MONITORING *LEGIONELLA PNEUMOPHILA* IN DRINKING WATER DISTRIBUTION SYSTEMS IN SOUTHERN CROATIA

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ABSTRACT

In drinking water distribution systems (DWDSs) opportunistic bacteria of environmental origin such as *Legionella pneumophila* (*L. pneumophila*) represent a potential source of water contamination, resulting in a potential health risk for humans. The main objective of this study includes an integrated approach based on hazard assessment, identification and monitoring of control factors in order to characterize the influence of physical-chemical parameters on *L. pneumophila* presence in DWDSs as well as to determine possible seasonal effects, with the purpose of improving the prevention measures.

The contamination of hot water samples with *L. pneumophila* was studied in relation to temperature, pH, free residual chlorine, and metal ions concentrations (iron, copper, zinc, manganese, calcium, and magnesium). The results of microbiological and physical-chemical characteristics were analyzed in order to identify the factors that can effectively contribute to reduce legionellae proliferation and risk of human infection.

The samples were collected between March 2009 and December 2011 from three hotels and two homes for the elderly and disabled in the Split-Dalmatian County, Croatia. *Legionella pneumophila* was isolated in 99 out of 304 samples (32.6%). The seasonal *L. pneumophila* occurrence trends in drinking water distribution systems were observed, with the highest positive samples percentage of 43.5% found within the 3rd quarter (7–9 month). *L. pneumophila* contamination was found to be positively associated with Ca, Mg, Fe and Cu concentrations, and negatively associated with Mn concentrations and temperature.

KEYWORDS: iron, copper, manganese, Legionella pneumophila, drinking water distribution system, risk assessment

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1. INTRODUCTION

Water is an essential prerequisite for life, therefore water safety in drinking water distribution systems is of public importance. The examination of drinking water distribution systems (DWDS) reveals the complexity and the heterogeneity of such a technical system and the fate of autochthonous microbial populations and contaminant pathogens is related to this complex system generating a variety of situations where microbial activity may develop [1]. In DWDSs, the presence of microorganisms relevant to public health was regularly monitored and the occurrence and survivor of pathogens such as *Legionella pneumophila* was among significant ones [2,3].

The type of pipes in DWDSs, loose materials, sediment and corrosion can play a significant role in the dynamics of bacterial growth [4]. Generally, the corrosion leads to metal dissolution, biofouling leads to the undesirable accumulation of microbiological deposits at the interface and biofilms are formed in distribution system pipelines. The formation of the biofilms leads to re-contamination of water after disinfection and to micro-corrosion of metal tube surface under the biofilm layer. In DWDSs biofilms are ecological niches in which *Legionella* species survive and proliferate and therefore present a health risk [5,6].

Legionella pneumophila is a Gram-negative bacterium that belongs to the genus *Legionella* spp. It is the one most frequently related to human disease, especially pneumonia (Legionnaire´s disease) [3,7,8]. *L. pneumophila* has been shown to be harbored within biofilms formed within cooling towers, swimming pools, hot-water tanks drinking water pipelines and other parts of DWDSs [9-11]. Evidence has also been presented indicating that amoebae and other protozoa may be natural hosts and “amplifiers” for *L. pneumophila* in different water systems [12-15]. Therefore, the maintenance of water quality as well as hygienic conditions in such environments is important [16].

Water temperature is perhaps the most important rate controlling factor regulating microbial growth. Temperature influences microbial growth rate, disinfection efficiency, corrosion rates and distribution system hydraulics [17]. *L. pneumophila* multiplies at temperatures between 25 and 42°C with an optimal growth temperature of 35°C

[18, 19]. Furthermore, the survivor and growth of *L. pneumophila* depends on other physicochemical properties of water such as pH, hardness, organic materials, nutrients, disinfection residual concentrations and the presence of heavy metals, water flow velocity, corrosion of distribution system pipes and fittings [20, 21]. Some metal ions inhibit while others have a bio-stimulating effect on the growth of *L. pneumophila*. For example, copper ions could slow down the development of *L. pneumophila* in DWDS but they do not retard mycobacteria [22-24] and iron was shown to have a positive relationship with the presence of protozoa and *L. pneumophila* [25,26]. The correlation of *L. pneumophila* presence and growth at different temperatures and pH values and in the presence of various free residual chlorine concentrations have been studied [27-29]. Generally, higher pH values, lower temperatures and lower chlorine content increase the survival rate of *L. pneumophila*.

The aim of this study was to correlate the *Legionella pneumophila* presence in hot water distribution systems in the Dalmatian County of Croatia with physicochemical properties of water such as temperature, pH, free residual chlorine, and metal ions concentrations (iron, copper, zinc, manganese, calcium, and magnesium). The main objective of this study was to analyze and characterize the influence of chemical parameters on the presence of *L. pneumophila* in DWDSs and to determine possible seasonal effects on the *L. pneumophila* presence in order to improve prevention measures that can effectively contribute to reduce legionellae proliferation and risk for human infection.

2. MATERIALS AND METHODS

2.1. Water samples

The water samples were obtained from four stations along the Adriatic coast in Southern Croatia (Figure 1). The samples were collected during 3 years, between March 2009 and December 2011. Hot water samples were collected in sterile 1 L bottles containing 180–200 mg sodium thiosulphate to neutralize any chlorine or other oxidizing biocides. At sampling port, the outside of the pipe was disinfected with a flame, water was flushed for 2 minutes and then water was sampled. For each sample, hot water (1L) was collected in duplicate in sterile plastic bottles from hot-water faucets. Samples were stored in the dark, in an insulated container (cool box) at 4°C, transported to the laboratory as soon as possible.

FIGURE 1 – Sampling sites at the Adratic Coast.

TABLE 1 – Experimental conditions for metal determinations by AAS.

Metal	Fe	Mn	Cu	Zn	Ca	Mg
Conditions						
Flame	C_2H_2/Air				C_2H_2/ N_2O_2	C_2H_2/ N_2O_2
Wavelength (nm)	248.3	279.3	324.8	213.9	422.7	285.2
Slit width (mm)	0.8	0.2	0.5	0.5	0.5	1.3
AAS detection limit (μg/L)	1	0.2	0.12	0.1	0.5	0.02

A total of 304 samples were used for the analysis. The hot water samples were immediately analyzed for temperature, pH and free residual chlorine concentrations and within 24 h for *L. pneumophila* concentration and concentrations of iron, copper, zinc, manganese, calcium, and magnesium.

2.2. Physical and chemical analyses

The water temperature, pH and free residual chlorine (DPD method) were measured immediately at sampling port by an electronic thermometer (EcoScan Temp 5, Thermo Fisher Scientific, UK), a direct reading pH meter (pHmeter 827 pH lab, Metrohm, Switzerland) and a photoLab WTW free Cl_2 (WTW, Germany) respectively.

The water samples were acidified to pH < 2 with nitric acid (1% nitric acid) for determination of element concentrations (Fe, Mn, Cu, Zn, Ca and Mg). Then an aliquot was injected into the atomic absorption spectrometer (AAS Model Z-2000, Hitachi, Japan) at predetermined experimental conditions (Table 1). Standard calibration solutions were prepared from commercial solutions.

2.3. Microbiological analysis

Legionella pneumophila were enumerated and identified according to the Croatian standard method which is equivalent to ISO 11731-2 [30]. Water samples were concentrated by filtration through 0.20 µm pore size (a polyamide filter, Millipore, Bedford, MA, USA) and cultured before and after heating treatment. For that purpose, membranes were transferred into 10 mL of the same water sample and vortexed. The samples (5 mL) were treated at 50°C for 30 min and the concentrates (0.1 mL) were plated onto buffered charcoal yeast extract (BCYE-a) agar with cysteine (bioMériux, Marcy l`Etoile, France) and charcoal yeast extract agar (cysteine-free) (bioMériux, Marcy l`Etoile, France). The remaining 5 mL were cold seeded using the same technique. After incubation at 36°C during 72 h, a quantitative assessment was conducted. The suspect colonies were subcultured on a BCYE medium and those ascribable to the *Legionella* genus were then determined by means of agglutination (Legionella latex test, Oxoid, Basingstoke, UK). The agglutination test enabled separate determination of *L. pneumophila* sg 1 and *L. pneumophila* sg 2 to 14 and the detection of seven species of non-*L. pneumophila* legionellae (polyvalent) that have been implicated in human disease. The results (mean of two plates) were expressed as CFU/L, and the detection limit of the procedure was 25 CFU/L.

2.4. Statistical analysis

Statistical calculations were performed using MedCalc 11.3.0.0; Windows 2000/XP/Vista/7 versions (Copyright 1993-2010, MedCalc Software byba). Prior to the statistical analysis the normality tests were performed to check the data distribution. Spearman's Rho coefficient was used to test the association between measured elements and microbiological test results. A statistical analysis was performed by using the non-parametric Mann-Whitney U test [31] with the aim of determining the connection between *L. pneumophila* and the previously described variables. Statistical results were interpreted at the level of significance $p < 0.05$. The chi-square test or χ^2 was calculated to compare the proportions of *L. pneumophila* contamination and nonparametric statistical methods were applied to determine statistically significant differences.

3. RESULTS AND DISCUSSION

The results of this study have shown a widespread environmental contamination of water systems by *L. pneumophila*. A total of 304 water samples were analysed and the *L. pneumophila* was found in 32.6% of which 20.3% in hotels and 12.7% in homes for the elderly and disabled.

The premise identification, sampling ports and total number of samples that were collected during different quarters are described in Table 2, and the analysis of results, according to the seasonal period of sampling, revealed that within the 3rd quarter (7–9 month) 43.5% of the samples were *L. pneumophila* positive (Table 2). Moreover, within the 3rd quarter the observed concentration of *L. pneumophila* was in the range of 500–13,000 CFU/L and was significantly higher than in other seasons. The occurrence of *L. pneumophila* in hot water samples shown in Table 2 indicated that *L. pneumophila* was found in 58 and 35 samples taken from the bathroom taps in hotels and in homes for the elderly and disabled respectively. Among 99 *L. pneumophila* positive samples, 93 samples were taken from the bathroom taps indicating that the risk of legionellosis was significantly increased in such a location. On the contrary, in a recent report Marchesi et al. [28], during their study conducted in an Italian hospital observed no differences according to sampling port or season, but shower aerosols have been identified as a potential pathway for exposure and recently, the conditions within in-premise plumbing that could result in an infection from inhalation of aero-

TABLE 2 – Seasonal distribution of *Legionella pneumophila* in hot water samples from hotels and homes for the elderly and disabled in the Split region.

Premises identification	Sampling port	Number of samples	Occurrence of *L. pneumophila* Quarters (determination range; CFU/L) Q1 N:48 (50-2000)	Q2 N:133 (200-8400)	Q3 N:62 (500-13000)	Q4 N:61 (150-8000)
Hotels	Bathroom tap	188	8 (16.7%)	19 (14.3%)	20 (32.2%)	11 (18%)
	Kitchen and bar tap	25	0	1(0.7%)	0	1 (1.6%)
	Other (jacuzzi and wellness)	23	1 (2.1%)	0	0	1 (1.6%)
Homes for the elderly and disabled	Bathroom tap	57	11 (22.9%)	8 (6%)	6 (9.7%)	10 (16.5%)
	Kitchen tap	11	0	1 (0.7%)	1 (1.6%)	0
All the sampling stations		304 32.6%	20 41.7%	29 21.8%	27 43.5%	23 37.7%

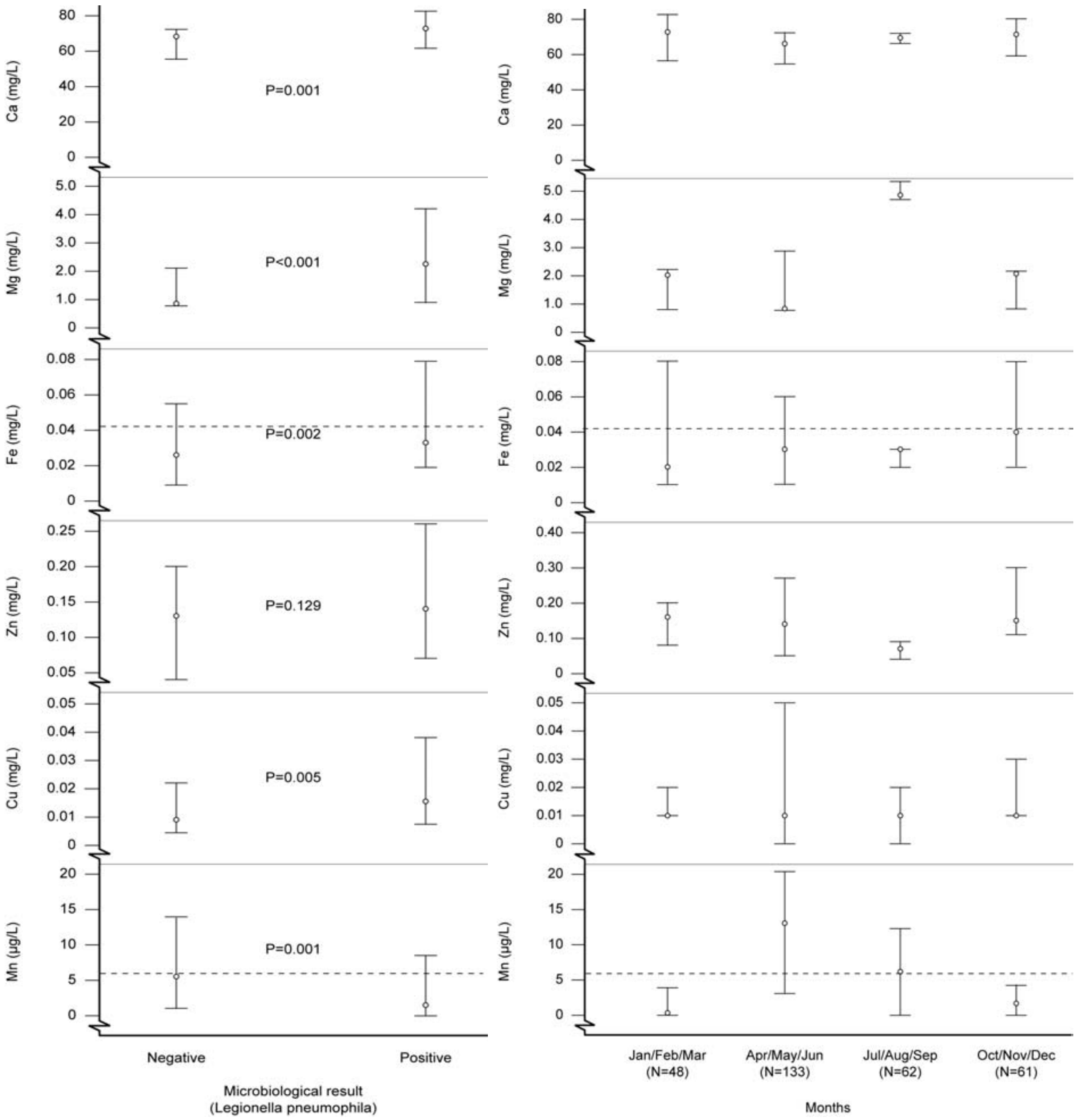

FIGURE 2 a-b – Occurrence of *Legionella pneumophila* in correlation with metal ions concentrations (a) and seasonal period of sampling (b).

TABLE 3 – Spearman's Rho coefficient, physical and chemical characteristics of 304 hot water samples.

Parameters		Ca (mg/L)	Mg (mg/L)	Fe (mg/L)	Zn (mg/L)	Cu (mg/L)	Mn (µg/L)	Temp. (°C)	FRC (mg/L)	pH
Spearman`s coefficient		0.266*	0.432*	0.182*	0.087	0.162*	-0.185*	-0.362*	0.090	0.103
P		0.001	0.000	0.001	0.129	0.005	0.001	0.000	0.116	0.205
The quarters Q1	Median	72.52	1.99	0.02	0.16	0.01	0.19	45.2	0.2	8.2
	Percentile 25	56.13	0.77	0.01	0.08	0.01	0.00	44.0	0.2	8.2
	Percentile 75	82.41	2.19	0.08	0.20	0.02	3.84	48.8	0.2	8.2
Q2	Median	65.87	0.80	0.03	0.14	0.01	13.02	54.5	0.2	8.0
	Percentile 25	54.33	0.75	0.01	0.05	0.00	3.02	51.8	0.2	8.0
	Percentile 75	72.04	2.84	0.06	0.27	0.05	20.35	58.8	0.3	8.1
Q3	Median	69.22	4.83	0.03	0.07	0.01	6.12	51.8	0.3	8.3
	Percentile 25	66.00	4.69	0.02	0.04	0.00	0.00	50.3	0.2	7.7
	Percentile 75	71.74	5.31	0.03	0.09	0.02	12.25	56.7	0.3	8.4
Q4	Median	71.19	2.04	0.04	0.15	0.01	1.61	49.7	0.2	7.9
	Percentile 25	58.92	0.79	0.02	0.11	0.01	0.00	44.3	0.2	7.8
	Percentile 75	80.05	2.13	0.08	0.30	0.03	4.17	55.1	0.2	8.0

*-Correlation is significant at the 0.01 level.

sols containing the pathogen while showering, were investigated and modeled [32,33]. Nevertheless, the presence of *L. pneumophila* was confirmed in hot water samples in many countries, and some studies demonstrated the findings of *Legionella pneumophila* with a contamination range from 63.6% to 75% [24, 29, 34-37]. Borella et al. [38] also reported that 22.6% to 30.5% of the samples were *Legionella* positive in water from apartments in Italy. Obviously, the presence of *Legionella* species was detected in a wide range depending on the physicochemical properties of water. In our study, the *Legionella* presence of 32.6% was in accordance to similar studies conducted in Italy, Finland and Germany where 33.3%, 30% and 26% of the samples were *Legionella* positive**,** respectively [39-41].

The correlation of microbiological results with measured metal ions concentrations (iron, copper, zinc, manganese, calcium, and magnesium) shown in Figure 2 indicate that positive microbiological findings were linked to higher values of Ca, Mg, Fe and Cu and with lower values of Mn. The presented results confirm the hypothesis about the linkage between the tested risk factors and the *Legionella pneumophila* presence in the hot water from the DWDSs. The calculated Spearman's rank correlation coefficient, ρ and the seasonal variability of physical and chemical parameters in DWDSs were presented in Table 3.

The presence of Ca and Mg in hot water samples was in a wide range of 46.78–137.61 mg/L and 0.44–5.93 mg/L respectively, but the statistically significant positive correlation was observed (Table 3). Generally, correlation between *Legionellae* and calcium and magnesium concentrations was somewhat more difficult to explain and conflicting reports were published. For example, Leoni et al. [41] found a statistically significant inverse correlation between the *L. pneumophila* presence and Ca and Mg content. The comparison to results obtained during this study revealed that the level of Ca was similar, but Mg concentrations reported in their study were almost twice as high, indicating that detail analysis of the correlation between *Legionellae* and Ca and Mg content should be further investigated.

Furthermore, they reported the distribution of *L. pneumophila* positive samples according to Cu concentrations (0.01–0.05 mg/L) and statistically significant inverse correlation was observed. Accordingly, the copper was represented as a limiting factor for *L. pneumophila* development with the possible explanation that copper is able to effectively penetrate into the biofilm which provides the basis for the colonization of water distribution systems. On the contrary, the results obtained during this 3 years study (Figure 2 and Table 3) with similar levels of Cu, indicate the statistically significant positive correlation and a similar observation was recently reported [42]. The positive correlation was unexpected since the protective effect of copper was reported and the higher Cu levels (> 50 µg/L) were associated with a lower risk of *Legionella* proliferation [23,24]. In addition, in recent study of Mathys et al. [43], authors compared pipe materials and reported that plumbing systems with Cu pipes were more contaminated than those made of synthetic materials or galvanized steel. Obviously, the role of copper and the association with *L. pneumophila* strongly depends on its concentrations and lower Cu concentrations could have a positive or negative correlation while higher levels of copper have been shown to be effective against *Legionellae* [44].

Fe is an important component of oxidation-reduction systems and a cofactor of some important enzymes. The results presented in Figure 2a indicated the median Fe concentration values for negative and positive samples were in the range of 0.01–0.058 and 0.02–0.08 mg/L respectively. The higher values obtained in samples with *L. pneumophila* (ρ = 0.182; P = 0.002) indicated positive association of *L. pneumophila* with Fe concentration (Table 3). The similar Fe values and positive association with the presence of *Legionellae* were recently reported [23]. In comprehensive statistical analysis, the cut off value of 0.042 mg/L was discussed as sufficient to increase the colonization risk. Thus, a large number of samples that had a value higher than 0.042 mg/L was congruent to this assumption and confirmed the *Legionella* risk. Hence, our results indicate that metal plumbing components and associated corrosion products are important factors in the survival and growth of *L. pneumophila* in DWDSs. Corrosion can develop crevices and cracks on pipe walls [45], which can shelter *Legionellae* and other pathogenic bacteria, and increase turbidity in DWDSs, which can promote bacteria regrowth [46]. It can exhaust residual chlorine at a faster rate [47], which may lead to the increased formation of biomass at the extremities of the DWDSs. Corrosion scales can actively modify physicochemical parameters of water in the DWDSs not only by releasing Fe oxyhydroxides but also by reactions with e.g. chlorinated disinfection by-products [48].

Measurement of pH and free residual chlorine concentration are essential to ensure an efficient disinfection procedure. As such, higher doses of disinfectants (e.g. chlorine) are needed in such scenarios, since water chlorination effectively reduces *Legionellae* contamination [45]. In this study, the monitoring of pH, free residual chlorine concentration and Zn indicated that the differences were not statistically significant (Table 3). A slight increase of pH observed during the 3rd quarter indicated that the relation between *L. pneumophila* positive samples and pH was positively associated as previously reported [41].

The presence of *L. pneumophila* was negatively associated with Mn concentrations and the Mn level in *L. pneumophila* positive samples was significantly lower, mainly in the range of 0.0–9.0 µg/L (Figure 2). The Mn data series presented in Figure 2a, clearly indicate the difference of medians between samples in which *L. pneumophila* was proven and those in which it was not found. The Mn median concentrations of 6.12 and 13.2 µg/L were found in hot water samples within the 3rd and 2nd quarters respectively (Table 3). The least positive samples (21.8%) were found within the 2nd quarter and the obtained twice as high manganese values confirmed that the presence of increased Mn levels contributes to lower *L. pneumophila* presence. Furthermore, Table 3 and Figure 2b show that lower Mn concentrations were determined during the 3rd quarter and generally with the exception of the 2nd quarter, manganese concentrations were mostly lower than 6 µg/L. Bargellini et al. [24] set Mn concentration lower than 6 µg/L as a cut off value and discussed that it could be a good indicator of *Legionella* absence. These findings and the fact that Mn was an essential element for the growth and pathogenesis of the bacteria indicate that the role of manganese and its involvement in the *Legionella* risk should be further investigated.

The obtained results (Figure 3) show convincingly that the temperature of the hot water is probably the most important determinant for the multiplication of *L. pneumophila*. Water with a temperature between 44–54°C was most frequently colonized and contained the highest concentrations of *Legionellae* (Figure 3). Furthermore, according to obtained results, the median temperature values for the 3rd quarter (7–9 month) were 51.8°C (Table 3), on the contrary, within the 2nd quarter (4–6 month), the water temperature median, 25 and 75 percentile values were 54.5°C, 51.8°C and 58.8°C respectively and those increased values confirming the protective role of such temperatures. In agreement, several authors have demonstrated that lower hot water temperatures are also closely associated with the contamination of domestic hot water systems [34,43,46]. The literature and observed results clearly indicate that the water temperature higher than 54°C was protective, while a temperature range of 44–54°C is one of the factors responsible for *Legionella* colonization in hot water systems. Those temperatures favor growth of *Legionellae* in water systems, and very high counts present a legionellosis risk for elderly and immuno-compromised members of the community.

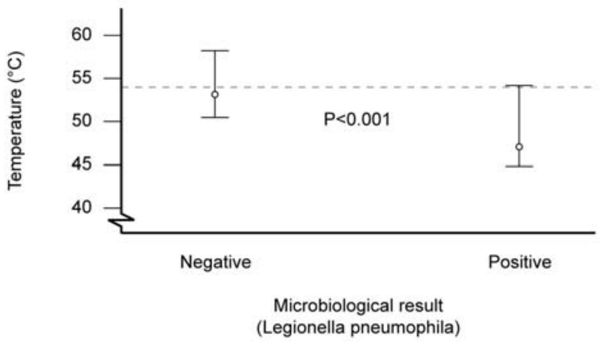

FIGURE 3 – Occurrence of *Legionella pneumophila* in function of temperature.

4. CONCLUSIONS

The 3 years monitoring of *Legionella pneumophila* in drinking water distribution systems in Southern Croatia revealed that within 304 hot water samples 99 were *L. pneumophila* positive and among them 58 and 35 samples were taken from the bathroom taps in hotels and in homes for the elderly and disabled respectively. Observed results indicated that the risk of *Legionellae* presence was significantly increased in the bathrooms in comparison to the kitchen, bar or other taps. The seasonal *L. pneumophila* occurrence trends in drinking water distribution systems were observed and the highest positive samples percentage of 43.5% was found within the 3rd quarter (7–9 month). Furthermore, within this quarter, the presence of *L. pneumophila* was determined in the range of 500–13,000 CFU/L which was significantly higher than in other seasons. Obviously, total of 32.6% *L. pneumophila* positive samples present an increased potential health risk that should be effectively reduced.

The obtained results of microbiological and physical-chemical characteristics were analyzed in order to identify reliable indicators for prediction of *L. pneumophila* risk and statistical analysis indicated that *L. pneumophila* contamination were positively associated with Ca, Mg, Fe and Cu concentrations, and negatively associated with Mn concentrations and temperature. The water samples positive for *L. pneumophila* exhibited significantly higher Fe and Mg concentrations compared to the negative samples. The observed Fe concentrations higher than 0.042 mg/L indicated that this value could be the good predictor of increased *L. pneumophila* risk, contributing that the corrosion in the DWDSs favor conditions for the *L. pneumophila* proliferation. Similarly, the monitoring of Cu level indicated the statistically significant positive correlation with *L. pneumophila*. On the contrary, the higher Mn levels contributed to lower *L. pneumophila* presence. The statistical analysis showed that zinc, free residual chlorine and pH have no significant influence on the presence of *L. pneumophila*, confirming the low efficacy of free chlorine on microbe eradication. The water temperature higher than 54°C revealed as protective, while the temperature range of 44–54°C is one of the factors responsible for *Legionella* colonization in hot water systems.

The results of this study provide insight into *L. pneumophila* presence in the DWDSs and can provide a basis for protection of water quality and human health in Southern Croatia. Moreover, the development of DWDSs and maintenance programs especially in the bathroom taps can reduce and eliminate the presence of *L. pneumophila*.

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