The Difference of Water Quality in Permanently vs. Seasonally Used Water Supply Systems

ANITA RAKIĆ[1], LUCIJA FOGLAR[2]*
[1]Public Health Institute of Split,
Vukovarska 46, Split
anita.rakic@st.t-com.hr
[2]*Division of Industrial Ecology,
Faculty of Chemical Engineering and Technology, University of Zagreb,
Marulićev trg 20, Zagreb,
CROATIA
lfoglar@fkit.hr www.fkit.hr

Abstract: The presence of opportunistic bacteria such as *Legionellae* in water presents a public health problem especially in accommodation facilities due to the possible risk of infection and development of Legionnaires´ disease. The water quality in hotel water distribution systems (WDS) was monitored during 3 years in order to estimate *Legionella pneumophila* colonization risk and to determine the difference in *Legionellae* presence between the all year-round open hotel and the seasonal hotel (open only during the summer months) in an attempt to identify risk factors for *Legionella pneumophila* colonization in the Split Dalmatian County, Croatia. Furthermore to emphasize the possible impact and the role of metal ions, the iron, manganese, copper and zinc concentrations were monitored in 159 hot water samples taken from 2 hotels in the period from January 2009 to January 2012. The concentrations of metal ions and the presence of *Legionella pneumophila* were determined by AAS and by the BCYE agar cultivation method, respectively.
During this study, the water quality monitoring results were used for detail statistical analyses. The dependence between permanent and seasonally opened systems was investigated and the seasonal variations of Fe, Zn, Mn and Cu in those WDS were observed and correlated with *Legionella* presence. Differences in metal concentrations between the seasonal and year-round accommodation facilities were evident, and metal concentrations were generally higher in the seasonal hotel. *Legionella pneumophila* was isolated in 24 out of 77 hot water samples (31.2%) in the hotel opened only during the summer months and in 24 out of the 82 samples (29.3%) in the all year-round open hotel. Higher concentrations of heavy metals observed in samples from the hotel open only during the summer were in accordance with the corrosion of the metal piping system and with the reduced water flow in seasonal systems, which favoured conditions for the growth and breeding of *Legionella* spp. WDS in hotels can be heavily colonized by *Legionella pneumophila* and may increase a risk of Legionnaires´ disease for tourists, therefore the monitoring of some water quality parameters could be used for the prediction of possible *Legionellae* colonisation.

Key Words*: Legionella pneumophila*, copper, iron, manganese, zinc, water distribution systems.

1 Introduction

The presence and quality of water was crucial for life, health and development on Earth. The presence of different microorganisms in drinking water distribution systems (DWDS) presents a health risk [1]. In recent years, it has become evident that biofilms in drinking water distribution networks can become long-term habitats for different microorganisms such as *Legionella* spp., *Pseudomonas aeruginosa*, *Campylobacter* spp., *Cryptosporidium parvum* and others. These microorganisms can attach to pre-existing biofilms, where they become integrated and survive for days to weeks depending on the biology and ecology of the organism and the environmental conditions. Furthermore, their presence is especially critical for the increasing proportion of sensitive human populations such as infants, the very elderly and immunocompromised persons, which can be highly susceptible to infection by these bacteria [2].

In water distribution systems, interfaces for biofilm growth are usually solid-liquid as in piped WDS [3,4]. Important sources of infection are aerosols generation sites such as cooling towers, whirlpools and water outlets such as showerheads or

taps. The main transmission pathway is the inhalation of contaminated aerosols [5]. *Legionellae* were identified in biofilms on filter materials (swimming pool filters, sand filters), in plumbing systems of public and private buildings and in corroded products and sediments of water storage tanks [6,7]. Sediments in WDS can stimulate the growth of aquatic microflora, including the growth of *Legionella pneumophila* [8]. Corrosion and dead-ends promote *Legionellae* biofilm formation. Therefore, many researchers have focused on biofilms in WDS [9-12].

The colonisation of different microorganisms and the formation of biofilms were dependant on numerous factors. The influence of plumbing materials, the presence and concentrations of metal ions, the application of different disinfectants and the water temperature were some of the factors that were monitored and evaluated in earlier studies [13-15]. Their results along with results of recent studies indicate that the presence of *Legionella* species were 10× higher inside the pipes than in water samples alone [16]. The presence, survivor and proliferation of *Legionellae* in WDS was most often determined in heating units since higher temperatures especially those between 30-50 °C favoured *Legionellae* growth [17,18]. Due to the proved health risk of *Legionella* spp. presence in WDS and in order to decrease the presence of *Legionellae* in water, recent investigations intensively study the correlation of physicochemical parameters of water with the presence of *Legionella* species [19-21]. Generally, higher pH, lower temperatures and lower chlorine content in water favour *Legionellae* proliferation. The common chlorine concentrations applied for disinfection in WDS were often insufficient for complete eradication of *Legionellae*, therefore its presence was determined in public supply water samples [21]. Along with the presence of organic and inorganic pollutants in the water, the growth and survival of *Legionella* species was favoured by water stagnation in dead ends of WDS such as reservoirs, heaters, pools and others [22,23].

The presence and influence of different metal ions were also monitored, and the results indicated that some metal ions facilitate proliferation of *Legionellae,* while some others have a negative impact on *Legionella* presence. As an example, the positive correlation between the presence of *Legionella pneumophila* and iron ions present in water at concentrations higher than 0.042 mg/L was proven [24,25]. On the contrary, Cu concentrations higher than 0.05 mg/L and Zn, Fe and Mn concentrations lower than 0.1, 0.02 and 0.006 mg/L respectively, contribute to the lower risk of *Legionella pneumophila* proliferation in hot water systems [21,25,26]. Obviously, the contamination of hot water systems in public buildings has been found to be an important risk factor for *Legionella* infection [27,28].

2 Problem formulation

The purpose of this study was to investigate *Legionella pneumophila* colonization in water distribution systems. For that purpose, the 159 hot water samples were taken from two hotels, one was the all year-round open hotel and the second one was open only during the summer months and analysed. The obtained data was further statistically analysed in order to determine the difference and impact of such a working regime on the *Legionella pneumophila* presence and to identify the risk factors associated with the water characteristics.

2.1 Materials and methods

Hot water samples (159) were collected from two hotels in the period from January 2009 to January 2012 in the Split Dalmatian County. The first hotel is open only during the summer months and has a discontinuous water consumption and the second one is the all year-round open hotel. Both hotels have a central storage tank and have a one way distribution system for hot water from a storage water heater to the local tap with no return (central storage type).

2.1.1 Sample Collection

The collection of water samples for chemical and microbiological analyses was performed according to ISO 19458 [29]. The samples were drawn and placed in sterile plastic bottles (1 L), after a flow time of 5 min to eliminate any cold water present inside the tap or the flexible shower pipe. In order to neutralise the free residual chlorine, 10% sodium thiosulphate was added into the sterile bottles for microbiological analysis (1 mL/L). The samples were placed in portable coolers at 4 °C and transported to the laboratory for chemical and microbiological analyses.

2.1.2 Chemical and bacteriological analyses

Mass concentrations of iron, manganese, copper and zinc were measured in samples acidified with 1 % nitric acid, using flame atomic absorption spectroscopy (AAS Z-2000 Series, Hitachi, Tokyo, Japan). Instrument calibration standard solutions

were prepared from commercial materials. The AAS working conditions were given in Table 1.

Table 1 Operating conditions for the determination of metal ions.

Metal	**Fe**	**Zn**	**Cu**	**Mn**
Conditions				
Flame	C_2H_2/Air			
λ (nm)	248.3	213.9	324.80	279.30
Slit width (mm)	0.8	0.5	0.50	0.20
Detection limit (μg/L)	1.0	0.1	0.12	0.2

A microbiological analysis was carried out within 24 h and the isolation of *Legionella pneumophila* was performed by the cultural method according to the ISO 11731-2 standard technique [30]. The bacteria concentration was determined by membrane filtration using 0.20 μm filter paper (a polyamide filter, Millipore, Bedford, USA). The paper was re-suspended in a 10 mL water sample, and the bacterial cells were transferred in the sample solution. The sample solution (5 mL) was treated at 50 °C for 30 min and the concentrates (0.1 mL) were plated onto a buffered charcoal yeast extract (BCYE-a) agar with cysteine and charcoal yeast extract agar - cysteine free (bioMériux, Marcy l`Etoile, France) during 72 h at 36 °C. Colonies grown on BCYE agar with cysteine were further tested for *Legionella pneumophila* serogroup 1, serogroup 2-14 and *Legionella* non-pneumophila by the use of the agglutination test (Legionella Latex Test, Oxoid). The bacterial counts were expressed as the number of colony forming units (CFU) per litre and the detection limit of the procedure was 25 CFU/L (mean of two plates).

2.1.3 Statistical analysis

All calculations were performed using MedCalc 11.3.0.0; Software byba. Statistical analysis and the comparison of mean values, medians values, and percentiles (Per.) were performed by using the non-parametric Mann-Whitney U test [31] with the aim of determining the connection between *Legionella pneumophila* and the previously described variables. Results were considered statistically significant when the P value was <0.05.

3 Problem Solution

The results presented in this paper are a part of the water monitoring study of the Split Dalmatian County and were obtained in the period from January 2009 to January 2012. The average values of the monitored parameters were calculated for each institution and the differences of their distribution between permanently and seasonally opened hotels were analysed. The microbiological and chemical parameters variations of the hot water samples among permanently and seasonally opened hotels were observed to reflect on the water quality.

The microbiological findings of *Legionella* species obtained from permanently and seasonally opened hotels were positive for 24 out of 82 samples (29.3%) and for 24 out of 77 samples (31.2%) respectively (Table 2). The average values of *Legionella pneumophila* in positive samples of the all year-round open hotel and the seasonal hotel were 478 and 553 CFU/L respectively. Moreover, in the all year-round open hotel, the highest level of 6000 CFU/L was found in one sample and the lowest level of 50 CFU/L was found in 3 samples, while in the seasonal hotel, the highest level of 8000 CFU/L was found in two samples and the lowest level of 50 CFU/L was also found in two samples. Obtained results were similar to the data reported in earlier studies conducted in Germany, Finland, and Japan where *Legionella pneumophila* detection rates in hot water systems of public buildings were26%, 30% and 40% respectively [32-34].

The differences among the concentrations of iron and zinc were obvious (Table 3). The higher values were determined in the seasonally open hotel rather than in the all year-round open hotel with continual consumption. The presented results confirm the hypothesis about the linkage between the tested risk factors and the *Legionella* presence in the hot water from the WDS. It is known that water stagnation in the hot WDS might cause an increase of iron concentration and the corrosion products, so the concentrations of metal ions are important parameters in the survival and growth of *Legionellae* in WDS. Furthermore, iron and zinc are important components of oxidation-reduction systems and a cofactor of some important enzymes [35]. Therefore, chemical characteristics of hot water samples (metal ions concentrations) were evaluated for their association with *Legionella pneumophila* contamination.

Table 2 Findings of the *Legionella pneumophila* in hot water samples from the all year-round open hotel A) and the seasonal hotel B) in the Split region and statistical analysis within the quarters.

A)

			Quarters				
			1st quarter (1–3 month)	2nd quarter (4–6 month)	3rd quarter (7–9 month)	4th quarter (10-12 month)	**Total**
Presence of *Legionella pneumophila*	Negative samples	Count	16	18	12	12	58
		% within quarter	66.7%	60.0%	75.0%	100.0%	70.7%
	Positive samples	Count	8	12	4	0	24
		% within quarter	33.3%	40.0%	25.0%	0.0%	29.3%
	Total	Count	24	30	16	12	82

B)

			Quarters				
			1st quarter (1–3 month)	2nd quarter (4–6 month)	3rd quarter (7–9 month)	4th quarter (10-12 month)	**Total**
Presence of *Legionella pneumophila*	Negative samples	Count	1	34	16	2	53
		% within quarter	16.7%	79.1%	66.7%	50.0%	68.8%
	Positive samples	Count	5	9	8	2	24
		% within quarter	83.3%	20.9%	33.3%	50.0%	31.2%
	Total	Count	6	43	24	4	77

Table 3 The Fe, Zn, Cu and Mn concentrations in hot water samples collected from the all year-round open hotel A) and the seasonal hotel B).

A)

	C (mg/L)											
Quarters	1st quarter			2nd quarter			3rd quarter			4th quarter		
	Median	Per. 25	Per. 75	Median	Per. 25	Per. 75	Median	Per. 25	Per. 75	Median	Per. 25	Per. 75
Fe	0.02	0.00	0.04	0.03	0.02	0.06	0.03	0.02	0.03	0.01	0.00	0.02
Zn	0.15	0.04	0.17	0.16	0.07	0.44	0.04	0.04	0.06	0.13	0.12	0.14
Cu	0.01	0.01	0.01	0.01	0.00	0.01	0.00	0.00	0.00	0.01	0.01	0.01
Mn	0.00019	0.00	0.0018	0.0150	0.0121	0.0203	0.0229	0.0134	0.0235	0.00	0.00	0.00

B)

	C (mg/L)											
Quarters	1st quarter			2nd quarter			3rd quarter			4th quarter		
	Median	Per. 25	Per. 75	Median	Per. 25	Per. 75	Median	Per. 25	Per. 75	Median	Per. 25	Per. 75
Fe	0.09	0.08	0.11	0.03	0.02	0.07	0.03	0.03	0.09	0.16	0.12	0.29
Zn	0.18	0.09	0.25	0.14	0.03	0.18	0.14	0.08	0.23	0.05	0.03	0.08
Cu	0.01	0.01	0.01	0.02	0.00	0.09	0.01	0.00	0.01	0.06	0.03	0.09
Mn	0.00895	0.0083	0.0097	0.0128	0.0064	0.0279	0.00765	0.0061	0.0092	0.00001	0.00	0.00001

Table 3 represents chemical characteristics associated with *Legionella pneumophila* contamination in hot water samples during four seasons. As can be seen from Table 3 A) and B), during the study period, the highest median Fe concentration of 0.16 mg/L was recorded during the 10^{th}–12^{th} month (4^{th} quarter) in the seasonal hotel while the lowest value of 0.10 mg/L was obtained in the same quarter but in samples from the all year-round open hotel. The Fe concentration and the concentration of *Legionella pneumophila* in hot water samples were shown in Figure 1 A) and B) and the positive correlation of Fe concentration with *Legionella pneumophila* was observed. Similar results have been shown in recent investigations performed in the Mediterranean region [25,26].

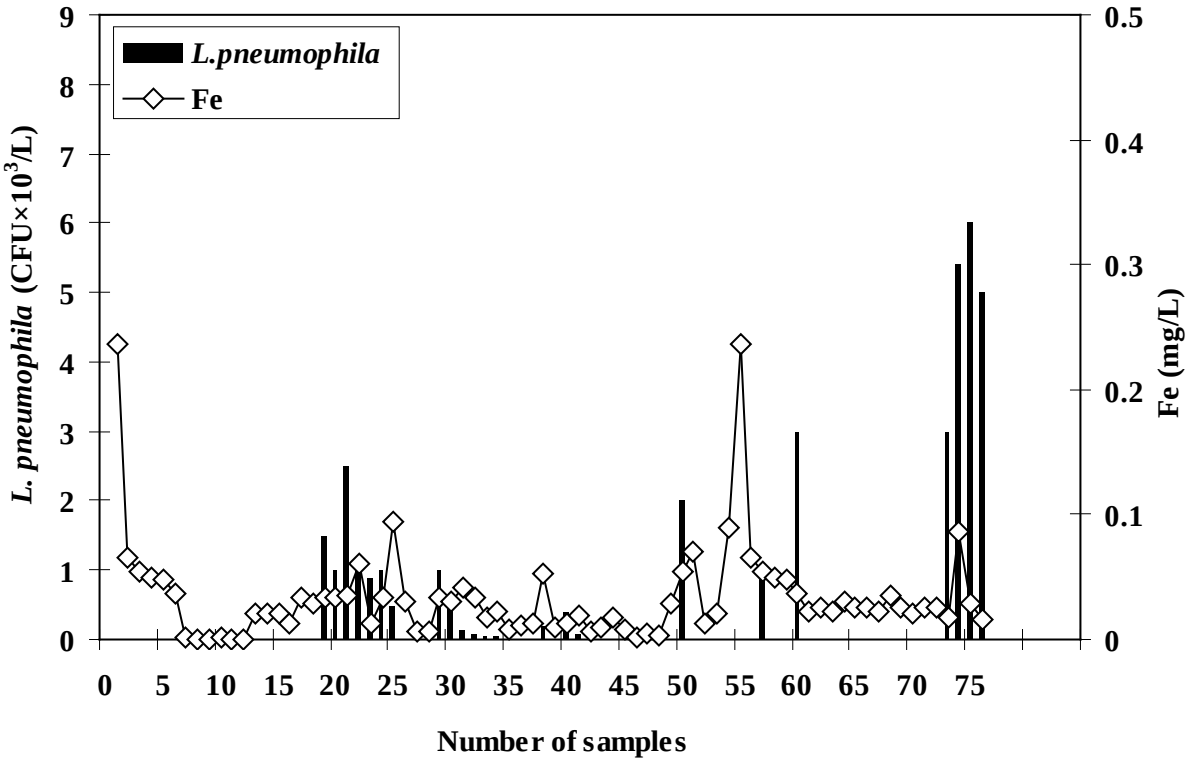
During the study period, the annual average Fe concentration in samples from the year-round accommodation facility was 0.033 mg/L, but the highest Fe concentration of 0.237 mg/L was determined in two samples and the minimum of 0 mg/L was recorded in four samples. At the same time in the seasonal facility, the annual average Fe concentration was 0.066 mg/L, the highest Fe concentration of 0.385 mg/L was determined in one sample and the minimum of 0.002 mg/L was also noticed in one sample. Obviously, higher Fe values detected in the seasonal hotel contributed to a higher *Legionella* contamination. In a recent study, the similar Fe values and positive association with the presence of *Legionellae* were reported [25]. In comprehensive statistical analysis, the cut off value of 0.042 mg/L was discussed as sufficient to increase the colonisation risk. Consequently, a large number of samples from the seasonal hotel that had a value higher than 0.042 mg/L was congruent to this assumption and confirmed the increased risk of *Legionellae* presence. In agreement with theoretical knowledge, our results confirmed that metal plumbing components and associated corroded products are important factors in the survival and growth of *Legionellae* in DWDSs. Corrosion can develop crevices and cracks on pipe walls which can shelter *Legionellae* and other pathogenic bacteria, and increase turbidity in DWDSs, and can consequently promote bacterial regrowth [1,36].

The highest median Zn concentration of 0.18 mg/L was observed during the 1^{st}–3^{rd} month in the seasonal hotel while the lowest value of 0.04 mg/L was determined in the all year-round open hotel during the 7^{th}–9^{th} month (Figure 2). The observed Zn concentrations were in the range of 0-0.486 mg/L, but statistical analysis of data showed no significant correlation of Zn and *Legionella pneumophila* presence. Furthermore, the comparison of Zn levels in samples from seasonal and all year-round open hotel showed no significant influence of Zn on *Legionella* presence. The higher Zn content in *Legionella* positive samples was reported [27] and values higher than 0.37 mg/L were indicated to have a positive association with *Legionella* presence [25]. Recently during another study, lower Zn content in *Legionella* positive samples and threefold higher value in *Legionella* negative samples with no statistically significant correlation were determined [33]. Obviously the role of Zn and its correlation with *Legionella* presence should be further investigated.

The highest Cu concentration of 0.06 mg/L was noticed during the 10^{th}–12^{th} month, also in the seasonal facility, while the lowest Cu value of 0.00 mg/L was obtained during the 7^{th}–9^{th} month in the all year-round open hotel. Generally, Cu content in hot water samples collected during the 3 years was low (Figure 2) and was not associated with *Legionella* presence. Similar results were previously determined in hot water samples from public buildings in Japan [33]. The previously mentioned inhibitory effect of copper discussed and confirmed during some studies was associated with Cu levels higher than 0.05 mg/L [25,27,37]. The values determined during our study in the seasonally open hotel and in the all year-round open hotel with continual consumption were similar and with respect to that, observed lower values of Cu were not found to be statistically significant and could not be associated with lower risk of *Legionellae* proliferation.

The Mn concentrations in the all year-round open and in seasonal hotels ranged from 0.00037 to 0.02916 mg/L and from 0.001 to 0.03166 mg/L respectively. But, the highest and the lowest median Mn concentration of 0.0229 mg/L and 0.00 mg/L were observed during the 7^{th}–9^{th} month and during the 10^{th}–12^{th} month respectively in the all year-round open hotel (Table 3). The comparison of Mn concentrations observed in analysed samples indicate that manganese concentration values were generally lower in the all year-round open hotel and more than 50 % of the samples containing less than 0.006 mg/L Mn. In literature, the presence and influence of Mn on *Legionella* presence was discussed and highlighted and the manganese concentrations in hot water samples lower than 0.006 mg/L were reported as a good indicator of *Legionella* absence [25]. Obviously, somewhat lower contamination with *Legionella* species observed in the all year-round open hotel (29.3%) was in accordance with lower Mn concentration.

A)



B)

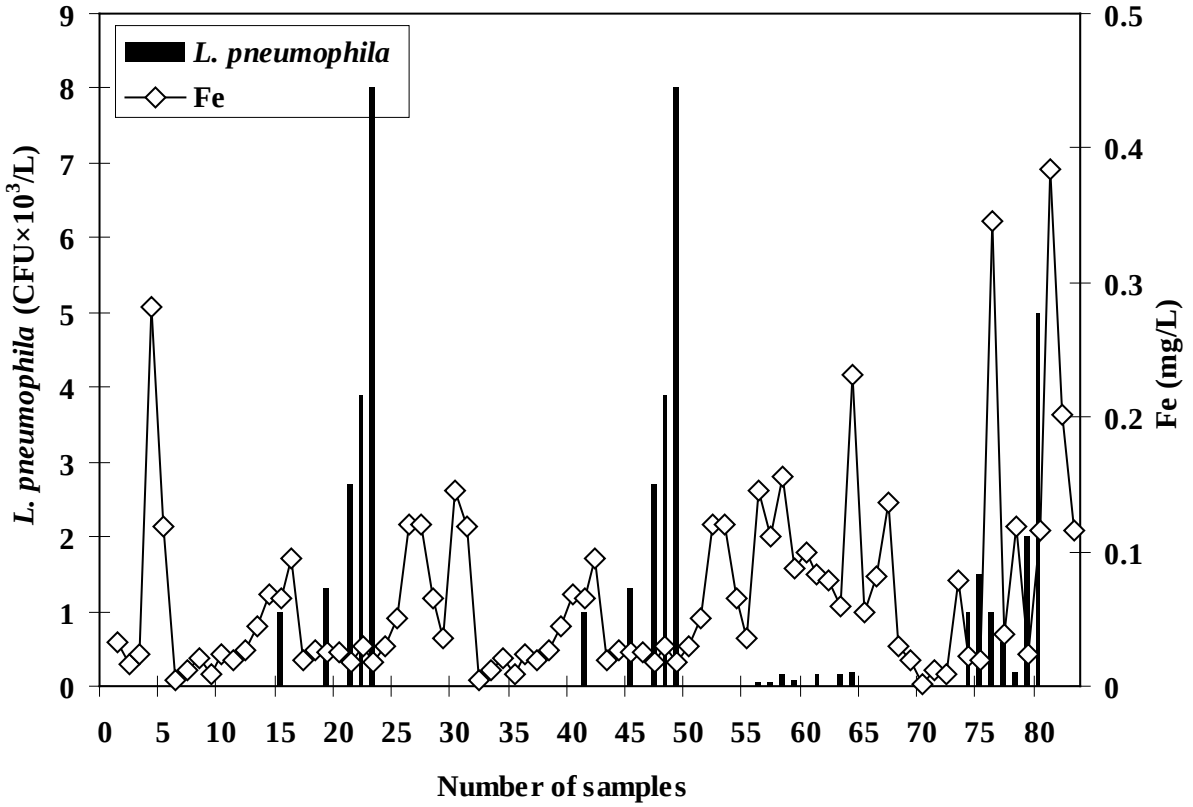
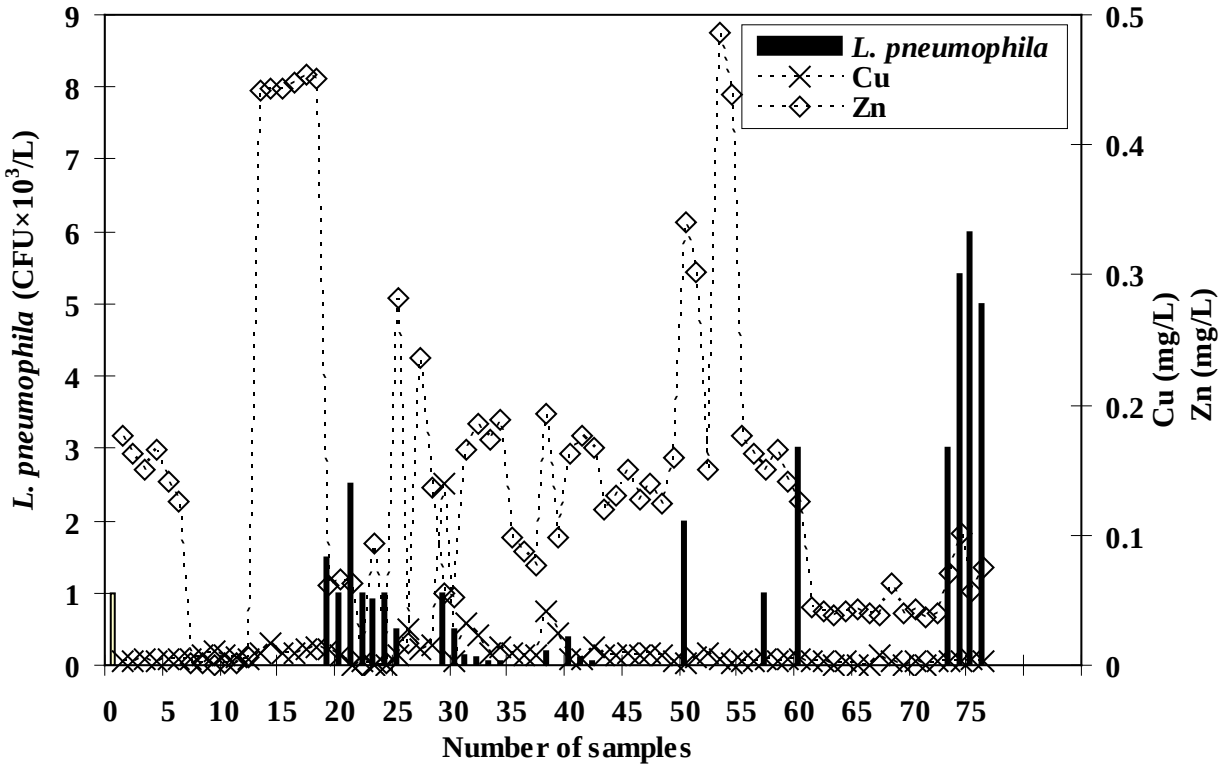


Figure 1 The presence of *Legionella pneumophila* and Fe concentrations in hot water samples in the all year-round open hotel A) and in the seasonal hotel B).

A)



B)

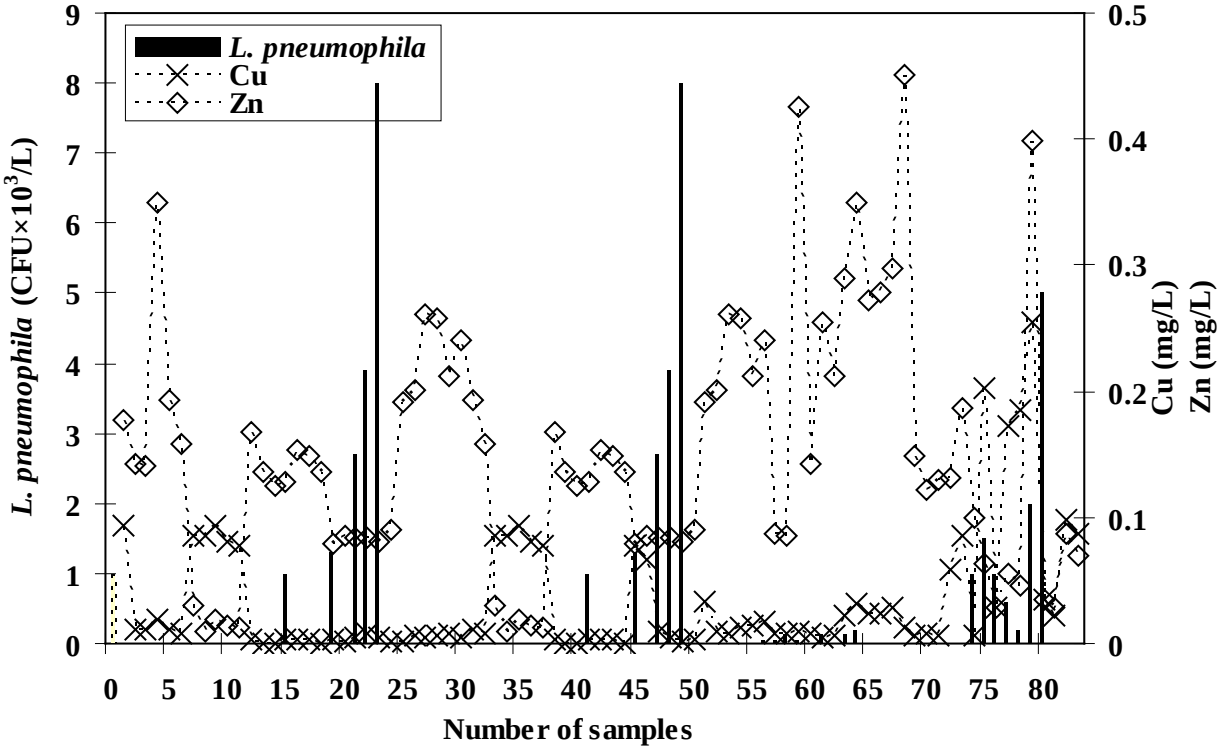


Figure 2 The presence of *Legionella pneumophila*, Cu and Zn concentrations in hot water samples in the all year-round open hotel A) and in the seasonal hotel B).

4 Conclusion

The results of Fe, Zn, Cu, Mn and *Legionella pneumophila* measurements in the WDS of the Split Dalmatian County, obtained from January 2009 to January 2012, were presented and the study demonstrated that the quality of water differs between the all year-round open hotel and seasonal hotel. Both hotels were colonized by *Legionella pneumophila* and its presence could represent a possible risk for Legionnaires´ disease.

The results of this study indicated that in the all year-round open hotel and the seasonal hotel, 29.3% and 31.2% of hot water samples, respectively were contaminated by*Legionella pneumophila.*

The metal ion concentration monitoring and the statistical analysis indicated that differences among the iron and zinc concentrations were obvious and the higher values were determined in the seasonally open hotel rather than in the all year-round open hotel with continual consumption. The comparison of observed Mn levels indicate that manganese concentration values were generally lower in the all year-round open hotel which contributed to the somewhat lower contamination with *Legionella* species. The inhibitory effect of Cu is known, but determined low levels of Cu showed no significant influence on *Legionella* presence.

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 WDS. These results suggest that the hot water in public buildings, especially in the seasonal accommodation facilities present a potentially higher risk for *Legionella* infection. Obviously, earlier mentioned stagnation of water should be further monitored and the prevention of water stagnation in hot water systems should be an important factor in reducing *Legionella* spp. contamination.

Acknowledgments

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

References

[1] Kolyshkin A., Case study: Bacterial regrowth in water distribution networks, *Proceedings of the 5th WSEAS International Conference on Education and Educational Technology*, Tenerife, Canary Islands, Spain, December 16-18, 2006, pp. 206-209.

[2] Haridas M., Experimental approaches and optical calculations in sensing biological pathogens, *WSEAS Transactions on Biology and Biomedicine*, Vol.2(2), 2005, pp. 235-242.

[3] Flemming H.C., *Biofilm Highlights*, Springer-Verlag Berlin Heidelberg, 2011.

[4] Declerck P., Behets J., van Hoef V., Ollevier F., Detection of legionella spp. and some of their amoeba host in floating biofilms from antropogenic and natural aquatic environments, *Water Research*, Vol.41, 2007, pp. 3159-3167.

[5] Taylor M., Ross K., Bentham R., *Legionella*, protozoa and biofilms: Interactions within complex microbial systems, *Microbial Ecology*, Vol.58, 2009, pp. 538-547.

[6] Loret J.F., Greub G., Free-living amooebae: biological by-passes in water treatment, *International Journal of Hygiene and Environmental Health*, Vol. 213, 2010, pp. 167-175.

[7] Bonadonna L., Briancesco R., Libera S.D., Lacchetti I., Paradiso R., Semproni M., Microbial characterization of water and biofilms in drinking water distribution systems at sport facilities, *Central European Journal* of *Public Health*, Vol.17, 2009, pp. 99-102.

[8] Stout J.E., Yu V.L., Best M.G., Ecology of *Legionella pneumophila* within water distribution systems, *Applied and Environmental Microbiology*, Vol.49, 1985, pp. 221-228.

[9] Långmark J., Storey M.V., Ashbolt N.J., Stenström T.A., Biofilms in urban water distribution system: measurement of biofilm biomass, pathogens and pathogen persistence within the Greater Stockholm area, Sweden, *Water Science and Technology*, Vol.52, 2005, pp. 181-189.

[10] Storey M.V., Ashbolt N.J., Stenström T.A., Biofilms, thermophilic amoebae and *Legionella pneumophila* – a quantitative risk assessment for distributed water, *Water Science and Technology*, Vol.50, 2004, pp. 77-82.

[11] Walker J.T., Rogers J., Keevil C.W., An investigation of the efficacy of a bromine-containing biocide on an aquatic consortium of planktonic and biofilm microorganisms including *Legionella pneumophila*, *Biofouling*, Vol.8, 1993, pp. 47-54.

[12] Koiv T-A., Voll H., Haniv A., Domestic hot water consumption in educational premises, apartment and office buildings, *WSEAS Transactions on Environment and Development*, Vol.6(1), 2010, pp. 43-52.

[13] Kuchta J.M., Stanley J., McNamara A.M., Wadowsky R.M., Yee R.B., Susceptibility of *Legionella pneumophila* to chlorine in tap water,

Applied and Environmental Microbiology, Vol.46 (5), 1983, pp. 1127-1134.
[14] Kusnetsov J., Iivanainen E., Eloma N., Zacheus O., Martikainen P.J., Copper and silver ions more effective against *Legionellae* than against mycobacteria in a hospital warm water system, *Applied and Environmental Microbiology*, Vol.35, 2001, pp. 4217-4225.
[15] Rogers J., Dowset A.B., Dennis P.J., Lee J.V., Keevil C.W., Influence of temperature and plumbing material selection on biofilm formation and growth of *Legionella pneumophila* in a model potable water system containing complex microbial flora, *Applied and Environmental Microbiology*, Vol.60(5), 1994, pp. 1585-1592.
[16] Bertram J., Chartier Y., Lee V.J., Pond K., Surman-Lee S., *Legionella and the prevention of Legionellosis*, WHO Press., Geneva, Switzerland, 2007, pp. 30-37.
[17] Moritz M.M., Flemming H.C., Wingender J., Integration of *Pseudomonas aeruginosa* and *Legionella pneumophila* in drinking water biofilms grown on domestic plumbing materials, *International Journal of Hygiene and Environmental Health*, Vol. 213, 2010, pp. 190-197.
[18] 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 Research*, Vol. 44, 2010, pp. 4805-4811.
[19] Mouchtouri V., Velonakis E., Hadjichristodoulou C., Thermal disinfection of hotels, hospitals, and athletic venues hot water distribution systems contaminated by *Legionella* species, *American Journal of Infection Control*, Vol.35, 2007, pp. 623-627.
[20] 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, *Journal of Hospital Infection*, Vol.77, 2011, pp. 47-51.
[21] Buse H.Y., Schoen M.E., Ashbolt N.J., *Legionella* in engineered systems and use of quantitative microbial risk assessment to predict exposure, *Water Research*, Vol.46, 2012, pp. 921-933.
[22] Muder R.R., Yun V.L., Infection due to *Legionella* species other than *L. pneumophila*, *Clinical Infectious Diseases*, Vol.35, 2002, pp. 990-998.
[23] Stout J.E., Rihs J.D., Yu V.L., *Legionella*. In: *Manual of Clinical Microbiology*, Murray, P.R., Baron, E.J., Jorgensen, J.H., Pfaller, M.A., Yolken, R.H. (eds). 8th edition. Washington, ASM Press, 2003, pp. 809-823.
[24] Cianciotto N.P., Iron acquisition by *Legionella pneumophila*, *Biometals*, Vol.20, 2007, pp. 323-331.
[25] 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 Research*, Vol.45, 2011, pp. 2315-2321.
[26] Borella P., Montagna M.T., Romano-Spica V., Stampi S., Stancanelli G., Triassi M., Neglia R., Marchesi I., Fantuzzi G., Tatò D., Napoli C., Quaranta G., Laurenti P., Leoni E., De Luca G., Ossi C., Moro M., D'Alcalà G.R., Risk factors associated with isolation of *Legionellae* from domestic hot water, *Emerging Infectious Diseases*, Vol.10, 2004, pp. 457-464.
[27] 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, *Journal of Applied Microbiology*, Vol.98, 2005, pp. 373-379.
[28] Borella P., Montagna M.T., Stampi S., Stancanelli G., Romano Spica V., Triassi M., Marchesi I., Bargellini A., Tatò D., Napoli C., Zanetti F., Leoni E., Moro M., Scaltriti S., D'Alcalà G.R., Santarpia R., Boccia S., *Legionella* contamination in hot water of Italian hotels, *Applied and Environmental Microbiology*, Vol.71, 2005, pp. 5805-5813.
[29] ISO 19458, *Water quality - Sampling for microbiological analysis*, International Standardization Organization, Geneva, 2006.
[30] ISO 11731, *Water quality – Detection and enumeration of Legionella*-Part 2: Direct membrane filtration method for waters with low bacterial counts, International Standardization Organization, Geneva, 2004.
[31] Kirkwood B.R., Sterne J.A.C. (2003). *Essential Medical Statistics*, 2nd ed. Oxford: Blackwell Science.
[32] Warren W.J., Miller R.D., Growth of Legionnaires disease bacterium (*Legionella pneumophila*) in chemically defined medium, *Journal of Clinical Microbiology*, Vol.10, 1979, pp. 50-55.
[33] Edagawa A., Kimura A., Doi H., Tanaka H., Tomioka K., Sakabe K., Nakajima C., Suzuki Y., Detection of culturable and nonculturable *Legionella* species from hot water systems of public buildings in Japan, *Journal of Applied Microbiology*, Vol.105, 2008, pp. 2104-2114.

[34] Shelton B.G., Flanders W.D., Morris G.K., Legionnaires` disease outbreaks and cooling towers with amplified *Legionella* concentrations, *Current Microbiology*, Vol. 28, 1994, pp. 359-363.
[35] Hutner S.H., Inorganic nutrition, *Annual Review of Microbiology*, Vol.26, 1972, pp. 313-346.
[36] Wullings B.A., Bakker G., van der Kooij, D., Concentration and diversity of uncultured *Legionella pneumophila* in two unchlorinated drinking water supplies with different concentrations of natural organic matter. *Applied and Environmental Microbiology*, Vol. 77(2), 2011, pp. 634–641.
[37] van der Kooij D., Veenendaal H.R., Scheffer W.J., Biofilm formation and multiplication of *Legionella* in a model warm water system with pipes of copper, stainless steel and cross linked polyethylene. *Water Research*, Vol.39, 2005, pp. 2789-2798.