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The influence of temperature, disinfection and water softening of drinking water on the multiplication of *Legionella pneumophila*

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ABSTRACT

The influence of temperature, disinfectant and water softener for drinking water preparation on the bacterial growth of *Legionella pneumophila* was examined. The optimal temperature for bacterial growth was 36 °C. At the incubation temperature of 15 °C the multiplying of bacteria slowed down, as confirmed by the bacteriostatic effect of this temperature. The number of bacteria was after the first 24 h of incubation at 55 °C reduced by more than 6 log in comparison to the culture at 36 °C. After 10 minutes of exposure to the disinfectant dichloroisocyanuric acid, the number of bacteria in the culture decreased for 1.4 log CFU mL^{-1}, followed by an intensive growth immediately after the disinfectant degradation. The number of bacteria was up to 72 hours incubation even higher than in the control sample without a disinfectant, and then the number in both samples equalled at approximately 7.3 log CFU mL^{-1}. Sodium polyphosphate used as a water softener stimulated the bacterial growth. The largest difference in growth was observed after 72 h of incubation, which was 1.9 log CFU mL^{-1} higher in samples with sodium polyphosphate in comparison to those without the softener.

Key words: *Legionella pneumophila;* growth curve; temperature; water softener; disinfectant

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INTRODUCTION

Legionellae are Gram-negative bacteria found in freshwater environments [1]. Problems can occur in buildings where the water temperature is between 25 °C and 42 °C, which are ideal conditions for the reproduction of these bacteria [2]. Many studies have shown that *Legionella pneumophila* may be found in water storage and distribution systems as well as in the recirculating cooling water of air-conditioning systems [3]. There are over 50 different species of *Legionella* and more than half of them can cause Legionnaires' disease and/or Pontiac fever [4, 5]. *L. pneumophila* causes more than 90 % of legionellosis [6, 7]. In 2015 ECDC reported 7034 cases of legionellosis in Europe. *L. pneumophila* serogroup 1 was the most often confirmed pathogen, accounting for 679 of 834 (81%) culture-confirmed cases [8]. The presence of these bacteria in water distribution systems, hospitals, hotels, and spas is a significant problem all around the world [9, 10].

L. pneumophila often survives drinking water preparation and processing procedures and can therefore be found in plumbing systems [11]. First it enters these systems in low numbers and then starts to reproduce rapidly due to favorable temperature conditions [12]. If the presence of *Legionella* bacteria in a facility's drinking water has been confirmed, different procedures for their elimination can be used. The most commonly carried out disinfection procedures use chlorine or heat shock. Heat shock involves flushing the system with water heated to temperature of at least 70 °C for 30 minutes. The water temperature must be 60 °C at the tap most distant from the storage tank [13]. Zhang et al. [14] reported that chlorination was an effective method for killing *L. pneumophila* in water distribution systems, however, the results suggest that the bacteria were not completely removed even after 40 months of constant chlorination. Other preparations for the chemical disinfection of water involve chloramine, chlorine dioxide, bromine, ionization of silver and copper ions, and ozone. Chloramine is more stable and penetrates deeper into biofilms in comparison to chlorine [15]. Ionization of copper and silver ions is an effective method of destroying bacteria [16] but Rohr et al. [17] pointed out that prolonged usage leads to reduced susceptibility of *Legionella* bacteria. In large plumbing systems, hot water is often added to soften water. Water softening is carried out with the intention of avoiding the precipitation of lime scale from the water which is deposited on the equipment, storage tanks, pipes, and taps [18].

Due to the above-described processes and preparation of drinking water in water supply systems, the aim of our study was to verify the influence of different conditions on the multiplication and survival of *Legionella pneumophila*. We studied the effects of various physical and chemical factors (temperature, added disinfectants and water softener) on the survival and reproduction of *Legionella pneumophila*.

The results can be used for further improvement of prevention measures against *Legionella* bacteria that could have more important public health and economic impacts. The limitation of the study is that we did not use different concentrations of softeners and disinfectants.

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METHODS

Bacterial strain

The standard strain *Legionella pneumophila* subsp. *pneumophila* ATCC 33153 (Czech Collection of Microorganisms, Brno, the Czech Republic) was used for studying the growth curve and for determining the impact of temperature, dichloroisocyanuric acid and sodium polyphosphate on bacterial growth. Cultivation took place according to the relevant ISO standard [19].

Bacterial growth

To study the growth curve of the test strain of *L. pneumophila* in different conditions, the method of counting bacterial colonies (CFU mL^{-1}) that grew aerobically after incubation on a solid medium was used [20]. First, bacteria were cultured in test tubes in a liquid medium of Yeast Extract Broth (YEB) (Fluca Analytica, India) with 5 mg L^{-1} of cysteine (Biolife, Italy) broth. During 120 h of incubation at 36 °C in aerobic conditions, 1 mL of overnight culture with a concentration of 5.7 log CFU mL^{-1} was taken from the tubes every 24 h. The serial 10-fold dilutions were prepared in a saline solution and 1 ml of each dilution was mixed with a melted Buffered Charcoal Yeast Extract (BCYE) medium (Biolife, Italy) with cysteine, using the plate count method [20]. After incubation, the colonies growing on the solid medium were counted and the results were expressed as CFU mL^{-1}.

The same procedure was used also for detecting bacterial concentration in the culture exposed to different incubation temperatures, a chlorine disinfectant and a water softener with sodium polyphosphate. The results were calculated and expressed as log CFU mL^{-1} [21].

The influence of temperature on bacterial growth

The growth curve of the bacterial strain was conducted at three different incubation temperatures: 36 °C, which was optimal for bacterial growth, and at the unsuitable temperatures of 15 °C, and 55 °C.

The effect of dichloroisocyanuric acid on bacterial growth

The effect of disinfectants on the growth of the test bacteria was measured by adding dichloroisocyanuric acid. The bacterial culture of *L. pneumophila* were added in YEB with a concentration of 5.7 log CFU mL^{-1}, with added dichloroisocyanuric acid (Oasis, England) with a concentration of 17 mg L^{-1}. This concentration of disinfectant is recommended by the producer of the product (Oasis), as effective for the destruction of micro-organisms in drinking water, so we did not change it. The pH values of bacterial cultures were measured after adding the disinfectant using the Cyberscan pH11 RS232 Meter (Eutech Instruments, Singapore) according to the producer's instructions. Samples were taken after 0 minutes, 10 minutes and then after 24, 48, 72, 96, and 120 h of incubation at 36 °C, to determine the number of bacteria (CFU mL^{-1}). For each selected time, two samples (two parallels) were taken, rep-

resenting 14 samples in an experiment with added dichloroisocyanuric acid and 14 samples without added dichloroisocyanuric acid. The experiment was repeated three times (42 samples with and 42 without disinfectant), which together represented 84 samples. The bacterial culture was exposed to dichloroisocyanuric acid for 10 minutes. For negative control, the bacteria were grown under the same conditions without exposing them to dichloroisocyanuric acid.

The effect of sodium polyphosphate softener on bacterial growth

The sodium polyphosphate (Microfos SH, TKI Hrastnik, Slovenia) water softener in standard concentrations [22] was added to the broth culture. The softener with a concentration of 5 mg L^{-1} was added to the fresh YEB broth with cysteine and overnight bacterial culture with a concentration of 5.7 log CFU mL^{-1}. We used the product Microfos SH (TKI Hrastnik, Slovenia) in a concentration, as prescribed by the softener manufacturer [23]. Samples were taken to determine the number of bacteria (CFU mL^{-1}) after 0, 24, 48, 72, 96 and 120 h of incubation at 36 °C. Free chlorine in the broth culture was measured with Colorimeter (Chlorine Test Kit, HACH, Floriffaux, Belgium) during incubation. For each selected time, two samples (two parallels) were taken, experiment was repeated three times.

Statistical analysis

R software version 3.1.3 was used for statistical analysis. The results were analysed using a paired Student t-test. The significance level was set at $p < 0.05$.

RESULTS AND DISCUSSION

The effect of temperature on the growth of *L. pneumophila*

Figure 1 shows the growth curve of *L. pneumophila* at different incubation temperatures. After the first 48 h of incubation at 36 °C, bacteria were in the logarithmic growth phase. From the beginning of the experiment to the stationary phase, the number of bacteria increased by about 3 log CFU mL^{-1}. Their number after 120 h of incubation was 7.4 log CFU mL^{-1}. At the incubation temperature of 15 °C the multiplying of bacteria slowed down, as the number of the initial value of 5.6 log CFU mL^{-1} culture did not significantly change, hence confirming the bacteriostatic effect of those temperatures. Bactericidal activity was defined as a 3 log10 decrease in CFU/ml (99.9 % kill-effect). Bacteriostatic activity was defined as <99.9 % kill of bacterial cells after exposure to an antimicrobial [24]. The reproduction of bacteria at 15 °C was minimal, which has also been confirmed by other studies [3, 25]. The number of bacteria incubated at 55 °C after the first 24 h was reduced by more than 6 log in comparison to the culture at 36 °C. After 48 h, their concentration decreased from 5.7 log CFU mL^{-1} to 0.1 log CFU mL^{-1}. After 72, 96, and 120 h of incubation, the number of colonies that grew on the solid media varied from 0 to 3 CFU mL^{-1}.

The reproduction of bacteria at 15 °C was minimal, which has also been confirmed by other studies.

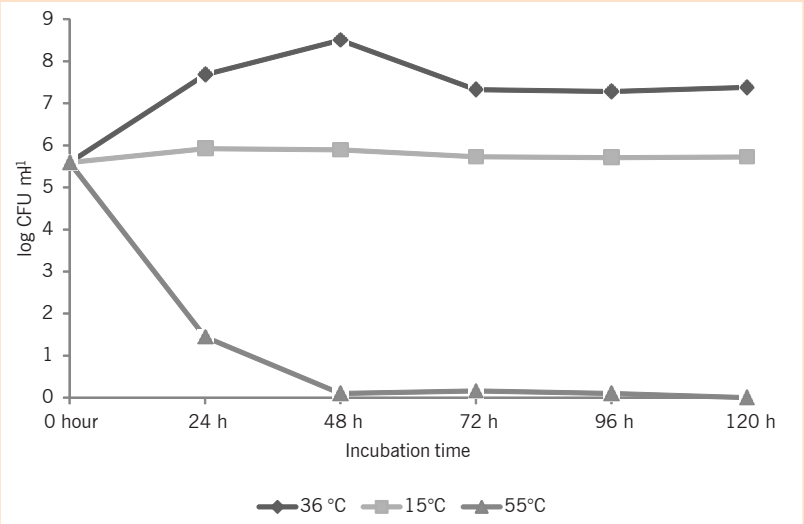

Figure 1.
Growth curve of the bacterium *L. pneumophila* subsp. *pneumophila* ATCC 33152 at three different incubation temperatures.

The bactericidal effect of this temperature was also reported by Cooke [26], who determined that 90 % of *Legionella* cells at a temperature of 50 °C died within 2 h. Serrano-Suarez et al. [27] noted that at a temperature above 55 °C, about 12 % of the population died, as confirmed by Carvalho et al. [28]. Differences in the productivity of the bacteria of *L. pneumophila* in the incubation at 36 °C, 15 °C and 55 °C were statistically significant ($p < 0.05$).

It is clear that the bacteria surviving the disinfection of water replicate more intensely than in the medium without the added dichloroisocyanuric acid.

The effect of dichloroisocyanuric acid disinfectant on the growth of *L. pneumophila*

Among the possible measures for preventing the growth of *Legionella* bacteria in water distribution systems is the use of different disinfectants, especially chlorine [16]. Totaro et al. [29] confirmed in their study the connection between the number of *Legionella* bacteria in water and the concentration of total chlorine and temperature. The growth curve of *L. pneumophila* in a medium with and without the added dichloroisocyanuric acid disinfectant at a concentration of 17 mg L^{-1} was examined. The results showed (Figure 2) that after 10 minutes of exposure to the disinfectant, the number of bacteria decreased from 5.7 log CFU mL^{-1} to 4.3 log CFU mL^{-1} (reduced by 36 %) and the concentration of free chlorine was 0 mg L^{-1}. The change in the pH value of the medium immediately after adding the disinfectant was reduced by only 0.1 grade, from 6.9 to 6.8. Followed by an intensive reproduction of the bacterial number, which had already exceeded the initial value after 24 h of incubation. After 72 h of incubation, the number of bacteria in both samples was about 7.3 log CFU mL^{-1} and did not change much even after 120 h of incubation. The resistance of *Legionella pneumophila* to hydrogen peracetic acid was also confirmed by Farhat et al. (30). Bonadonna et al. [31] determined that the concentration of free chlorine 0.08 mg L^{-1} confirmed the presence of *Legionella* spp. at a level of 400 CFU mL^{-1}. Despite the high concentration of added chlorine, the number of surviving bacteria was high, mainly due to the extremely high concentration of bacteria at the beginning of the experiment, which is not common in most water supply systems.

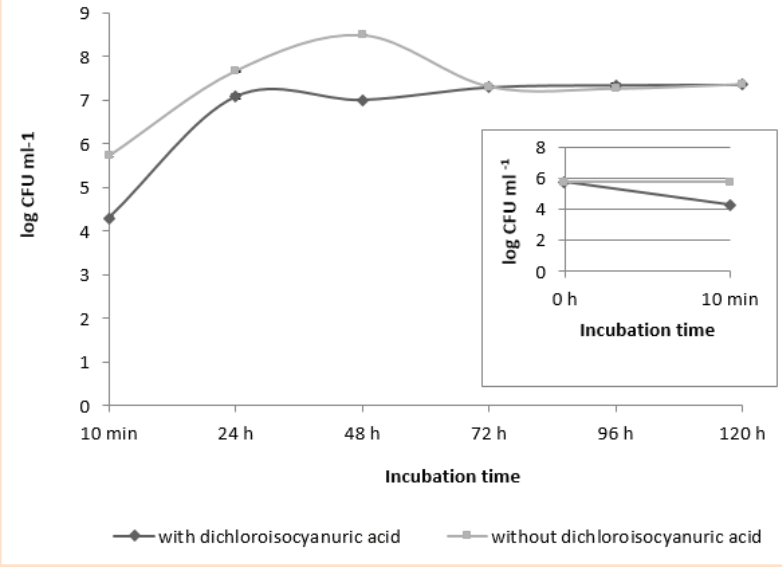

Figure 2.
Growth curve of bacterium *L. pneumophila* subsp. *pneumophila* ATCC 33152 in the presence of dichloroisocyanuric acid, a chlorine concentration of 17 mg L^{-1}.

The number of bacteria in the samples with the addition of a disinfectant increased in the first 24 h of incubation and was only 8 % lower than in the samples that were not exposed to dichloroisocyanuric acid. After 72 h, the number of bacteria was almost the same as in the control. It is clear that the bacteria surviving the disinfection of water replicate more intensely than in the medium without the added dichloroisocyanuric acid. There was a statistically significant difference between the number of bacteria that had been exposed to the disinfectant and the number of bacteria without the disinfectant ($p < 0.05$).

The effect of sodium polyphosphate on the growth of *L. pneumophila*

Figure 3 shows growth curves of *L. pneumophila* in a medium with and without sodium polyphosphate. Water softeners are often used in apartment buildings and large facilities (hospitals, hotels) to prevent calcification in the system [19, 32].

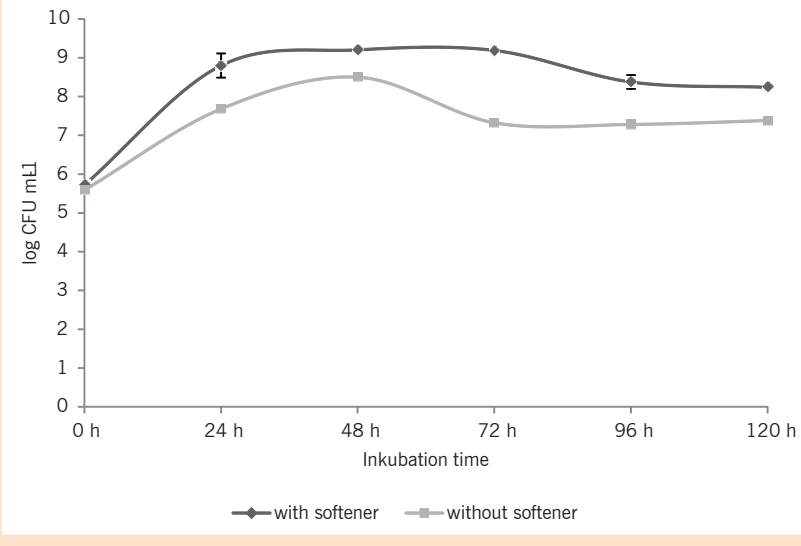

Figure 3.
Growth curve of the bacterium *L. pneumophila* subsp. *pneumophila* ATCC 33152 with adding sodium polyphosphate for water softening in a concentration of 5 mg L^{-1}.

Our results have shown that the incubation temperature of 15 °C has a bacteriostatic effect and a temperature of 55 °C has a bactericidal effect on these bacteria. We concluded that the temperature of 55 °C, even after a long time of incubation, does not completely eliminate all *L. pneumophila* bacteria since we confirmed the presence of bacteria in samples even after 96 h of incubation.

In the current study, sodium polyphosphate was used at a concentration of 5 mg L^{-1} as prescribed by the manufacturer of the product. The pH value of the culture after adding the softener did not change noticeably. Bacteria responded positively to the presence of the softener as their number was much higher at all incubation times. The maximum difference occurred after 72 h of incubation, when the number was higher by about 1.9 log CFU mL^{-1}. After 120 h of incubation, bacteria concentration was reduced to approximately 0.8 log CFU mL^{-1} but was still higher than in the control culture. The shape of the growth curve is similar in both cases, the difference being the number of bacteria in the samples with added sodium polyphosphates, because even after a longer period of incubation the number of bacteria was higher than in the culture without it. The reason for the intensive growth of bacteria might have been the phosphate in the polyphosphate solution as phosphates can also be a nutrient for microorganisms, which Drev et al. also mentions (19). Between the average values, overgrown colonies on the solid medium with sodium polyphosphate showed a statistically significant difference ($p < 0.05$) to the results without the softener.

CONCLUSION

Opinions about the influence of different factors on the reproduction and survival of *Legionella* bacteria in indoor water supply environments vary noticeably. Our results have shown that the incubation temperature of 15 °C has a bacteriostatic effect and a temperature of 55 °C has a bactericidal effect on these bacteria. We concluded that the temperature of 55 °C, even after a long time of incubation, does not completely eliminate all *L. pneumophila* bacteria since we confirmed the presence of bacteria in samples even after 96 h of incubation. We have proven that the bacteria that survived disinfection by water with dichloroisocyanuric acid reproduced as intensively as the bacteria that were not exposed to chlorine. This means that the incomplete or improper disinfection of drinking water in water supply systems may cause an intensive reproduction of bacteria. Sodium polyphosphate in a water softening product caused an intensive multiplication of the bacteria since the reproduction was more intense in the samples with added softener. The process of preventing lime scale build up in water supply systems by adding softeners can thus stimulate the growth of bacteria, which can cause major problems due to the large numbers of bacteria in the drinking water. It should be emphasized that all experiments were carried out under controlled laboratory conditions. Bacteria were grown in liquid medium (yeast extract with added cysteine), which means that we provided them with nutrients for their reproduction. Conditions in plumbing systems are less favourable, so the results would probably be slightly different in the real conditions. In the future, we would like to check the reproduction of bacteria under different temperature conditions, and we are also wondering if other water softeners have a positive effect on the growth of *Legionella* bacteria. This would be very important since we could recommend the use of non-phosphate-based softeners.

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