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**Spatial distribution of *Legionella pneumophila* MLVA-genotypes in a drinking water
system**

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Running head: *Legionella pneumophila* MLVA-genotypes in a drinking water system

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Abstract

Bacteria of the genus *Legionella* cause water-based infections, resulting in severe pneumonia. To improve our knowledge about *Legionella* spp. ecology, its prevalence and its relationships with environmental factors were studied. Seasonal samples were taken from both water and biofilm at seven sampling points of a small drinking water distribution system in Israel. Representative isolates were obtained from each sample and identified to the species level. *L. pneumophila* was further determined to the serotype and genotype level. High resolution genotyping of *L. pneumophila* isolates was achieved by Multi-Locus Variable Number of Tandem Repeat analysis (MLVA). Within the studied water system, *Legionella* plate counts were higher in summer and highly variable even between adjacent sampling points. *Legionella* was present in six out of the seven selected sampling points, with counts ranging from 1.0×10^1 to 5.8×10^3 cfu/l. Water counts were significantly higher in points where *Legionella* was present in biofilms. The main fraction of the isolated *Legionella* was *L. pneumophila* serogroup 1. Serogroup 3 and *L. sainthelensis* were also isolated. *Legionella* counts were positively correlated with heterotrophic plate counts at 37 °C and negatively correlated with chlorine. Five MLVA-genotypes of *L. pneumophila* were identified at different buildings of the sampled area. The presence of a specific genotype, “MLVA-genotype 4”, consistently co-occurred with high *Legionella* counts and seemed to “trigger” high *Legionella* counts in cold water. Our hypothesis is that both the presence of *L. pneumophila* in biofilm and the presence of specific genotypes, may indicate and/or even lead to high *Legionella* concentration in water. This observation deserves further studies in a broad range of drinking water systems to assess its potential for general use in drinking water monitoring and management.

Keywords: *Legionella*, drinking water, biofilm, MLVA genotyping, bacteria monitoring 36

1. Introduction 37

Legionella pneumophila, the causative agent of legionellosis, was first discovered in 1976, 38
following an outbreak of pneumonia among army veterans attending an American Legion 39
convention in Philadelphia (Fraser et al. 1977). Sporadic and epidemic legionellosis has a 40
worldwide distribution (Phin et al., 2014). *Legionella* is a genus of aquatic bacteria 41
comprising many species which are able to infect human lung macrophages (Swanson and 42
Hammer, 2000). This infection results in Legionnaires' disease (LD), a severe form of 43
pneumonia, or in Pontiac fever, a self-limited flu-like illness. Up to date, more than 50 44
Legionella species, including more than 70 serogroups have been described (Diederer, 2008; 45
Lee et al., 2010). However, *L. pneumophila* serogroup 1 has been reported responsible for 46
over 84% of legionellosis cases world-wide (Cao et al. 2013; Marston et al. 1997; Yu et al. 47
2002). 48

Legionella species are considered to be a part of the natural freshwater microbial community 49
(Veríssimo et al., 1991; Fliermans et al., 1981). They are capable of existing in water with 50
varied temperatures, pH levels and oxygen contents (Nguyen, Stout and Yu, 1991). Its life 51
cycle is complex, including multiplication inside its natural host, protozoa (Fields, Benson 52
and Besser, 2002). In water, *Legionella* survives planktonically. In biofilms (e.g. pipes of 53
drinking water systems) it lives and replicates within protozoa (Molmeret et al., 2005; 54
Fields, 1996; Steinert et al., 2007). *Legionella* infections in humans occur as a result of 55
inhalation of bacteria-containing aerosols. It is well established that *Legionella* is able to 56
colonize man-made water systems and in order to reduce the risk of *Legionella* transmission, 57

many countries enacted guidelines in order to prevent the regrowth of this bacterium in drinking water systems. Some of the key factors that contribute to the prevalence, proliferation and persistence of the *Legionella* in water systems are: temperature; disinfectants and biofilm formation (Buse, Schoen and Ashbolt, 2012). *Legionella* control focuses on creating unfavourable conditions for *Legionella* presence in water systems. Keeping water temperature out of its survival range (below 20°C in cold water and above 50°C in hot water), adding disinfectants and removing biofilm are some of the used strategies (World Health Organization, 2007). However, despite these control measures, there are still cases and outbreaks related with water systems in buildings (Lee et al. 2013). Thus, further studies on the conditions favouring *Legionella* occurrence in water systems world-wide are needed in order to improve the control strategies in water.

Molecular epidemiology of pathogens is a rather recent extension of epidemiology. A molecular methodology is needed to connect the environmental identification of *L. pneumophila* with clinical material or isolates from specific patients. High resolution genotyping of clinical material can be used to identify the environmental source of a *Legionella* infection (Lück et al., 2007) and provide clues on its infection routes (Olsen et al., 2010; Reuter et al., 2013). One of these methods is the Multiple-Locus Variable-number tandem-repeat Assay (MLVA) (Pourcel et al., 2007, Kahlisch et al. 2010). This method relies on the variability found in some tandemly repeated DNA sequences which represent sources of genetic polymorphism (mini-satellites).

Yarom et al. (2010) carried out a five year survey of drinking water systems in Israel. They sampled the water randomly at different locations and found that the most frequently isolated

Legionella strain was *L. pneumophila* serogroup 3. These results were different from what
 was reported in the literature for other worldwide *Legionella* surveys in drinking water
 systems (Campese, Jarraud and Che, 2007; Helbig et al., 2002; Yu et al., 2008). Moran-
 Gilad et al. (2013) studied clinical isolates in Israel between the years 2006 and 2011. The
 authors found that the legionellosis epidemiological trend in Israel was similar to that in the
 EU, except that there were a larger proportion of nosocomial cases. Seasonal distribution of
 cases showed a slightly increased occurrence during the warm season, with 51% of cases
 diagnosed between July and November. Most clinical isolates (74%) were identified as *L.*
pneumophila serogroup 1 whereas 14.3% of them belonged to *L. pneumophila* serogroup 3.
 However, serogroup 3 was the most prevalent strain (52.2%) when the environmental strains
 from the drinking water system were monitored (Moran-Gilad et al., 2013).

Beyond the above mentioned investigations, this study, aimed at a comprehensive analysis
 of *Legionella*, by comparing water and biofilm in a small drinking water system and by
 including different taxonomic levels, i.e. from the genus *Legionella* up to high resolution
 genotypes of *L. pneumophila*. Together with a comprehensive set of environmental
 parameters, we tried to elucidate the ecology of *Legionella* monitoring a seasonal cycle. To
 achieve our aim the following approach was pursued: i) total *Legionella* cfus were
 determined and representative isolates were obtained, ii) in parallel, *Legionella* isolates were
 obtained from adjacent biofilms, iii) genotypes of *L. pneumophila* isolates were determined
 by MLVA, and iv) correlations between location, environmental parameters and molecular
 genotypes were revealed by statistical analyses. Taken together, these analyses enabled an
 ecotyping of the dominant *L. pneumophila* genotypes leading to an improved understanding

of *L. pneumophila* ecology and potentially new tools for drinking water monitoring and management.

2. Materials and Methods

2.1. Water and biofilm sampling points

Mekorot, the Israeli water company, uses Lake Kinneret (Sea of Galilee) water as well as groundwater as the drinking water source (Aizenberg-Gershtein, Vaizel-Ohayon and Halpern, 2012). Seven sampling points were selected at a premise plumbing system at Kiryat Tivon, Northern Israel (coordinates: 32° 42' 43.17" N, 35° 6' 28.66" E). The points were selected in order to cover the water route in the sampled zone and they are described in Figure 1. Point A is the closest point to where the drinking water enters the sampling area and points B-G follow the water course. Cold water and biofilm were sampled seasonally at all seven sampling points, starting from summer 2012 until spring 2013. Seasons, according to the Israel weather were defined as: Summer (mid June until mid September), Autumn (mid September until mid December), Winter (mid December until mid March) and Spring (mid March, until mid June). Water from the hot water system was sampled when possible. Due to the characteristics of the system, these samples never reached temperatures above 55°C, therefore, throughout this article they will be referred to as "warm water" samples.

2.2. Water and biofilms sampling

In all the sampling points, biofilms were sampled first. When showers were sampled, the shower head was removed and biofilm samples were taken from the interior part of the

shower tube by wiping off along the whole diameter of the tube with a sterile swab (Heinz
 Herenz, Germany). When faucets were sampled, the aereator was removed and biofilm
 samples were taken from the interior of the tube in the upstream sector of the aereator.
 After biofilm sampling, faucets were left open for ten minutes to allow the exit of all the
 residual material from the biofilm sampling. That flushing also avoided the effect of
 different stagnation times in the bacterial counts.

Water sampling for *Legionella* culture and for heterotrophic plate counts (HPC) was done
 in sterile bottles supplemented with 100 mg thiosulfate for each liter of water. Two liters of
 water were sampled according to the instructions for sampling, handling and preservation
 given in ISO 5667-1:2006 (International Organization for Standardization, 2010), and ISO
 5667-3:2012 (International Organization for Standardization, 2012). One liter water was
 used for the *Legionella* culture and the other liter for the HPC.

2.3. Biotic and abiotic analysis of the water

Abiotic parameters were monitored as soon as water was sampled to ensure correct values.
 Temperature, conductivity and pH were measured in all samples using a MultiMeter
 MM40+ (Crison, Spain). Chlorine is the disinfectant used in drinking water supply systems
 in Israel. Free chlorine was analyzed using the Aquaquant Colorimetric DPD test (Merck,
 Germany) with a lower limit of detection (LOD) of 0.01ppm. Analysis of ammonia,
 copper, nitrite, nitrate, iron, total water hardness and carbonic hardness were included in
 the second semester of the sampling period using Quantofix semi-quantitative test strips
 (Macherey-Nagel, Germany). LODs were 10, 10, 1, 10 and 5 mg/l for the ammonia,

copper, nitrite, nitrate and iron respectively. The LODs of the total water hardness and carbonic hardness were 5°d in both cases.

Heterotrophic plate counts (HPC) were determined according to ISO 6222: 1999 (International Organization for Standardization, 1999) with some modifications: The analyzed volumes were either 0.5ml and 10ml or 10ml and 100ml, depending on the expected cell densities in each specific sampling point. Yeast extract agar (YEA) was the media used and it was prepared as specified in the ISO 6222: 1999 (International Organization for Standardization, 1999). Volumes of 0.5ml were plated directly on YEA plates. 10ml and 100ml samples were filtered through cellulose nitrate 0.2µm filter (Sartorius Stedim Biotech, Germany) and placed on YEA. Five replicates were incubated both at 20 °C and 37 °C. Colonies were counted after 48 and 72 hours.

2.4. Enumeration and isolation of *Legionella* spp. from water samples

One liter of water sample was filtered through cellulose nitrate 0.2 µm filter (Sartorius Stedim Biotech, Germany). Filters were analyzed following the ISO 11731:1998 (International Organization for Standardization (1998). Each filter was transferred to a 15 ml falcon tube containing 10 ml sampled water and vortexed at maximum speed for ten minutes. Two plates of the *Legionella* selective media GVPC (Glycine-Vancomycin-Polymyxin- Cycloheximide medium, Beckton Dickinson GmH, Germany), were plated with 0.5 ml and 0.1 ml of untreated water. One ml was thermally treated (50 °C for 30 min) and 0.5 ml samples were plated on two GVPC plates. The LOD for *Legionella* was 10 cfu/l. All the plates were incubated at 37 °C. Colonies were counted after seven and fifteen

days of incubation. Five colonies of each positive sample were selected and isolated five
more times. Isolates were kept in LB supplemented with 30% glycerol at -80 °C

2.5. Isolation of *Legionella* spp. from biofilm samples

To isolate *Legionella* from biofilm samples we used a sampling method based on ISO
11731:1998 (International Organization for Standardization (1998) with modifications. The
swab used for sampling was transferred into a 15ml tube containing 5ml of the sampled
water. The tube was then vortexed at maximum speed for 10 minutes. The obtained
suspension was analyzed by following the ISO 11731:1998 (International Organization for
Standardization (1998), in the same way that the filters obtained from the water samples.
Results were qualitative and were expressed like *Legionella* present or absent in the
biofilm. *Legionella* was considered present in the biofilm when the numbers of colonies
obtained after culture were higher than the ones that correspond to the 5ml of the water
used to resuspend the swab.

2.6. *Legionella* identification and typing

2.6.1. 16S rRNA gene sequencing. Isolates were identified by amplifying and sequencing
an internal fragment of the 16S rRNA gene in accordance to Senderovich *et al.*, 2008. The
obtained sequences were compared to those available in the EzTaxon server
(<http://eztaxon-e.ezbiocloud.net/>) (Kim et al. (2012), to ascertain their closest relatives. The
sequences were submitted to the GenBank database (Table S1).

2.6.2. Serotyping. The serogroup typing of *Legionella* isolates was done using a
Legionella latex test (Oxoid, UK). This test allows a separate identification of *Legionella*

pneumophila serogroup 1 and serogroups 2-14 and detection of seven other *Legionella* species (*L. longbeachae* serogroup 1 and 2, *L. bozemanii* serogroup 1 and *L. dumoffii*, *L. gormanii*, *L. jordanis*, *L. micdadei* and *L. anisa*). For the identification of *L. pneumophila* serogroup 3, a specific *L. pneumophila* serogroup 3 latex reagent was used (Pro-lab Diagnostics, Canada).

2.6.3. Molecular typing. MLVA-8 genotyping using capillary electrophoresis was conducted on 62 of the environmental *Legionella* isolates as described by Kahlisch *et al.* 2010 using the primers according to Pourcel *et al.* (2007). Briefly, 1 to 2 ng of template DNA was used in 25 µl PCR reactions containing 1x Multiplex PCR Master Mix (Qiagen, Hilden, Germany) and 1.25 pmol of each primer (VIC[®]-, NED[®]-, FAM- and NET- labelled forward primers from Applied Biosystems, Foster City, CA). After amplification, the PCR products were pooled, denatured and the fragments were sequenced (Applied Biosystems). Each *L. pneumophila* mini-microsatellite locus (Lpms), was identified by color and assigned a size by GeneMapper software, version 3.7 (Applied Biosystems), using settings for microsatellite analysis. The final repeat profile was compared with the MLVA-8 database for *Legionella* (<http://bacterial-genotyping.igmors.u-psud.fr/Legionella2006/help.htm>).

2.7. Statistical analysis

All bacterial counts were adjusted by the addition of 1.0 to convert zeros to positive numbers and then transformed to log₁₀. Log₁₀ values of bacterial counts were used in order to simplify figures. Statistical analyses were performed using the SPSS 20 and the JMP[®] 10 software on raw data (i.e not on transformed values). *Legionella* counts values

were not normally distributed. Thus, a nonparametric Spearman rank-order correlation coefficient was calculated between the different parameters analyzed. In addition, Chi-square test was applied in order to determine seasonal differences between *Legionella* spp. counts at different seasons. Repeated-measures ANOVA with post-hoc analysis using the Bonferroni test were conducted for determining seasonal differentiation of water conductivity, chlorine, pH and temperature. Paired T-test was applied in order to compare HPC incubated at 20 °C and 37 °C. To determine the main parameters that affect water quality, a Principal Component Analysis (PCA) was chosen in order to provide a two dimensional representation of multidimensional data set that enables an easy visual cluster identification (Jolliffe, 2012). PCA was conducted on covariance and included the following variables: temperature (°C), chlorine (mg/l), conductivity (µs), pH and bacterial counts (HPC and *Legionella*- [log cfu/l]), season.

To study the correlation between *Legionella* presence/absence in biofilms and *Legionella* concentrations in bulk water, samples were classified into two groups: (i) *Legionella* positive biofilm samples (ii) *Legionella* negative biofilm samples. A Mann-Whitney test was conducted to compare the median latencies of *Legionella* counts in water of each one of these two groups. Non-parametric comparisons for each pair of the detected genotypes were conducted using the Wilcoxon method. All tests were applied at a 95% level of confidence. Graphs were built with JMP® 10 Software.

3. Results 228

3.1. Abiotic characteristics of the drinking water 229

In order to study the prevalence of *Legionella* in a drinking water system in Israel, we 230
selected seven sampling points (figure 1. A-G) that followed the water course in a premise 231
plumbing system at Kiryat Tivon, Israel. Sampling point A was the water system entering 232
point and point G was the closest point to the end of the water course (Figure 1). Water and 233
biofilms were sampled seasonally. 234

The following parameters were measured in the sampled water: Temperature, pH, 235
conductivity, and chlorine (Table 1 and Table S2). The average temperatures of cold water 236
in summer and winter were 28.6 °C and 16.8 °C, respectively. Cold water temperatures in 237
spring and autumn were similar, 21.1 and 20.8 °C respectively. Repeated-measures 238
ANOVA test confirmed significant differences in temperatures of cold water 239
[$F(3,18)=55.35$, $P<0.01$] between the different seasons. Bonferroni post-hoc test showed 240
that water temperatures were significantly higher during summer. Spring and autumn 241
temperatures were not significantly different from each other; however, both were 242
significantly higher than water temperatures during winter. The warm water system was 243
not available for the summer samplings. The average temperature of warm water samples 244
taken in autumn, winter and spring was 42 °C, 51 °C and 53 °C, respectively. 245

In the cold water, the pH average values ranged between 7.4 and 8.2, with significant 246
differences between seasons [Repeated-measures ANOVA with Greenhouse-Geisser 247
correction: $F(1.25,7.49)=8.31$, $p<0.05$]. Summer and spring pH values were similar and 248
significantly higher than autumn and winter values (Bonferroni post-hoc test). Conductivity 249

was also seasonal [Repeated-measures ANOVA with Greenhouse-Geisser correction: 250
 $F(1.38, 8.29) = 10.89$, $p < 0.01$]. Bonferroni post-hoc test showed that values were similar 251
during spring and summer and significantly higher than in autumn and winter (Table 1 and 252
Table S2). In the warm water, point A had an average pH of 7.5 and point E was the point 253
with the highest pH in the water system, with an average of 8.6. Conductivity values were 254
higher in spring (Table 1 and Table S2). 255

Chlorine concentrations varied between the sampling points. In cold water samples, a 256
minimum of two sampling points presented every season, values higher than 0.3ppm 257
(Table 1 and Table S2). Chlorine average was calculated from the sampling points with 258
detectable concentrations. In autumn, winter and spring, the average was 0.16, 0.13 and 259
0.18ppm, respectively. In summer, chlorine concentrations were lower (0.05ppm). No 260
significant differences were found between seasons (Friedman's test: $n=7$, $df=3$, $X^2=1.58$, 261
 $p > 0.05$). The highest chlorine concentration in the warm water was 0.025ppm. 262

Ammonia, copper, iron, total water hardness and carbonic hardness were measured in the 263
second semester of the sampling period. Copper, iron, ammonia and nitrite were not 264
detected both in cold or warm water. Total hardness and carbonate hardness did not show 265
any variation between the sampling points, type of water and the two sampling seasons. 266
Total hardness was high in all the points, ranging between 3.56 and 4.45mmol/l. Carbonate 267
hardness was also high, 3.56 mmol/l in all the cases. 268

3.2. Biotic characteristics of the drinking water 269

Heterotrophic plate counts (HPC) of cold water incubated at 22 °C and 37 °C ranged between 4.0 and 7.6 x 10³ cfu/l and between 2.0 x 10¹ and 1.1 x 10⁶ cfu/l respectively (Figure 2 and Table S2). Paired t-test revealed that HPC incubated at 37 °C had higher values than HPC incubated at 22 °C (n=27, df=12, t=4.82, p<0.01). No significant differences were found between the HPC in the different seasons. In warm water, HPC at 22 °C and 37 °C ranged between 80 and 2.8 x 10² cfu/l and between 50 and 3.31 x 10⁵ CFU/l, respectively.

3.3. Prevalence and persistence of *Legionella* spp. in water and biofilms along one year

The occurrence of *Legionella* in cold water in the different sampling points (n=7) and seasons was 71% in summer and spring with counts ranging between 1.1 x 10² and 5.8 x 10³ cfu/l and 2.0 x 10¹ and 2.4 x 10³ cfu/l, respectively (Figure 2, Table 2 and Table S2). *Legionella* was detected in 57% of the samples in autumn and winter, with counts between 3.0 x 10¹ and 4.0 x 10² cfu/l and 1.0 x 10¹ and 2.3 x 10³ cfu/l, respectively. *Legionella* was detected in autumn and spring in both warm water sampling points and just in one warm sampling point in the winter. In warm water *Legionella* highest counts were 1.2 x 10² cfu/l (Table 2).

Seasonality of *Legionella* counts was analyzed in cold water by using the Chi-square test (Table 3). Significant differences were found by season in the *Legionella* counts when all the points were analyzed as a group ($\chi^2=1173.9$, DF=3, P<0.01). Summer *Legionella* concentration was the highest and the most contributing to the chi-square total value

($\chi^2=801.12$, mean= 1132.9 cfu/l). Seasonality was also found in all the sampling points when analyses was performed for each point separately (Table 3). In point B, *Legionella* was not detected in any of the seasons. *Legionella* counts in water and its presence or absence in biofilm in relation to their sampling points and season of sampling, demonstrated different seasonal trends (Figure 3 and Table 2). Cold water sampling points D, F and G presented the same U-shaped seasonal pattern. Counts were higher at summer time, decreased during autumn and winter and increased again in spring. Point C presented a similar U-shape trend, with the exception that counts in winter were as high as the ones in spring. All these points corresponded to faucets. Points A and E which were shower sampling points, presented a non-U-shaped trend. In point A, *Legionella* was not detected in summer and autumn, whereas in point E it was not detected in winter and spring. As it will be explained further on, different *Legionella* species and serogroups were isolated in these two points compared with the other sampling points. In warm water, both sampling points presented higher counts in autumn and spring than in winter.

Legionella was isolated from biofilms, in 71%, 57%, 29% and 71% of the samples in summer, autumn, winter and spring, respectively (Table 2 and Figure 3). *Legionella* counts in water were significantly higher in points where *Legionella* was present in biofilms, compared to *Legionella* counts in samples where the biofilm was negative for *Legionella* (Mann-Whitney test: n=28, U=11, p<0.01, Figure 4). When *Legionella* was absent in biofilm, its counts in water were lower than 30 cfu/l (equivalent to 1.48 log cfu/l).

3.4. *Legionella* identification

Sequencing of the 16S rRNA gene of the different isolates confirmed that most of the isolates were *L. pneumophila pneumophila* (>99.8% 16S rRNA gene similarities) except for some *Legionella* strains that were isolated from point E, where a mixture of *Legionella* species were identified, i.e. *L. pneumophila* and *L. sainthelensis* (Appendix Table A1). *L. sainthelensis* was isolated from two samples one cold water and one biofilm, representing a 3.2% of the total isolates from the studied water system. In point E, some differences were observed between the biofilm and the water samples (Table 4). In summer, *L. pneumophila* serogroup 1 was detected in both the water and the biofilm whereas *L. sainthelensis* was detected only in the biofilm. In autumn, *L. sainthelensis* was detected both in the water and in the biofilm but *L. pneumophila* serogroup 1 was found just in biofilm.

3.5. The effect of biotic and abiotic parameters on *Legionella* prevalence

Multiple correlations were analysed between *Legionella* presence and the background parameters in the cold water. No correlation was found between water temperature and *Legionella* counts when the data from the seven sampling points were analysed together ($r_s=0.157$, $n=27$, $p>0.05$). Conductivity and pH did not exhibit significant correlation with *Legionella* counts in bulk water. Significant negative correlation was found between *Legionella* spp. counts and chlorine levels ($r_s=-0.82$, $n=27$, $p<0.01$). Non-detectable *Legionella* samples presented chlorine concentrations equal or above 0.2ppm. A significant positive correlation was found between *Legionella* counts and heterotrophic plate counts at 37 °C ($r_s=0.45$, $n=27$, $p<0.05$). Significant negative correlations were found between HPC counts at 22 °C and 37 °C and chlorine levels ($r_s=-0.56$, $r_s=-0.59$, $n=27$, $p<0.01$ respectively).

A principal component analysis was conducted in order to get a global picture on the relationships between the seven abiotic and biotic parameters that were analysed in this study. After dimension reduction using the PCA method, two principal components were chosen to describe most of the variability in the water samples (Figure 5). 84.4% of the data variation was explained by Axis 1 (PC 1) and 9.77% by Axis 2 (PC2). PC1 was composed for temperature and conductivity and PC2 for chlorine, *Legionella* and HPCs. The microbiological parameters grouped together and differed from the effect of the physicochemical parameters, especially chlorine which was situated far from the bacterial effect. Temperature and chlorine presented contrary effects (Figure 5). Water quality in summer presented a different pattern in the physicochemical and microbiological properties compared to the other seasons. It was affected more by the temperature and the microbiological parameters and less by chlorine.

3.6. MLVA-8 genotyping

In total, 62 isolates from all sampling points, were subjected to genotype analyses by MLVA-8. The isolates could be classified into five different genotypes according to their pattern for the eight different loci of the MLVA-8 analysis, i.e. genotypes (Gt) 4, 6, 15, 17, and 18 (Tables 5 and S3). Two genotypes (Gt4 and Gt6), were dominant and were observed in isolates both from water and biofilm (Figure 6A and 6B and tables S3 and S4). The strains identified as Gt4, Gt6, and Gt18 belonged to *L. pneumophila* serogroup 1. The strains identified as Gt15 and Gt17 belong to serogroup 3 (Figure 7, Table S3). *L. pneumophila* genotype 4 (Gt4) was the most abundant in the system, with a prevalence of 62% (Table S4). Gt4 was detected both in water and biofilm with 58% and 70%

abundances, respectively (Figure 7, Table S4). Differences in the genotypes were observed between the sampling points. All the strains from sampling point A which were previously identified as *L. pneumophila* serogroup 3, belonged to two genotypes: Gt15 (86%) and Gt17 (14%). The strains from the other sampling points, which were identified as serogroup 1, showed a spatial distribution. In point C, all of the strains belonged to Gt4. At the next point (D), the predominant genotype was still Gt4 (88%), but three other genotypes were also detected: Gt 6 and Gt 18 (6% each one of them). At points E, F and G, Gt6 was the only observed genotype (Figure 7, Tables S3 and S4).

Water environmental conditions were summarized for each one of the three main MLVA-8 genotypes (Table 6). Non-parametric comparisons for each pair of the detected genotypes showed that Gt15 was detected in water samples with significantly higher temperatures (average of 42°C) than the other two genotypes (Wilcoxon test: $Z=3.67$, $Z=2.75$, $p<0.01$, for Gt4 and Gt6, respectively) and with significantly lower pH (average 7.4) (Wilcoxon test: $Z=-3.19$, $Z=-2.89$, $p<0.01$, for Gt4 and Gt6, respectively). Interestingly, Gt4 was detected in water samples with significantly higher *Legionella* counts than samples where Gt6 and Gt15 were isolated from (Wilcoxon test: $Z=3.00$, $Z=2.92$, $p<0.01$, respectively). In summary, Gt4 was predominant when relatively low temperatures (20°C) and high *Legionella* counts were measured in water, whereas Gt15 was related to the highest water temperatures and lowest the pH. Moreover, *Legionella* counts were lower in samples where Gt15 was detected than in samples with Gt4 or Gt6. None of the other abiotic parameters or HPC counts showed an effect on the presence of a specific genotype in the water samples.

Genotype relations with the two studied niches, water and biofilm, demonstrated that the two most abundant genotypes Gt4 and Gt6 were highly competitive in biofilm establishment. Whenever water samples were positive for Gt4, it was also isolated from the biofilm samples (Table 6 and S4). Gt6 also showed a good establishment in biofilm (sites E and F). Gt15 and Gt17 were observed only in water (Figure 6), while Gt18 was observed only in biofilm.

4. Discussion

The current study was carried out in northern Israel, a region with a Mediterranean climate, characterized by hot dry summers and cool rainy winters. It is known that water temperatures between 20 °C to 45 °C favour *Legionella* growth (Health and Safety executive UK, 2013), so this climate is considered to provide an ideal scenario for *Legionella* survival most of the year. The current investigation was designed as a seasonal *Legionella* study in both water and biofilm within one small drinking water system, targeting *Legionella* from the genus to a high resolution sub-species level for *L. pneumophila*.

Legionella spp. was detected in the monitored water system along the whole year in six out of the seven sampling points. *Legionella* was undetected in point B at all seasons, while point C, located only at a 250 m distance, showed high counts ($\sim 10^3$ cfu/l). Thus, we need to be aware of the fact that one sampling point in a sampled area is not representing the accurate picture regarding *Legionella* prevalence in the water system. Compared with other studies, the percentage of *Legionella* detection in the current study was relatively high (68.5% positive water samples). The prevalence of positive samples in other studies was only 53.3%, 42% and 12.9, in Poland, Italy and Israel, respectively (Koziol-Montewka et al., 2008; Tesauero et al., 2010; Yarom et al., 2010). Nevertheless, the prevalence of positive samples can change depending on the site, disinfection protocol, etc.

Moran-Gilad et al. (2013) described a seasonal distribution of legionellosis in Israel, where cases showed a very slightly increase during the warm season, although there was not a clear *Legionella* cases peak. In the current study, we have demonstrated that *Legionella*

counts changed seasonally (Table 2 and 3, Figure 2, 3 and 5). Thus, we suggest taking
seasons into account when designing a *Legionella* monitoring plan for water systems in
Israel and in other countries with a Mediterranean climate.

Influence of physical and chemical characteristics of water, on the presence of *Legionella*
in water systems has been extensively studied in the last years. However, some studies
show contradictory results regarding the association of *Legionella* counts with chlorine,
temperature or HPC (Lasheras et al., 2006; Rakić, Perić and Foglar, 2012; Serrano-Suárez
et al., 2013; Zanetti et al., 2000). In our study, heterotrophic plate counts at 37 °C were
positively correlated with *Legionella* counts, indicating a similar respond to environmental
conditions. A strong negative correlation was found between *Legionella* spp. counts and
chlorine concentrations along the whole sampling year ($r_s=-0.82$, $n=27$, $p<0.01$) and the
threshold for *Legionella* detection was 0.2ppm. However, water quality in summer,
presented a different pattern than the other seasons, with a higher effect of temperature and
a smaller effect of chlorine (Figure 5).

Legionella is a biofilm colonizer which replicates via parasitization of amoeba cells, with
severe consequences for legionellosis transmission (Lau and Ashbolt, 2009; Watnick and
Kolter, 2000). Biofilm removal from the water system is one of the legionellosis control
measures recommended by the WHO (WHO, 2007). Despite that, biofilm monitoring is not
included in *Legionella* regulations because there are only few biofilm monitoring studies
(Mäkinen et al., 2013; Wang et al., 2012; Koziol-Montewka et al., 2008). This is probably
the reason for the lack of a standard method regarding biofilm monitoring. In the current
study, we established a culture method, based on a modification of ISO 11731:1998 (see

section 2.5) (International Organization for Standardization, 1998). Our results demonstrated high percentage of positive samples compared to Makinen et al. (2013), Koziol-Montewka et al. (2008) and Wang et al. (2012), possibly due to our different method. Nevertheless, our method had some limitations. Only the biofilm of the last part of the premise of the plumbing system was swabbed. When our biofilm sample was negative for *Legionella*, it could have still been positive further back in the plumbing system. That is why further research to improve the method is needed. In the current study, a correlation was found between *Legionella* presence in biofilm and its counts in water (Figure 3 and Table 2). Whenever the biofilm samples were negative for *Legionella*, *Legionella* was undetectable (less than 10 cfu/l) in the water samples or its counts were lower than 30 cfu/l. Thus, the results obtained from biofilm monitoring of our water system were a useful indicator for *Legionella* presence in its water.

In the current study, *L. pneumophila* serogroup 1 was the most abundant. Serogroup 3 was detected only at point A and a mixture of *L. sainthelensis* and *L. pneumophila* serogroup 1 were detected at point E (Fig. 1, Appendix Table A1). Point E is a shower that is not frequently used and this fact may explain its unique species composition compared to the other sampling points. *L. sainthelensis* was first isolated from freshwater by Campbell et al. (1984). It has been associated with pneumonia outbreaks however, it has not been frequently isolated worldwide (Loeb et al. 1999). As far as we know, *L. sainthelensis* has not been recorded as a cause of legionellosis in Israel.

Studies in Israel described serogroup 3 as the most prevalent serogroup in drinking water (Yarom et al., 2010; Moran-Gilad et al., 2013). Although serogroup 1 is the most prevalent

serogroup in our study, our results do not contradict Yarom *et al.* (2010) or Moran-Gilad *et al.* (2013), because we focused on the seasonality of seven sampling points within one small drinking water system, while they sampled water from different random locations all around Israel and in random seasons. Serogroup 3 was the most predominant in water systems in Kuwait (Al-Matawah *et al.* 2012) but not in Iraq and Jordan where serogroup 1 was the most abundant (Al-Matawah *et al.*, 2012; Al-Sulami, Al-Tae and Yehyazarian, 2013; Khleifat, Hanafy and Al-Omari, 2014). The dominance of one serogroup versus others seems to be water system dependent and cannot be generalized to a whole region (Middle East).

In the current study, a high resolution typing method (MLVA-8), allowed genotype analysis of the *L. pneumophila* strains at the clonal level and the assessment of *L. pneumophila* genotypes' spatial distribution in the drinking water distribution system. Overall, five different genotypes switched gradually along the water system (Fig. 7). A detailed analysis of the environmental and microbiological parameters at the site of isolation allowed a characterisation of the genotypes with a larger group of representatives, i.e. Gt4, Gt6 and Gt15 (Table 6). These genotypes showed a different water temperature preference and a different abundance of *Legionella*. Most noteworthy is Gt4 that has the lowest temperature spectrum, but always the highest *Legionella* counts (Table 6). The mechanism behind this phenomenon remains to be elucidated, and we only can speculate if and how Gt4 may “trigger” *Legionella* abundance in drinking water. It is interesting to compare Gt4 with Gt15. Gt15 is dominant at high temperatures (45°C), but is accompanied by *Legionella* counts that are about two orders of magnitude less (Table 6). Thus, we see a new mechanism influencing *Legionella* counts in water that seem to be dependent on *L.*

pneumophila genotypes and not on high temperatures. An important mechanism for the success of Gt4 may be a good and competitive establishment in biofilms and/or the generation of biofilms that allow a permanent inoculation of the adjacent drinking water.

The MLVA-8 analysis showed furthermore that Genotype 4, 6 and 18 can be considered to form a clonal complex. The latter differ by one allele, i.e. with respect to Lpms 35 for Gt6 and Lpms 13 for Gt18 (Table 5). Gt17 differs with respect to two alleles (Lpms 13 and 19) from Gt4, 6 and 18. Gt15 is considered as most distantly related to the four genotypes 4, 6, 17 and 18 (Table 5). The MLVA pattern provides a genetic code that allows surveillance and comparison with worldwide observed *L. pneumophila* strains and their respective features. In comparison with other studies and the MLVA-database on *L. pneumophila* strains (Sobral et al., 2011; Visca et al, 2011; <http://mlva.u-psud.fr>), the MLVA-8 pattern of genotypes 4 and 6 is identical to strains pertaining to the MLVA clonal complex VACC1. For the other genotypes no identical pattern was observed. Gt18 shows one allele difference, and Gt17 two allele differences to strains of VACC1. Gt15 showed one allele difference (Lpms35) to strain 03-004, a clinical serogroup-3 strain isolated 1989 in France (Visca et al. 2011), classified as VACC5. We therefore conclude that the major fraction of the isolated strains is affiliated with VACC1.

In comparison to Europe wide observations, strains with a MLVA-8 pattern identical to Gt4, were mostly clinical strains isolated from a broad climatic range from all over Europe (Scotland to Greece) (Visca et al. 2011). Strains identical to Gt6 were clinical isolates from Denmark. The strains with identical pattern to genotypes 4 and 6 belong to VACC1, and Sequence Type (ST) 1. VACC1 comprises a broad set of worldwide retrieved strains that

mostly belong to serogroup 1 and serotype 1. The most prominent representative of VACC1 is *L. pneumophila* strain “Paris” CIP107629. Strains of the MLVA clonal complex VACC1 not only have a large fraction of clinical strains but also are often found in man-made freshwater systems, especially cooling towers (France, Japan, Germany) (Amemura-Maekawa et al. 2005; Kahlisch et al. 2010; Sobral et al. 2011). VACC1 strains were observed as causative agent of sporadic cases as well as for outbreaks reported since 1981 and are considered as being highly persistent in anthropogenic freshwater systems (Cazalet et al. 2008; Sobral et al. 2011).

With respect to the high abundance of Gt4 compared to Gt6 and Gt15 at the respective sites, it is unclear which features make the difference for the closely related *L. pneumophila* strains with respect to their growth and competitiveness within the bacterial community of the water network. Though Gt4 and Gt6 are considered as a clonal complex within VACC1, they can be perceived as different “ecotypes” with features rendering them competent in different niches. Genome analyses and physiological studies of representative strains of the different genotypes are foreseen to allow more insight into these issues as demonstrated for other genotypes (Cazalet et al. 2008; Khan et al. 2013; Lévesque et al. 2014).

The finding of this study that different parts of the water network were dominated by different genotypes, contrasts with findings by Sobral et al. (2011). In this large-scale study performed after two outbreaks in a French city (Rennes) it was shown that the entire hot water supply system was highly dominated by a single genotype of VACC6. Furthermore, it was demonstrated that this abundant VACC6 genotype was not responsible for the

outbreaks; the latter was caused by two VACC2-genotypes that were also retrieved from cooling towers. However, both studies showed a spatial distribution of the *Legionella* genotypes and the importance of using a high resolution typing in order to better understand the *Legionella* colonization of the water system and the relationship between colonization and disease. The spatial distribution of the genotypes in our study (Figure 7), might be due to differences in the adaptability of the genotypes to certain environmental conditions or to differences in resistance to disinfection. Further research is required in order to fully understand the risk posed to public health by their presence in the water system. To the best of our knowledge, this is the first time that this association between prevalence of *Legionella* counts in the environment and its genotype presence is described.

5. Conclusions

In conclusion, the analysis of a seasonal cycle of *Legionella* in water and biofilm in a local drinking water system showed a variable spatial and seasonal abundance of *Legionella*. The variability of the *Legionella* counts correlated positively with heterotrophic plate counts and negatively with the chlorine content of the bulk water. When *Legionella* was present in biofilms, significantly higher *Legionella* counts (>30 cfu/l) were detected in the bulk water. The main fraction of the *Legionella* population was *L. pneumophila* serogroup 1. A better understanding of the ecology of *L. pneumophila* was obtained when specific MLVA genotypes, were considered. From a total of five genotypes, three had a high abundance and could be characterized by the physico-chemical and biotic parameters at the site of their isolation. These three genotypes could be considered as different “ecotypes” being characterized by distinct temperature ranges, highly distinct *Legionella* counts and

dominated different sites of the drinking water system. Most noteworthy is genotype 4 that
correlated with high total *Legionella* counts and low temperature. Its presence in the
drinking water and biofilm is considered as indicator for high *Legionella* counts in the
respective water. *L. pneumophila* MLVA-genotypes, such as Gt4, could be used as general
indicator for drinking water monitoring as early warning “system” and/or for drinking
water management and therefore deserve further studies in a broader set of drinking water
systems comprising different climate zones.

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Table 1. Seasonal summary of physicochemical parameters of cold water.

	Statistics	Temp (°C)	pH	Chlorine ** (ppm)	Conductivity (µS/cm)
Summer	A. mean*	28.6	7.8	0.05*	1165
	SD	1.1	0.2	0.06	72
	Max	30.1	8.0	>0.30 (2)	1256
	Min	27.5	7.5	0.01	1090
Autumn	A. mean	20.8	7.6	0.16*	933
	SD	1.6	0.2	0.14	130
	Max	23.5	7.9	>0.30 (3)	1094
	Min	18.8.	7.5	<0.01 (1)	766
Winter	A. mean	16.8	7.4	0.13*	909
	SD	2.3	0.1	0.12	45
	Max	19.4	7.5	>0.30 (3)	972
	Min	12.8	7.3	0.06	847
Spring	A. mean	21.1	8.2	0.18	1033
	SD	2.9	0.6	0.11	58
	Max	24.3	9.5	>0.30 (2)	1127
	Min	17.4	7.8	<0.01 (1)	939

Legend:

Temp, Temperature; A.mean, Arithmetic mean; SD, standard deviation; Max, Maximum; Min, Minimum.

*For mean calculation, only values in the range of the method detection were taken into account.

** For the min. and max. chlorine values, the number of samples with >0.30 ppm chlorine or <0.01ppm are indicated (in parenthesis).

Table 2. *Legionella* detection by culture in water (cfu/l) and in biofilm (presence or absence), by sampling point and season. The detection limit of the *Legionella* isolation was 10 cfu/l.

Sampling points	Type of sample	Summer	Autumn	Winter	Spring
A	Cold water	ND*	ND*	1.0x10 ¹ *	5.0x10 ¹
	Warm water	NA	1.2x10 ²	1.0x10 ¹	8.0x10 ¹
	Biofilm	-	+	-	+
B	Cold water	ND*	ND*	ND*	ND*
	Biofilm	-	-	-	-
C	Cold water	5.8x10 ³	4.0x10 ²	2.3x10 ³	2.4x10 ³
	Biofilm	+	+	+	+
D	Cold water	1.1x10 ³	7.0x10 ¹	8.0x10 ¹	1.2x10 ² *
	Biofilm	+	+	+	+
E	Cold water	1.1x10 ²	2.4x10 ² *	ND*	ND
	Warm water	NA	1.0x10 ¹	ND	1.0x10 ¹
	Biofilm	+	+	-	-
F	Cold water	8.8x10 ²	3.0x10 ¹	2.0x10 ¹	3.0x10 ²
	Biofilm	+	-	-	+
G	Cold water	1.2x10 ²	ND*	ND*	2.0x10 ¹ *
	Biofilm	+	-	-	+
% positive samples	Cold water	71	57	57	71
	Warm water	NA	100	50	100
	Biofilm	71	57	29	71

NA, not analysed; ND, not detected. +, *Legionella* present; -, *Legionella* absent

*Chlorine concentration 0.3 ppm or higher. The samples in which *Legionella* was not detected in biofilm are shaded in grey.

Table 3. Chi-square test results for *Legionella* counts in cold water by season, both in the whole water system (all points) and point by point.

	Chi-Square value				Total Chi-square	p-value	df
	Summer	Autumn	Winter	Spring			
All points	801.12	310.94	47.62	14.21	1173.90	0.00	3
Point A cold	15.00	15.00	1.67	81.67	113.33	0.00	3
Point A warm	-	35.85	51.36	1.45	88.66	0.00	2
Point C	3375.33	1978.82	61.80	33.09	5449.04	0.00	3
Point D	1612.61	209.63	194.10	137.99	2154.33	0.00	3
Point E cold	5.79	265.79	87.50	87.50	446.57	0.00	3
Point E warm	-	1.68	6.66	1.68	10.01	0.0067	2
Point F	1065.87	250.43	268.80	0.18	1585.28	0.00	3
Point G	206.43	35.00	35.00	6.43	282.86	0.00	3

3

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Table 4. *Legionella pneumophila* serogroups which were identified by agglutination latex kit.

Sampling points	Type of sample	Summer	Autumn	Winter	Spring
A	Cold water	NI	NI	sg3	sg3
	Warm water	NA	sg3	sg3	sg3
	Biofilm	NI	sg3	sg3	sg3
B	Cold water	NI	NI	NI	NI
	Biofilm	NI	NI	NI	NI
C	Cold water	sg1	sg1	sg1	sg1
	Biofilm	sg1	sg1	sg1	sg1
D	Cold water	sg1	sg1	sg1	sg1
	Biofilm	sg1	sg1	sg1	sg1
E	Cold water	sg1 and Ls	Ls	NI	NI
	Warm water	NA	NI	NI	sg1
	Biofilm	sg1	sg1 and Ls	NI	NI
F	Cold water	sg1	sg1	sg1	sg1
	Biofilm	sg1	NI	NI	sg1
G	Cold water	sg1	NI	NI	sg1
	Biofilm	Cont	NI	NI	sg1

NA, not analysed; NI, not isolated; sg1, *Legionella pneumophila* serogroup 1; sg3, *Legionella pneumophila* serogroup 3; Ls, *Legionella sainthelensis*; Cont, contaminated strain.

Table 5. Results of the eight loci of the Multilocus Variable Number of Tandem Repeat analysis (MLVA-8) of the five different MLVA-8 genotypes obtained from all isolated strains. For more details see Table S3.

MLVA-8 genotype	No. of repeats at each VNTR locus							
	Lpms*1	Lpms3	Lpms13	Lpms17	Lpms19	Lpms33	Lpms34	Lpms35
4	7	7	10	2	4	4	2	17
6	7	7	10	2	4	4	2	18
15	9	8	8	2	5	2	2	21
17	7	7	8	2	3	4	2	17
18	7	7	7	2	4	4	2	17

*Lpms, *L. pneumophila* mini-microsatellite locus

Table 6. Summary of water physicochemical and biotic parameters at the site and time of isolation for the strains of the three most abundant MLVA-8 genotypes. Strains isolated from water and biofilm were grouped by MLVA-8 genotyping. The environmental and microbiological parameters (mean, SD) are calculated per genotype based of the data of the respective sampling sites (from which the strains were isolated).

Genotype (number of strains)		Temp (°C)	pH	Conductivity (µS/cm)	Chlorine (ppm)	HPC 20°C (cfu/l)	HPC 37°C (cfu/l)	Legionella (cfu/l)	Biofilm positive samples (%)**
Gt4 (n=25)	A. mean	20.6	7.6	1025	0.04*(2)	1.8x10 ³	4.7x10 ⁵	2.5x10 ³	100.0
	SD	6.2	0.1	150	0.04	8.4x10 ²	4.3x10 ⁵	2.5x10 ³	
Gt6 (n=10)	A. mean	27.9	7.6	1118	0.06	4.2x10 ³	5.4x10 ⁴	2.4x10 ²	80.0
	SD	3.5	0.1	210	0.07	3.2x10 ³	1.2x10 ⁵	3.5x10 ²	
Gt15 (n=6)	A. mean	45.1	7.3	997	0.03* (4)	1.9 x 10 ²	2.2x10 ⁵	8.3x10 ¹	66.7
	SD	3.8	0.02	9	0.00	1.3x10 ²	1.7x10 ⁵	5.7x10 ¹	

*When the mean was calculated, only the values in the range of the method detection were taken into account. Between parenthesis, number of samples under the detection limit of the method (<0.01ppm)

**% of samples per genotype with Legionella-positive biofilm

Figure 1.

Drinking water system sampling map at Kiryat Tivon, Israel. Seven sampling points were selected in order to cover the drinking water system route. Sampling points are marked with capital letters. Points where cold and warm water was sampled are underlined. A, Shower B, Garden irrigation faucet C, Sink faucet D, Toilet sink faucet E, Shower F, Sink faucet G, Toilet sink faucet.

Figure 2.

Box plot of bacterial counts in the four sampled seasons. For each season, *Legionella* spp. plate counts and total heterotrophic bacterial plate counts incubated at 20°C and 37°C for 72h are shown. Significant differences were found between seasonal *Legionella* counts when all the points were analyzed as a group ($\chi^2=1173.9$, DF=3, $P<0.01$) or point by point. For more details see Table 3.

Figure 3.

Dynamics of *Legionella* plate counts in the different water sampling points throughout all four seasons. Both triangles and circles denote *Legionella* counts in water according to the Y-axis. A triangle represents the fact that at this particular sampling point, *Legionella* was also positive in the biofilm. A circle represents the fact that at this particular sampling point, *Legionella* was not detected in the biofilm. At all sampling points (except for point A, Autumn), *Legionella* plate counts for water were equal or above 3.0×10^1 cfu/l when the respective biofilms were positive for *Legionella*. Su, summer; A, autumn; W, winter and Sp, spring). See also Table 2.

Figure 4.

***Legionella* plate counts in water in the vicinity of *Legionella* positive or negative biofilms.** When *Legionella* was present in the respective biofilms significantly higher *Legionella* counts in the bulk water were detected (Mann-Whitney test: $n=28$, $U=11$, $p<0.01$).

Figure 5. Principal component analysis of the biotic and abiotic characteristics of cold water, by season. After dimension reduction using the PCA method, two principal components were chosen to describe most of the variability in the water samples; 84.4% of the data variation was explained by Axis 1 (PC 1) and 9.77% by Axis 2 (PC2). PC1 was composed for temperature and conductivity and PC2 for chlorine, *Legionella* and HPCs. HPC, Heterotrophic plate count; EC, Conductivity. Ellipses represent the 95% confidence interval of the log normal distribution of principle components values in each of the seasons.

Figure 6. MLVA-8 genotypes of environmental *L. pneumophila* isolated from water (A) and biofilm (B). Pie chart percentages show relative abundances of *L. pneumophila* genotypes in each sample type.

Figure 7. Spatial distribution of *L. pneumophila* MLVA-8 genotypes along the different sampling points. Pie chart percentages show relative abundances of *L. pneumophila* genotypes in each sample site. The *Legionella* serogroup (sg) obtained by agglutination test is indicated below the pie chart.

Highlights

Legionella was abundant through all seasons in water and biofilm

L. pneumophila serogroup 1 was the predominant identified serogroup

When *Legionella* was present in biofilm its counts in water were >30 cfu/l

Five *L. pneumophila* MLVA-genotypes were identified at different buildings

MLVA-genotype 4, consistently co-occurred with high *Legionella* counts in cold water

Supplementary content

Table S1. A list of *Legionella* isolates by season, sampling point and type of sample, that were sequenced for the 16S rRNA gene. *All isolates were identified by 16S rRNA gene sequencing as *L. pneumophila*, except for strain O90, that was identified as *L. sainthelensis*.

Season	Strain	Sampling point	Type	16S rRNA Genbank accession number
Summer	O1	C	CW	KF537539
	O12	C	B	KF537558
	O16	F	CW	KF537543
	O21	F	B	KF537560
	O28	E	CW	KF537541
	O35	G	CW	KF537544
	O45	D	CW	KF537540
	O55	D	B	KF537559
Autumn	O58	A	WW	KF537546
	O63	A	B	KF586535
	O66	C	CW	KF537545
	O73	C	B	KF537563
	O75	D	CW	KF537547
	O85	D	B	KF537561
	O90*	E	CW	KF537569
	O92	E	B	KF537562
	O93	F	CW	KF537548
Winter	O100	A	WW	KF537549
	O103	C	CW	KF537550
	O112	C	B	KF537565
	O118	D	CW	KF537551
	O129	D	B	KF974480
	O132	F	CW	KF974481
Spring	O133	C	CW	KF537554
	O13	F	CW	KF537556
	O140	F	B	KF537567
	O144	G	CW	KF537557
	O146	G	B	KF537568
	O149	D	CW	KF537555
	O152	D	B	KF974482
	O157	A	CW	KF537552
	O160	A	WW	KF537553
	O164	A	B	KF537566

B, biofilm; CW, cold water; WW, Warm water.

Table S2. Abiotic and biotic detailed data of the drinking water. The detection limit of the *Legionella* isolation was 10 cfu/l.

Sampling point	Temp (°C)	pH	Chlorine (ppm)	Conductivity (μS/cm)	HPC 22/37 (cfu/l)	<i>Legionella</i> (cfu/l)
Summer						
A	28.6	7.84	>0.3	1138	1.18x10 ² /2.80x10 ³	ND
B	28.4	7.97	>0.3	1090	4.60x10 ¹ /2.66x10 ⁵	ND
C	27.5	7.56	0.01	1219	1.66x10 ³ /5.38x10 ⁵	5.75x10 ³
D	28.1	7.87	0.08	1113	6.95x10 ¹ /5.07x10 ²	1.07x10 ³
E	30.9	7.62	0.01	1256	7.64x10 ³ /2.84x10 ⁴	1.10x10 ²
F	29.1	7.5	0.01	1246	2.60x10 ³ /6.00x10 ³	8.80x10 ²
G	27.7	7.75	0.15	1098	2.88x10 ¹ /2.53x10 ²	1.20x10 ²
Autumm						
A	18.0	7.48	0.3	1010	1.48x10 ² /3.83x10 ⁴	ND
A warm	42.6	7.36	<0.01	1003	2.77x10 ² /3.31x10 ⁵	1.20x10 ²
B	21.5	7.5	>0.3	1073	4.40x10 ¹ /4.80x10 ¹	ND
C	18.8	7.52	<0.01	983	1.12x10 ³ /6.00x10 ³	4.00x10 ²
D	21.1	7.66	0.15	833	2.80x10 ³ /3.80x10 ⁵	7.00x10 ¹
E	20.2	7.88	>0.3	766	5.20x10 ¹ /3.54x10 ³	2.40x10 ²
E warm	42.1	8.97	0.01	994	1.80x10 ¹ /5.60x10 ¹	1.00x10 ¹
F	23.5	7.6	0.0175	775	6.80x10 ³ /3.50x10 ³	3.00x10 ¹
G	20.3	7.75	>0.3	1094	6.40x10 ¹ /1.30x10 ²	ND
Winter						
A	18.5	7.31	>0.3	960	3.00x10 ¹ /2.40x10 ²	1.00x10 ¹
A warm	50.0	7.31	0.025	985	1.80x10 ¹ /8.00x10 ²	1.00x10 ¹
B	19.4	7.33	0.3	847	1.12x10 ² /2.60x10 ¹	ND
C	12.8	7.49	0.025	901	1.39x10 ³ /1.11x10 ⁶	2.31x10 ³
D	16.8	7.5	0.06	920	4.00x10 ⁰ /2.80x10 ³	8.00x10 ¹
E	15.7	7.5	>0.3	873	2.18x10 ² /1.72x10 ³	ND
E warm	51.0	8.74	0.025	831	8.00x10 ⁰ /3.65x10 ²	ND
F	18.7	7.3	0.15	972	7.02x10 ² /9.00x10 ²	2.00x10 ¹
G	16.0	7.31	>0.3	892	1.80x10 ¹ /2.00x10 ¹	ND
Spring						
A	24.3	7.82	0.2	1070	6.00x10 ¹ /6.00x10 ²	5.00x10 ¹
A warm	50.0	7.77	0.01	1079	3.50x10 ¹ /2.20x10 ²	8.00x10 ¹
B	22.5	8.07	>0.3	1029	2.20x10 ¹ /3.20x10 ¹	ND
C	17.8	7.79	<0.01	1002	6.90x10 ¹ /1.60x10 ⁴	2.42x10 ³
D	17.4	7.97	>0.3	1045	4.40x10 ¹ /6.00x10 ¹	1.20x10 ¹
E	19.1	9.45	0.2	939	1.40x10 ² /1.28x10 ⁴	ND
E warm	56.0	8.11	0.025	1093	5.40x10 ¹ /5.00x10 ¹	1.00x10 ¹
F	24.0	8.06	0.025	1127	6.00x10 ² /6.20x10 ²	3.00x10 ²
G	22.3	8	0.5	1022	3.20x10 ¹ /4.20x10 ¹	2.00x10 ¹

ND, not detected.

Table S4. MLVA-8 genotypes percentages by sampling point and sample type. Percentages were calculated with the results obtained after genotyping 63 *Legionella pneumophila* strains isolated both from water and biofilm.

Sampling point	A			C			D			E			F			G			Total		
Genotype (%)	W	B	T	W	B	T	W	B	T	W	B	T	W	B	T	W	B	T	W	B	T
Gt4				100	100	100	75	100	88										58	70	62
Gt6							8		6	100	100	100	100	100	100	100		100	23	25	24
Gt15	100		86																16		11
Gt17		100	14																	5	2
Gt18							8		6										2		2
n	6	1	7	16	9	25	11	5	16	3	3	6	4	2	6	2	0	2	42	20	62

W, water; B, biofilm; T, total; Gt, genotype; n, number of analyzed strains

<i>L. pneumophila</i> isolate	Serogroup	Sampling point	Sample type	MLVA-8 type	No. of repeats at each VNTR locus							
					Lpms1	Lpms3	Lpms13	Lpms17	Lpms19	Lpms33	Lpms34	Lpms35
O2	Sg1	C	Water	4	7	7	10	2	4	4	2	17
O3	Sg1	C	Water	4	7	7	10	2	4	4	2	17
O4	Sg1	C	Water	4	7	7	10	2	4	4	2	17
O5	Sg1	C	Water	4	7	7	10	2	4	4	2	17
O6	Sg1	C	Water	4	7	7	10	2	4	4	2	17
O8	Sg1	C	Water	4	7	7	10	2	4	4	2	17
O9	Sg1	C	Water	4	7	7	10	2	4	4	2	17
O10	Sg1	C	Water	4	7	7	10	2	4	4	2	17
O11	Sg1	C	Biofilm	4	7	7	10	2	4	4	2	17
O12	Sg1	C	Biofilm	4	7	7	10	2	4	4	2	17
O13	Sg1	C	Biofilm	4	7	7	10	2	4	4	2	17
O15	Sg1	C	Biofilm	4	7	7	10	2	4	4	2	17
O16	Sg1	F	Water	6	7	7	10	2	4	4	2	18
O17	Sg1	F	Water	6	7	7	10	2	4	4	2	18
O21	Sg1	F	Biofilm	6	7	7	10	2	4	4	2	18
O22	Sg1	F	Biofilm	6	7	7	10	2	4	4	2	18
O26	Sg1	E	Water	6	7	7	10	2	4	4	2	18
O28	Sg1	E	Water	6	7	7	10	2	4	4	2	18
O29	Sg1	E	Water	6	7	7	10	2	4	4	2	18
O33	Sg1	E	Biofilm	6	7	7	10	2	4	4	2	18
O34	Sg1	E	Biofilm	6	7	7	10	2	4	4	2	18
O35	Sg1	G	Water	6	7	7	10	2	4	4	2	18
O36	Sg1	G	Water	6	7	7	10	2	4	4	2	18
O45	Sg1	D	Water	4	7	7	10	2	4	4	2	17
O50	Sg1	D	Water	4	7	7	10	2	4	4	2	17
O55	Sg1	D	Biofilm	4	7	7	10	2	4	4	2	17
O56	Sg1	D	Biofilm	4	7	7	10	2	4	4	2	17
O58	Sg3	A	Water	15	9	8	8	2	5	2	2	21
O59	Sg3	A	Water	15	9	8	8	2	5	2	2	21
O60	Sg3	A	Water	15	9	8	8	2	5	2	2	21
O61	Sg3	A	Water	15	9	8	8	2	5	2	2	21
O63	Sg3	A	Biofilm	17	7	7	8	2	3	4	2	17
O64	Sg1	C	Water	4	7	7	10	2	4	4	2	17
O70	Sg1	C	Water	4	7	7	10	2	4	4	2	17
O72	Sg1	C	Biofilm	4	7	7	10	2	4	4	2	17
O73	Sg1	C	Biofilm	4	7	7	10	2	4	4	2	17
O75	Sg1	D	Water	4	7	7	10	2	4	4	2	17
O76	Sg1	D	Water	6	7	7	10	2	4	4	2	18
O80	Sg1	D	Water	4	7	7	10	2	4	4	2	17
O81	Sg1	D	Water	18	7	7	7	2	4	4	2	17
O85	Sg1	D	Biofilm	4	7	7	10	2	4	4	2	17
O86	Sg1	D	Biofilm	4	7	7	10	2	4	4	2	17
O92	Sg1	E	Biofilm	6	7	7	10	2	4	4	2	18
O93	Sg1	F	Water	6	7	7	10	2	4	4	2	18
O94	Sg1	F	Water	6	7	7	10	2	4	4	2	18
O100	Sg3	A	Water	15	9	8	8	2	5	2	2	21
O101	Sg3	A	Water	15	9	8	8	2	5	2	2	21
O102	Sg1	C	Water	4	7	7	10	2	4	4	2	17
O103	Sg1	C	Water	4	7	7	10	2	4	4	2	17

O104	Sg1	C	Water	4	7	7	10	2	4	4	2	17
O107	Sg1	C	Water	4	7	7	10	2	4	4	2	17
O108	Sg1	C	Water	4	7	7	10	2	4	4	2	17
O109	Sg1	C	Water	4	7	7	10	2	4	4	2	17
O112	Sg1	C	Biofilm	4	7	7	10	2	4	4	2	17
O113	Sg1	C	Biofilm	4	7	7	10	2	4	4	2	17
O114	Sg1	C	Biofilm	4	7	7	10	2	4	4	2	17
O117	Sg1	D	Water	4	7	7	10	2	4	4	2	17
O118	Sg1	D	Water	4	7	7	10	2	4	4	2	17
O119	Sg1	D	Water	4	7	7	10	2	4	4	2	17
O122	Sg1	D	Water	4	7	7	10	2	4	4	2	17
O123	Sg1	D	Water	4	7	7	10	2	4	4	2	17
O128	Sg1	D	Biofilm	4	7	7	10	2	4	4	2	17

Table S3 .- *Legionella pneumophila* isolates examined by MLVA-8 included in the present study (n=63). The number of tandem repeats observed in each marker is indicated in the corresponding cells. Lpms, *L. pneumophila* mini-microsatellite locus.