute of water pre-flush, which is more representative for the characterisation of water quality in the system (Quaranta, 2012; Bedard, 2015).

In our study it was observed that samples taken directly from the tap before flushing had higher levels of colonisation with *L. pneumophila* than samples taken after flushing. As described in previous studies, water stagnation for more than four hours may significantly increase the number and diversity of bacteria in the water (Sartory, 2004; Lehtola, 2007). Average colonisation of *L. pneumophila* before flushing was two times higher (minimum increase 5×10^1 CFU/L, maximum increase 6×10^3 CFU/L). Although differences of colonisation levels were not statistically significant ($p = 0.07$), it has to be considered that water stagnation, as well as other favourable conditions for *L. pneumophila,* may significantly increase the risk of infection, and water pre-flushing before use may be considered as preventive action to avoid the risk of Legionnaire's disease and Pontiac fever (Suchomel, 2013).

Our data showed that temperature of the hot water had a significant influence on *L. pneumophila* contamination in the water system. Optimum temperature range for proliferation of legionellae is 32–35 °C; however, they are able to proliferate up to 45 °C (Wadovsky, 1985; Levesque, 2004). Our data showed that contamination with *L. pneumophila* was observed much more frequently in water at temperatures below 45 °C. Meanwhile, no contamination was detected in samples at temperature 55 °C or higher. At temperatures higher than 55 °C there is a break point and this finding agrees with observations from other studies, who report that the range 55–60 °C is a critical temperature region, above which the proliferation of legionellae in the water supply systems is inhibited (Wadowsky, 1982; Darelid, 2002). In this study, after ten minutes of flushing, hot water temperature at the tap ranged between 25.2–52.0 °C, with average temperature 45.9 °C, while other studies in Germany showed that average temperature after short flush was 47.5 °C and temperature at constancy was 52.9 °C (Volker, 2015). Such large differences of the temperature can be caused by different technical parameters of the water supply systems. Due to the structure of the hot water supply system, circulation of the hot water is not possible in all buildings, which means that the maximum hot water temperature at the point of consumption is reached after longer time of flushing.

Temperature control on the regular basis and implementation of water safety plan (Anonymous, 2007) is widely recognised as the first mitigation measure for *L. pneumophila* control in hot water distribution systems (Bedard, 2015). Effective strategies for preventing legionellosis need to involve establishment of risk-based reference values for *Legionella* in the water. Building management plays a crucial role in facilitating preventive actions against contamination of water at the point of consumption in apartments. Building managers ensure disinfection of the water system in buildings and maintenance of the appropriate circulation temperature. However, the low economic status in some countries, including Latvia (Rozentale, 2011), causes situations whereby the temperature of hot water is voluntarily reduced. In accordance with the Residential Property Law in Latvia, the community of apartment owners is entitled to decide any matter, which relates to the existing joint property share, and residents employ this opportunity to make decisions in order to reduce hot water supply costs.

Our study emphasises the important role of active preventive actions and regular monitoring of both, water temperature and *Legionella* load, in order to achieve better understanding of the methods for control of the spread of water pathogens, and it highlights the necessity of considering WHO recommendations in implementing the complex and interdisciplinary approach for *Legionella* control in Latvia.

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PARAUGU ŅEMŠANAS SEZONALITĀTES UN PARAUGU ŅEMŠANAS METODES IETEKME UZ *LEGIONELLA PNEUMOPHILA* KONTAMINĀCIJAS NOTEIKŠANU KARSTAJĀ ŪDENĪ

Legionella pneumophila ir ūdens inženiersistēmās sastopams vides patogēns, kas var izraisīt dažādas legionelozes formas, sākot no viegla drudža līdz potenciāli letālai pneimonijai. Dabiskā vidē sastopamās zemās *Legionella* koncentrācijas var būtiski pieaugt ūdens inženiersistēmās, ja ūdens temperatūra nepārsniedz 55 °C. Šī pētījuma mērķis bija noskaidrot paraugu ņemšanas sezonalitātes, karstā ūdens temperatūras un paraugu ņemšanas metodes ietekmi uz *L. pneumophila* noteikšanu. No 20 ēkām tika paņemti 120 karstā ūdens paraugi, paraugu ņemšana tika veikta divos posmos — 2014. gada ziemā (n = 60) un 2015. gada vasarā (n = 60). Vasarā ņemtajos paraugos tika novērota statistiski būtiski augstāka *L. pneumophila* sastopamība. Būtiskas temperatūras atšķirības starp negatīvajiem un pozitīvajiem paraugiem netika konstatētas, ko var izskaidrot ar zemajām ūdens temperatūrām ūdens patērēšanas vietās. Temperatūra virs 55 °C tika novērota tikai vienu reizi, un pārējo paraugu ņemšanas laikā karstā ūdens temperatūra bija diapazonā no 14 °C līdz 53 °C.