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Occurrence of *Legionella* in groundwater used for sprinkler irrigation in Southern Italy

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ABSTRACT

Legionellae are opportunistic bacteria that cause various conditions after exposure to contaminated aerosols, ranging from a serious type of pneumonia to a mild case of an influenza-like illness. Despite the risks of exposure, little is known about the occurrence of *Legionella* in natural environments and, even though studies have shown that there is a potential risk of transmission via inhalation, it does not have to be detected in groundwater that is used for irrigation. The culture methods traditionally used to detect *Legionella* have several limits that can be partly solved by applying molecular techniques.

Samples from 177 wells in Apulia, Southern Italy, were collected twice, in winter and in summer, and analyzed. When compared with the guidelines, 145 (81.9%) of the sampled wells were suitable for irrigation use. The culture-based method highlighted the presence of different species and serogroups of *Legionella* in 31 (21.2%) of the 145 wells that were shown to be suitable for irrigation use.

A greater number of wells returned positive results for *Legionella* in summer than in winter ($p = 0.023$), and the median concentrations were mostly higher in summer (500 CFU/L) than in winter (300 CFU/L). The median temperature in the *Legionella* positive well waters was significantly higher than that in the negative ones, both in winter and in summer ($p < 0.001$).

Using molecular techniques, *Legionella non-pneumophila* was found in 37 of the 114 wells earlier detected as suitable for irrigation use but negative for *Legionella* by the culture-based methods. The distribution of *Legionella* differ significantly in porous aquifers compared to the karst-fissured ones both with quantitative polymerase chain reaction (qPCR) ($p = 0.0004$) and viable cells by propidium monoazide (PMA-qPCR) ($p = 0.0000$). *Legionella* concentrations were weakly correlated with temperature of water both with qPCR ($\rho = 0.47$, $p = 0.0033$) and PMA-qPCR ($\rho = 0.41$, $p = 0.0126$).

Our data suggest that water that aerosolizes when sprinkled on plants represents a potential source of Legionellosis, with a higher risk from exposure in summer. On a practical level, this finding is important for workers (farmers and gardeners) who are in contact with waters used for irrigation.

Abbreviations: CFUs, colony forming units; DM 185/03, Ministerial Decree No. 185 of 12 June 2003; MID, minimum infectious dose; Lpn, Legionella pneumophila; sg, serogroups; GA, Gargano; MU, Murgia; SA, Salento; TAV, Tavoliere; JON, Arco Jonico Tarantino; GU, genomic units

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1. Introduction

Legionella is an intracellular Gram-negative microorganism that is ubiquitous in natural and artificial water systems. It grows at temperatures between 25 and 42 °C, but can survive in temperatures between 5.7 and 63 °C, especially if the water is stagnant (Borella et al., 2005; Napoli et al., 2010). To date, 61 species and more than 70 serogroups (sg) have been classified, of which *Legionella pneumophila* (Lpn) is the most common species detected in clinical cases (Euzéby, 2018 http://www.bacterio.cict.fr/l/legionella.html, Montagna et al., 2018b). Legionellosis, the collective term for diseases caused by *Legionella*, occurs when aerosols produced in contaminated water sources are inhaled and is characterized by different clinical forms ranging from a serious type of pneumonia (Legionnaires' disease) to a mild case of an influenza-like illness known as Pontiac fever (Borella et al., 2005; Napoli et al., 2010). Sporadic and epidemic Legionellosis are distributed worldwide (Phin et al., 2014).

There are frequent reports of *Legionella* contamination in artificial water systems of hotels, gyms, private apartments, schools, offices, churches, swimming pools (Borella et al., 2004, 2005; Delia et al., 2007; Napoli et al., 2010), and also in fountains and spas (Palmore et al., 2009; Costa et al., 2010; Haupt et al., 2012; Montagna et al., 2012). *Legionella* contamination in healthcare facilities is a cause for particular concern because the water supply and hospital equipment can be a potential source of infection (Montagna et al., 2016, 2017a, 2017b, 2018a).

In contrast however, there are limited records of *Legionella* in natural environments (Amemura-Maekawa et al., 2012; Gomez-Alvarez et al., 2015; Guo et al., 2015; Cassell et al., 2018; Steege, Moore, 2018), and information about its occurrence in groundwater is rare (Riffard et al., 2001; Brooks et al., 2004; Costa et al., 2005; Gomez-Alvarez et al., 2015; Inoue et al., 2015), probably because it is generally accepted that groundwater quality is preserved by soil filtration processes, or protected by overlying impermeable strata (O'Reilly et al., 2007). To date, studies have reported *Legionella* in well waters or in artificial water networks served by wells, but these studies have mostly focused on the different analysis methods (Riffard et al., 2001; Brooks et al., 2004; Costa et al., 2005; Beer et al., 2015, Gomez-Alvarez et al., 2015, Inoue et al., 2015, Richards et al., 2018). Few authors have considered the potential biological risk to humans if *Legionella*-contaminated water is used for purposes other than drinking (Rota et al., 2011).

According to Italian Ministerial Decree No. 185 of 12 June 2003 (DM 185/03), water can be used for irrigation if the fecal indicators *Escherichia coli* and *Salmonella* are present either at concentrations of less than 100 colony forming units per 100 mL (CFU_s/100 mL) or not detected in a sample of 1000 mL, respectively. Because the standards relate only to *E. coli* and *Salmonella*, in Italy data for other microorganisms in water for irrigation use are limited (De Giglio et al., 2016, 2017) and water does not have to be tested for *Legionella*, even though the potential risk of transmission via inhalation is widely documented (Pasquarella et al., 2010, 2012; Montagna et al., 2018b).

To date, no known outbreaks of Legionellosis have been linked directly to natural waters. Although the *Legionella* concentrations in groundwater reported so far in the literature are usually low and are presumably below the minimum infectious dose (MID), the MID is difficult to define precisely because it depends on many factors, including the virulence of the organism and the susceptibility of the host (Brooks et al., 2004). The risks may also vary seasonally as Legionnaires' disease reaches a maximum during the warm season in temperate regions, perhaps because of the high temperatures and heavy rainfall (Sakamoto, 2015).

As reported in the 6th Italian General Census of Agriculture, when compared with other regions, Apulia has one of the largest areas irrigated by groundwater in Italy. Sprinkler irrigation is the most common method (39.6%), followed by surface flow irrigation (30.9%), and micro-irrigation (17.5%) (Istat Data, 2013), for irrigating cultivated fields and public and private ornamental gardens. Sector operators, such as farmers and gardeners, could be at risk of contracting Legionellosis if they inhale contaminated aerosols generated by sprinkler irrigation (Stojek and Dutkiewicz, 2002; Thomas et al., 2014; Hamilton et al., 2018; Papadakis et al., 2018; Pepper, Gerba, 2018).

This article forms part of a large study about groundwater pollution in Apulia (Montagna et al., 2016, Montagna et al., 2017). The aims of this part of the study were i) to evaluate, for what is thought to be the first time in Italy, the presence of *Legionella* in groundwater suitable for irrigation use (DM 185/03) by culture-based methods and the relationships between *Legionella* and *E. coli/Salmonella*; ii) to test well waters that were negative for *Legionella* for the presence of vital but non-cultivable cells with molecular methods, and iii) to determine whether *Legionella* contamination was related to a particular aquifer type or season (winter or summer).

2. Materials and methods

2.1. Study area

Apulia, a region in southeastern Italy, covers about 20,000 km^2 and has 4 million inhabitants. Because of its Mediterranean climate with mild and dry winters, hot summers, and irregular annual rainfall, agriculture plays an important role in the economics of the region. Apulia ranks second in Italy for the production of several foods, particularly fresh fruits and vegetables to be eaten raw (e.g., salad greens, tomato, fennel, and celery). Large amounts of water are therefore required for irrigation and about 75% of the water demand for agriculture is met by groundwater; the groundwater wells are supplied by the Gargano (GA), Murgia (MU), and Salento (SA) karst-fissured aquifers that are up to 400 m deep and by the Tavoliere (TAV) and Arco Jonico Tarantino (JON) porous aquifers that are less than 60 m deep (De Giglio et al., 2016).

2.2. Water sampling

Groundwater samples were collected —after flushing for 10 min— twice from 177 of the artesian wells used by the Regional Environmental Protection Agency for emergency purposes, in winter and summer (January and July 2014, respectively), making a total of 354 water samples (Fig. 1). In winter, 1.5 L of water was collected from each well and used to test for the compulsory bacteria (*E. coli* and *Salmonella*) according to DM 185/03; moreover, 1 liter of water was collected to test *Legionella* with culture-based methods. In the summer, during the second sampling phase, the water samples from wells that were suitable for irrigation use (i.e. *E.coli* < 100 CFU/100 mL and *Salmonella* absent in 1 L) were tested for *Legionella* both with culture-based methods (1 L) and molecular techniques (2 L) to verify the presence of vital but non-cultivable cells.

These wells draw groundwater from two different hydrogeological settings, as follows: 113 (63.8%) draw water from Mesozoic karst-fissured aquifers in the areas around Gargano (GA, n = 14), Salento (SA, n = 41), and Murgia (MU, n = 58), while 64 (36.1%) draw water from Quaternary porous aquifers that represent the hydrogeological settings of Arco Jonico Tarantino (JON, n = 20) and Tavoliere (TAV, n = 44).

Following the procedure of De Giglio et al. (2016), the water samples were collected in sterile containers between 9:00 and 12:00 in calm atmospheric conditions with no rain. They were transported to the laboratory in a refrigerator at + 4 °C. Samples to be tested for *E. coli* and *Salmonella* were kept at the ambient temperature while those to be tested for *Legionella* by culture-based and molecular techniques were protected from direct light.

Data for rainfall (mm) and the mean atmospheric temperature (T °C) from December 2013 until July 2014 were acquired by the Regional Environmental Protection Agency (Meteo Service, 2018).

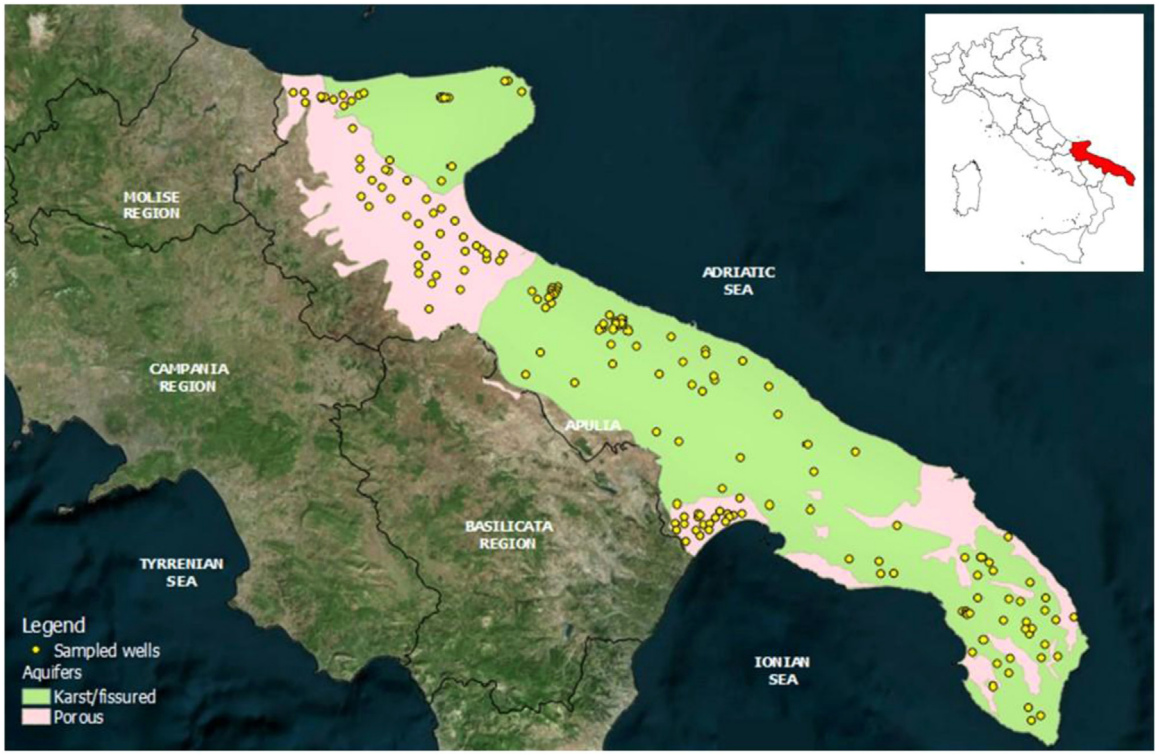

Fig. 1. Distribution of sampled wells in Apulia, Italy.

2.3. Bacteria detection as required by DM 185/03

2.3.1. Escherichia coli

Aliquots (100 mL) of the water samples were filtered through a cellulose ester membrane with a diameter of 47 mm and a pore size of 0.45 μm (Millipore, Milan, Italy). They were then placed on Tergitol-7 Triphenyl Tetrazolium Chloride agar (Biolife Srl, Milan, Italy) and incubated at 36 ± 2 °C for 24 ± 2 h. If there were no typical colonies after this initial incubation, the samples were incubated for a further 24 ± 2 h. Lactose-positive colonies were sub-cultured onto a tryptone tryptophan medium (Sigma-Aldrich, St. Louis, USA) and incubated at 37 ± 1 °C for 24 ± 2 h. The resulting colonies were assumed to be *E. coli* if they were oxidase negative and indole positive (EN ISO, 9308-1: 2000).

2.3.2. Salmonella spp

The water samples (1 liter) were filtered through a cellulose ester membrane with a diameter of 142 mm and a pore size of 0.45 μm (Millipore, Milan, Italy). These membranes were placed in 100 mL of sterile 0.1% (w/v) peptone water (Thermo Scientific Oxoid, Milan, Italy) and homogenized for 1 min. Subsequently, aliquots of the homogenized material were mixed with a selective enrichment medium, consisting of selenite cystine broth (Biolife Italiana srl, Milan, Italy). After incubating for 24 h at 35 °C, the samples were sub-cultured on two agar plates, brilliant green and xylose lysine deoxycholate (Becton Dickinson, Heidelberg, Germany), and incubated for another 24 h at 35 °C. At least one colony from each plate that was suspected of being *Salmonella* was inoculated on triple sugar iron and lysine iron agar (Biolife Italiana srl, Milan, Italy), incubated for 24 h at 35 °C, and typed with specific serological tests (Standard Methods 9260D).

Water was determined to be unsuitable for irrigation use if levels for at least one microorganism of fecal origin exceeded the DM 185/03 limits in at least one of the two samples from either winter or summer.

2.4. Legionella detection

2.4.1. Culture-based investigation

The water samples (1 liter) were filtered with 0.2 μm isopore polycarbonate membranes (Millipore Corporation, Bedford, MA, USA). The membranes were then suspended in 10 mL of the same water sample and vortexed, after which 0.2 mL of each sample were cold-seeded on plates containing a glycine vancomycin polymyxin cycloheximide medium (GVPC, Liofilchem Srl, Teramo, Italy). The plates were incubated at 36 °C for 10 days in a humid environment under 2.5% CO_2, following which suspect colonies were then sub-cultured on a charcoal yeast extract medium (Liofilchem Srl, Teramo, Italy) without L-cysteine and a buffered charcoal yeast extract medium (BCYE; Liofilchem Srl, Teramo, Italy) with L-cysteine. Colonies that only grew on BCYE agar plates were assumed to belong to the *Legionella* genus; these were then identified using a latex agglutination test with polyvalent (Oxoid Spa, Milan, Italy) and monovalent antisera (Biogenetics Srl, Tokyo, Japan). As recommended by the Italian Guidelines, a minimum theoretical mathematical detection limit equal to 100 *Legionella* bacteria per liter of sample was applied. Samples in which the concentrations were greater than or equal to 100 CFU/L were considered positive (UNICHIM :14, 1037).

2.4.2. Molecular investigation

Of the wells that were suitable for irrigation use in both winter and in summer, and for which the culture-based method for *Legionella* was negative, 37 were randomly selected from the whole sampling area. Molecular techniques were applied to the water samples from these wells to discriminate between viable but non-cultivable cells and non-viable cells.

Two liters of water were filtered through two 0.2-μm polycarbonate filters (Millipore, Billerica, MA, USA); one liter was analyzed for viable/non-viable cells by quantitative polymerase chain reaction (qPCR), and the other liter was analyzed for viable cells by propidium monoazide (PMA-qPCR). The membrane filter from the first 1 L aliquot was directly added to the lysis solution of an Aquadien Kit (Bio-Rad, Hercules, CA,

USA) and DNA were extracted according to the manufacturer's protocol. The filter from the second aliquot was first overlaid with 500 µl of PMA dye (50 µM) (Biotium Inc., USA) in 90 mm Petri dishes and incubated in the dark for 10 min. It was then exposed to a 500 W light on ice for 10 min at 20 cm from the light source. After irradiation, the second filter was added to the lysis solution for DNA extraction (Aquadien Kit, Bio-Rad) (Ditommaso et al., 2014).

Next, qPCR was performed in 96-well plates on 5 µl of the DNA extracted from the PMA-treated and untreated samples with iQCheck Quanti Legionella pneumophila and Legionella species kits (Bio-Rad). The number of genomic units per well (GU/well) were determined with a deep well real-time detection system (CFX 96, BioRad) and CFX Manager Software (BioRad). The total GU was calculated by dividing the obtained GU/well by 132, a factor that accounted for the volumes analyzed, purified, and subjected to PCR.

2.5. Statistical analysis

Since data did not follow a Gaussian distribution, quantitative variables were reported as medians and inter-quartile ranges and non-parametric Mann-Whitney *U* test was used to compare the independent variables. Correlations between the concentrations of *Legionella* (by culture-based method or molecular investigations), other microorganisms and water temperature were determined using the Spearman correlation test. Compliance with the national legislation on irrigation (Y/N), the type of aquifer (karst or porous), sampling season (summer or winter), and the mean concentrations of the microorganisms detected in this study were used as the covariates in the analysis. All analyses were conducted using STATA 12 statistical software.

3. Results

3.1. Legionella vs compulsory bacteria (DM 185/03)

The medians and ranges of the concentrations of *E. coli* and *Salmonella* detected in the 177 well waters are reported in Table 1. Regarding to *Legionella*, the distribution of its concentration in winter and in summer is showed in Fig. 2. The concentrations of *Legionella* were not significantly correlated with either *E. coli* or *Salmonella* ($p = 0.7934$ and $p = 0.5280$, respectively), and the suitability for irrigation use was not significantly correlated with the presence of *Legionella* ($p = 0.4527$).

When compared with DM 185/03, 145 (81.9%) of the 177 wells were suitable for irrigation use; *Legionella* was isolated in 31 (21.4%) of these 145 wells, 18 (58%) and 13 (42%) of which drew groundwater from karst-fissured and porous aquifers, respectively. The *Legionella* distribution did not differ significantly between the two types of aquifer ($p = 0.980$) and the different areas examined ($p = 1.003$) (Table 2).

Of the different serogroups, L. *pneumophila* sg 6 (26%) was the most frequently isolated, followed by *Lpn* sg 8 (24%), *Lpn* sg 1 (22%), *Lpn* sg 14 (4%), and *Lpn* sg 15 and *Lpn* sg 3 (2% each), all in pure cultures. The cultures were mixed in the remaining 20% of the samples (*Lpn* sg 1 + L. *gormannii*; *Lpn* sg 1 + L. *bozemanii*; *Lpn* sg 8 + sg 15).

Table 1
Medians and ranges of the concentrations of fecal bacterial indicators carried out on 177 well water samples.

Isolates	Season	Median	Min-Max	Iqr[1]
E. coli (CFU/100 mL)	winter	0	0–3000	5
	summer	0	0–1700	6
Salmonella (CFU/1000 mL)	winter	0	0–120	0
	summer	0	0–20	0

[1] Interquartile range.

3.2. Molecular investigation for Legionella detection

The results of the culture-based investigation showed that, of the 145 wells that were suitable for irrigation use, 114 tested negative for *Legionella*. Of these, 37 of the wells that were in porous aquifers (TAV, n = 8; JON, n = 4) and karst-fissured aquifers (GA, n = 3; SA, n = 9; MU, n = 13) were also examined with molecular techniques. *Legionella non-pneumophila* was detected in all 37 wells with qPCR (median = 41700 GU/L, range 261.3–2870000) and PMA-qPCR (median = 3566.8, range 91.2–364000) (Table 3). The *Legionella non-pneumophila* concentrations decreased significantly after treatment with PMA (PMA-qPCR) ($p = 0.003$) and increased significantly in porous aquifers compared to the karst-fissured ones both with qPCR ($p = 0.0004$) and PMA-qPCR ($p = 0.0000$). *Legionella pneumophila* was not detected in any of the wells.

3.3. Climatic evaluation

Regarding cultural based method, more wells tested positive for *Legionella* in summer than in winter ($p = 0.023$). Nineteen (62.3%) wells were positive in both seasons, 11 (35.5%) were positive only in summer, and only one was positive in winter (3.2%).

The mean atmospheric monthly temperatures in Apulia from December 2013 until July 2014 differed significantly between months and ranged from 11.5 ± 1.3 °C in December 2013–25.4 ± 0.5 °C in July 2014 ($p < 0.05$). In contrast, there was no significant difference between the monthly rainfall amounts ($p > 0.05$). The rainfall was lowest in January 2014 (10.6 ± 11.2 mm) and July 2014 (18.6 ± 15.9 mm), and was highest in April 2014 (51.0 ± 15.1 mm).

Regarding the temperatures of water in the sampling months, the mean temperature ranged from 17.9 ± 1.7 °C in January to 19.8 ± 2.1 °C in July 2014. *Legionella* concentrations were correlated with temperature of water both in winter ($\rho = 0.24$, $p = 0.003$) and in summer ($\rho = 0.41$, $p < 0.001$) (Fig. 3).

With the exception of the JON aquifer, the median concentrations of *Legionella* in groundwater were higher in summer (500 CFU/L) than in winter (300 CFU/L). The *Legionella* concentrations were highest in the MU aquifer in winter (20,000 CFU/L) and in summer (50,000 CFU/L).

The median temperature was significantly higher in the *Legionella* positive well waters than in the negative ones in winter ($p < 0.001$) and in summer ($p < 0.001$).

Regarding molecular investigation, *Legionella* concentrations were weakly correlated with temperature of water both with qPCR ($\rho = 0.47$, $p = 0.0033$) and PMA-qPCR ($\rho = 0.41$, $p = 0.0126$) (Fig. 4).

4. Discussion

Agriculture and tourism, stimulated by resorts and hotels adorned with lawns and ornamental gardens, are the most important sectors in Apulia, Southern Italy. Groundwater is the principal source of water for irrigation because there are no significant rivers or lakes. Given the possible risk of oral-fecal transmission diseases, the hygienic quality of agricultural products, and particularly of vegetables to be eaten raw, is very important for public health (De Giglio et al., 2017). Unfortunately, food borne illnesses are not the only diseases that are linked to agricultural practices. Other bacteria that occur naturally in water may be transmitted via inhalation or aspiration of aerosols (e.g. *Legionella*) or by contact (e.g. *P. aeruginosa*), but they are not included among the parameters specified in the Italian Ministerial Decree 185/03.

Numerous studies, carried out in Apulia (Montagna et al., 2006; Napoli et al., 2009, 2010) and elsewhere (Borella et al., 2005; Pasquarella et al., 2010, 2012; Montagna et al., 2016, 2017a, 2017b, 2018b) have reported that *Legionella* colonizes artificial water environments, perhaps because of the development of biofilms in man-made aquatic environments (Declerck et al., 2009). In contrast, there is limited information about its presence in natural water (including

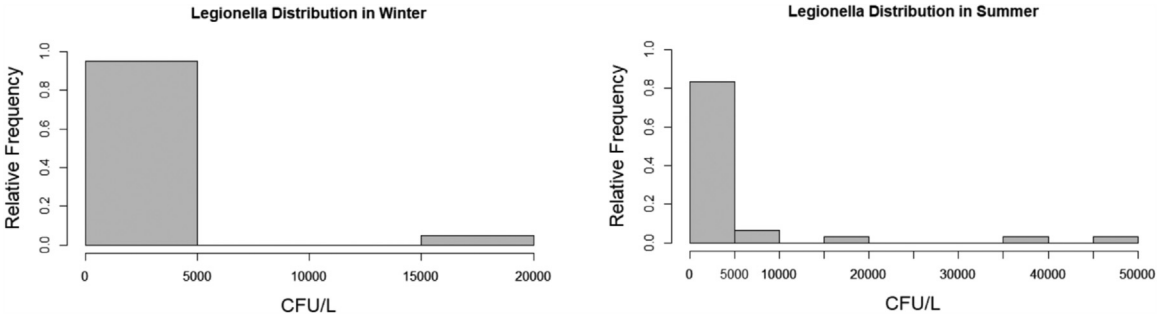

Fig. 2. Distribution of *Legionella* concentration (CFU/L) from cultural based method carried out on 50 well water samples resulted positive.

Table 2
Legionella presence in wells suitable for irrigation (DM 185/03) in different areas and aquifers by culture-based investigation.

	Wells suitable for irrigation use (No = 145)				
Aquifers	Karst-fissured (n = 98)			Porous (n = 47)	
Areas	GA (n = 12)	SA (n = 32)	MU (n = 54)	JON (n = 17)	TAV (n = 30)
Wells positive for Legionella (No = 31)	5 (41.6%)	4 (12.5%)	7 (12.9%)	7 (41.2%)	8 (26.7%)

GA, Gargano; SA, Salento; MU, Murgia; JON, Arco Jonico Tarantino; TAV, Tavoliere.

Table 3
Median and range values of molecular investigations of *Legionella* by qPCR and PMA-qPCR on 37 wells water.

Aquifer	**No.wells for aquifer**	***Legionella* (GU/L)**	
		qPCR median (range)	PMA-qPCR median (range)
TA	8	145,392 (41,237–2,870,000)	49,431 (11,100–304,000)
JON	4	905,500 (120,000–1,930,000)	124,000 (78,851–364,000)
GA	3	123,000 (14,900–186,000)	6917.6 (485.5–7801.1)
SA	9	10,200 (261.3–44,837)	548.2 (91.2–3045.7)
MU	13	8384.7 (280.8–2240,000)	557 (140.2–124,000)

GA, Gargano; SA, Salento; MU, Murgia; JON, Arco Jonico Tarantino; TAV, Tavoliere.

groundwater) and how it is dispersed by water aerosolized during sprinkling of plants (Riffard et al., 2001; Stojek and Dutkiewicz, 2002; Brooks et al., 2004; Costa et al., 2005).

To the best of our knowledge, this is the first study in Italy that has reported the presence of *Legionella* in groundwater for irrigation use. Our data suggest that natural waters may be a potential source of *Legionella*, especially when used to irrigate cultivated fields, greenhouses, or ornamental gardens. The irrigation method should be considered; sprinkler irrigation is one of the most common methods, but water aerosolized and dispersed during sprinkling can be inhaled readily.

As also reported in other studies (Riffard et al., 2001; Brooks et al., 2004; Declerck et al., 2009), the concentrations of *Legionella* in groundwater were usually low. While *Legionella* was not closely associated with a particular aquifer type by cultural investigation, the contamination was higher with a statistical significantly difference in the porous aquifer than in the karst-fissured aquifer by molecular investigation. These results are not in agreement with other reports (Embrey and Runkle, 2006; Goeppert and Goldscheider, 2011) and we believe the differences may be owing to well depths (Rudolph et al., 1998) and local characteristics, as reported in a previous study (De Giglio et al., 2016). The porous aquifers (TA and JON) are shallow and characterized by a succession with thickness of a few meters of permeable sandy—gravelly sediments intercalated by less permeable silt and clay layers, which facilitate microbiological contamination. By comparison, the karst-fissured aquifers (GA, MU and SA) are deeper and characterized by seasonal and climatic factors. As a thermophile microorganism, the concentrations of *Legionella* were higher in summer (median value 500 CFU/L) than in winter (median value 300 CFU/L), and the median water temperature was higher in the *Legionella*-positive wells than in the negative wells. reported that, when isolated from cold groundwater, the concentrations of *Legionella* were similar to those found in warmer water, which suggests that *Legionella* in natural systems may survive in a range of conditions and, for example, may tolerate temperatures of up to 60 °C (Leoni et al., 2001). While its

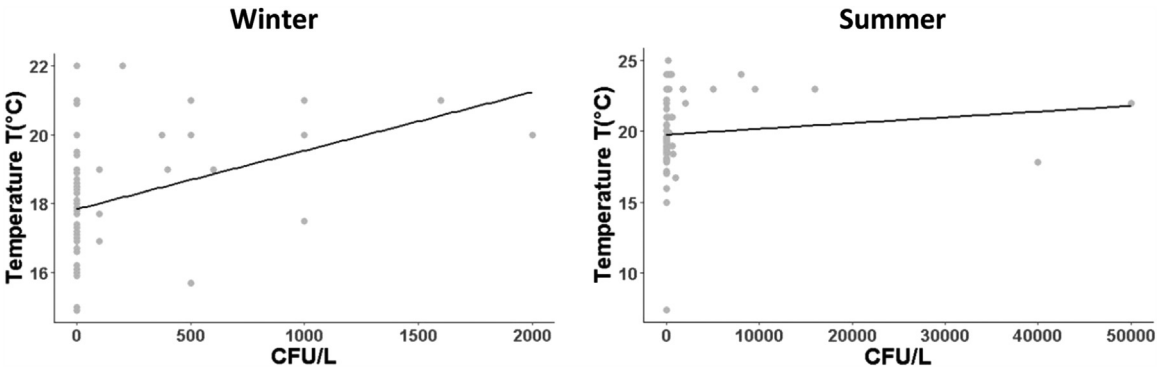

Fig. 3. Correlation among water temperature (T°C) and *Legionella* concentration (CFU/L) by cultural based method (both in winter and in summer).

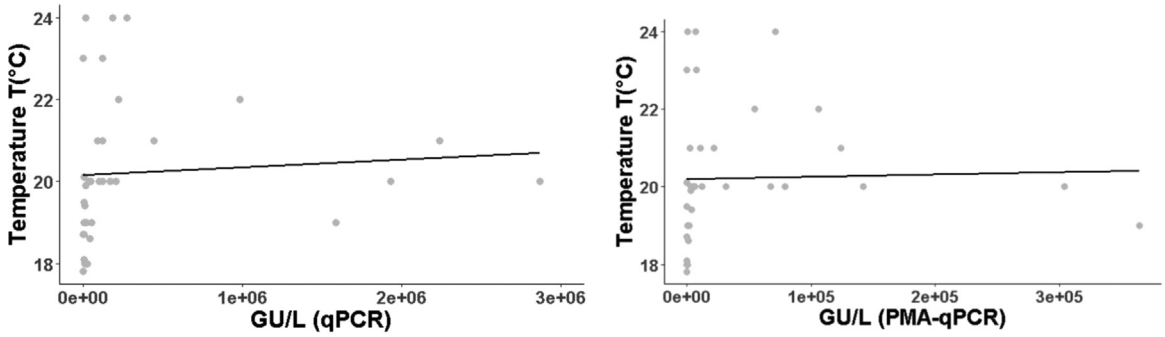

Fig. 4. Correlation among water temperature (T°C) and *Legionella* concentration (GU/L) by molecular investigation (only in summer).

minimum infectious dose is not precisely known and is difficult to define because it is influenced by many factors (e.g. the microorganism virulence and the susceptibility of the host), fatal pneumonia may result from even low bacterial loads (Montagna et al., 2007); Legionellosis however, occurs frequently during summer and is often linked to hot water systems (Rota et al., 2013).

In this study, 21.4% of the wells that were suitable for irrigation use (DM 185/03) tested positive for *Legionella,* independent of any association between *Legionella* and *E. coli* or *Salmonella*. Similarly, other researchers found that concentrations of fecal indicator bacteria were not always correlated with protozoa and viral pathogens (Laganà et al., 2014; De Giglio et al., 2017; Sunger et al., 2018) but did not investigate the correlations between the concentrations of *Legionella* and *E. coli* or *Salmonella*.

The species most frequently detected in this study by the culture-based method was Lpn. Found in 26% of wells, Lpn sg 6 was the most common species, followed by Lpn sg 8 (24%), and Lpn sg 1 (22%). While studies previously reported Lpn sg 1 as the species most commonly identified in humans, cases are increasingly attributed to other species and serogroups that adapt more easily to the natural environment (Brooks et al., 2004; Montagna et al., 2007; Rota et al., 2013). After Lpn sg 1, Lpn sg 6 is the second most identified species in human cases (Yu et al., 2002). These findings suggest that contamination of the environment by a range of serogroups and species of *Legionella* should be evaluated.

Consistent with other authors (Lye et al., 1997; Villari et al., 1998; Brooks et al., 2004), our results showed that studies of *Legionella* based only on the culture method are somewhat limiting, as this method underestimates the number of viable microorganisms. In fact, even though the culture-based method is still preferred, *Legionella* was detected by molecular methods (both qPCR and PMA-qPCR) in all the 37 wells that tested negative for the culture-based method. Molecular methods, such as qPCR, also have limitations and can overestimate the risk of infection (Ditommaso et al., 2014). However, our data showed that, when PCR is combined with viability measurements, (for example, DNA can be pre-treated with ethidium monoazide viable dye and propidium monoazide), the *Legionella* concentrations decreased significantly (Montagna et al., 2017b).

Unfortunately, only the water samples were examined for their microbiological characteristics, and people engaged in irrigation with groundwater were not serologically examined in this study. More sensitive methods for detecting *Legionella* should be sought and exposed and non-exposed country workers (e.g. farmers and gardeners) should be serologically tested in future studies.

5. Conclusions

The results of this study indicate that water aerosolized when plants are irrigated by sprinkling is a potential source of Legionellosis, and that the risk of exposure is higher in summer than in winter. These results are very important for workers who are regularly in contact with well waters used for irrigation. Consequently, *Legionella* should be included among the mandatory parameters in the standards for groundwater to be used for irrigation. We also believe that *Legionella* should be included in water monitoring programs.

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Conflict of interest

The authors declare they have no actual or potential competing financial interests.

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