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Prevalence of *Legionella spp.* in the water distribution systems of Northern Greece

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ABSTRACT

Legionella spp. are the etiological agent of legionnaire's disease, a severe respiratory disease, which affects mostly the vulnerable groups of the population. In the present study, we investigated the presence of *Legionella* according to ISO 11731:2017 in water samples, collected from five regions of Northern Greece. The results showed that 64 (8.9%) out of the 595 collected samples were positive for *Legionella*. Furthermore, 23 (35.9%) and 14 (21.9%) out of the 64 isolated *Legionella* strains were confirmed as *L. pneumophila* serogroup *1* and *L. pneumophila* serogroup *2-15*, respectively, while the remaining 27 (42.2%) isolates were characterized as non-pneumophila *Legionella* spp. The findings of this study offer proof that *Legionella* remains a significant public health concern. They will aid in enhancing our comprehension of Legionella's epidemiology in Greece and assist in implementing efficient control measures to minimize its occurrence in water meant for human consumption.

Keywords: public health, *legionella*, water, epidemiology

INTRODUCTION

Legionella spp. are gram-negative, aerobic, non-spore forming, unencapsulated bacilli that measure 0.5 μm in width and 2 μm in length [1]. They are part of the natural aquatic environment, and the etiological agent of a severe pneumonia called legionnaires' disease. A milder form of the infection with a flu-like outcome, which is called pontiac fever is also connected to *Legionella* [2]. The investigation of epidemic and sporadic pneumonia cases has shown that *Legionella* spp. and more specifically *L. pneumophila* is a common cause of both community-acquired and nosocomial pneumonia affecting mostly the immunocompromised individuals [3]. Higher risk groups like the elderly population with comorbidities are more likely to suffer from severe symptoms. Furthermore, another study [4] showed that 36.8% of the samples collected from Italian retirement homes were positive for the presence of *Legionella*. This finding is of particular importance since elderly people belong to the high-risk group of the population. Cases of pneumonia associated with *Legionella* spp. were also observed among other groups like neonates [5]. The case-mortality rate of adequately treated legionnaires' disease varies from 7.0% to 24.0% [6]. The transmission occurs primarily through the inhalation of the bacteria, mainly via aerosolized water from engineered systems [7]. There is only one reported case of probable human-to-human transmission of *Legionella* [8]. *Legionella* spp. persist in freshwater reservoirs, watercourses, moist soil, and composted material and since they are ubiquitous in aquatic environments it is possible to enter man-made water systems [6, 9]. They can survive temperatures ranging from 0 to 68 °C, while their physical growth is supported in temperatures from 20 °C to 42 °C [10]. *Legionella* bacteria have developed strategies to survive, and in some cases to replicate within a diverse group of protozoa like amoebozoa, percolozoa, and ciliophora [11]. Moreover, although they can exist in planktonic form in freshwater systems, they are most often found as active components within existing biofilms. It is worth mentioning that *L. pneumophila* has the ability to acquire nutrients by forming synergistic relationships with other members of the biofilms [12-18]. The fact that *Legionella* strains can also exist in multispecies biofilms makes them a threat to aquatic ecosystems and a serious public health hazard [19].

The importance of *Legionella* spp. regarding public health and its increasing involvement in severe cases of pneumonia has raised the need for modifications in water safety legislation. More specifically, in 2017 the World Health Organization (WHO) regional office for Europe conducted a detailed review of the parameters in Directive 98/83/EC and suggested that due to the technical and scientific progress there is a need to modify the tested parameters for the water

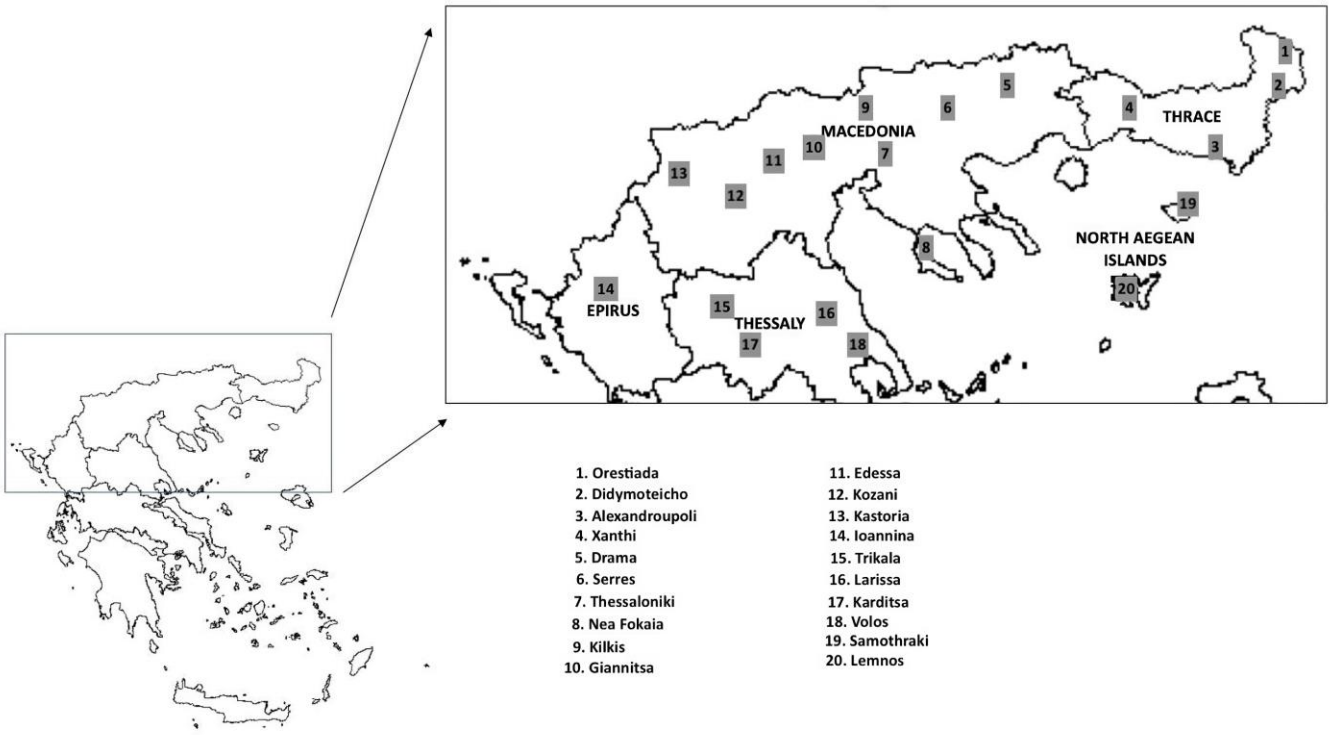

Figure 1. Five regions of Greece in which the water samples were collected during this study (Source: Authors' own elaboration)

supplies. Therefore, according to the new EU Directive 2020/2184 on the quality of the water intended for human consumption, *Legionella* should be also controlled as it causes the highest health burden of all waterborne pathogens and more specifically, the number of *Legionella* in water samples should be less than 10^3 cfu/L.

The aim of this study was to investigate the prevalence of *Legionella* spp. in the water samples from five regions of Northern Greece and to determine the number of samples, where *Legionella* exceeded the limit of 10^3 cfu/L. Furthermore, an epidemiological study was conducted regarding the trends of *Legionella* spp. in colonizing specific premises and facilities.

MATERIALS AND METHODS

A total of 595 water samples from five regions of Northern Greece were tested for the presence of *Legionella* spp. from 01 January of 2019 until 31 August 2020. The samples were collected from 20 cities of the above regions, as shown in **Figure 1**.

The distribution of the samples in the different regions is presented in **Table 1**.

Sample Collection

Water samples were collected in 500 ml sterile glass containers. Before the sterilization 0.5 ml sodium thiosulfate had been added to each container to inactivate any residual chloride. The water facilities sampled were potable water (kitchen, showers) (n=553), dental unit water lines (n=25) and other sources (four cooling towers and 13 tanks) (n=17). The potable water samples were collected as cold and hot samples according to the relevant National legislation and ISO 19458:2006 (water quality–sampling for microbiological analysis). More specifically, from each sampling point two cold and two hot samples were collected. Regarding the cold samples (500 ml each), the first was collected after the release of the first drops (direct cold sample) and the second two minutes after the water started flowing (indirect cold sample). The same procedure was followed for the collection of the hot samples (direct and indirect).

In **Table 2**, there is data regarding the number of the collected direct and indirect cold and hot water samples. Regarding the dental unit's water lines, samples were obtained from the taps located on the dental chairs. Before collecting the samples, the end of each tap was disinfected with 70.0%

Table 1. Number of samples collected from different regions of Greece

Region	Facilities	Sampling points	Samples
Macedonia	38	93	334
Thessaly	8	22	99
Thrace	18	25	110
Epirus	2	4	16
North Aegean Islands	4	8	36
Total	**70**	**152**	**595**

Table 2. Number of collected samples from different sampling points

Type of sample	Number of samples
Direct cold	130
Indirect cold	147
Direct hot	131
Indirect hot	145
Dental unit water lines	25
Other (cooling towers & tanks)	17
Total	**595**

alcohol. The samples were then collected two minutes after the flow commenced. Following collection, the samples were transported to the laboratory in isothermal boxes with temperatures ranging between 5-10 °C and tested on the same day.

Sample Testing

The samples were tested for the enumeration of *Legionella* spp. according to ISO 11731:2017. More specifically, the samples were filtered through 0.45 μm pore-sized membrane filters (Pall Corporation, New York, NY, USA) through which the water passed by using a vacuum pump (Merck, Darmstadt, Germany). Each membrane was transferred in a sterilized container with 10 ml Ringer's solution (Oxoid, Hampshire, UK) and glass beads, and was vortexed for two minutes. To reduce the number of other bacteria three ml of this suspension was heat-treated in a water bath at 50 °C for 30 minutes; In addition, an aliquot of each suspension was treated for five minutes with an acid solution consisting of hydrochloric acid and potassium chloride. To prepare the acid solution 3.9 ml of hydrochloric acid (0.2 mol/l) were mixed with 25 ml of potassium chloride (0.2 mol/l) and the pH was adjusted to 2.2±0.2 with the addition of potassium hydroxide. 0.1 ml of the untreated, heat-treated and acid-treated suspension each was spread on plates with Buffered charcoal yeast extract (BCYE) agar (VWR, Pennsylvania, PA, USA) and another 0.1 ml of each suspension was spread on plates with glycine vancomycin polymyxin B cycloheximide (GVPC) agar (VWR, Pennsylvania, PA, USA). The plates were incubated at 37 °C for 10 days in a humified environment and they were examined on the fifth day and at the end of the incubation period for the presence of *Legionella* suspected colonies. Three of the suspected colonies from each plate were subcultured on nutrient agar (Biolife, Milan, Italy) and incubated at 37 °C for three days. Only colonies that did not grow on nutrient agar were considered as *Legionella* and were further characterized as *Legionella* spp., *L. pneumophilla* serogroup 1 or serogroup 2-15 by an agglutination test (Biolife, Milan, Italy). The limit of detection for the used method was 200 cfu/L.

Molecular Identification

For the confirmation of suspected colonies as *Legionella*, a conventional PCR protocol was used for the amplification of 16SrRNA gene fragments. The bacterial DNA was isolated from the suspected colonies using Qiamp DNA Mini-Kit (Qiagen, MA, USA). The sequences of the selected primers were, as follows:

Forward primer: 5'-AGGGTTGATAGGTTAAGAGC-3'

Reverse primer: 5'-CCAACAGCTAGTTGACATCG-3'

For the reaction mix, the KAPA Taq HotStart PCR kit (Sigma-Aldrich, St Louis, USA) had been used. More specifically, the 20 μl reaction mix contained: 4 μl 5X KAPA Taq HotStart Buffer, 2 μl $MgCl_2$ (25 mM), 0.4 μl dNTP Mix (10 mM), 0.3 μl of each primer (10 mM), 0.2 μl 5 U/μl KAPA Taq HotStart DNA Polymerase, 2 μl of sample DNA and 10.8 μl nuclease-free water. The amplification was carried out in a T100 Thermal Cycler (Biorad, Hercules, USA) under the following conditions: initial denaturation at 95 °C for three minutes, 35 cycles, each comprising of denaturation at 95 °C for 30 seconds, annealing at 57 °C for 30 seconds and elongation at 72 °C for one min, and a final elongation step at 72 °C for seven minutes.

The products of the amplification were identified in 2.0% agarose gel after electrophoresis in standard conditions and stained with ethidium bromide solution (2 μl/ml). The size of the amplified DNA product was 386 bp.

Sequencing

To compare the sequences of *L. pneumophila* isolates to other circulating strains, two of the isolated strains were sent for Sanger sequencing to Eurofins Scientific (Luxenbourg). The two *Legionella pneumophila* strains CKNO-th-19 and CKNO-tr-19 had been isolated in the regions of Macedonia and Thessaly, respectively. We chose strains from these regions as they have the largest population compared to the other regions in the selected part of Greece. Another reason was that these strains were isolated from premises hosting more than 300 people on a consistent basis. The sequences were submitted to GenBank under the accession numbers MT743037.1 and MT743249.1. The phylogenetic tree was constructed using MEGA X software. For that reason, apart from the two Greek isolates from this study, other *L. pneumophila* 16SrRNA gene sequences from the GenBank (https://www.ncbi.nlm.nih.gov/genbank) were used.

RESULTS

From a total of 595 water samples collected in 5 different Greek regions, 64 (10.8%) were positive for the presence of *Legionella*. Data presented in **Table 3** refer to the number of positive samples in different regions and the counts of the isolated *Legionella*. More specifically, in the region of Macedonia 32 (9.6%) out of 334 samples were positive while in Thessaly and Thrace 18 (18.2%) out of 99 and 5 (4.5%) out of 110 samples were positive for the presence of *Legionella*, respectively.

In addition, our study found that the positive samples for the Epirus region and the Islands of North Aegean Sea, were 1

Table 3. Counts of confirmed *Legionella* in different regions of Greece

Region	Number of samples	Counts of *Legionellas* in positive samples						Total positive samples
		$<10^3$	$10^3<X<5\times10^3$	$5\times10^3\leq X<10^4$	$10^4\leq X<1.5\times10^4$	$1.5x10^4\leq X<2\times10^4$	$\geq2\times10^4$	
Macedonia	334	7 (21.8%)	11 (34.3%)	3 (9.4%)	4 (12.5%)	1 (3.2%)	6 (18.8%)	32
Thessaly	99	4 (22.2%)	8 (44.3%)	1 (5.6%)	1 (5.6%)	1 (5.6%)	3 (16.7%)	18
Thrace	110	1 (20.0%)	2 (40.0%)	1 (20.0%)	0 (0.0%)	0 (0.0%)	1 (20.0%)	5
Epirus	16	0 (0.0%)	0 (0.0%)	1 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1
North Aegean Islands	36	0 (0.0%)	6 (75.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (25%)	8
Total	**595**	**12 (18.8%)**	**27 (42.2%)**	**6 (9.4%)**	**5 (7.7%)**	**2 (3.1%)**	**12 (18.8%)**	**64**

Note. *Numbers in parentheses refer to percentages regarding positive samples for each region

Table 4. *Legionella* counts in different type of samples

Type of sample	Counts of *Legionellas* in positive samples						Total positive samples
	$<10^3$	$10^3<X<5\times10^3$	$5\times10^3\leq X<10^4$	$10^4\leq X<1.5\times10^4$	$1.5\times10^4\leq X<2\times10^4$	$\geq2\times10^4$	
Direct cold	3 (27.2%)	6 (54.5%)	0 (0.0%)	0 (0.0%)	1 (9.15%)	1 (9.2%)	11
Indirect cold	2 (33.3%)	1 (16.7%)	0 (0.0%)	1 (16.7%)	0 (0.0%)	2 (33.3%)	6
Direct hot	2 (11.1%)	8 (44.4%)	1 (5.6%)	2 (11.1%)	0 (0.0%)	5 (27.8%)	18
Indirect hot	4 (21.1%)	7 (36.8%)	5(26.3%)	0 (0.0%)	0 (0.0%)	3 (15.8%)	19
Dental unit water lines	1 (10.0%)	5 (50.0%)	0 (0.0%)	2 (20%)	1 (10.0%)	1 (10.0%)	10
Other (cooling towers & tanks)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0
Total	**12 (18.8%)**	**27 (42.2%)**	**6 (9.4%)**	**5 (7.7%)**	**2 (3.1%)**	**12 (18.8%)**	**64**

Note. *Numbers in parentheses refer to percentages regarding positive samples for each type

Table 5. Characterization of isolated *Legionella* in selected regions of Greece

Region	*Legionella* spp.	*Legionella pneumophila* serogroup 1	*Legionella pneumophila* serogroup 2-15	Total
Macedonia	13 (40.0%)	16 (52.0%)	3 (8.0%)	32
Thessaly	7 (35.7%)	5 (28.6%)	6 (35.7%)	18
Thrace	1 (20.0%)	2 (40.0%)	2 (40.0%)	5
Epirus	0 (0.0%)	0 (0.0%)	1 (100.0%)	1
North Aegean Islands	6 (75%)	0 (0.0%)	2 (25.0%)	8
Total	**27 (42.2%)**	**23 (35.9%)**	**14 (21.9%)**	**64**

Note. *Numbers in parentheses refer to percentages regarding total positive samples confirmed for each region

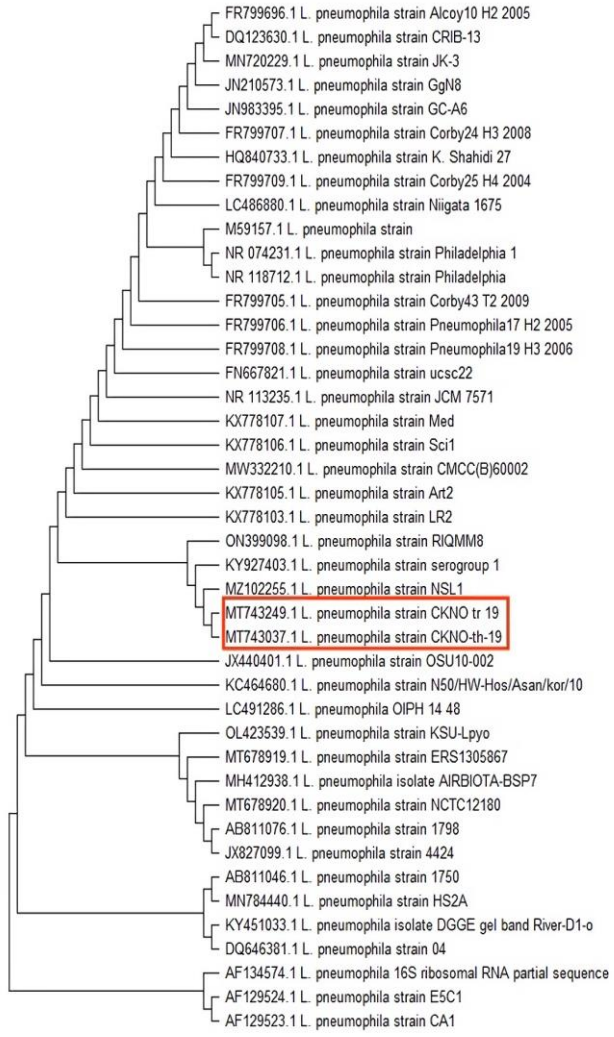

Figure 1. Phylogenetic tree based on partial 16SrRNA gene sequences (tree was created using maximum likelihood method of MEGA tool & two selected strains from our study are indicated with the red colour frame) (Source: Authors' own elaboration)

(6.3%) out of 16 and eight (22.2%) out of 36, respectively. **Table 4** presents further data on the numbers of the isolated *Legionella* bacteria. More specifically, it is noteworthy that 12 (18.8%) out of 64 samples contained more than $2x10^4$ cfu/L *Legionella* while in 52 (81.3%) out of the 64 positive samples the counts of isolated *Legionella* bacteria were above the limit determined by the European Directive (10^3 cfu/L).

According to the data presented in **Table 4**, from a total of 130 and 147 direct and indirect cold samples, 11 (8.5%) and 6 (4.1%) were positive for the presence of *Legionella* spp., respectively. In addition, from a total of 131 and 145 direct and indirect hot samples, 18 (13.7%) and 19 (13.1%) were positive, respectively. Concerning the dental unit water line systems, 10 (40%) out of 25 were positive while none of the 17 samples collected from cooling towers and tanks were positive for the presence of *Legionella*.

The results of the agglutination test for the characterization of the isolated *Legionella* bacteria are presented in **Table 5**. More specifically, 23 (35.9%) and 14 (21.9%) out of the 64 isolated *Legionella* strains were confirmed as *L. pneumophila* serogroup *1* and *L. pneumophila* serogroup 2-15, respectively. The remaining 27 (42.2%) out of 64 isolates were characterized as non-pneumophila *Legionella* spp.

The phylogenetic tree presented in **Figure 2** is the outcome of the phylogenetic analysis of the two *L. pneumophila* strains from this study and the *L. pneumophila* strains, which were selected from GenBank.

DISCUSSION

Legionella is widely distributed in aquatic environments and is correlated with Legionnaire's disease, a severe respiratory disease, which appears to be increasing in recent years.

More specifically, according to the European Center for Disease Prevention and Control (ECDC) 8,372 cases of legionellosis had been identified in the European Union (EU)

and the European Economic Area (EEA) Territory in 2020. The number of notifications had decreased to 1.9 per 100,000 population, which was lower than in the two preceding years. However, according to the ECDC report, from 2016 to 2019, notification rates in the EU/EEA increased yearly from 1.4 per 100,000 population in 2016 to 2.2 in 2019 [20]. The Centers for Disease Control and Prevention (CDC) also reports that data from surveillance and reporting systems indicate that the incidence of legionellosis has been increasing in the United States of America, with a report of a nearly 3.5-fold increase between 2000 and 2011 [21-23].

EU Directive 2020/2184 on the quality of the water intended for human consumption determines *Legionella* as an important factor for public health especially for those who belong to vulnerable groups such as the immunocompromised. The re-emergence of *Legionella* spp. as well as the new legislation has raised awareness to the authorities regarding the presence of the bacteria in the potable water and other water sources [24]. In our study an attempt was made to investigate the presence of *Legionella* in different types of water samples from five regions of Northern Greece. The results of our study showed that in total 10.8% of the collected samples were positive for the presence of *Legionella*.

50% of the positive samples were collected in the region of Macedonia, while the 28.1%, 7.8%, 1.6% and 12.5% positive samples were collected from the regions of Thessaly, Thrace, Epirus, and North Aegean Islands, respectively. The differences in the percentages of the positive samples might be explained by the different number of samples collected from the different regions eg. 334 from Macedonia and 16 from Epirus (**Table 1**). Data presented in **Table 5**, shows that 42.2% of the 64 positive samples were confirmed as non-pneumophila *Legionella* spp., while 35.9% and 21.9% were characterized as *L. pneumophila* serogroup 1 and serogroup 2-15, respectively. A study regarding the *Legionella* presence in care homes from two Danish municipalities, showed that only *L. pneumophila* was detected in the positive samples and most systems were colonised with *L. pneumophila* serogroups 2-14, while serogroup 1 was only found in a few premises (8%) [25]. Another study conducted in Greece involved the collection of 1,870 water samples from various locations such as hotels, athletic venues, cruise ships and ferries. Results indicated that 172 (9.2%) of the samples tested positive for *L. pneumophila* serogroup 1, while 171 (9.1%) samples tested positive for *L. pneumophila* serogroups 2-14. Additionally, 45 (2.4%) samples were found to be positive for *L.* non-*pneumophila* [26].

According to the data presented in **Table 4**, 54 (9.8%) out of 553 samples that were collected from hot and cold distribution systems, were positive for the presence of *Legionella*. Furthermore, in 43 (7.8%) out of 553 collected samples of that type, the number of *Legionella* exceeded the EU Directive's limit of 10^3 cfu/mL. Our results are in line with the results of a study conducted in the UK, which demonstrated that 6% of bathroom fixtures in households were positive for Legionella [27]. Another study in Spain determined the presence of Legionella in 4.7% and 4.6% of potable cold and hot-water tanks, respectively [28]. These results differ to the results of our study in which we found that the number of positive hot samples is higher compared to the number of positive cold samples. More specifically, we found that 37 (13.4%) out of 276 hot samples and 17 (6.1%) out of 277 cold samples were positive for the presence of *Legionella* bacteria. A 2012 study revealed that *Legionella* was predominately isolated from hot water samples obtained from hospitals in Greece [29]. The study found that 27.3% of the hot water samples and 8.2% of the cold-water samples were positive for *Legionella*. Similarly, a study conducted on the island of Crete in southern Greece found that 26.29% of the hot water samples and 16.66% of the cold-water samples were positive for *Legionella* [30]. These results align with our study's findings regarding the higher prevalence of positive hot water samples. However, the aforementioned studies reported higher positive rates for both hot and cold-water samples compared to our study. It is significant to note that *Legionella* presence in hot water samples is particularly important as most of the samples were from showers, which facilitates the generation of water droplet aerosols and increases the likelihood of Legionella inhalation by individuals.

Other man-made systems in which *Legionella* bacteria were isolated are fountains, pools, and cooling towers [31-34]. It is worth mentioning that a contamination analysis of public buildings in Greece showed that *Legionella* was identified in 48.9% out of the 96 cooling towers that were tested and moreover 30% of them were classified as heavily contaminated ($\geq 10^4$ cfu/L) [35]. In addition, investigations of Legionnaire's disease outbreaks in Germany in 2013 revealed that cooling towers were a possible source of contamination, due to the isolation of *Legionella* strains from these units [36]. In our study we did not isolate *Legionella* from the samples collected from cooling towers probably because the number of the samples from these premises was significantly low (n=4). Therefore, it is not possible to decide on the importance of the cooling towers as *Legionella* reservoirs based only on the results of this study.

Another interesting finding of our study was the high incidence of positive samples in dental unit's water lines. Data presented in **Table 4** show that 10 (40.0%) out of 25 samples were positive for *Legionella* bacteria. More specifically, three (12.0%) and seven (28.0%) out of the 25 samples were positive for the presence of *Legionella* spp. and *L. pneumophila* serogroup 1, respectively. This finding is significant, as water spray in dental units favours the creation of aerosol thus it may be a hazard for the inhalation of bacteria by the patients and the dentists. In a Middle East systematic review and meta-analysis, it was showed that *L. pneumophila* was present in 23.5% of the sampled dental unit water lines [37]. In another study [38], it was provided evidence that the number of the dental unit water line systems, which were found positive for the presence of *Legionella* bacteria depended on the sample collection time. More specifically, *L. pneumophila* was detected in 86.7% and 53.3% of the dental units at the beginning of the working day and at midday, respectively. In our study, after informing the relevant staff about the presence of *Legionella* in the dental unit water lines, the subsequent maintenance of the units and correction actions up taken, led to the elimination of these bacteria after re-sampling and examining the water from the infected units.

CONCLUSIONS

In conclusion, the risen awareness regarding *Legionella* as a public health hazard in recent years has led the Authorities in investigating its presence in the water distribution systems. The presence of *L. pneumophila* and non-pneumophila *Legionella* spp. in the water facilities indicates the importance of the control and maintenance procedures in these premises. We believe that our study provides useful information to help the better understanding of the ecology of the bacterium, which is important for the safety of the water intended for human consumption.

Author contributions: KP: conceptualized & drafted manuscript; **IT:** edited tables; **EC, AT, VS, DK, IT, & KP:** provided important references & revised manuscript; & **SK:** obtained funding. All the authors participated in the examination of the water samples, read, and approved the final manuscript. All authors agree with the results and conclusions.

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Data sharing statement: Data supporting the findings and conclusions are available upon request from corresponding author.

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