ASSESSMENT OF *LEGIONELLA PNEUMOPHILA* DEVELOPMENT THROUGH IRRIGATION SYSTEM MATERIALS

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Abstract. *Legionella pneumophila* can cause a type of pneumonia called Legionnaires' disease. The bacterium is responsible for most cases of the disease and can multiply in man-made water systems, such as irrigation systems. The study analyzed the potential development of *Legionella pneumophila* in irrigation system materials of Spanish hotels. Sprinkler irrigation systems were analysed in hotel golf courses and compared with the Spanish regulation standards in order to establish sanitation criteria for the prevention and control of legionellosis. An analytical study and principal component methods were employed in the context of materials, type of water and legislative compliance. Lead, polybutylene and wastewater treatment plant had great relevance. There was a strong association between wastewater treatment plant-copper and well water-polyvinyl chloride. Polybutylene, iron, polyethylene, stainless steel and lead showed a great magnitude in the contribution biplot, while lead facilities had a very low level of compliance in the correspondence analysis. Materials and legislative compliance were not statistically significantly associated ($p<0.05$). Considering the material and type of water, deficiencies were identified in the hygienic-preventive maintenance of the facilities. Attending the sanitary control criteria, ease of colonization and results obtained, it´s suggested that there is a relationship between the different materials and the risk of bacterial development.
Keywords: *legionellosis, prevention and control, Spain, golf, construction materials*

Introduction

Legionellosis is an acute respiratory infection linked to the environment. It´s a disease caused by a bacterium belonging to the Legionellaceae family. It comprises a genus, *Legionella*, with a total of 52 species and 70 serogroups. For the *Legionella pneumophila* species 14 serogroups have been described. More than a half of the species have been implicated in human infection, however the most common legionellosis is cause by *Legionella pneumophila* serogroup 1 which is the most frequent in the environment. *Legionella pneumophila* is responsible for 90% of cases, being serogroups 1, 4 and 6 the most relevant, followed by *Legionella micdadei* (responsible for 10%), *Legionella bozemanii*, *Legionella longbeachae* and *Legionella dumoffii*. The most common form of transmission of the microorganism is through aerosolization of water (Nguyen et al., 2006) although more studies are required in this regard (O'Connor et al., 2007; Den Boer et al., 2015; Correia et al., 2016). The agent was initially identified in 1976 following an outbreak in Philadelphia (USA) (Cunha et al., 2016). Although the sources of development of the bacterium comprise a variety of devices related to human activity (Moritz et al., 2010), it has a natural presence in non-anthropic media (Van Heijnsbergen et al., 2015). Within the term "legionellosis" different forms are included: Legionnaires' disease, Pontiac and Lochgoilhead fever. Regarding clinical forms of presentation (Cunha, 2010), the most important are the first two.

Legionellosis shows a worldwide distribution with representation in North and South America, Asia, Africa, Australia and Europe. The purpose of the epidemiological surveillance is, among others, to control the occurrence of cases and outbreaks linked to risk facilities, which could be related to the environment (Kozak et al., 2013) or work

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environment (Kozak-Muiznieks et al., 2014). The infection can be acquired fundamentally in two main areas: the community and nosocomial (Irons et al., 2013). In both the disease may be associated with various types of facilities and buildings, and can occur in the form of outbreaks, group of cases, and isolated or sporadic cases. It is worth noting cases link with travellers.

In Spain, legionellosis is a topic of special health interest, being a disease that is subject to specific monitoring by the government, essential (NNDSS Annual Report Working Group, 2016) for its prevention and control (Maini et al., 2012; Lee, 2018). The epidemiological surveillance of legionellosis is preferably based on the National Epidemiological Surveillance Network. Further, the purpose of legionellosis legislation is none other than control the appearance of cases and outbreaks linked to risky facilities. In recent years, the evolution and improvement of Royal Decree 909/2001 (Real Decreto 909, 2001) conduced to Royal Decree 865/2003 (Real Decreto 865, 2003), which established the hygienic-sanitary criteria for the prevention and control of legionellosis. Thus, legislative implementation together with the epidemiological surveillance system are synergistic elements that result in a better health protection (Campese et al., 2011).

In Spain around 50% of the hotels are somewhere colonized by *Legionella* spp. in the water network. This quantity is like those detected in Europe and other parts of the world and denote that it is a globally distributed and extraordinarily ubiquitous bacterium in natural and anthropic environments. The presence in these media of other microorganisms (bacteria, protozoa, algae) brings on their development by colonization, as well as iron, which acts as a development enhancer in some systems and facilities. The formation of biofilms, corrosion materials and incrustations in pipes is essential for the bacterium to find niches (Atlas, 1999; Ji et al., 2015) that protect it against certain conditions adverse in the aqueous medium.

This study was aimed to determine if the potential development of *Legionella pneumophila* may be related to irrigation system materials from hotels and the compliance of Spanish health legislation for prevention of legionellosis.

Materials and Methods

Sprinkler irrigation systems were analyzed in hotel golf courses (n=31) located in both inland and coastal communities of Malaga (Andalusia, Spain), comparing the results obtained with the Spanish regulation standards through which sanitary criteria is established for the prevention and control of legionellosis (Real Decreto 865, 2003).

The design of the study suggested gathering information through a self-administered survey (*Table A1* in the *Appendix*) aimed at directors and persons in charge of buildings with facilities at risk, as well as drawing water samples for a later analysis on laboratories authorized by the Spanish Sanitation Authority, in accordance to standardized regulations (ISO 11731, 2017). Forty variables were selected (*Table A1* in the *Appendix except * and non-selected in I*) for the sanitary control of the *Legionella pneumophila* development.

Exploratory data analysis and contingency tables were used to assess whether there was a dependence between categories. These tables drew a graphical matrix where each cell contained a dot whose size reflects the relative magnitude of the corresponding component. Particularly, materials with type of water and legislative compliance. To interpret the significant dependence a chi-square statistic was used between materials and the last. To obtain the nature of the dependence between the row and the column variables Pearson residuals (standardized residuals) were calculated and the contribution (%) of

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each cell to the total chi-square score. Also, eigenvalues to determine the number of axes were considered in subsequent analysis.

An analytical study and principal components methods was carried out to explain the *n* observations (materials) and the *p* original variables (type of water), recycled water (Rw), wastewater treatment plant (Wwtp) and well water (Ww), according to a *biplot* graphic display (symmetric, asymmetric and contribution). To achieve this, their scores were represented in the coordinate axes of the principal components that characterize the *p* variables. To obtain factorial solutions, the principal component analysis considered the total variance, estimating the factors that contain low proportions of unique variance and, in specific cases, error variance. The study's interest focused on the prediction or on the minimum number of necessary factors to justify the maximum variance portion, represented on the original series of variables. As an objective means of interpretation, a vector model was used to capture the position degrees of irrigation pipe materials in the perceptual map. The bacterium potential development was considered in function of the material and physicochemical and microbiological water controls, in addition to the frequency of said controls and, in particular, of *Legionella* spp. and total aerobes. In the symmetric plot only the distance between row or column points can be interpreted, not the inter-distance between them. That´s why to interpret the mentioned distance an asymmetric plot was done, with column profiles presented in row space or vice versa. In the standard symmetric biplot it´s difficult to know the most contributing points to the solution of the correspondence analysis (Kassambara, 2017). In the asymmetric biplot, rows (or columns) points are plotted from the standard coordinates and the profiles of the columns (or the rows) are plotted from the principal coordinates (Bendixen, 2003). The argument used is a logical vector specifying if the plot should contain points (false, default) or arrows (true). The first value sets the rows and the second value sets the columns. The contribution biplot incorporates the contribution of points. In this display, points that contribute very little to the solution, are close to the center of the biplot and are relatively unimportant to the interpretation. We interpreted the contribution of rows to the definition of the axes. In this case, emphasize that columns are in principal coordinates and rows in standard coordinates multiplied by the square root of the mass. For a given row, the square of the new coordinate on an axis i is exactly the contribution of this row to the inertia of the axis i (Greenacre, 2013).

Likewise, the relation (correspondence analysis) between "materials" and "legislation fulfillment" was analyzed according to the observations corresponding to the number of hotels that include facilities at risk for bacterium. To this end, as we indicated above, a chi-square test of independence was carried out, where the null hypothesis was the independence between both variables. For the correspondence analysis, chi-square test of independence was used to analyze the frequency table formed by these variables, evaluating whether there was a significant association between the categories ($p<0.05$). Afterwards, the interdependence was presented through the dimensional reduction and perceptual map, the latter of which was based on the association between hotel fulfillment and the materials, expressing the correspondence of variable category with a unique capacity to represent rows and columns in a unique multidimensional space, and offering a multivariant interdependence expression for non-metric data. The observations were ordered according to the type of legislative compliance with the Spanish regulation standards to establish sanitation criteria for the prevention and control of legionellosis: very low (Vl, 0-10%), low (Lo, 11-40%), medium (Me, 41-70%), high (Hi, 71-90%) and very high (Vh, 91-100%). The analysis of the observations was carried out for the

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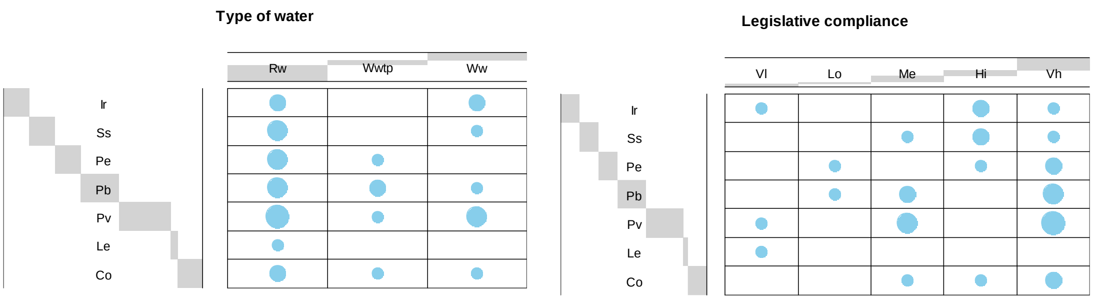
materials of the different systems: copper (Co), lead (Le), iron (Ir), stainless steel (Ss), polybutylene (Pb), polyethylene (Pe) and polyvinyl chloride (Pv).

Analysis was conducted in R (R Core Team, 2019) using FactoMineR and FactoExtraR (ggplot2-based elegant visualization) packages.

Results

To assess whether there is a dependence between "type of water" and "legislative compliance" referred to "materials" two contingency tables were designed (*Figure 1*). Additionally, chi-square test of independence shows a Pearson λ value=27.04 (p-value=0.30), so materials and legislative compliance are not statistically significant associated. Pearson residuals can be visualized in *Figure 2*, in which cells with the highest absolute standardized residuals contribute the most to the chi-square score (size of the circle is proportional to the amount of the cell contribution). The positive values in cells specify a positive association between the corresponding row (materials) and column (legislative compliance) variables. The negatives values imply a repulsion among the corresponding. In *Figure 2A* it´s evident that there are an association between Vl-Ir, Hi-Ir and Hi-Ss. There is a strong positive association between Vl-Le. Negative residuals expressed that Vl and Lo are negatively associated with Co. There is a repulsion between Me-Ir and lighter between Vh-Ir and Vh-Ss. The contribution in percentage is in *Figure 2B*. The relative contribution of each cell to the total chi-square score gives some indication of the nature of the dependency between materials and legislative compliance of the contingency table. The most contributing cells to the chi-square are: Vl-Le (31.17%, strongly associated), Lo-Pe (7.88%), Hi-Ir and Hi-Ss (7.17%). These cells contribute about 53.39% to the total chi-square score. In this case, the contribution of one cell to the total chi-square score becomes a useful way of establishing the nature of dependency.



(A) (B)

Figure 1. *Dependence.* ***(A)*** *Materials and type of water.* ***(B)*** *Materials and legislative compliance*

The results of applying classification data to the perceptual compositional map are shown in a biplot (*Figure 3A*), in which three distinct types of attribute dimensions can be identified (each type of water), all in different directions. To highlight the most contributing row points for each dimension *Figure 3B* was done. The most contributing row points is spotlight on the scatter plot giving an idea of what pole of the dimensions the row categories are actually contributing to. It´s evident that row categories Pe and Pb have an important contribution to the positive pole of the first dimension, while the

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categories Ir and Ss have a major contribution to the negative pole of the first dimension (Kassambara, 2017). Well water is almost perpendicular when compared to wastewater treatment plant, suggesting a separate and distinct dimension of its own. This representation is expressed on the biplot graphic display according to the initials corresponding to different materials and original variables. Regarding each type of water, the variance for the variables is very similar, since they have associated near length vectors. A correlation likewise exists, since the angle that separates the corresponding vectors is small. The direction of the axis corresponding to the first principal component arranges the data and it can be observed, through the scores obtained by the first two principal components in the observations, that the best materials regarding preventive compliance are Pv and Pb, always in respect to what the first principal component represents, which will be the most important one and will hold the largest data matrix variability. The approximation order is well and recycled water (Pv > Co/Ss > Pe > Ir > Pb > Le) and wastewater treatment plant (Pb > Pe > Co > Ss > Ir > Pb > Le).

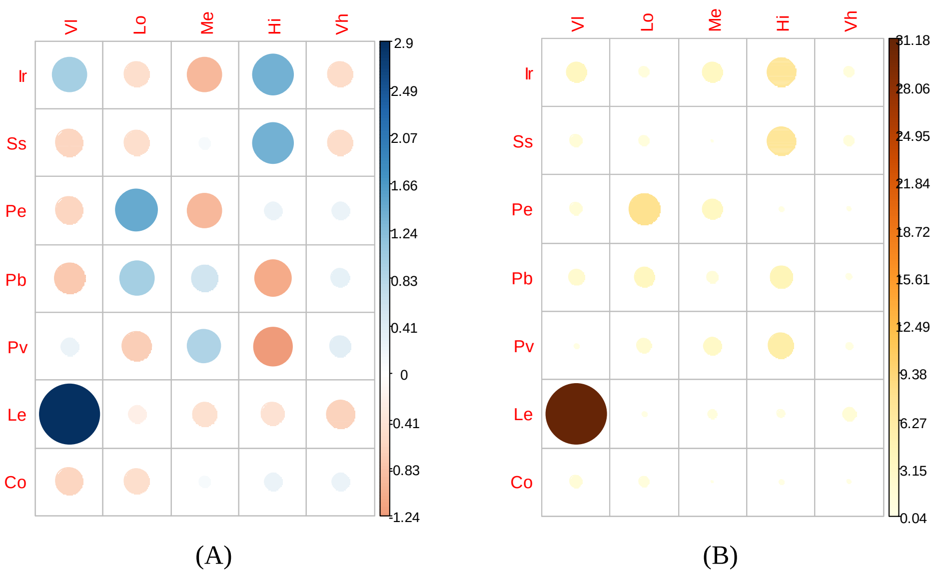

***Figure 2.** **(A)** Pearson residuals. **(B)** Contributions (%). Positive residuals are in blue and negative in red*

As mentioned before, first we represented the standard plot of correspondence analysis called symmetric biplot (*Figure 3C*). Materials (blue points) and type of water (red triangles) were represented in the same space using the principal coordinates (profiles). After, we calculated an asymmetric biplot (*Figure 3D*) to interpret the distance between column points and row points. As seen in contribution biplot (*Figure 3E*) the position of the column profile points is unchanged relative to that in the conventional biplot and the distances of the row points from the plot origin are related to their contributions to the two-dimensional factor map.

Through the analysis in two coordinates, the correspondence graph (*Figure 4*) was obtained. The representation of the two dimensions holds 77.8%, which is enough to conclude the two-dimensional representation is adequate. This means that the total inertia value is held at a 98.7% rate by the two-dimensional representation, which leads to the conclusion that said representation is valid. It is worth noting that the perceptual map obtained during the correspondences analysis is weighted by the existing inherent

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interdependences and potential biases of a possible omitted attribute in a given “material”, or of a single inappropriate attribute (or system). In the last *Figure*, irrigation system materials are located near the legislative compliance with which they are highly related, and apart from other categories with lower correspondences.

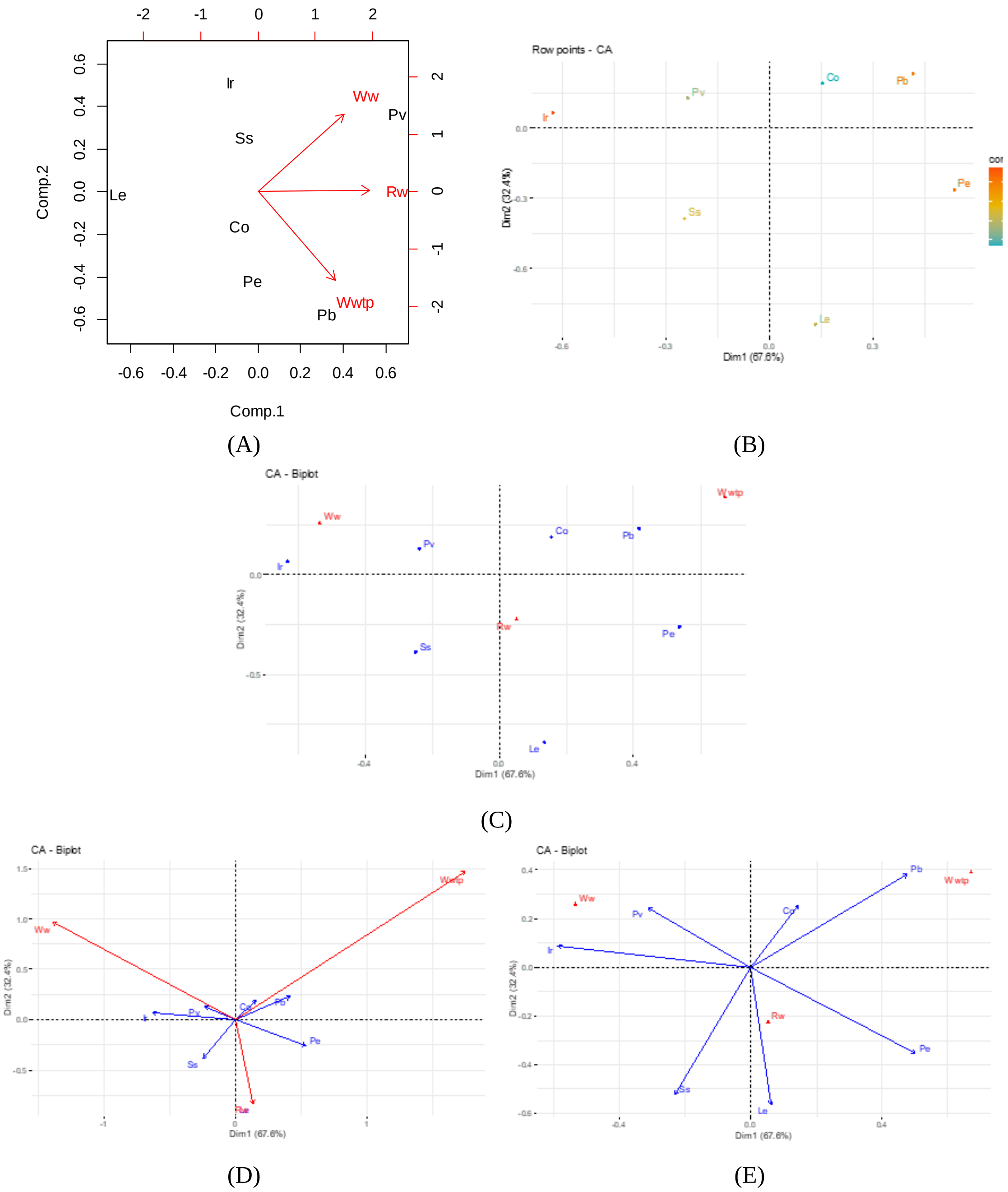

***Figure 3.** Graphs of materials-type of water. **(A)** Biplot. **(B)** Contributions of rows (materials) to the dimensions. **(C)** Symmetric biplot. **(D)** Asymmetric biplot. **(E)** Contribution biplot*

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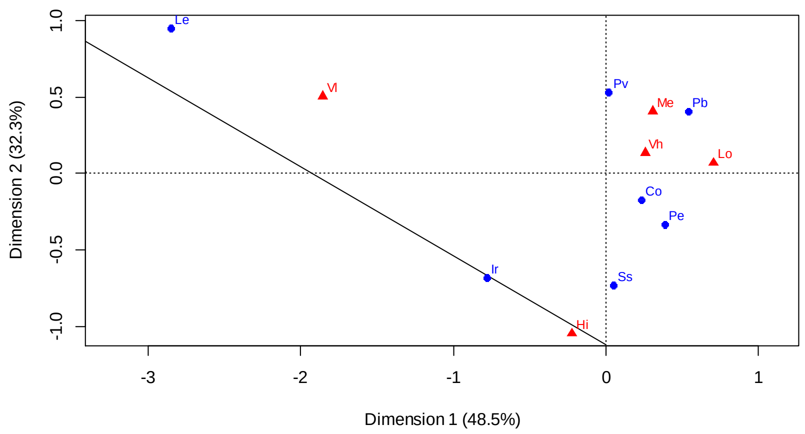

Figure 4. *Correspondence analysis: materials-legislative compliance*

Discussion

Considering the materials studied, it is mandatory to indicate that iron is a very critical material about *Legionella pneumophila*, easily colonizable, but in our study the facilities are protect by a high sanitary legislative compliance. The same occurs for stainless steel (van der Kooij et al., 2020), with less risk than the previous material. Likewise, polyethylene is a substrate that can be easily colonized by the bacterium, although to a lesser extent than iron, with intermediate preventive levels for the irrigation facilities studied. Regarding polybutylene, for the tests carried out for the survival and growth of *Legionella pneumophila* in the planktonic phases and in biofilm at 20ºC, the bacterium appears with a low proportion in the flora of the biofilm and in chlorinated polyvinyl chloride, but it is absent on copper surfaces. The pathogen is more abundant in biofilms in plastics at 40ºC, where it represents 50% of the total flora. Copper surfaces appear to be inhibitory to biological contamination and include only a low number of microorganisms, and polyvinyl chloride has low ease of being colonized (Lu et al., 2014). In fact, the pathogen can survive in biofilms on the surface of plastic materials at 50ºC but is absent from copper surfaces at the same temperature. In the presence of copper surfaces, biofilms formed on (adjacent) glass control surfaces are capable of "incorporating" copper ions, which subsequently inhibit colonization on their surfaces (Rogers et al., 1994), added to the low adhesion of the glass (Assaidi et al., 2018). It´s suggested that the use of copper pipes in water systems can help limit their colonization by *Legionella pneumophila*, an aspect that is acceptably controlled at a preventive level in the facilities analysed. It is worth noting the lead facilities, most of which are very old and deteriorated, which are not being performed hygienic-preventive maintenance, and could be subject to the formation of biofilms with growth of the specific bacterium studied. In fact, there is a relationship between this critical material and poor hygiene standards in Spanish neglected older irrigation systems, low-class hotels, and rural areas.

The chi-square test indicated whether there is a significant association between "materials" and the “preventive compliance intervals”. According to the Pearson´s chi-square test and because the p-value of the test is high, the null hypothesis of independence between the two variables was accepted. Likewise, it can be deduced that a certain material has no dependency to compliance or how these approaches can affect the growth and development of the bacterium.

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The symmetric biplot showed a global pattern within the data. The distance between any material points or type of water points gave a measure of their similarity or not (general statements). In this way it can be stated that row and column points with similar profile were closed on the factor map. *Figure 3C* showed that Ir and Pv are more related to Ww, and Pb and Co with Wwtp. Additionally, the row variables with the larger value, contributed the most to the definition of the dimensions. Ir, Pe, Pb are the rows that contributed the most to dimension 1 and 2 and are the most important explaining the variability in the data set. Dimension 1 was mainly defined by the opposition of Pb-Co and Ir-Pv (positive and negative pole respectively). In the asymmetric biplot if the angle between two arrows was acute, then there was a strong association between the corresponding row and column, but to interpret the distance between rows and columns we perpendicularly project row points on the column arrow. In this biplot it´s evident that there was a strong association between Wwtp-Co and Ww-Pv. Wwtp had great relevance. Notice that in the contribution biplot the closer an arrow was (in terms of angular distance) to an axis the greater was the contribution of the row category on that axis relative to the other axis. If the arrow was near halfway between the two, its row category contributed to the two axes to the same extent (Kassambara, 2017). The variables that less stand out in magnitude were Co and Pv (explanatory power resides in the remaining), Ir was the most. Pe was the one that contributed the most to dimension 1.

From the correspondence analysis, the main conclusions were obtained due to the proximity or not of the representation of the variables. It should be noted that lead facilities had a very low level of compliance, being this material a nutrient that favors the development of the bacterium. Polybutylene and polyvinyl chloride materials presented a high risk (medium level of compliance) as well as copper (Rhoads et al., 2017) and polyethylene (medium-high level), not being associated with the bacterial growth studied.

Conclusions

Therefore, it can be concluded about the presence of materials easily colonizable by *Legionella pneumophila*, which do not have adequate hygienic-preventive maintenance, and which can be sources of development and proliferation of bacterium. It is worth highlighting Le and Pb with Rw (Fahrenfeld et al., 2013; Pepper and Gerba, 2018) and Wwtp (Kulkarni et al., 2018; Lequette et al., 2019), respectively. It´s estimated that the need for treatments according to the material (compatibility between the type of material, treatment used, condition and design) should be considered for a better control of the bacterium. Likewise, it is noteworthy the consideration of the origin of the irrigation water of the golf courses in the hotels studied (Papadakis et al., 2018; Gea-Izquierdo, 2020) and its need for treatment according to the physicochemical and microbiological characteristics of water (Gea-Izquierdo, 2018).

Recommendations

Possibility of expanding the Spanish sanitary legislative requirements for the prevention and control of legionellosis in irrigation systems is proposed (Mori et al., 2021), improving technical surveillance, increasing water quality controls, substituting materials for others with less risk of development and proliferation of the bacterium (Wang et al., 2012; Cullom et al., 2020), and vigilance regarding cross contamination with other facilities or water distribution systems (Blanky et al., 2015; De Giglio et al., 2019).

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APPENDIX

***Appendix 1.** The questionnaire used for monitoring prevention and control of legionellosis*

I)- Do you have any of the following facilities? Mark with a cross what applies.

	AVAILABILITY			OPERATION		WORKING	
FACILITY	YES	NO	**Number**	SEASONAL	NOT SEASONAL	DIURNAL	NIGHT
1.-Sanitary hot water							
2.-Accumulators (“boilers”)							
3.-Sanitary cold water							
4.-Water tanks							
5.-Cooling towers							
6.-Evaporative condensers							
7.-Adiabatic condensers							
8.-Bathtubs without recirculation (single use)							
9.-Recirculating pools (collective use, spas o similar)							
10.-Humidifiers/evaporative cooling/ others							
11.-Water lines in dental units							
12.-Ornamental fountains							
13.-Tank trucks							
14.-Humidification in air ducts							
15.-Sprinkler irrigation (including aerosol)							
16.-Other industrial processes (with aerosol)							
17.-Thermal facilities							
18.-Aquariums (uncovered)							
19.-Fire fighting water systems							

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20.- Origin of the water: Public ☐ Private ☐

Recycled water ☐ Wastewater treatment plant ☐ Well water ☐

	YES	NO
II) 21.- In the case of cooling towers, evaporative condensers or adiabatic, have they been notified to the competent health authority?		
22.- Have any modifications or improvements been made since its installation?		
23.- Do you usually carry out any control of the quality of the water?		
24.- If so, are the analysis carried out in an approved laboratory?		

III)-25.- What is the distance between the cooling towers or similar systems (unprotected) and the people who may be exposed to the aerosols?___meters.

26.- And regarding air conditioning or ventilation intakes?___meters.

	YES	NO
IV) 27.- The cooling system, can it be emptied completely?		

28.- What flow of circulating water exists?_______l/h.

29.- And of dragged water?______l/h.

	YES	NO
30.- Do you have a continuous biocide dosing system?		

31.- Do you have any other disinfection system? Which one?

	YES	NO
- Chlorination		
- Ultraviolet radiation		
- Ozonation		
- Bromation		
- Copper-silver ionization		

V)- In cooling towers, evaporative and adiabatic condensers, others do you carry out water controls? (Please indicate facility type)

	YES	NO
32.-Physical-chemical		
33.-Microbiological		

With what periodicity?

	Annual	Biannual	Quarterly	Three-month	Bimonthly	Monthly	Weekly	Daily	Undefined
34.-Physical-chemical									
35.-*Legionella*									
36.-Total aerobes									

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	YES	NO
VI) 37.- Do you have a facilities Maintenance Program?		
38.- And a Maintenance Registry?		

	YES	NO
VII) 39.-Staff performing hygienic-sanitary maintenance operations; have they completed the training courses, to that effect, approved by the Ministry of Health?		
40.- Do you have an external company to carry out the different treatments?		
41.- If so, is said company registered in the Official Register of Biocidal Establishments and Services?		

VIII) 42.- In the facilities marked by you in the box of the first question, is there any control of the water temperature?

43.- Is there water disinfection? What kind of disinfection?

	YES	NO
IX) 44.- Do you have a filtration system for drinking water?		

	YES	NO
X) 45.- Is access to drinking water equipment safe?		
46.- And for cooling towers and similar systems?		

XI) 47.- What material are the drinking water pipes made of?

Polybutylene ☐ Polyethylene ☐ Iron ☐ Lead ☐ Stainless steel ☐ Polyvinyl chloride ☐

Copper ☐ Other _______________.

XII) 48.- What is the temperature of the sanitary cold water?________ºC.

	YES	NO
49.- Where are water tanks located?		
- Inside		
- Outside		
50.- Are they covered?		
51.- Do they have any access inside?		
52.-Are they isolated?		

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XIII) 53.- What is the temperature of the sanitary hot water system?__ ºC.

54.- What is the temperature of the water in the accumulators?___ºC.

55.- Do accumulators have a drain valve?

YES	NO

XIV) 56.- Do you have respiratory therapy equipment?*

YES	NO

57.- How often do you disinfect reusable equipment?*

Monthly	Weekly	Daily	After use	Undefined	Never

XV) 58.- Do ornamental fountains have an automatic chlorination system?

YES	NO

XVI) 59.- Are the chemical products used in different water treatments approved?

60.- Do they have safety data sheet?

YES	NO

***STUDY CENTER*:**____

OBSERVATIONS:

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