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Short Communication

LEGIONELLA SPECIES IN YEAR-ROUND *VS.* SEASONAL ACCOMMODATION WATER SUPPLY SYSTEMS

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The purpose of this study was to compare the quality of hot water between eleven hotels in the Split-Dalmatia County, Croatia that are open year round and 10 summer season hotels and retirement homes with irregular use of water. We took 122 samples between May and December 2009. Water temperature and free residual chlorine were measured *in situ*. Physical and chemical analysis included pH, electrical conductivity, and concentrations of iron, manganese, copper, zinc, calcium, and magnesium that were measured using atomic absorption spectrophotometry, while the *Legionella* species were determined using a cultivation method on buffered charcoal yeast extract agar.

Differences in metal concentrations between the seasonal and year-round accommodation facilities were negligible, save for zinc that was higher in year-round (0.341 mg L^{-1}) than in seasonal facilities (0.130 mg L^{-1}).

Samples from all year-round and six summer season hotels were negative to the *Legionella* species, but four seasonal facilities turned up with positive samples to *Legionella pneumophila*.

Our study has demonstrated that water quality differs between year-round and seasonal accommodation facilities. These findings suggest that metal plumbing components and associated corrosion products are important factors in the survival and growth of *Legionella* species in water distribution systems.

KEY WORDS: *atomic absorption spectrophotometry, bacteria, biofilm, legionellosis, metals in water*

Water is a critical medium for the transmission of a large number of diseases (1). The reports of the World Health Organization (WHO) relate 80 % of human illnesses to water-transmitted diseases.

For water to act as a vehicle for a disease, it must be contaminated with pathogens and impurities such as dissolved minerals and decaying organic matter. Water quality index for the Split-Dalmatia County waters includes nine parameters: temperature, mineral content, corrosion coefficient K_1, dissolved oxygen, biochemical oxygen demand, total nitrogen, protein N, total phosphorus, and total coliform bacteria (the most probable number, MPN coli per 100 mL) (2).

Legionellosis is a group of infections caused by *Legionella pneumophila (L. pneumophila)* and related *Legionella* species, which are Gram-negative, rod-shaped bacteria. Populations which are the most susceptible to this kind of infection are the elderly, the immunosuppressed, and the smokers (3, 4). *Legionella* species prefer water temperatures between 25 °C and 55 °C for breeding. Other favourable conditions include bacterial or other organic contamination, corrosion, and stagnation of water (5). Sanitary requirements for potable water (6) exclude the presence of *Legionella* and scientific research conducted by various organisations such as the World

Health Organization (WHO) is focussed on their prevention.

Contagion is often spread through water supply in hotels or buildings used by a large number of people. In most cases, the plumbing is not well-maintained and/or used irregularly, so that water is stale and warm enough to provide ideal conditions for *Legionella* breeding. Moreover, these bacteria grow in warm and humid environments and can be transmitted by inhalation (7).

Research of certain metals influencing the development of *Legionella* species has shown that corrosion of the metal parts of plumbing systems and cooling towers can favour the survival and growth of the *Legionella* species (8). Potable water contains a low number of heterotrophic microorganisms, which can colonise hard surfaces and create biofilms, that is, stable eco-systems with large numbers of bacteria, protozoan parasites, and enteric hepatitis viruses. Organisms in biofilms often have increased tolerance to biocides and are hard to remove, especially from hard-to-reach surfaces. Biofilms re-contaminate purified water and micro-corrode metal surfaces beneath. Biofilm formation is the least pronounced on the surfaces of plastic tubes, which are smoother than metal materials, do not corrode and have a very long service life (9).

The aim of our study was to see whether there were differences between hot water quality in year-round and seasonally used plumbing systems, based of physical, chemical, and microbiological parameters.

METHODS

Water sampling

We took 122 hot water samples from 21 hotels and retirement homes in the Split-Dalmatia County, Croatia between May and December 2009. The median number of samples per facility was six (range 3 to 9). Eleven year-round hotels used water regularly throughout the year. Ten facilities used water only during the summer season (seasonal hotels) or had a restricted or irregular access to hot water (retirement homes), usually over weekends and holidays when the tenants would leave to visit families.

More frequent sampling was carried out in the retirement homes, because this population has a high percentage of chronic illnesses and weakened immune systems. In contrast, water samples from year-round hotels were taken once a year. Water in seasonal hotels was sampled before the season opened. In case of positive results, sampling was repeated.

Hot water samples were taken from bathroom outlets (shower heads or bathroom taps) in sterile polyethylene bottles (V=1 L) after we let it run for a while to eliminate cold water. To neutralise residual free chlorine, we added sodium thiosulphate ($Na_2S_2O_3$=0.01 mol L^{-1}; Merck, Germany) to bottles intended for microbiological analysis. To bottles intended for chemical analysis we added an acid preserver (0.5 mL of 65 % HNO_3 per 50 mL sample; Merck, Germany). The bottles were immediately stored in portable refrigerators at 4 °C. If analyses would not begin within 24 hours, the samples were kept at 4 °C to 8 °C and analysed within 48 hours of collection.

Physical and chemical analysis

pH was measured with a 826 pH mobile meter (Metrohm, Switzerland) according to the ISO 10523:1998 standard (10). Electrical conductivity was determined with a conductivity pocket meter (WTW, Germany) according to the ISO 7888:2001 standard (11). Water temperature was measured with a digital thermometer (Thermo Fischer Scientific, UK). Free residual chlorine was recorded using a portable digital apparatus (Thermo Fischer Scientific, UK). All instruments used were calibrated.

The concentrations of iron, manganese, zinc, calcium, copper, and magnesium were determined by atomic absorption spectrometry on a model Z-2000 instrument (Hitachi, Japan) using the graphite cuvette technique (12). The absorbance was measured at a wavelength of 248.3 nm for iron, 279.6 nm for manganese, 213.9 nm for zinc, 324.8 nm for copper, 422.7 nm for calcium, and 285.2 nm for magnesium. The limits of detection were 0.001 mg L^{-1} for iron, 0.0002 mg L^{-1} for manganese, 0.0001 g L^{-1} for zinc, and 0.00012 mg L^{-1} for copper.

Microbiological analysis

Microbiological analysis was focussed on the *Legionella* species (expressed in colony forming unit, CFU L^{-1}). Bacteria of the genus *Legionella* are important causes of both community-acquired and nosocomial pneumonia. According to the antigenic structure, *L. pneumophila* is divided in 14 serogroups. Human infections are caused by the *L. pneumophila* serogroup 1.

Microbiological analysis was performed in a specialised laboratory within 24 hours after the samples were taken. Cultivation and identification followed the ISO 11731 standard (13). One-litre water samples were filtered (0.20-µm-pore-sized polyamide filter, Millipore, Billerica, Massachusetts, USA) and filter paper re-suspended in 10 mL of the original sample water. Thereafter, 100 µL of the concentrated samples were inoculated onto GVPC selective medium (bioMérieux, Lyon, France). Plates were incubated with 2.5 % CO_2 at (36±1) °C for 7 days and analysed on day four with a dissecting microscope. Colonies with a morphology similar to that of the family *Legionellaceae* were subcultured on a buffered charcoal yeast extract (BCYE) medium with cysteine (bioMérieux, Lyon, France) and on a cysteine-free BCYE medium (bioMérieux, Lyon, France). The plates were incubated at (36±1) °C for two days. The colonies grown on BCYE were lucent, round, and had an integral rim. They were identified with the latex agglutination test (Oxoid, Cambridge, UK). This test enables separate identification of *L. pneumophila* serogroup 1 and serogroups 2 to 14, and detection of seven species of non *L. pneumophila* legionellae that have been implicated in human disease. The number of colonies is expressed in CFU L^{-1} (limit of detection = 10 CFU L^{-1}). Samples with confirmed presence of the *Legionella* species were considered positive.

Statistics

We calculated the averages for every accommodation facility and analysed the differences in parameters between year-round and seasonal facilities. To test the statistical significance we used non-parametric statistics. The results were analysed using the Mann-Whitney U-test and the chi-square test. The probability level of $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

This study seems to be the first of its kind to be performed in Croatia. It has included 80 % of all the hotel systems in Split and Split-Dalmatia County.

All the analysed water samples were clear, colourless, and odourless. The samples did not differ in pH and conductivity; mean pH was 7.61 and conductivity at 25 °C was 404 µS cm^{-1}.

However, we have established differences in microbiological and chemical properties between accommodation facilities that use hot tap water year-

Table 1 *Findings of the* Legionella *species in hot water samples by the type of accommodation facility. The average count of L. pneumophila in the positive samples was 450 CFU L^{-1}. The highest count (1000 CFU L^{-1}) was found in two and the lowest (150 CFU L^{-1}) in one sample.*

		Hotels		
		Open year-round	**Open seasonally**	**Total**
Presence of	Negative samples	11	6	17
Legionella species	Positive samples	0	4	4
Total		11	10	21

Note: the Croatian regulation does not allow the Legionella *species in drinking water (6).*

Table 2 *Physical and chemical findings in hot water samples collected in 11 facilities open year-round, and 10 open only during the summer months. Median values are reported.*

Parameters	Open	
	Year-round	**Seasonally**
T / °C	55.0	48.2
Ca / mg L^{-1}	52.94	54.87
Mg / mg L^{-1}	0.791	0.867
Fe / mg L^{-1}	0.039	0.052
Zn / mg L^{-1}	0.341	0.130
Cu / mg L^{-1}	0.012	0.008
Mn / µg L^{-1}	5.08	5.64

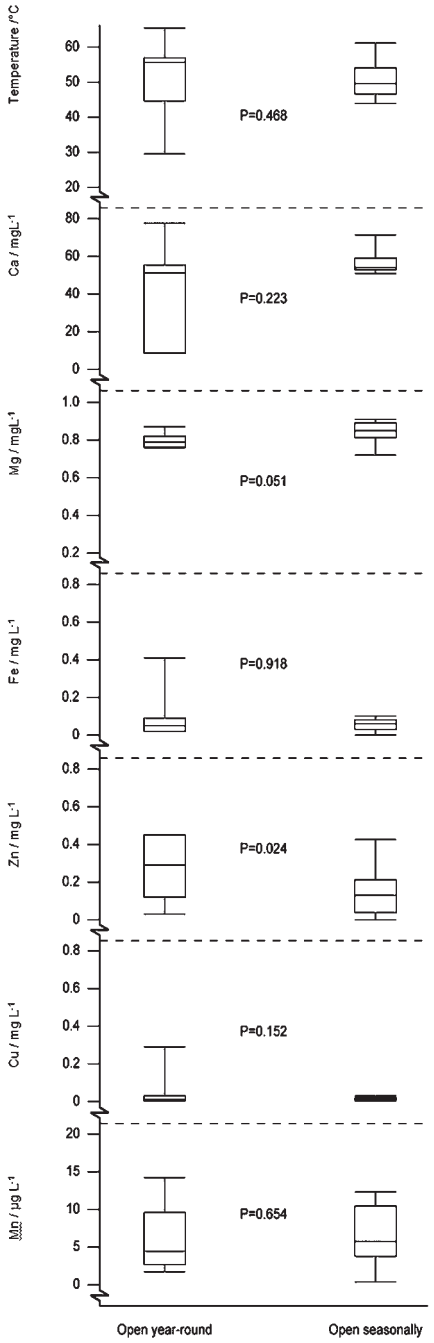

Figure 1 *The concentration of manganese (Mn), copper (Cu), zinc (Zn), iron (Fe), magnesium (Mg), calcium (Ca) and the temperature measured in hot water samples collected in year-round and seasonal accommodation facilities.*
Graphs show the lowest and the highest values (represented by the whiskers), the median (a line across the box), and the interquartile range, which contains 50 % of the values (represented by the box length).
P- level of significance
Maximum allowed concentrations of the metals in water are: Fe=0.2 mg L^{-1}; Zn=3 mg L^{-1}; Cu=2 mg L^{-1}; Mn=0.05 mg L^{-1} (6).

round and seasonally. The *Legionella* species were only found in the 16 samples taken from four seasonal facilities (P<0.001, chi-square test) (Table 1). Of the 16 positive samples, 14 were positive to the *L. pneumophila* serogroup 1 (87.5 %) and two to the *L. pneumophila* serogroups 2 to 14 (12.5 %).

Yu et al. (14) found that the *L. pneumophila* serogroup 1 was responsible for 80 % to 90 % of the Legionnaires' disease cases. A study conducted in Italy (15) found that the *L. pneumophila* of serogroups 2 to 14 was the main cause of legionellosis in hospitals. Compared to the *L. pneumophila* serogroup 1, the serogroups 2 to 14 in the study by Yu et al. (14) were isolated in water samples with lower temperatures and lower free residual chlorine concentration. Our findings are similar; samples with *L. pneumophila* serogroups 2 to 14 had temperatures ranging from 42.2 °C to 43.1 °C, while the median water temperature for samples with the serogroup 1 was 49 °C. In addition, samples with the *L. pneumophila* serogroups 2 to 14 had lower free residual chlorine concentrations (average 0.19 mg L^{-1}) than samples with the serogroup 1 (0.22 mg L^{-1}). However, we found no statistically significant correlation between microbiological findings and residual chlorine concentration (Spearman's (ρ=-0.06, p=0.499). The *Legionella* species were found in the water with free residual chlorine as well as in water without chlorine. This confirms the resistance of the species to free residual chlorine.

Figure 1 shows the differences in physical and chemical parameters between year-round and seasonal facilities. The only significant difference was in significantly higher zinc concentrations in year-round facilities.

CONCLUSIONS

To lower the risk of legionellosis, special care should be taken to implement plumbing materials that to not foster breeding of the *Legionella* species or other bacteria. Recommendations include efficient cleaning, regular spare part replacement, and the use of autonomous heating systems.

Notwithstanding the limited number of samples in our study, it provides a good starting point for further epidemiological research, which is justified by the serious potential of the *Legionella* species as a waterborne pathogen.

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Sažetak

PRISUTNOST VRSTA RODA *LEGIONELLA* U VODOOPSKRBNOM SUSTAVU U OBJEKTIMA KOJI SU STALNO OTVORENI I OBJEKTIMA SEZONSKOG TIPA

Svrha ovog istraživanja bila je procijeniti kakvoću tople vode s pomoću određenih fizikalnih, kemijskih i mikrobioloških parametara, analizirajući uzorke vode na prisutnost vrsta roda *Legionella*, u ustanovama koje su otvorene tijekom cijele godine i onih koje su otvorene samo tijekom ljetnih mjeseci u Splitsko-dalmatinskoj županiji, Hrvatska.

U istraživanju su određene koncentracije željeza, mangana, bakra, cinka, kalcija i magnezija u toploj vodi u 122 uzorka iz 21 ustanove u razdoblju od svibnja 2009. do prosinca 2009. Deset ustanova bilo je otvoreno tijekom ljetnih mjeseci, dok je 11 ostalo otvoreno tijekom godine. Temperatura i slobodni rezidualni klor mjereni su *in situ* prijenosnim digitalnim termometrom i prijenosnim digitalnim aparatom za mjerenje slobodnoga rezidualnog klora. Koncentracije željeza, mangana, bakra, cinka, kalcija i magnezija određene su metodom atomske apsorpcijske spektrofotometrije.

Legionele su određivane u istim uzorcima metodom kultivacije na BCYE-agaru 72 h pri 36 °C.

Nalaz vrste *Legionella pneumophila* bio je negativan u 11 ustanova koje rade kroz cijelu godinu i u šest ustanova koje su otvorene sezonski. Rezultati su bili pozitivni u 4 ustanove koje su otvorene sezonski.

U ustanovama koje su otvorene tijekom cijele godine određene su koncentracije u vodi: željeza 0,039 mg L^{-1}, magnezija 0,791 mg L^{-1}, kalcija 52,94 mg L^{-1}, cinka 0,341 mg L^{-1}, bakra 0,012 mg L^{-1} te mangana 5,08 µg L^{-1}. U ustanovama otvorenim sezonski također su u vodiodređene koncentracije: željeza 0,052 mg L^{-1}, magnezija 0,867 mg L^{-1}, kalcija 54,87 mg L^{-1}, cinka 0,130 mg L^{-1}, bakra 0,008 mg L^{-1} i mangana 5,64 µg L^{-1}. Samo je koncentracija cinka bila povišena u hotelima koji su radili tijekom cijele godine. Naše je istraživanje pokazalo da se kvaliteta vode razlikuje u vodovodnim sustavima koji se trajno rabe u odnosu prema sustavima sezonskog tipa. Ovo saznanje ima veliko značenje za kontrolu kakvoće vode u turističkim područjima.

KLJUČNE RIJEČI: *atomska apsorpcijska spektrofotometrija, biofilm,* Legionella *spp., metali u vodi*

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