ORIGINAL ARTICLE

Annals of Agricultural and Environmental Medicine 2012, Vol 19, No 3, 431-436
www.aaem.pl

Influence of temperature, chlorine residual and heavy metals on the presence of *Legionella pneumophila* in hot water distribution systems

Anita Rakić[1], Jelena Perić[2], Lucija Foglar[3]

[1] *Public Health Institute, Split, Croatia*
[2] *Faculty of Chemistry and Technology, University of Split, Split, Croatia*
[3] *Division of Industrial Ecology, Faculty of Chemical Engineering and Technology, University of Zagreb, Zagreb, Croatia*

Rakić A, Perić J, Foglar L. Influence of temperature, chlorine residual and heavy metals on the presence of *Legionella pneumophila* in hot water distribution systems Ann Agric Environ Med. 2012; 19(3): 431-436.

Abstract

The microbiological colonisation of buildings and man-made structures often occurs on the walls of plumbing systems; therefore, monitoring of opportunistic pathogens such as *Legionella pneumophila* (*L. pneumophila*), both in water distribution mains and in consumers' plumbing systems, is an important issue according to the international and national guidelines that regulate the quality of drinking water. This paper investigates the presence of *L. pneumophila* in the Dalmatian County of Croatia and the relationship between *L. pneumophila* presence and heavy metals concentrations, free residual chlorine and water temperature in hot water distribution systems (WDS). Investigations were performed on a large number of hot water samples taken from taps in kitchens and bathrooms in hotels and homes for the elderly and disabled in the Split region. Of the 127 hot water samples examined, 12 (9.4%) were positive for *Legionella* spp. with median values concentration of 450 cfu × L^{-1}. Among positive isolates, 10 (83.3%) were *L. pneumophila* sg 1, and two of them (16.6%) belonged to the genera *L. pneumophila* sg 2–14. The positive correlation between the water temperature, iron and manganese concentrations, and *L. pneumophila* contamination was proved by statistical analysis of the experimental data. On the contrary, zinc and free residual chlorine had no observed influence on the presence of *L. pneumophila*. The presence of heavy metals in water samples confirms the corrosion of distribution system pipes and fittings, and suggests that metal plumbing components and associated corrosion products are important factors in the survival and growth of *L. pneumophila* in WDS.

Key words

heavy metals, hot water distribution systems, *Legionella pneumophila*, risk factors

INTRODUCTION

Bacteria of the *Legionella* species can be found in the natural environment and in spring waters, including surface and underground waters [1, 2, 3]. Their presence in nature is caused by their ability to survive in water under different conditions, including temperatures of 15-60 °C and pH values from 5.0-8.5 [4, 5]. *L. pneumophila* is a species of the genus *Legionella* most commonly associated with human disease, especially pneumonia. It is known that Legionnaires disease is caused by *L. pneumophila* [6]. *Legionella* infect humans, susceptible animals and single cell protozoa. They are a ubiquitous intracellular opportunistic microbe, widespread in the environment, especially in aquatic habitats, persisting for extended periods in single cell paramecia such as amoeba, and occasionally cause acute infection in humans, especially immune-compromised individuals in institutions [7]. *L. pneumophila* is a Gram-negative coccobacillus belonging to the gamma-subgroup of proteobacteria. Today, the family Legionellaceae consists of a single genus, Legionella, but contains 56 species/subspecies belonging to over 70 serogroups [8]. Most legionellosis are linked to serogroup 1 (sg 1) and serogroups 2–14 (sg 2–14). The history of Legionnaires' disease began already in 1947. The reanalysis of sera of a patient from an outbreak of pneumonia of unknown aetiology in 1947, showed the organism to be *L. pneumophila*, but the epidemic in Philadelphia in 1976 provided the scientific focus and resources necessary to determine that *L. pneumophila* caused epidemics [9]. Humans can be infected by inhalation of contaminated aerosol which is formed within taps and shower heads [10].

L. pneumophila have a high tolerance to elevated temperatures which enhances their growth and breeding in hot water. Along with other micro-organisms they form biofilms on the inner surfaces of water pipes which re-contaminate water after disinfection. In water distribution systems (WDS), *L. pneumophila* bacteria can colonize solid surfaces of cooling towers, hot-water tanks, taps and shower heads, and form biofilms. Biofilm is composed of a large number of bacteria including *Legionella spp., Klebsiella spp., Pseudomonas spp., Mycobacterium, E. coli* and other organisms such as protozoa (amoebae), parasites and enteroviruses [4, 11, 12, 13]. The rate of biofilm growth depends on the physicochemical properties of the water (e.g. temperature, pH, hardness, organic materials, nutrients,

Address for correspondence: Anita Rakić, Public Health Institute, Vukovarska 46, 21000 Split, Croatia.
E-mail: anita.rakic@st.t-com.hr

Received: 30 March 2012; accepted: 09 July 2012

disinfection residual concentrations, and heavy metals), water flow velocity, corrosion of distribution system pipes and fittings [14]. The corrosion of metal pipes increases the concentration of heavy metal ions which are harmful to human health. It has been found that important factors for the occurrence of *L. pneumophila* are the drinking water distribution system and the corrosion of pipes, pumps, valves, other appurtenances and cooling towers [15]. It has been proved that some metal ions retard while others have a bio-stimulating effect on the growths of *L. pneumophila*. Research studies have shown that copper pipes, i.e. copper ions, slow down the development of *L. pneumophila* in WDS, but they do not retard mycobacteria [16, 17, 18]. The positive relationship between iron concentration and the presence of protozoa and *L. pneumophila* has been proved [19, 20]. Besides increasing the concentration of metal ions in water, corrosion also increases the roughness of the inner pipe surfaces. Micro-organisms therefore have access to more surfaces in contact with water. Furthermore, biofilm adheres more to rough surfaces and is more resistant to ablation in turbulent flows [19]. Micro-organisms in biofilm are often resistant to biocides and it is hard to remove them, especially from hard-to–access places such as pipe edges, T-joints and rough inner surfaces. This has been proven by research which found the number of *L. pneumophila* to be 10 times higher within the tap than in the water from the tap itself [21].

Research studies comparing the sensitivity of *L. pneumophila* to various concentrations of free residual chlorine, temperature and pH values have been conducted [22, 23, 24] and *L. pneumophila* has been found to be much more resistant to chlorine, and also able to survive at low levels of chlorine for a relatively long time [22]. It has been generally concluded that higher pH values, lower temperatures and lower chlorine content increase the survival rate of *L. pneumophila*. This supports the assumption that *L. pneumophila* can pass through public water supply systems, and that it can subsequently infect the water in the supply system. Ultraviolet rays influence inactivation of *L. pneumophila* if applied to the extent that is normally used to disinfect potable water [25]. Temperatures between 20-50 °C are suitable for the formation of *L. pneumophila* colonies. Accelerated growth occurs at a temperature of approximately 42 °C, while measurable slow growth commences at temperatures above 50 °C [25].

The aim of the presented study was to investigate the occurrence of *Legionella spp.* colonization in hot water distribution systems in the Dalmatian County of Croatia, and to investigate the relationship between the hot water *Legionella* contamination and the physical-chemical characteristics of water which included temperature, residual free chlorine and heavy metals concentrations (Fe, Cu, Zn, Mn).

MATERIALS AND METHODS

Sample Collection. A total of 127 hot water samples were collected from 19 hotels and 2 homes for the elderly and disabled in the Split region. The location of sampling sites is displayed in Figure 1. The samples were taken from taps in kitchens and bathrooms in the period March - December 2009. The collection of water samples for physical, chemical and microbiological analyses was performed according to ISO 19458 [26]. Water was sampled without flaming after a few minutes of flushing. Hot water samples were drawn from the bathroom outlets (showers or taps) and placed in 1 L sterile glass bottles after a flow time of 3–5 min in order to eliminate any cold water present inside the tap or flexible shower pipe. For each sample, hot water (1L) was collected in duplicate in sterile plastic bottles from a hot-water tap. In order to neutralize the residual free chlorine, 10% sodium thiosulphate was added in sterile bottles for bacteriological analysis, whereas acid-preserved glass bottles were used for chemical determinations. The samples were immediately transported in portable coolers (at 4 °C) to the laboratory for chemical and microbiological analyses and processed within 24h.

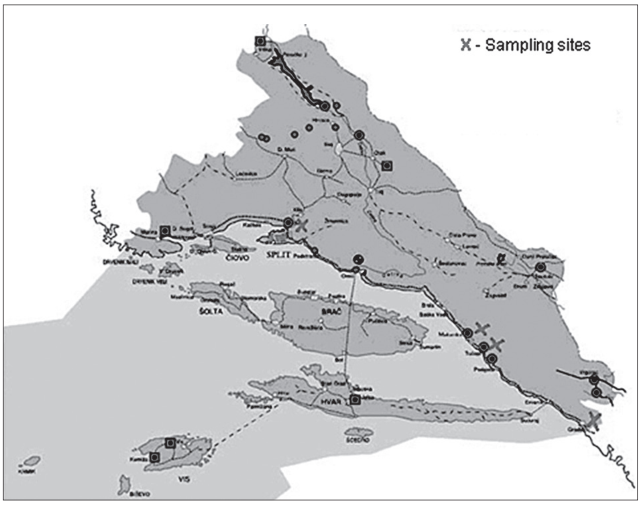

Figure 1. The sampling sites in the Dalmatian County

Physical and Chemical Analyses. During the sampling, the water temperature was monitored by use of a calibrated thermometer (WTW Multi 3500i Multi-Parameter Water Quality Meter, Denver, USA) placed in the middle of the water stream. The residual free chlorine was measured by the standard colorimetric technique – the N, N-diethyl-p-phenylenediamine method (HANNA Instruments 96701, Rhode Island, USA) at the time of sample collection.

Mass concentrations of Fe, Mn, Zn, Cu were determined in acidified samples (1% HNO_3) using an atomic absorption spectrophotometer (Hitachi, Z-2000 Series, Tokyo, Japan) [27]. The absorption rates are measured at 248.3 nm for Fe, 279.6 nm for Mn, 213.9 nm for Zn, and 324.8 nm for Cu. Standard calibration solutions were prepared from commercial solutions. The lower detection level of the absorption spectrophotometer was 1 $\mu g \times L^{-1}$ for Fe, 0.2 $\mu g \times L^{-1}$ for Mn, 0.1 $\mu g \times L^{-1}$ for Zn, and 0.12 $\mu g \times L^{-1}$ for Cu.

Microbiological Analysis. Microbiological analysis of samples was performed in the reference microbiological laboratory immediately after the delivering of samples. Analyses for the detection and quantification of *Legionella* were carried out in accordance with the ISO 11731 method [28]. Water samples were filtered through a 0.20 µm polyamide filter, (Millipore, Bedford, USA). Filtration paper was in turn re-suspended in a 10 mL water sample, followed by cultivation on Buffer Charcoal Yeast Extract Agar (BCYE–bioMériux, Marcy l`Etoile, France) during 72 h, at 36 °C. The BCYE agar base is recommended and often used for growth of *Legionella.* [18, 29]. The BCYE agar consisted of yeast extract (10.0 g×L^{-1}), ACES buffer (5.0 g×L^{-1}), activated charcoal (2.0 g×L^{-1}) and agar (15.0 g×L^{-1}), further enriched with *Legionellae* supplement solutions (lyophilized enrichment supplement and mixture for selective isolation). The enrichment supplement solution contained essential growth factors, ferric pyrophosphate (0.25 g×L^{-1}) and cysteine hydrochloride (0.4 g×L^{-1}), while the mixture for selective isolation contained vancomycin (0.001 g×L^{-1}) and colistin (45,000 IU), which prevent growth of most of the flora found in association with *Legionella.* Charcoal is the distinguishing element in the medium and has been described as a detoxifier, and the ACES buffer stabilizes the pH at 6.9 which is the optimal condition for growth.

Colonies grown on BCYE agar were subsequently determined by means of an agglutination test (*Legionella* latex test, Oxoid, Basingstoke, UK). The agglutination test enabled separate determination of *L. pneumophila* serogroup 1 and *L. pneumophila* serogroups 2–14. The results were expressed as colony-forming units per litre (cfu×L^{-1}), on the basis of plate counts of *Legionella* colonies, and the detection limit of the procedure was 25 cfu×L^{-1} (mean of 2 plates).

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 statistical analysis, normality tests were performed to check the data distribution. Statistical analysis was performed by using the non-parametric Mann-Whitney U test [30] 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 χ^2 was calculated to compare the proportions of *L. pneumophila* contamination.

RESULTS

In order to estimate the *Legionella* risk in the Dalmatian County, 127 hot water samples taken from 19 hotels and 2 homes for the elderly and disabled in the Split region were analyzed. The presence and number of *L. pneumophila* were determined with a simultaneous determination of temperature, concentration of free residual chlorine, and concentrations of Fe, Mn, Zn and Cu in order to evaluate the influence of those physical-chemical parameters on the presence of *L. pneumophila*.

The results of this study revealed that the luminescent, glossy, white-grey colonies (2-3 mm in diameter) developed on BCYE agar after 3-4 days of incubation, belonging to the genera *Legionella* and confirmed the presence of *L. pneumophila* (Tab. 1, Fig. 2). At the time of the study (March - December 2009), *Legionella* spp. were isolated in 12 out of 127 water samples (9.4%) (p<0.001, χ^2-test), with a mean number of *Legionellae* in positive samples of 450 cfu×L^{-1} (geometric mean). The peak value of 1,000 cfu×L^{-1} was found in 2 samples, and the lowest value of 150 cfu×L^{-1} in one sample (Fig. 2). Of the total 12 positive samples, *L. pneumophila* sg 1 was detected in 10 samples (83.3%), and *L. pneumophila* sg 2–14 was present in the remaining 2 positive samples (16.6%).

Table 1. Descriptive data on the occurrence of *Legionella pneumophila* in hot water samples from hotels and homes for the elderly and disabled in Split region

Premises identification	Sampling port	No. of sample	Positive samples: **L. pneumophila sg 1** (determination range)	**L. pneumophila sg 2-14** (determination range)
Hotels	Bathroom tap	66	2 (150-450 cfu×L^{-1})	0
	Kitchen and bar tap	13	2 (200-250 cfu×L^{-1})	2 (200-250 cfu×L^{-1})
	Other (well and jacuzzi)	18	0	0
Homes for the elderly and disabled	Bathroom tap	28	5 (250-1000 cfu×L^{-1})	0
	Kitchen tap	2	1 (500 cfu×L^{-1})	0

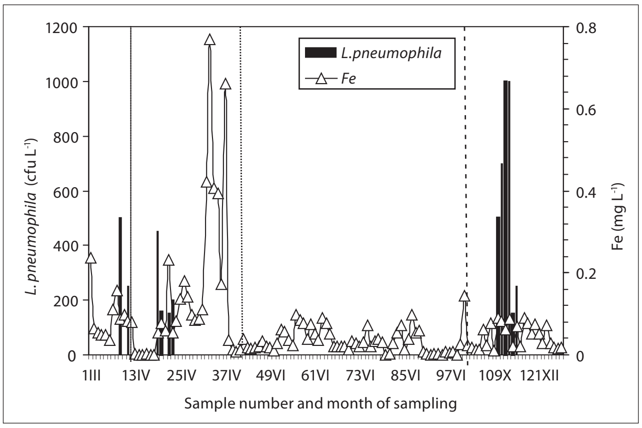

Figure 2. The presence of *L. pneumophila* and Fe concentrations in water samples

The values of examined physicochemical parameters, their relationship to *L. pneumophila* and results of statistical analysis (Mann-Whitney U test; Z and p), are presented in Table 2. The Mn data series presented in graphical form (Figure 3) by means of box and whiskers diagrams [30], summary plots based on the median, quartiles, and extreme values were used for statistical descriptions of Mn influence. Additionally, the temperature of samples was regularly determined, and observed values depicted in Figure 4.

Table 2. Median values and ranges of the measured parameters in examined water samples in relation to *L. pneumophila*

	Legionella pneumophila: Negative (n=115) Geometric mean (range)	Pozitive (n=12) Geometric mean (range)	Z	p
Chlorine residual (mg×L^{-1})	0.20 (0.10–0.30)	0.20 (0.10–0.30)	0.62	0.499
T (°C)	54.10 (22.3–65.4)	49.00 (42.2–53.4)	2.19	0.028
Zn (mg×L^{-1})	0.17 (0–0.59)	0.24 (0.13–0.43)	0.90	0.364
Fe (mg×L^{-1})	0.04 (0–0.77)	0.08 (0.02–0.23)	2.30	0.021
Cu (mg×L^{-1})	0.01 (0–0.44)	0.02 (0–0.03)	2.41	0.016
Mn (µg×L^{-1})	4.60 (0.37–18.45)	8.37 (5.51–17.20)	3.48	0.001

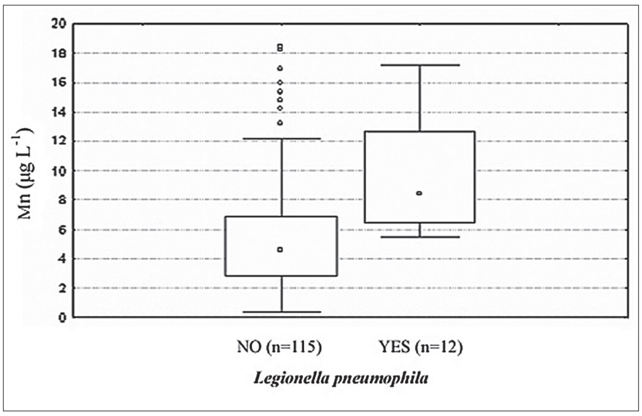

Figure 3. Median of Mn concentrations in water samples in relation to the presence of *L. pneumophila*

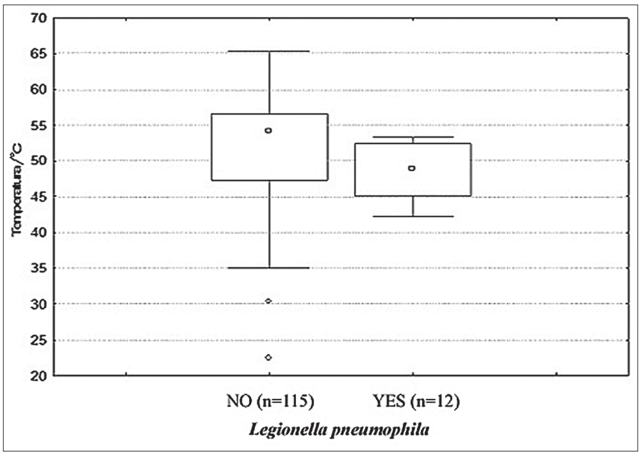

Figure 4. Median of water samples temperature values in relation to the presence of *L. pneumophila*

DISCUSSION

The monitoring of opportunistic pathogens such as *L. pneumophila*, both in water distribution mains and in consumers' plumbing systems, is an important issue according to the international and national guidelines that regulate drinking water quality. In recent years, the presence of *L. pneumophila* has been proved in hot water samples in countries such as Italy, France, Germany, Taiwan, and many others [5, 18, 22, 29], but the data for Croatia is rare. Therefore, the presence of *L. pneumophila* was investigated with a simultaneous determination of physical-chemical parameters, such as temperature, concentration of free residual chlorine, and concentrations of Fe, Mn, Zn and Cu in order to evaluate their influence on the presence of *L. pneumophila*.

The presence of *L. pneumophila* sg 1 was detected in 6 samples out of 30 samples originated from two homes for the elderly and disabled in comparison to 4 positive samples out of 97 samples that was analysed from total of 19 hotels (Tab. 1) Furthermore, the peak value of 1,000 cfu$\times$L^{-1} was found in 2 samples from bathroom taps at the homes for the elderly and disabled in Split region, indicating an increased health risk for such a population.

Generally, the determined values of examined physicochemical parameters were lower than maximum contaminant levels (MCLs), only a few samples contained Fe concentrations that were above MCLs. The water samples contaminated by *L. pneumophila* (positive samples) showed higher metal concentrations and lower temperature values in comparison to negative *L. pneumophila* samples. Table 2 shows that no statistically significant connection existed between the concentrations of Zn ($Z=0.90$; $p=0.364$) and free residual chlorine ($Z= 0.62$; $p=0.499$) with the appearance of *L. pneumophila*. It has been proved that *Legionella* species can be present in water with free residual chlorine as well as in water where it was not found, confirming the *Legionella* species resilience to free residual chlorine [21].

Although statistical analysis showed no significant connection between Zn and *L. pneumophila* presence, observed Zn concentrations were in the range of 0-0.59 mg$\times$L^{-1} and the *L. pneumophila* positive samples, if compared to negative ones, had a slightly higher Zn content (Tab. 2). Similarly, higher Zn content in *Legionella* positive samples was reported [31], but higher concentrations (values higher than 0.37 mg$\times$L^{-1}) were reported to have positive association with *Legionella* presence [18]. On the contrary, lower Zn content in *Legionella* positive samples and threefold higher value in *Legionella* negative samples with no statistically significant correlation were determined during another study [32]. With respect to this, generally lower values (geometric mean of 0.24 mg$\times$L^{-1}) obtained during this study could be the reason for statistical non-significance.

Out of 127 analyzed samples, 10 contained Fe concentrations above maximum contaminant levels (Fig. 2). Corrosion (higher concentrations of Fe ions) increases the surface roughness of inner pipe surfaces, thus increasing the contact surface between the water and the water pipe, as well as the possible formation of ecological niches which are protected from disinfectants. Corrosion also increases the concentration of nutrients for the growths of biofilms which firmly attach themselves to the rough surfaces. Uncontrolled biofilm growth can cause pipe blockage, particularly in parts of the water system with low flow-rates. The above factors foster *L. pneumophila* presence in water distribution systems [20].

The statistical analysis of Fe concentration spans showed the largest differences in spans in *L. pneumophila* negative samples (the span was 0.77), in comparison to samples with proven *L. pneumophila* (the span was 0.21), but the median Fe concentration values for negative and positive samples were 0.04 and 0.08 mg$\times$L^{-1}, respectively (Tab. 2). The twice as big values obtained in samples with *L. pneumophila* ($Z=2.30$; $p=0.021$) indicated positive association of *L. pneumophila* with Fe concentration. In a recent comprehensive study, Bargellini et al. [18] reported similar Fe values and positive association with the presence of *Legionella*. In detailed statistical analysis, they set cut-off values of 42 μg$\times$L^{-1}, which was discussed as being sufficient to increase the colonisation risk. Similarly, a large number of samples had a value higher than 42 μg$\times$L^{-1}, confirming the *Legionella* risk (Fig. 2).

In water samples with and without *L. pneumophila*, Cu median concentrations were 0.02 and 0.01 mg$\times$L^{-1}, respectively. Statistical analysis indicated the data span for Cu in positive samples of 0.03, a larger span (0.44) for samples without *L. pneumophila* and the positive association with the presence of *L. pneumophila* ($Z=2.41$; $p=0.016$). This was unexpected since the inhibitory effect of copper was reported and the higher Cu levels (> 50 μg$\times$L^{-1}) were associated with a lower risk of *Legionella* proliferation [16, 18, 31], but according to the literature, the lower Cu concentrations were not associated with *Legionella* presence [32].

The Mn data series presented in graphical form (Fig. 3) clearly indicate the difference of medians between samples in which *L. pneumophila* was proved and those in which it was not found. The Mn concentrations of 4.6 μg×L^{-1} (geometric mean) were found in *L. pneumophila* negative samples, while the geometric mean of positive samples was 8.37 μg×L^{-1}. The span of Mn concentration values in water samples with and without *L. pneumophila,* indicates variations amounting to 11.69 and 18.08, respectively; however, the median of Mn concentrations in samples with *L. pneumophila* is almost twice as large as in the negative samples (Z=3.48; p=0.001). Accordingly, the presence of *L. pneumophila* was positively associated with Mn concentrations, and *L. pneumophila* was mainly present in samples with Mn levels higher than 6 μg×L^{-1}(Fig. 3). This level was in agreement with the previously reported study in which the correlation of Mn levels and *Legionella* occurrence were discussed in detail and highlighted, and the Mn concentration lower than 6 μg×L^{-1} was found to be a good indicator of *Legionella* absence [18].

The water temperature is known to be a decisive factor in Legionella colonization, since its growth is inhibited as the temperature exceeded 42 °C; therefore, temperatures higher than 65 °C have even been used in a disinfection study [24]. During this investigation, in 62 samples out of 127 (48.82%), a temperature higher than 55 °C was determined (Fig. 4), and *L. pneumophila* was not determined in any samples with temperatures above this value. The observed protective effect of this temperature value has been reported previously [18]. In addition, the median temperature for samples without *L. pneumophila* is 5 °C higher than in those in which it was found (Z=2.19; p=0.028) (Tab. 2) and the temperature span of 30 °C was determined in negative samples (without *L. pneumophila*), while in positive samples it was 11.2 °C (Fig. 4). The lowest measured temperatures of samples in which the *L. pneumophila* sg 1 was isolated, ranged from 42.2-43.1 °C, as opposed to samples with proven *L. pneumophila* sg 2-14 where the median of measured temperatures was 49 °C. Borella et al. [33] also found *L. pneumophila* sg 2-14 to be present in water samples with higher temperatures and smaller concentrations of free residual chlorine. The same study also determined *L. pneumophila* sg 2-14 as the main cause of legionelosis in hospitals. Obviously, observed higher temperature values (>55 °C) were one of the main factors that contribute to the lower *L. pneumophila* presence and lower *Legionella* risk.

CONCLUSIONS

Out of 127 hot water samples analyzed, the presence of *L. pneumophila* was found in 9.4% of the samples. Heavy metals concentrations, temperature and free residual chlorine were measured in order to identify reliable indicators of hot water quality able to predict *L. pneumophila* risk. Water samples positive for *L. pneumophila* exhibited significantly higher Fe and Mn concentrations compared to negative samples. At the same time, statistical analysis showed that zinc and free residual chlorine has no influence on the presence of *L. pneumophila*, confirming the low efficacy of free chlorine on microbe eradication. The presence of *L. pneumophila* was proved at lower sanitary water temperatures (42.2 °C -53.4 °C) and higher concentrations of Fe and Mn. Higher concentrations of Fe indicate the corrosion of the metal piping system and favour conditions for the growth and breeding of *L. pneumophila*. Observed results indicate that the presence of Fe and manganese in concentrations higher than 42 μg×L^{-1} and 6 μg×L^{-1}, respectively, could be good predictors of *L. pneumophila* presence, while temperatures above 55 °C lower *Legionella* risk.

The presented study provides an insight into the quality of hot water distribution systems. The obtained results can provide a basis for decisions concerning protective measures for water quality assurance and for the promotion of human health. Moreover, the development of water system and cooling tower maintenance programmes (thorough cleaning practices and periodical replacement of system components) as well as the use of separate water heaters can reduce and eliminate the presence of the *Legionella* spp. and the occurrence of legionary disease can be reduced.

Acknowledgments

We would like to thank our colleagues at the Public Health Institute of Split & Dalmatian County, who contributed in different ways to this research, as well as Prof. Nađa Dešpalj, Lecturer of English at Zagreb University, for the translation of this paper into English.

REFERENCES

1. Carvalho FR, Vazoller RF, Foronda AS, Pellizari VH. Phylogenetic study of legionella species in pristine and polluted aquatic samples from a tropical Atlantic forest ecosystem. Curr Microbiol. 2007; **55**: 288-293.
2. Hsu BM, Lin C.L, Shih FC. Survey of pathogenic free-living amoebae and Legionella spp. in mud spring recreation area. Water Res. 2009; 43: 2817-2828.
3. Parthuisot N, West NJ, Lebaron P, Baudart J. High diversity and abundance of Legionella spp. in a pristine river and impact of seasonal and anthropogenic effects. Appl Environ Microbiol. 2010; 76: 8201-8210.
4. Moritz M M, Flemming H C, Wingender J. Integration of Pseudomonas aeruginosa and Legionella pneumophila in drinking water biofilms grown on domestic plumbing materials. Int J Hyg Envir Heal. 2010; 213: 190-197.
5. Huang S W, Hsu B M, Wu S F, Fan C W, Shih F C, Lin Y C, Ji D D. Water quality parameters associated with prevalence of Legionella in hot spring facility water bodies. Water Res. 2010; 44: 4805-4811.
6. Gomez-Valero L, Rusniok C, Buchrieser C. *Legionella pneumophila*: Population genetics, phylogeny and genomics. Infect Genet Evol. 2009; 9: 727-739.
7. Hoffman P, Friedman H, Bendinelli M. *Legionella Pneumophila:* Pathogenesis and Immunity, New York, Springer, 2008.
8. Bacterial Nomenclature Up-to-date. http://www.dsmz.de/bactnom/bactname.htm (acess: 2012.03.29).
9. Edelstein P A. Legionnaires' disease: history and clinical findings. In: Heuner K, Swanson M. Legionella: Molecular Microbiology. Norfolk, Caister Academic Press, 2008. p.1-19.
10. Schoen M E, Ashbolt N J. An in-premise model for Legionella exposure during showering events. Water Res. 2011; 45: 5826-5836.
11. Bagh L K, Albrechtsen H J, Arvin E, Ovesen K. Distribution of bacteria in a domestic hot water system in a Danish apartment building. Water Res. 2004; 38: 225-235.
12. Wingender J, Flemming HC. Contamination potential of drinking water distribution network biofilms. Water Sci Technol. 2004; 49: 277-286.
13. Payment P, Robertson W. The microbiology of piped distribution systems and public health. In Ainsworth R. Safe Piped Water: Managing Microbial Water Quality in Piped DistributionSystems. World Health Organization, London, IWA Publishing, 2004.
14. EPA, Water Distribution System Analysis: Field Studies, Modelling and Management. A Reference Guide for Utilities. Ohio, U. S. Environmental Protection Agency, 2005.
15. Eboigbodin K E, Seth A, Biggs C A. A review of biofilms in domestic plumbing. J. Am Water Works Assoc. 2008; 100: 131-138.

16. van der Kooij D, Veenendaal HR, Scheffer WJ. Biofilm formation and multiplication of Legionella in a model warm water system with pipes of copper, stainless steel and crosslinked polyethylene. Water Res. 2005; 39: 2789-2798.
17. Kusnetsov J, Iivanainen E, Elomaa N, Zacheus O, Martikainen P J. Copper and silver ions more effective against legionellae than against mycobacteria in a hospital warm water system. Appl Environ Microbiol. 2001; 35: 4217-4225.
18. Bargellini A, Marchesi I, Righi E, Ferrari A, Cencetti S, Borella P, Rovesti S. Parameters predictive of Legionella contamination in hot water systems: association with trace elements and heterotrophic plate counts. Water Res. 2011; 45: 2315-2321.
19. Habich W, Müller H E. Occurrence and parameters of frequency of legionella in warm water systems of hospitals and hotels in Lower Saxony. Zentralbl Bakteriol Mikrobiol Hyg B. 1988; 186: 79-88.
20. Cianciotto NP. Iron acquisition by *Legionella pneumophila*. Biometals. 2007; 20: 323-331.
21. Bertram J, Chartier Y, Lee J V, Pond K, Whipple S L. Legionella and the prevention of legionellosis. Geneva, Switzerland, *WHO Press*., World Health Organization, 2007. p. 30-37.
22. Buse H Y, Schoen M E, Ashbolt N J. Legionellae in engineered systems and use of quantitative microbial risk assessment to predict exposure. Water Res. 2012; 46: 921-933.
23. Marchesi I, Marchegiano P, Bargellini A, Cencetti S, Frezza G, Miselli M, Borella P. Effectiveness of different methods to control legionella in the water supply: ten-year experience in an Italian university hospital. J Hosp Infect. 2011; **77**: 47-51.
24. Mouchtouri V, Velonakis E, Hadjichristodoulou C. Thermal disinfection of hotels, hospitals, and athletic venues hot water distribution systems contaminated by *Legionella* species, Am J Infect Control. 2007; 35: 623-627.
25. WHO Guidelines for drinking - water quality. 2nd ed. Addendum: Microbiological agents in drinking water. Geneva, Switzerland, WHO Press, World Health Organization, 2007.
26. HZN (Hrvatski Zavod za Norme) HRN EN ISO 19458: Water quality – Sampling for microbiological analysis, 2008.
27. APHA Standard Methods for the Examination of Water and Wastewater, 19th ed. Washington DC, American Public Health association/American Water Works Association/Water Environment Federation, 1995.
28. HZN (Hrvatski Zavod za Norme) HRN EN ISO 11731-2: Water quality - Detection and enumeration of Legionella - Part 2: Direct membrane filtration method for waters with low bacterial counts, 2008.
29. Ditommaso S, Giacomuzzi M, Gentile M, Ruggenini Moiraghi A, Zotti C M. Effective environmental sampling strategies formonitoring *Legionella* spp contamination in hot water systems. Am J Infect Control. 2010; 35: 344-349.
30. Kirkwood BR; Sterne JAC. Essential Medical Statistics, 2nd ed. Oxford, Blackwell Science, 2003.
31. Leoni E, De Luca G, Legnani P P, Sacchetti R, Stampi S, Zanetti F. Legionella waterline colonization: detection of Legionella species in domestic, hotel and hospital hot water systems. J Appl Microbiol. 2005; 98: 373-379.
32. Edagawa A, Kimura A, Doi H, Tanaka H, Tomioka K, Sakabe K et al. Detection of culturable and nonculturable Legionella species from hot water systems of public buildings in Japan. J Appl Microbiol. 2008; 105: 2104-2114.
33. Borella P, Montagna M T, Romano-Spica V, Stampi S, Stancanelli G, Triassi M, et al. Legionella infection risk from domestic hot water. Emerg Infect Dis. 2004; 10: 457-464.