

Detection of *Legionella* spp. by a nested-PCR assay in air samples of a wastewater treatment plant and downwind distances in Isfahan

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Abstract

Background: Wastewater contains a variety of pathogens and bio-aerosols generated during the wastewater treatment process, which could be a potential health risk for exposed individuals. This study was carried out to detect *Legionella* spp. in the bio-aerosols generated from different processes of a wastewater treatment plant (WWTP) in Isfahan, Iran, and the downwind distances.

Materials and Methods: A total of 54 air samples were collected and analyzed for the presence of *Legionella* spp. by a nested-polymerase chain reaction (PCR) assay. A liquid impingement biosampler was used to capture bio-aerosols. The weather conditions were also recorded.

Results: *Legionella* were detected in 6% of the samples, including air samples above the aeration tank (1/9), belt filter press (1/9), and 250 m downwind (1/9).

Conclusion: The result of this study revealed the presence of *Legionella* spp. in air samples of a WWTP and downwind distance, which consequently represent a potential health risk to the exposed individuals.

Key Words: Air, Bio-aerosol, *Legionella* spp., nested-PCR, wastewater treatment plant

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INTRODUCTION

Legionella species are gram negative and non-spore bacilli, which are ubiquitous in natural and man-made aquatic environments, such as, lakes, rivers, reservoirs, cooling towers, and

whirlpools.^[1-4] *Legionella* can survive in extreme ranges of environmental conditions, with a variety of different physiochemical factors. This is related to the fact that some protozoa and blue green algae support the growth of the *Legionella* spp. This association increases the resistance of *Legionella* to extreme ranges of environmental conditions, such as, high temperature, low pH, and biocides.^[3-5]

Several species of *Legionella*, especially *L. pneumophila*, are human pathogens and have been associated with Legionnaires' disease, a type of acute pneumonia, with a relatively high fatality rate, or Pontiac fever, a milder non-fatal form of *Legionella* infection.^[3,5-7]

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Legionella is transmitted mainly by aerosolization of contaminated water. Inhalation of aerosols from contaminated water sources leads to *Legionella* infections or legionellosis.^[2,4,8]

Several studies have reported the detection of *Legionella* spp. in the wastewater of various units of WWTPs.^[1-3,9,10] Therefore, *Legionella* aerosols can be produced whenever aeration or mechanical agitation is used in wastewater treatment processes. There is evidence that the presence of *Legionella* in wastewater has been linked with increased levels of antibodies among wastewater irrigation workers.^[11] Kusnetsov et al., (2010) have reported two cases of severe pneumonia in employees working at two separate industrial WWTPs in Finland.^[12]

A few studies have reported the emission of *Legionella* spp. in bio-aerosols generated from WWTPs.^[2,7,9,13] However, the presence of *Legionella* in bio-aerosols of WWTPs could be influenced by several parameters, including, the concentration of these bacteria in the wastewater, characteristics of WWTP units, and the aerosolization at the different steps of treatment and weather conditions.^[11,14] This study was carried out to detect *Legionella* spp. in the bio-aerosols generated from different processes of an activated sludge WWTP and downwind distances, using a nested-PCR method. The aim of this study was to improve our understanding about health hazards due to inhalation of bio-aerosols generated from a wastewater treatment plant in our region.

MATERIALS AND METHODS

Site description and sampling procedure

The study was carried out in a municipal WWTP of Isfahan, Iran. The WWTP has 48-hectare area, receiving a maximum wastewater flow rate in the final design of 130,000 m³/day produced by 800,000 inhabitants. The plant is operated by using the activated sludge biological system, with horizontal surface aeration.

Air samples for detection of *Legionella* bio-aerosols were collected from three points in the WWTP, 100 m upwind, as a background sample, and 100 and 250 m downwind from the treatment plant. A total of 54 air samples were collected during nine months, from September 2012 to July 2013. A liquid impingement biosampler (SKC Biosampler) was used to capture the airborne *Legionella*. The biosampler was calibrated for a flow rate of 12 l/minute at a height of 1.5 m above ground level, to simulate the breathing zone. The location of the sampling points and the average volume

of air samples are presented in Table 1. The samples were transferred to the laboratory in an insulated box with cooling packs.

The weather conditions including temperature (°C), relative humidity (%), and wind speed (m/s) were also monitored and recorded by use of a portable weather station (Kimo), at each sampling location during collection of the *Legionella* bio-aerosol samples.

Detection of *Legionella* spp. in air samples by polymerase chain reaction assay

The impingement solution was concentrated by centrifugation. To extract the DNA, pellets were resuspended in distilled water and transferred to FTA-cards (Whatman, USA). DNA was then extracted from the FTA cards and used in the PCR. In the first PCR step, a ~1,420 base pair (bp) fragment of the bacterial 16S rRNA gene region was amplified using the primer set Eubac 27F (5' -AGAGTTTGATCCTGGCTCA <G>) and 1492R (5' -TACGGYTACCTTGTTACGACT <T>)^[15] to verify the nucleic acid extraction as well as check the probable presence of inhibitors. For detection of the *Legionella* species, a nested-PCR technique was applied as described previously by Baghal-Asghari et al. (2013).^[16] Briefly, PCR amplification was conducted in a final volume of 25 µl consisting of 2.5 µl of 10 × PCR buffer, 0.2 µM of LEG 448 and JRP primers, 0.2 mM of each dNTP, two units of Taq DNA polymerase, and 1 µl of DNA. All PCR assays contained a positive (DNA of *L. pneumophila*) [*L. pneumophila* NCTC 12821, FEPTU, HPA center for infections, United Kingdom, London] and a negative control (molecular grade water without DNA). The PCR was performed with an initial denaturation for five minutes at 95°C, followed by 30 cycles at 94°C for 45 seconds, at 55°C for 1 minute, at 72°C for 1.30 minutes, and a final extension at 72°C for 5 minutes. The PCR products were analyzed by agarose gel electrophoresis. The gels were viewed on a ultraviolet (UV) transilluminator (UV Tech, France), and the DNA fragment sizes were compared with the 50 and 100 bp ladder DNA.

Table 1: Sampling sites and air samples volumes tested in this study

Sampling sites	Process description	Sample volume (liters)
Background	100 m upwind of WWTP	160-170
Pump station	Pumping sewage from depth to surface	120-130
Horizontal surface aeration	Biological oxidation	120-130
Belt filter press	Sludge dewatering	120-130
100 m downwind	Downwind of WWTP	160-170
250 m downwind	Downwind of WWTP	160-170

WWTP: Wastewater treatment plant

RESULTS

A total of 54 bioaerosol samples were taken at three locations in a WWTP (pump station, aeration tank, and sludge dewatering process), upwind, and downwind distances, and examined for the presence of *Legionella* spp. The nested -PCR assay revealed that about 6% (three out of 54 samples) of the air samples were positive for *Legionella*. Samples of air above the aeration tank (1/9), the belt filter press (1/9), and 250 m downwind distance (1/9), were positive for *Legionella*. However, all the samples were found to be positive with the PCR assay with the Eubac 27F -1492R primer set and PCR inhibition was seen in none of the 54 samples tested. Table 2 shows the results of *Legionella* spp. detection at the sampling points. The weather conditions are also presented in Table 3. The ambient temperature ranged from 11.7 to 28.1°C and the relative humidity from 23 to 38%. The temperature, wind speed, and humidity values during air sampling showed no relationship with the presence of positive or negative results.

DISCUSSION

Wastewater contains a variety of pathogenic microorganisms and WWTP workers may be exposed to these pathogens from various routes. Some of wastewater treatment processes, such as activated sludge, lead to the production of bio -aerosols, which could result in workers exposure to pathogens. Exposure to the bio -aerosols is associated with a wide range of adverse health effects and is a concern from a public-health point of view. In the present study the occurrence of *Legionella* bio -aerosols in the air of an activated sludge treatment plant and downwind distances was investigated.

As shown in Table 2 *Legionella* spp. were detected in air samples of an activated sludge and a belt filter press, simultaneously. Aeration of wastewater or application of mechanical devices could assist in the aerosolization

of wastewater. As wastewater and sludge provide a suitable environment for the presence and growth of *Legionella* spp., aerosolization of wastewater could lead to the emission of *Legionella* spp. into the environment. Medemu *et al.*, (2004), detected *Legionella* spp. in air samples of 10 m³ at three of the five WWTPs tested by PCR. Similar to our study, they found *Legionella* in the air samples of aeration tanks (2/2) and the belt filter press (1/1).^[14] In the study of Pascual *et al.*, (2001), in a WWTP; three samples from the pretreatment point (30%) and one from the biological treatment (11%) were positive for *Legionella*.^[9] However, both studies found *Legionella* in air samples with only PCR, but not with a culture. Although, culture on a selective medium has commonly been used for detection of *Legionella* spp. in environmental samples, but the method has low sensitivity, and overgrowth by other bacteria, especially in environmental samples, could hamper the identification of *Legionella*.^[16,17] Besides, the presence of *Legionella* in a viable, but non -culturable (VBNC) state, can be caused by aerosolization of airborne microorganisms, which cannot be detected by the culture method. To overcome these shortages, molecular methods such as polymerase chain reaction (PCR) amplification of DNA sequences, specific for *Legionella* spp., were used as sensitive and rapid methods for detection of the bacteria in the environmental samples.^[18,19]

We used a PCR assay with the LEG448 -JRP primer set, with high sensitivity and specificity for detection of the *Legionella* spp.^[16] The nested -PCR assay was also applied to further increase the sensitivity. However, the nested -PCR assay is a qualitative assay that only recognizes the presence or absence of *Legionella* spp., and quantitative techniques such as real- time PCR should be taken into use for the analysis of health risks posed by the *Legionella* bio -aerosols in the WWTPs.

The results of the study also showed the presence of *Legionella* in an air sample at a 250 m downwind

Table 2: The number of positive samples for detection of airborne *Legionella* spp. in WWTPs with regard to seasons and points of sampling

Season	Sampling points					
	Background	Pump station	Aeration basin	Belt filter press	100 m downwind	250 m downwind
Autumn						
Total samples	3	3	3	3	3	3
Positive	0	0	1	1	0	1
Winter						
Total samples	3	3	3	3	3	3
Positive	0	0	0	0	0	0
Spring						
Total samples	3	3	3	3	3	3
Positive	0	0	0	0	0	0

Note: The positive samples for the *Legionella* bioaerosol are shown in bold, WWTP: Wastewater treatment plant

Table 3: Meteorological parameters in the different seasons

	Temperature (°C)	Wind velocity (m/s)	Relative humidity (%)
Autumn	15.5	0.6	38
Winter	11.7	0.55	30
Spring	28.1	1.0	23

distance from the WWTP. Some studies have reported that a downwind distance of 250 m or higher from the microbial emission sources is a safe distance from the viewpoint of safety.^[20] However, bio-aerosol dispersion is affected by a number of factors such as individual bio-aerosol properties and meteorological conditions.^[19,20] The presence of *Legionella* at a 250 m downwind distance could be a potential risk to neighbors of WWTP, however, more studies in this field are required.

As shown in Table 2 all the positive samples were related to autumn. Comparison of meteorological parameters in different seasons of sampling show higher values of relative humidity for positive samples than for negative samples [Table 3]. Bacteria generally survive better at higher relative humidity.^[13] This relationship is also comparable with the results of Pascual *et al.*, (2001), which showed that the humidity values of the *Legionella* positive samples at a WWTP were significantly higher than for negative samples.^[9]

CONCLUSION

The results of this study revealed the presence of *Legionella* spp. in air samples of a WWTP and downwind distances, which consequently represent a potential health risk for exposed individuals. Further studies, however, are needed for risk assessment of inhalation of *Legionella* bio-aerosols from WWTPs.

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