The transmission mode of Legionella from its source

Jun Li (lijun681116@163.com)
Zhejiang Gongshang University https://orcid.org/0000-0003-3123-5673
Lin Liu
Zhejiang Gongshang University
Kunquan Li
Nanjing Agricultural University
Xuebin Li
Nantong Shipping College
Tao Tao
Huazhong University of Science and Technology

Research article

Keywords: Legionella, vapor, aerosol, qPCR, membrane, surface tension

Posted Date: October 3rd, 2019

DOI: https://doi.org/10.21203/rs.2.15489/v1

Abstract

Background

Legionella pneumonia has a fatality rate of 28%.

Methods

microscope, fluorescence Quantitative Polymerase Chain Reaction (qPCR), force mathematical analysis.

Results and Conclusion

The transmission mode of *Legionella* from its source was analyzed by microscope and qPCR. The *Legionella* removal efficiency by a membrane composed of water molecules was 94.5%, and *Legionella* had difficulty in penetrating through the surface of the water membrane. A deflection point at the interface between water and air indicated a cluster of *Legionella* that was bonded to the contact surface by some unknown emplastic media. Force analysis showed that the surface tension of water is 10^6 orders of magnitude larger than the net force from the sum of the buoyancy and the weight of *Legionella*, and revealed that the surface tension of water is so large that a *Legionella* bacterium cannot break away from the water surface membrane and escape. The qPCR results showed that no *Legionella* was found in the air from a *Legionella* incubator or the *Legionella* laboratory. The results demonstrate that *Legionella* cannot be transmitted to people through water vapor or aerosol. The experimental results also indicate that water was able to remove most *Legionella* bacteria.

Introduction

National Broadcasting Company (NBC) News reported an outbreak of Legionnaires' disease in New York City in the USA on August 3, 2015, which resulted in seven deaths. *Legionella* pneumonia has a fatality rate of 28% (Stout et al., 2007). Therefore, it should be a public health priority to perform additional studies and gather a greater amount of data on Legionnaires' disease (NBCnews, 2015; Jun Li et al., 2009, 2013, 2017, 2018a, 2018b; Shengkun Dong et al., 2017, 2018).

Inhalation of aerosols containing *Legionella* spp. is presumed to be the primary means of acquiring Legionellosis. Aerosolized water that can cause infection comes from sources such as cooling towers and evaporative condensers (Osawa et al., 2014), showers (Breimen et al., 1990), ice-making machines (Graman et al., 1997), refrigerated cabinets, whirlpool spas (Coetzee et al., 2012; Campeseet al., 2010), hot springs (Kurosawa et al., 2010), fountains (Palmore et al., 2009), and dental equipment (Kadaifciler et al., 2014). The most common source of *Legionnaires'* disease outbreaks is cooling towers. *Legionella* becomes airborne and is transmitted via respiratory droplets containing the bacteria. Upon inhalation, the bacteria can infect alveolar macrophages (Leonardo et al., 2012; Sahid et al., 2013). A previous study showed that *Legionella pneumophila* could spread at least 6 km from its source by air (Nguyen et al., 2006).

Cohesive forces act on water molecules to create surface tension, which holds together a layer of water molecules on top of more loosely connected water molecules so that this layer behaves like an elastic membrane. The objectives of this study were to investigate if *Legionella* can break out of a surface layer of water molecules that form a membrane, and spread with vapor or aerosol.

Materials And Methods

An optical microscope was used (LEICA DM 5000M, Germany), with 3”×1”×1.0 mm microscope slides, and 18×18 mm cover glasses (Fisher Scientific, USA). *Legionella pneumophila* subsp. (ATCC® 33152TM) was obtained from the American Type Culture Collection (ATCC®, Manassas, VA, USA). *Legionella* was cultured overnight using #1099 Broth (casamino acids-yeast extract (CYE) buffered) at 37 °C for 12 h.

Legionella was confirmed by a polymerase chain reaction (PCR) assay based on the protocol of Van der Zee (Van der Zee et al., 2002). The forward primer (5’-GAAAATAAAGTAAAAGGGGAAGCC–3’) and reverse primer (5’-ATCAATCAGACGACCAGTGTATTC–3’) were designed according to the 16S rRNA gene sequence in order to amplify a 100-bp DNA fragment specific for *Legionella* species. The fluorescent probe was designed with 5’-fluorescein-CE-phosphoroamidite (FAM) on one end, and the sequence is: 5’FAM-AGGCGTTGTTGTATTGCCAAGTGGTT-BHQ1–3’. The primers were purified with ULTRAPAGE, which consists of a combination of polyacrylamide gel electrophoresis (PAGE) separation and mass spectrometry (MS) analysis. High-performance liquid chromatography (HPLC) was used to purify the fluorescent probe.

The quantitative PCR (qPCR) reaction mixture, 20 µL final volume, contained 10 µL of 2×SuperRealPreMix Plus, 0.6µL of the forward primer, 0.6 µL of the reverse primer, 0.4µL of fluorescent probe, 1µLof DNA template, and 7.4 µL of RNase-free ddH_2O (Tiangen, China). The quantitative PCR equipment used CFD–3220, DNA Engine Opticon, MJ Research Inc., USA. The real-time qPCR protocol is as follows: samples were preheated for 15 min at 95 °C, samples were heated to denature for 3 seconds at 95 °C, samples were cooled to extend for 30 seconds at 60 °C, read fluorescence strength, cycle 40 times from to . A negative control was also analyzed in each real-time qPCR run. Amplified DNA was detected by agarose gel electrophoresis with Gene Green nucleic acid staining (RT210) (Tiangen, China). The test was performed on all colonies that tested positive with the 2×SuperReal PreMix Plus (probe) (FP206) (Tiangen, China).

The forward and reverse primer, and fluorescent probe were purchased from Sangon Biotech (Shanghai) Co., Ltd. DNA sequencing of the specific PCR DNA fragments was performed by Sangon Biotech (Shanghai) Co., Ltd.

Results

3.1 Legionella observation by microscope at t = 0, 1, 2 min

An optical microscope that automatically captured images at a rate of one image per minute was used to investigate the movement of *Legionella.* Figure 1(a) shows the first image of *Legionella* at the initial time t = 0 min, where each dot represents one *Legionella* bacteria. In the upper left corner, the wavy line represents the interface between water and gas. Atmospheric gas was present on the left side of the interface, and a solution of water and *Legionella* was on the right side of the interface. At the interface, there was a single row of *Legionella* at t = 0 min. The water in the observed *Legionella* solution was continuously vaporizing under the influence of the ambient temperature (22 °C) in the laboratory. In the upper left corner, the wavy line represents the interface between water and gas, and runs from left to right. Figure 1 shows that *Legionella* was evenly distributed in solution, and after counting under the microscope, it was determined that there were 775 *Legionella* bacteria in this image. The actual size of this image is 0.394667×0.2960 mm. To investigate the mobility of *Legionella,* the microscope was focused on the interface line.

Figure 1 (b) was captured at t = 1 min and shows that most of the *Legionella* was absorbed on the right side of the interface line between water and gas; only 19 *Legionella* bacteria remained on the left side of the interface line. Microscopic observation indicated that there were three clusters of *Legionella* on the left side of the interface line, showing that the clusters of *Legionella* bacteria could not have been removed by water. The mechanism that caused these phenomena was still not clear. It was presumed that *Legionella* was absorbed on the surface of incompletely dissolved broth (#1099 broth, CYE) or certain emplastic media. Five clusters of *Legionella (*24 *Legionella* in total) were found to remain on the right side of the interface, as shown in Figure 1 (b). Considering that 19 *Legionella* remained on the left side of the interface, 43 *Legionella* in total were not removed out of 775 *Legionella* in Figure 1 (b). Hence, the *Legionella* removal efficiency was 94.5%, with only clusters of *Legionella* remaining. A single *Legionella* bacterium could be 100% removed by water, showing that the water surface tension is stronger than the movement force of a single *Legionella* bacterium. The circles on the upper left side of Figure 1 (b) came from an unclear microscope lens, and were not *Legionella* because they appeared in every image.

Figure 1 (c) was captured at t = 2 min and shows that the interface moved towards the right side. Comparing Figure 1 (c) to Figure 1 (b), the moving distance of the interface was 0.060639 mm in one minute. Therefore, the moving rate of water ($V_{t=1}$) was 0.060639 mm min^{-1}. Figure 1 (c) also showed that there was an inflection in the interface when it met a cluster of *Legionella,* suggesting that the force of a cluster of *Legionella* was stronger than the water surface tension.

3.2 Legionella observation by microscope from t = 12 to 23 min

The lens of the microscope was maintained at the same position to perform observations from t = 12 to 23 min. Images were automatically captured every minute. Figure 2 (a) was captured at t = 12 min. Clusters of *Legionella* that were not removed by water were labeled from 1 to 9. Clusters that were labeled with 1, 2, 3, 4, 5, 6, 7, 8, and 9 contained 2, 1, 3, 1, 3, 2, 1, 4, and 1 *Legionella* bacteria bacterium that remained, respectively. Thus, 18 *Legionella* bacteria in total were not removed in Figure 2 (a).There was a

minimum of two rows, an average of 3.5 rows, and a maximum of five rows of *Legionella* at the interface in Figure 2 (a). The quantity of *Legionella*at the interface at t = 12 min was 3.5 times more than that at t = 1 min because there was less than a row of *Legionella* at the interface at t = 1 min.

Figure 2 (b) was captured at t = 13 min. Cluster Number 7 of *Legionella* obviously affected the shape of the interface, showing that the force of a cluster of *Legionella* was stronger than the water surface tension. Through calculation, it was determined that the moving rate of water at t = 13 min was 0.044101 mm min^{-1} ($V_{t = 13}$ = 0.044101 mm min^{-1}), which was less than the moving rate at t = 1 min ($V_{t = 1}$ = 0.060639 mm min^{-1}).

Figure 2 (c) was captured at t = 14 min. In Cluster Number 10, two *Legionella* bacteria appeared, as shown in Figure 2 (c), but did not appear in Figure 2 (b). This result showed that the *Legionella* of Cluster Number 10 broke away from the water surface membrane and escaped. The moving rate of water at t = 14 min was 0.038589 mm min^{-1} through calculation ($V_{t = 14}$ = 0.038589 mm min^{-1}), which was less than the moving rate at t = 1 and 13 min.

Figure 2 (d) was captured at t = 15 min. The moving rate of water at t = 15 min was 0.025726 mm min^{-1} through calculation ($V_{t = 15}$ = 0.025726 mm min^{-1}), which was less than the moving rate at t = 1, 13, and 14 min.

Figure 2 (e) was captured at t = 18 min. The moving rate of water at t = 18 min was 0.023341 mm min^{-1} through calculation ($V_{t = 18}$ = 0.023341 mm min^{-1}), which was less than the moving rate at t = 1, 13, 14, and 15 min. There was a minimum of three rows, an average four rows, and a maximum of five rows of *Legionella* at the interface in Figure 2 (e).The *Legionella* quantity at the interface at t = 18 min was higher than at t = 1 and t = 12 min, showing that the *Legionella* quantity increased as time increased. Similar to two *Legionella* bacteria that escaped from the interface in Cluster Number 10, two *Legionella* bacteria in Cluster Numbers 11 and 12 also escaped from the interface. Thus, a total of four out of the initial 775 *Legionella* bacteria (0.52%) escaped from the water membrane, suggesting that *Legionella* does not easily break out of a water membrane.

Figure 2 (f) and (g) were captured at t = 21 and 22 min, respectively. The moving rate of water at t = 21 min was 0.021219 mm min^{-1} ($V_{t = 21}$ = 0.021219 mm min^{-1}), which was less than the moving rate at previous times of t = 1, 13, 14, 15, and 18min. There was an average of five rows of *Legionella* bacteria at the interface at t = 22 min, as shown in Figure 2 (g), suggesting that the quantity of *Legionella* at the interface increased as time increased.

Figure 2(h) shows the relationship of moving rate and time, suggesting that the moving rate decreased as time increased. This may be caused by increasing quantities of *Legionella* at the interface line, which can prevent water evaporation.

3.3 Legionella observation by microscope from t = 60 to 62 min

Figure 3 shows a microscopic view of *Legionella* bacteria from t = 60 to 62 min. The deflection points in Figure 3 (a) and (b) at the lower right corner show a cluster of *Legionella.* There was a minimum of 5 rows, an average of 9 rows, and a maximum of 13 rows of *Legionella* bacteria at the interface at t = 62 min, as shown in Figure 3 (c). The quantity of *Legionella* at the interface at t = 62 min was more than that before 62 min, showing that water can collect *Legionella.* There was very little escape of *Legionella* bacteria from the water membrane even if there was a maximum of 13 rows of *Legionella* bacteria aggregating at the interface line.

These experimental results showed that water could remove most of the *Legionella* bacteria, and very few *Legionella* bacteria could escape from this body of water. Thus, water can be used as a media of collection for *Legionella* and other bacteria, and based on this experiment, washing hands with water can remove most of the bacteria, thereby preventing it from spreading and subsequently maintaining public health.

3.4 Observation by microscope of vapor in a water bubble with Legionella

A microscope was used to observe vapor in a water bubble containing *Legionella,* as shown in Figure 4. There were some small water blocks, and *Legionella* was found in the large water bubble. Due to the effect of ambient temperature (22 °C), the large water bubble continuously vaporized. The vapor from the interface of the large water bubble membrane was clearly observed through the microscope, but it is not clear in Figure 4 because vapor has no color and cannot be shown in the image. Figure 4 shows a darker color near the water membrane and a lighter color farther away from the membrane. The color difference was caused by the vapor concentration, which was highest near the membrane and decreased with increasing distance from the membrane.

Almost all of the *Legionella* bacteria remained inside the membrane and did not exit from the water membrane, suggesting that *Legionella* cannot spread through contaminated water in the form of mist, steam, aerosol, or vapor. This result differs from previous studies and is an important finding. Previous reports showed that *Legionella* could spread through aerosol or vapor. A previous study showed that *Legionella* could spread at least 6 km from its source by air (Nguyen et al., 2006).

3.5 qPCR testing

Air samples were taken from the air inside a *Legionella* incubator and the *Legionella* laboratory four times per month in 2 years. The *Legionella* incubator continuously incubated *Legionella* on buffered charcoal yeast extract (BCYE) solid plates and in liquid BCYE medium in test tubes. The caps were removed from the 50- mL test tubes in which *Legionella* was growing in liquid BCYE medium. Seven days elapsed until the caps were put back on the test tubes. The sample points were at the air output of the incubator and window, door, and at the center of the laboratory. A total of 384 samples were obtained and were analyzed using qPCR. No sample was found positive, showing that no *Legionella* could be found in the air of the *Legionella* incubator or *Legionella* laboratory. This result suggests that no *Legionella* escaped out of solution and contaminated the air through any vapor, steam, or aerosol.

Discussion

Our results show that *Legionella* can be removed by water, but cannot escape from water by air, vapor, steam, or aerosol. The possible reasons are analyzed below. Water is a polar molecule and has a bent molecular geometrywith two hydrogen atoms on the oxygen vertex. In the following analysis, a water molecule is assumed to have the following characteristics: the H–O–H gas phase bend angle is 104.48°, as shown in Figure 5 (a), and the distance between the O and H is 95.84 pm (Hoy et al., 1979; Campbell et al., 2009). The positive hydrogen ends connect to the negative oxygen end to form a water molecule. The cohesive force among many water molecules leads to surface tension, and significant energy is needed to break these intermolecular bonds. Surface tension is also defined as the property of a liquid surface that resists an external force. There are no water molecules above the water surface, resulting in a stronger bond between the molecules in the surface than in the internal body of liquid. This surface layer creates a considerable barrier between air and water.

Water has the greatest surface tension of any other liquid except mercury. Water has a high surface tension of 0.0728 N m^{-1} at room temperature (20 °C), which is caused by the strong cohesion between water molecules. This phenomenon can be observed when a paper clip is able to float on the surface of water, as shown in Figure 5 (b) (Buzzle, 2015).Water molecules stay close to each other due to the collective action of hydrogen bonds (cohesion) between water molecules (Campbell et al., 2009), and these bonds also affect microorganisms in contact with them, including bacteria.

In biological cells, hydrophilic protein surfaces have a strong attraction to water. To dehydrate hydrophilic surfaces, a great deal of energy is required against the hydration forces that attract moisture to the surface. These forces are very large but rapidly decrease over a nanometer (Chiavazzo et al., 2014). According to a previous study (Chiavazzo et al., 2014), the influence of a water molecule extends to a distance of one nanometer. Figure 5 (c) is drawn based on the radius of a water molecule at 95.84 pm.

The perimeter of a *Legionella* bacterium is 9.42 μm based on the radius of *Legionella* of 1.5 μm. Therefore, there are 7970 water molecules on the sphere of *Legionella* based on a water molecule distance of 1nm and water molecule radius of 95.84 pm, as shown in Figure 5 (d). For clarity, Figure 5 (d) only shows 96 water molecules (R = 0.075 μm) on the sphere of *Legionella* out of 7970 water molecules. Figure 5 (d) shows that a single water molecule does not possess sufficient force to move *Legionella* away from it because *Legionella* is much larger than a single water molecule, which indicates that *Legionella* cannot spread out of the solution by air, vapor, steam, or aerosol.

The gravity acting on a bacterium is $1.07–1.19\times10^{3}$ kg m^{-3} (Docin, 2015). Therefore, the gravity acting on *Legionella* is presumed to be the average of that of a bacterium, i.e., 1.13×10^{3} kg m^{-3}. The volume occupied by a *Legionella* bacterium is 14.13×10^{-18} m^{3}, and the weight is 15.97×10^{-15} kg based on the radius of *Legionella* at 1.5μm. The buoyancy acting on a *Legionella* bacterium is 7.98×10^{-15} kg if half of a *Legionella* bacterium is immersed in water. The surface tension of water at 20 °C is 0.0727 N m^{-1}.

Assuming *Legionella* A is completely immersed in water, and half of *Legionella* B is immersed in water, as shown in Figure 5 (e), according to force analysis, *Legionella* A is affected by the adhesive force F_{adw} from the water molecules, the weight force F_w, and the buoyancy F_b. The adhesive forces F_{adw} act in all directions around *Legionella* A so that the adhesive force F_{adw} is zero, i.e., $\Sigma F_{adw} = 0$. The weight force F_w is 15.97×10^{-15} kg, and the buoyancy F_b is 7.98×10^{-15} kg. Therefore, the total net force F_n acting on *Legionella* A is 8.08×10^{-15} kg, and the force direction is downward, meaning that *Legionella* A cannot spread out of the water, as shown in Equation 1:

$$F_n = \Sigma F_{adw} - F_b + F_w = 0 - 7.98\times10^{-15} + 15.97\times10^{-15} = 8.08\times10^{-15} \text{ kg} \quad (1)$$

Where F_n denotes the total net force in kg, F_{adw} denotes the adhesive force from the water molecule in kg, F_b denotes the buoyancy in kg, and F_w denotes the weight force in kg.

Half of *Legionella* B is immersed in the water, and the other half is in the air. The surface tension F_t of water is equal to that the adhesive force F_{adw} from water molecules minus the adhesive force F_{adw} from air molecules. According to that, the surface tension of water at 20 °C is 0.0727 N m^{-1}, and the force F_t of *Legionella* B from the surface tension of water is 3.42×10^{-8} kg. The net force F_n acting on a *Legionella* bacterium is equal to that of force F_t from the surface tension of water minus buoyancy F_b of a *Legionella* bacterium plus weight F_w of a *Legionella* bacterium, as shown in Equation 2 and Figure 5 (e). Equation 2 shows the buoyancy F_b, and weight F_w can be ignored because the surface tension F_t of water is 10^6 orders of magnitude larger than the buoyancy F_b and the weight F_w. The net force F_n acting on a *Legionella*bacterium shows that the surface tension F_t of water is so large that a *Legionella* bacterium cannot break away from the surface membrane of water and escape, and *Legionella* B is pulled into the water due to the surface tension F_t:

$$F_n = F_t - F_b + F_w = 3.42\times10^{-8} - 3.99\times10^{-15} + 15.97\times10^{-15} \text{ kg} \quad (2)$$

where F_n denotes the total net force in kg, F_t denotes the surface tension of water in kg, F_b denotes the buoyancy in kg, and F_w denotes the weight force in kg.

Conclusions

Legionella bacteria can be easily removed by a membrane composed of water molecules, with a removal efficiency by the water membrane of 94.5%. It is very difficult for *Legionella* bacteria to break out of a water membrane and escape. Water can be used as a collection medium for *Legionella* and other bacteria. Washing hands with water can remove most of the bacteria and prevent it from spreading, thus maintaining public health. *Legionella* cannot be transmitted to people by mist, air, vapor, steam, or aerosol, but through splashing or touching. The real-time qPCR results suggested that no *Legionella* can escape from solution with air, vapor, steam, or aerosol.

Declarations

- Ethical Approval and Consent to participate

Not applicable.

- Consent for publication

All authors consent for publication.

- Availability of supporting data

Not applicable.

- Competing interests

The manuscript has no any conflict of interest.

- Funding

This project has been supported by the General Research Items of the Natural Science Foundation of Zhejiang Province, China (Grant No. Y5110280); the Key Research Items of Department of Education of Zhejiang Province, China (Grant No. Z201119987); the Natural Science Foundation of China (Grant No. 21876086); and the Primary Research and Development Plan of Jiangsu Province (Grant No. BE2018708).

- Authors' contributions

Authors' contributions are the same.

- Acknowledgements

The corresponding author would like to thank Professor Thanh Helen Nguyen for help, thank Professor Shaoting Du and Huijun Liu for instrument support, and also thank Denise R. for language editing. This project has been supported by the General Research Items of the Natural Science Foundation of Zhejiang Province, China (Grant No. Y5110280); the Key Research Items of Department of Education of Zhejiang Province, China (Grant No. Z201119987); the Natural Science Foundation of China (Grant No. 21876086); and the Primary Research and Development Plan of Jiangsu Province (Grant No. BE2018708).

- Authors' information

Jun Li: School of Environmental Science and Engineering, Zhejiang Gongshang University, 18 Xuezheng Street, Xiasha Gaojiaoyuan District, Hangzhou, Zhejiang, 310018, China, Email: lijun681116@163.com

Lin Liu: School of Environmental Science and Engineering, Zhejiang Gongshang University, 18 Xuezheng Street, Xiasha Gaojiaoyuan District, Hangzhou, Zhejiang, 310018, China, Email: 1341745792@qq.com

Kunquan Li: College of Engineering, Nanjing Agricultural University, 40 Dianjiangtai Road, Pukou District, Nanjing, Jiangsu, 210031, China, Email: kqlee@njau.edu.cn

Xuebin Li: Department of management and information technology, Nantong Shipping College, 185 Tongsheng Road, Nantong, Jiangsu, 226010, China, Email: lixuebin67@sina.com

Tao Tao: School of Environmental Science and Engineering, Huazhong University of Science & Technology, 1037 Luoyu Road, Wuhan, Hubei, 430074, China, Email: taocaiye@vip.sina.com

* Corresponding author

[1]School of Environmental Science and Engineering, Zhejiang Gongshang University, 18 Xuezheng Street, Xiasha Gaojiaoyuan District, Hangzhou, Zhejiang,310018, China

E-mail address: lijun681116@163.com

References

Breimen R. F., W. Cozen, B.S. Fields, T. D. Mastro, S. J. Carr, J. S. Spika, L. Mascola. Role of air sampling in investigation of an outbreak of Legionnaires' disease associated with exposure to aerosols from an evaporative condenser. Journal of Infectious Diseases, 1990, 161: 1257-1261.

Buzzle. http://www.buzzle.com/articles/surface-tension-meaning-and-practical-applications.html. (2015).

Campbell Neil A., Reece Jane B. Biology (8th ed.). Pearson. 2009, 47. ISBN 978-0-8053-6844-4.

Campese C., Roche D., Clément C., Fierobe F., Jarraud S., Waelle P. de, Perrin H., Che D. Cluster of Legionnaires' disease associated with a public whirlpool spa, France, April-May 2010. Euro Surveillance, 2010, 15 (26): 1-3.

Chiavazzo Eliodoro, Fasano Matteo, Asinari Pietro, Decuzzi Paolo. Scaling behaviour for the water transport in nanoconfined geometries. Natu. (2014).

Coetzee N., Duggal H., Hawker J., Ibbotson S., Harrison T. G., Phin N., Laza-Stanca V., Johnston R., Iqbal Z., Rehman Y., Knapper E., Robinson S., Aigbogun N. An outbreak of Legionnaires' disease associated with a display spa pool in retail premises, Stoke-on-Trent, United Kingdom, July 2012. Euro Surveillance, 2012, 17 (37): 1-3.

Docin. http://www.docin.com/p-506594503.html. (2015).

Graman P. S., Quinlan G. A., Rank J. A. Nosocomial Legionellosis traced to a contaminated ice machine. Infection Control and Hospital Epidemiology, 1997, 18: 637–640.

Hoy A. R., Bunker P. R. A precise solution of the rotation bending Schrödinger equation for a triatomic molecule with application to the water molecule. Journal of Molecular Spectroscopy, 1979, 74: 1-8.

Jun Li, Kunquan Li, Yan Zhou, Xuebin Li, Tao Tao, 2017. Kinetic analysis of Legionella inactivation using ozone in wastewater. Chemosphere, 168: 630-637.

Jun Li, Xuebin Li, Kunquan Li, Tao Tao. Plasmas ozone inactivation of Legionella in deionized water and wastewater. Environmental Science and Pollution Research, 2018a, 25(10): 9697-9707.

Jun Li, Kunquan Li, Erin Berns, Hanting Wang, Nora Sadik, Yun Shen, Xuebin Li, Tao Tao. Performance of nitrogen removal in an alternating activated sludge reactor for full-scale applications. Environmental Technology, 2018b, 8: 1-11. https://doi.org/10.1080/09593330.2018.1508251.

Jun Li, Tao Tao, Xue-bin Li, Li-min Wang, Hui Zheng. Effect of anaerobic time on biological nitrogen removal in a modified SBR. Desalination and Water Treatment, 2013, 51(19-21): 3691-3699.

Jun Li, Tao Tao, Xue-bin Li. A spectrophotometric method for determination of chemical oxygen demand using home-made reagents. Desalination, 2009, 239: 139-145.

Leonardo Gutierrez, Thanh H. Nguyen. Interactions between rotavirus and Suwannee River organic matter: Aggregation, deposition, and adhesion force measurement. Environmental science & technology, 2012, 46: 8705-8713.

Kadaifciler D. G., Cotuk A. Microbial contamination of dental unit water lines and effect on quality of indoor air. Environmental Monitoring and Assessment, 2014, 186: 3431-3444.

Kurosawa Hajime, Fujita Masahiro, Kobatake Satoshi, Kimura Hirokazu, Ohshima Mitsuko, Nagai Akira, Kaneko Shingaku, Iwasaki Yasuki, Kozawa Kunihisa. A case of Legionella pneumonia linked to a hot spring facility in Gunma Prefecture, Japan. Japanese Journal of Infectious Diseases, 2010, 63 (1): 78-79.

NBCnews. http://www.nbcnews.com/health/health-news/new-york-legionnaires-outbreak-sickens-71-n403301. (2015).

Tran Minh Nhu Nguyen, Daniele Ilef, Sophie Jarraud, Laurence Rouil, Christine Campese, Didier Che, Sylvie Haeghebaert, François Ganiayre, Frederic Marcel, Jerome Etienne, Jean-Claude Desenclos. A community-wide outbreak of Legionnaires disease linked to industrial cooling towers–how far can contaminated aerosols spread? The Journal of Infectious Diseases, 2006, 193 (1): 102-111.

Osawa Kayo, Shigemura Katsumi, Abe Yasuhisa, Jikimoto Takumi, Yoshida Hiroyuki, Fujisawa Masato, Arakawa Soichi. A case of nosocomial Legionella pneumonia associated with a contaminated hospital cooling tower. Journal of Infection and Chemotherapy, 2014, 20 (1-2): 68-70.

Palmore Tara N., Stock Frida, White Margaret, Ordner MaryAnn, Michelin Angela, Bennett John E., Murray Patrick R., Henderson David K. A cluster of cases of nosocomial Legionnaires disease linked to a

contaminated hospital decorative water fountain. Infection Control and Hospital Epidemiology, 2009, 30 (8): 764-768.

Sahid L. Rosado-Lausell, Hanting Wang, Leonardo Gutiérrez, Ofelia C. Romero-Maraccini, Xi-Zhi Niu, Karina Y. H., Gin Jean-Philippe Croué, Thanh H. Nguyen. Roles of singlet oxygen and triplet excited state of dissolved organic matter formed by different organic matters in bacteriophage MS2 inactivation. Water Research, 2013, 47: 4869-4879.

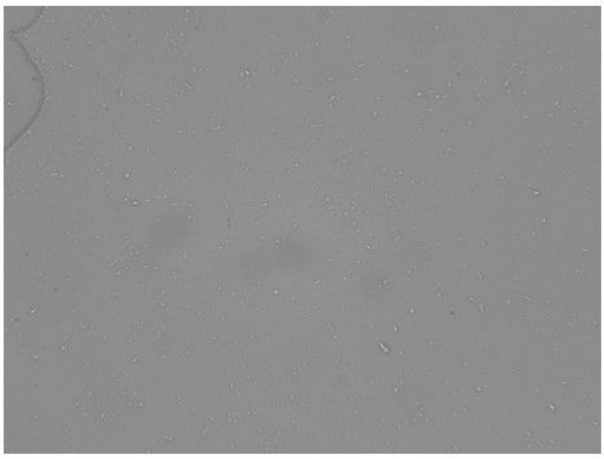
Shengkun Dong, Jun Li, Min-Hwan Kim, Sung-Jin Park, J. Gary Eden, Jeremy S. Guest, Thanh H. Nguyen. Human health trade-offs in the disinfection of wastewater for landscape irrigation: microplasma ozonation vs. chlorination. Environmental Science-Water Research & Technology, 2017, 3(1): 106-118.

Shengkun Dong, Jun Li, Min-Hwan Kim, Jinhoon Cho, Sung-Jin Park, Thanh H. Nguyen, J. Gary Eden. Deactivation of Legionella pneumophila in municipal wastewater by ozone generated in arrays of microchannel plasmas. Journal of Physics D: Applied Physics, 2018, 51(25): 1-9.

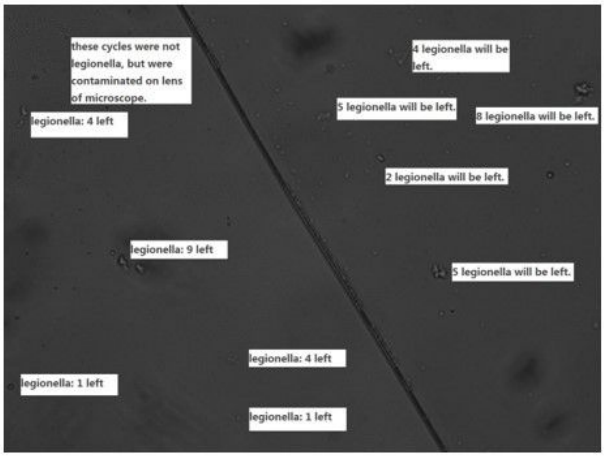
Janet E. Stout, Robert R. Muder , Sue Mietzner , Marilyn M. Wagener, Mary Beth Perri, Kathleen DeRoos , Dona Goodrich, William Arnold, Theresa Williamson, Ola Ruark, Christine Treadway, Elizabeth C. Eckstein, Debra Marshall, Mary Ellen Rafferty, Kathleen Sarro, Joann Page, Robert Jenkins, Gina Oda, Kathleen J. Shimoda, Marcus J. Zervos, Marvin Bittner, Sharon L. Camhi, Anand P. Panwalker, Curtis J. Donskey, Minh-Hong Nguyen, Mark Holodniy, Victor L. Yu, Legionella Study Group. Role of environmental surveillance in determining the risk of hospital-acquired Legionellosis: a national surveillance study with clinical correlations. Infection Control and Hospital Epidemiology, 2007, 28 (7): 818-824.

Van der Zee A., Verbakel H., De Jong C., Pot R., Bergmans A., Peeters M., Schneeberger P., Schellekens J. Novel PCR-probe assay for detection and discrimination between Legionella pneumophila and other Legionella species in clinical samples. Journal of Clinical Microbiology, 2002, 40(3): 1124-1125.

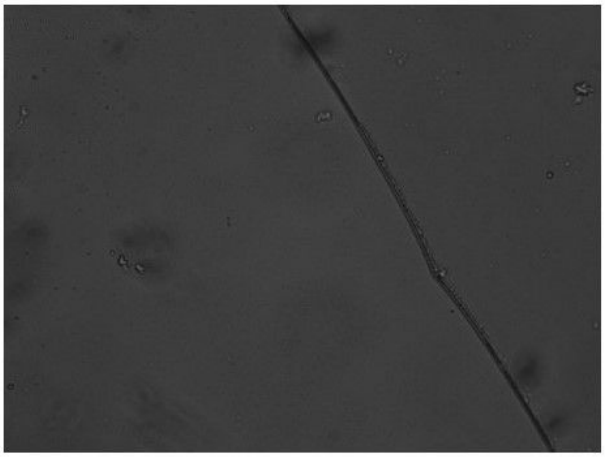
Figures



(a) *Legionella* at t=0 min



(b) *Legionella* at t=1 min



(c) *Legionella* at t=2 min

Figure 1

Legionella observation by microscope from t=0 to 2 min: (a) Legionella at t=0 min, (b) Legionella at t=1 min, and (c) Legionella at t=2 min.

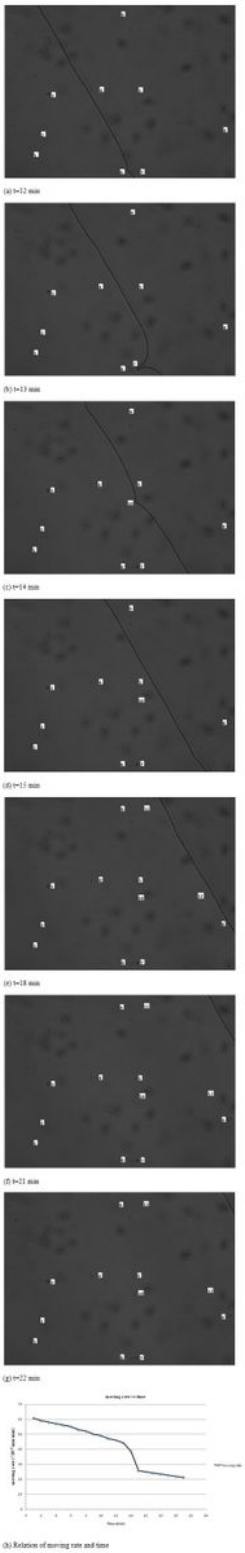
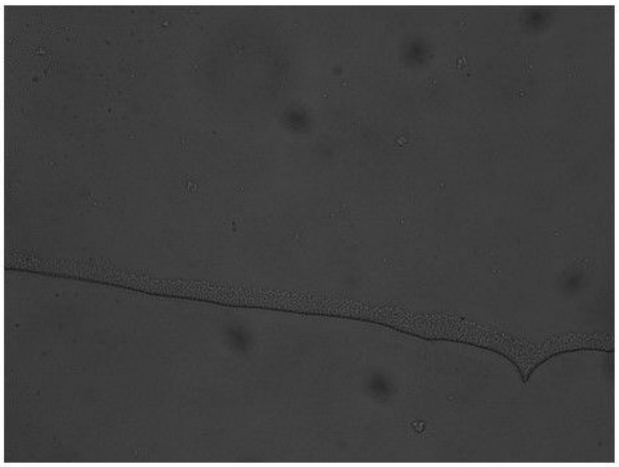
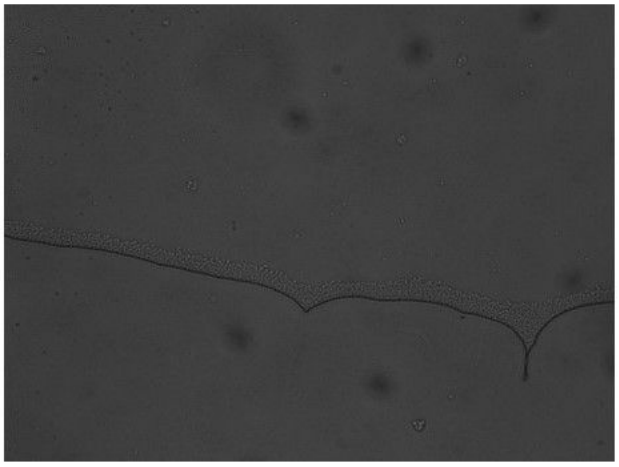


Figure 2

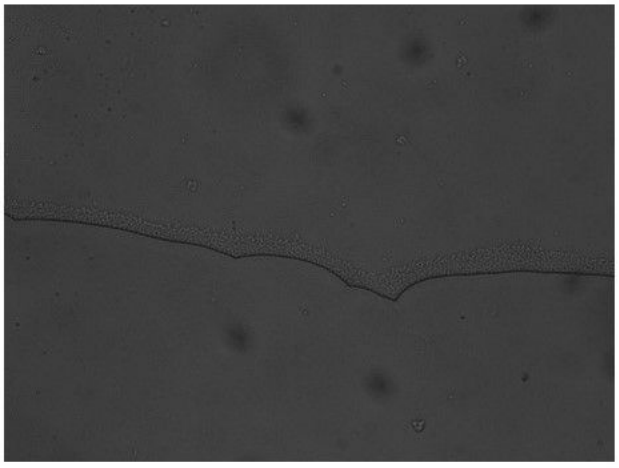
Legionella observation by microscope from t=12 to 23 min: (a) image of Legionella (t=12 min), (b) t=13 min, (c) t=14 min, (d) t=15 min, (e) t=18 min, (f) t=21 min, (g) t=22 min, and (h) relationship of moving rate and time.



(a) t=60 min



(b) t=61 min



(c) t=62 min

Figure 3

Legionella observation by microscope from t=60 to 62 min: (a) t=60 min, (b) t=61 min, and (c) t=62 min.

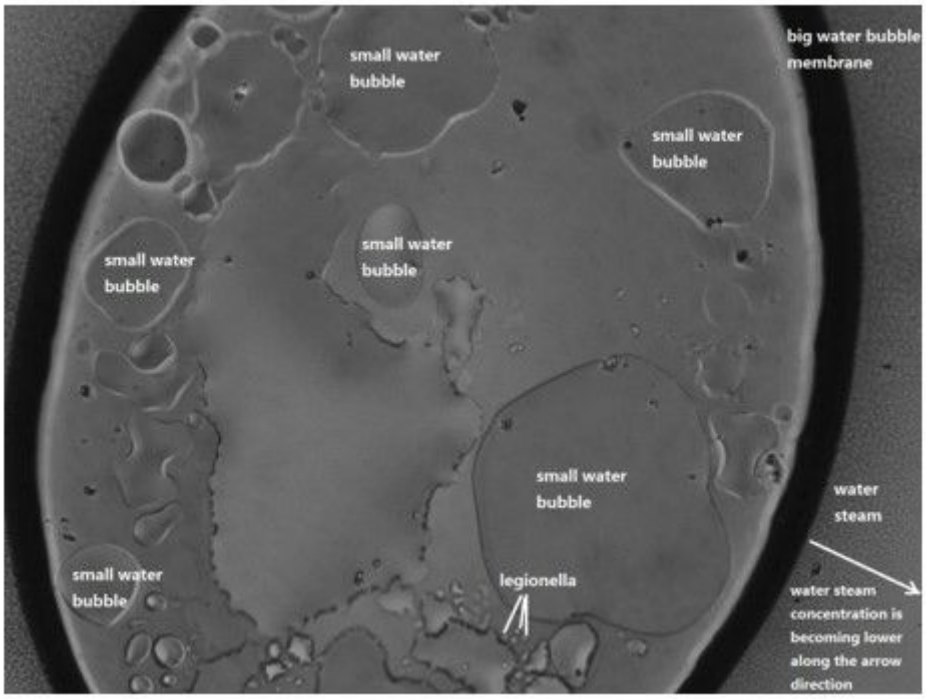


Figure 4

Microscopic observation shows Legionella and steam from contaminated water in a water bubble.

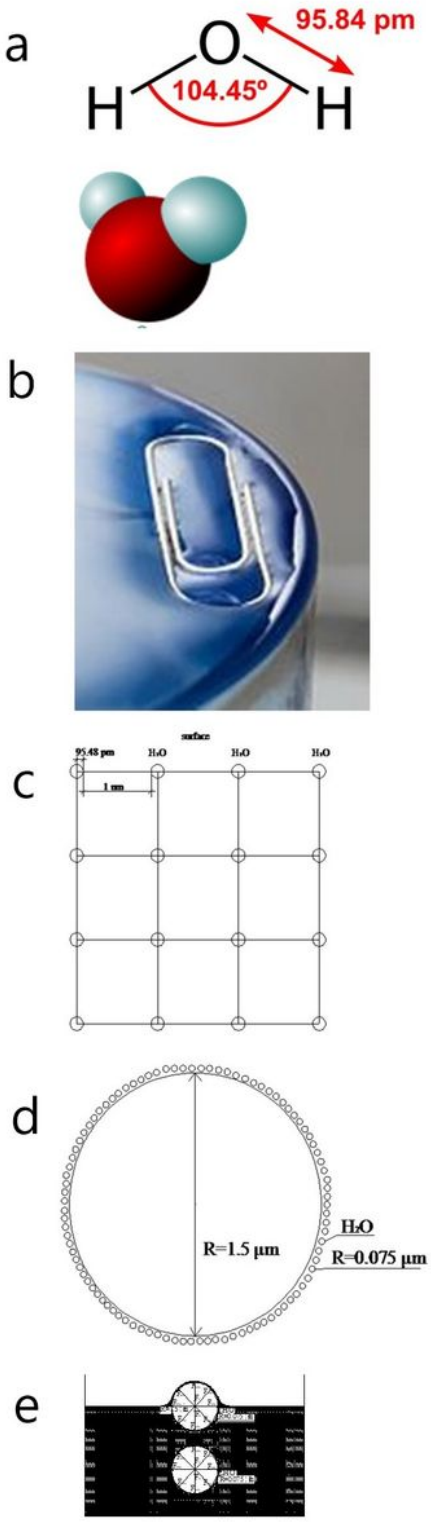


Figure 5

Surface tension of water, (a) Water molecule radius and model (Nguyen et al., 2006; Hoy et al., 1979), (b) a paper clip is able to float on the surface of water (Campbell et al., 2009), (c) molecules at the surface form stronger bonds, (d) Legionella bacteria and water molecule based on the actual scale of Legionella and 783 times the size of a water molecule, and (e) multiple force analysis of Legionella at different positions in water.