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Evaluation of the Effectiveness of a 4-Months Continuous Injection of a Gas Mixture (CO_2 and Inert Gases) on *Legionella* Contamination of a Hot Water Distribution System

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Abstract

Within the biofilm and scales *Legionella* is less far susceptible to the effects of the most frequently used biocides. The objective of this study was to evaluate the effect of a 4-months continuous injection of a gas mixture (CO_2 and inert gas) in the hot water distribution system of a large hotel colonized by *L. pneumophila* sg3 on limiting biofilm formation and scales and in turn *Legionella* growth. Before the continuous injection of the gas mixture, out of the 15 sampling points examined every month 60% were colonized by *Legionella* (mean concentrations of 10^2 cfu/L in the boilers and the return loop, and 10^4 cfu/L in taps and showers). One week after the injection of the gas mixture and daily fluxing of the distal outlets, the level of colonization decreased ($<10^3$ cfu/L). When it was decided to flux all the distal outlets only 1 day per week the mean concentration of *Legionella* increased again ($>10^4$ cfu/L) in all the sampling points. Thus, cleaning of the boilers was performed and distal outlets were again fluxed daily. One week after the level of contamination decreased again ($<10^2$ cfu/L). Nonetheless, the colonization was not eliminated and when fluxing of the distal outlets was not performed every day the mean concentrations of *Legionella* raised up to $>10^4$ cfu/L. Results indicate that the gas mixture was able to reduce the level of colonization by *Legionella* only because associated to the fluxing of the distal outlets.

Keywords

***Legionella*, Gas Mixture, Fluxing, Water Distribution Systems**

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1. Introduction

Legionnaires' disease is known to cause community-acquired pneumonia and may occur, sporadically or as part of an outbreak, as travel-associated infection [1] [2]. The most frequently route of transmission is by inhalation of contaminated aerosols through contaminated showers and whirlpool spas. Elderly adults, smokers and people with weakened immune systems or chronic lung diseases are particularly susceptible to the infection.

Factors that most enhance colonization of water environments are the water temperature (between 20˚C and 45˚C), obstruction and stagnation of the flow of the water, pipe materials, degree of pipe corrosion, as well as the presence of other microorganisms that support the growth of the bacteria. *Legionella* is also able to parasitize a range of protozoa, particularly free-living amebae in biofilm, and to multiply within them [3]. It is also well known that biofilm formation in plumbing systems is a microbial survival strategy that uses extracellular secretions to entrap and concentrate nutrients. At the moment, among the antimicrobial agents monochloramine seems to be more effective for decreasing *Legionella* within the biofilms *in vitro* as well as in model plumbing systems [4]-[6]. Anyway, it has been suggested that also water characteristics, such as trace element concentrations, hardness and accumulation of inorganic contaminants can cause many negative effects on drinking water quality, by offering nutrients and surface area for *Legionella* to grow and by protecting bacteria from disinfectants [7] [8]. Thus, it could be supposed that treatments targeting the removal of scales and biofilm on water distribution systems (WDS) may be effective at reducing *Legionella* colonization.

Research in oral biofilm removal suggests that one apparently effective approach to remove mature biofilms such as oral plaque is to pass large numbers of air bubbles over the oral surfaces [9] [10]. Thus, one novel approach to remove biofilms could involve the passage of a bubble stream throughout the WDS. The objective of this study was to evaluate the effectiveness of the continuous injection of a gas mixture in a hotel hot WDS on limiting particle accumulation and in turn *Legionella* growth.

2. Methods

2.1. Building Characteristics

A large hotel in the south-east of Sicily (Southern Italy) was considered. Built in 2010, the hotel has 4 restaurants, 1 spa, 192 rooms and 10 suites with similar architecture, from 1st level to 2nd level. The hotel uses groundwater as source of water, which is distributed throughout a 1.5 km-long WDS after filtration, softening and chlorination. The water piping is made of galvanized steel. Domestic hot water is produced at 55˚C and accumulated in three different 5000 L boilers in parallel.

2.2. The Gas Generator Device

An experimental equipment continuously injecting a gas mixture in the hot WDS was installed from April to August 2013 at the point where the water enters the building. The gas generator device has been specifically designed to inject into the hot WDS a gas mixture of carbon dioxide (CO_2) and inert gases (argon and nitrogen), which has previously proved to be efficacious in removing biofilm and scales in industrial wells (data not shown). To create a continuous stream of gas mixture in the hot water system, the apparatus was dimensioned to generate a gas fraction of 17.5 L/m^3 of water.

2.3. Sample Collection

In total, 165 hot water samples were collected from January to August 2014. The following water sampling plan was adopted: i) before the installation of the gas generator device, from January to March, one sampling per month; ii) after the installation of the gas generator device, from April to August, 4 samplings every week the first month, one sampling every 2 weeks the second month and one sampling the third and the fourth month. Due to the high number of distal outlets, it was decided to collect always the same 15 sampling sites throughout the WDS. In particular, water from boilers, return loop and distal outlets (bathroom and spa showers or taps) were collected in order to verify *Legionella* contamination throughout the hot WDS.

Water was sampled without flaming and after 1 minute of fluxing. Sterile bottles added with sodium thiosulphate in order to neutralize residual free chlorine were used for bacteriological analyses. According to ISO 19458 [11], procedures for sample collection, transport and storage were established. Bottles were returned to the laboratory immediately after sampling for bacteriological analyses.

2.4. Microbiological Analysis

Isolation and enumeration of *Legionella* was performed by cultural method according to the ISO 11731 [12] standard technique. The isolates were identified on the basis of cultural and serological features. In brief, 1 L of each sample of water was filtered through cellulose acetate filter of 0.2 µm-pore size (Sartorius AG, Goettingen, Germany) and the filtrate was resuspended in 10 ml of the original sample water by vortexing for 10 min, while 5 ml of each sample was treated with heat at 50˚C for 30 min in a water bath in order to reduce contamination with other bacteria. Two aliquots of heat-treated and untreated concentrated samples were plated onto selective GVPC medium (Oxoid Ltd., Basingstoke, Hampshire, UK). The plates were incubated at 37˚C ± 2˚C with 2.5% CO_2 for 8 - 10 days and colonies were counted at least 3 times (first at 4 days). *Legionella* species were confirmed by transferring suspected colonies to BCYE with L-cysteine and CYE without L-cysteine media (Oxoid). These cultures were incubated at 37˚C ± 2˚C for 48 h. Only colonies growing on BCYE medium were confirmed as *Legionella* and subsequently identified using an agglutination *Legionella* latex test (Oxoid).

2.5. Physical and Chemical Analyses

For each water sample the water temperature in ˚C was registered at the time of the sampling using a calibrated thermometer placed in the middle of the water stream.

The total water hardness, pH, electric conductivity (at 20˚C), residual free chlorine, total concentrations of cations (Na, K, Mg, Ca, Fe, Mn, NH_4^+), as well as anions like NO_3^-, NO_2^-, SO_4^- Cl^-, and Total Organic Carbon (TOC) were measured before the injection of the gas mixture (at baseline) and at the end of the experimental period.

3. Results and Discussion

No variation in chemical and physical characteristics of the water due to the injection of the gas mixture was observed at the end of the experimental period. Due to the length of the WDS the heat loss was high, so the temperature of the monitored hot water samples ranged between 45˚C and 55˚C. Only *L. pneumophila* sg3 was isolated. The detection limit for legionellae was ≥100 cfu/L.

Figure 1 compares the levels of contamination (reported as means of cfu/L) recorded at the various sampling points before (at baseline) and after the continuous injection of the gas mixture.

At baseline, out of the total 15 sampling points examined every month from January to March, 9 (60%) were colonized by *Legionella* at mean concentrations of 10^2 cfu/L in the boilers and return loop and 10^4 cfu/L in taps and showers. One week after the installation of the gas generator device and the performing of 20 minutes of fluxing every day of the contaminated taps and showers, the levels of *Legionella* decreased to mean concentrations <10^3 cfu/L.

In the author's opinion, the decrease of the *Legionella* load was only due to the daily fluxing of the distal outlets and not to the injection of the gas mixture. Probably the gas mixture contributed to the elimination of biofilm and scales but it could be supposed that without fluxing the *Legionella* load could not have been decreased. In fact, CO_2 dissolves in water to become dissolved CO_2, which combine with water molecules to form carbonic acid (H_2CO_3). H_2CO_3 can then dissociate to form hydrogen (H+) and bicarbonate (HCO_3^-) ions. Thus, it could

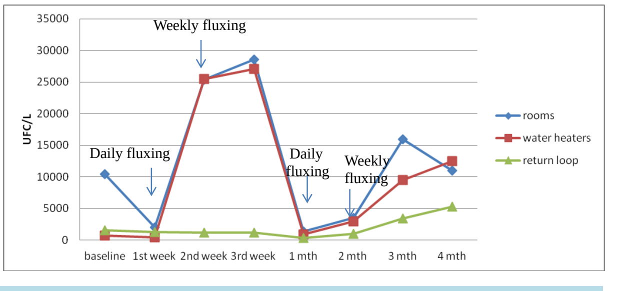

Figure 1. Trend of *L. pneumophila* contamination before (baseline) and after the continuous gas injection.

be argued that CO_2 is a weak acid that could dissolve scales from pipes. Moreover, hot water supersaturated by the inert gas mixture delivered micro-bubbles which probably helped the detachment of biofilm and scales which were successively easily eliminated by fluxing.

In order to verify if fluxing was the unique factor involved in the decrease of the *Legionella* load, it was decided to flux all the distal outlets only 1 day per week. After two and three weeks, the levels of *Legionella* increased at mean concentrations ≥ 10^4 cfu/L in the boilers and taps. This could be related to the fact that fluxing was not performed daily and that the gas mixture, according to its effect that has already been proved in industrial wells (data not shown), began to dissolve and detach biofilm and scales from the WDS. This, in turn, probably determined the release of legionellae. For this reason cleaning of the boilers and daily fluxing for 20 minutes of the contaminated distal outlets were performed. As expected, after one week the level of contamination by *Legionella* decreased again to mean concentrations < 10^2 cfu/L. Nonetheless, the colonization was not eliminated and when fluxing of the distal outlets was not performed every day, the mean concentrations of *Legionella* continued to rise from <10^4 cfu/L (second month) up to >10^4 cfu/L (third and fourth month).

Finally, during the first two months of the experimental period, mean concentrations of *Legionella* from the return loop remained stable and similar to those at baseline (<10^2 cfu/L), while they began to rise up to 10^3 cfu/L during the third and the fourth month. This could be probably related to two main reasons. First of all, fluxing was not performed in all the distal outlets but only in the contaminated ones. Secondly, the return loop is very long, thus legionellae released by the boilers under the effect of the gas mixture required more time to reach the point at which the sampling was performed.

4. Conclusions

In this study, the results of 4-months continuous injection of a gas mixture in the WDS of a large hotel colonized by *Legionella* are shown. The study was aimed at verifying if the use of a gas mixture which had previously proved to be effective in eliminating biofilm and scales in industrial wells, could be effective also in eliminating *Legionella*.

Results indicate that the gas mixture was able to reduce the mean concentrations of *Legionella* only because associated to tap or shower fluxing and cleaning of the bolilers. Anyway, *Legionella* was not eradicated.

Competing Interests

The author declares that she has no competing interests.

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