



Research Article

Detection of pathogenic bacteria in hot tap water using the qPCR method: preliminary research



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Abstract

Drinking water should be safe for health and free from amounts of pathogenic microorganisms and parasites posing a threat to human health. In recent years, particular attention has been paid to the threat associated with the operation of hot water installations, potentially providing favourable conditions for the development of bacteria of genera *Legionella* and *Escherichia*. Such bacteria are commonly found in the natural environment, but should not reach the consumers' taps. In this experiment, hot water samples were collected from six public buildings, and the detection of pathogenic microorganisms, namely *Legionella* spp., *Legionella pneumophila*, and *Escherichia coli* bacteria, was performed by means of qPCR analysis. The sequences specific for these bacteria were quantified with TaqMan probes in total DNA extracts of the hot tap water samples. Bacteria of the *Legionella* spp. type were detected at five sampling sites in the range from 4.52 to 15.59 genomes/mL of hot tap water. *Legionella pneumophila* was detected at four sampling sites in the range from 0.98 to 11.99 genomes/mL. *E. coli* was recorded at four sites, but in quantities around 0.00 genomes/mL (less than one genome/mL). This preliminary research points to the need to use molecular techniques as an additional source of information in standard water analyses to obtain shorter detection time and more accurate results.

Keywords qPCR · *Legionella* spp. · *Legionella pneumophila* · *Escherichia coli* · Water distribution system · Pathogens

1 Introduction

In favourable environmental conditions, microorganisms can adhere to abiotic surfaces, creating a biofilm structure. This phenomenon often occurs in water supply systems [1]. The presence of biofilm allows the reproduction of potentially pathogenic bacteria due to its organised structure and increased resistance to disinfectants [2, 3]. Despite extensive research conducted on the subject of biofilm adhesion [4], no methodology has been developed that would prevent its development in real water distribution systems. Therefore, it is necessary to monitor potentially pathogenic microorganisms whose presence can have negative results for consumers. The quality of drinking water, mostly in the case of microbial contamination, is regarded as a serious human health

issue [5]. The health risk associated with the presence of opportunistic pathogens in tap water systems attracts more and more scientific attention [6]. Drinking water microbiome is extensively studied, but in the majority of papers the research concerns exclusively cold tap water [7–12]. WHO guidelines briefly mention the issue of hot tap water quality, indicating that hot tap water systems should be designed to minimise the proliferation of *Legionella* [5]. To date, several studies considered the bacterial biodiversity and microbial contamination in hot tap water samples [13–16]. The majority of them focus on the presence of genus *Legionella*. Gram-negative bacteria of the *Legionella* genus present in water supply systems can develop in a wide range of temperatures (25–43 °C, with an optimal spectrum of 30–43 °C) and can survive in a range of 55–60 °C. Such conditions

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eliminate the competition of other bacteria, facilitating the proliferation of *Legionella* spp. [17].

Many approaches are available for the detection and enumeration of representatives of *Legionella* genus. Standard methods are based on culture media. More advanced methods focus on molecular biology tools. The culture-dependent methods for *Legionella* detection require the isolation of bacteria on selective agar medium containing L-cysteine, such as buffered charcoal yeast extract (BCYE) agar, supplemented with substances preventing the growth of other genera. The plates should be incubated at ~ 37 °C for 14 day and examined every 2 or 3 days [18]. Some part of viable *Legionella* cells, however, may be hidden in amoebal hosts, giving false-negative results of incubation [18]. Therefore, the cultivation method is time-consuming (providing results after up to 2 weeks) and may result in underestimations. The other approach for enumeration of *Legionella* spp. (*L. spp.*) and *Legionella pneumophila* (*L. pneumophila*) in tap water samples involves Legiolert™/Quanti-Tray®, providing acceptable accuracy [19]. Immunodetection is another proposed method, offering the advantage of fast detection [20]. One of the most frequently adopted culture-independent methods for *Legionella* detection and enumeration is PCR/qPCR. Many DNA sequences have been used to detect *Legionella* in the PCR approach to date [21]. Moreover, commercial kits for bacterial species detection become more and more popular and widely available. The use of these kits may contribute to the standardisation of the performance of experiments, at least in the laboratory, which repeat the test using the same kits. Despite their reliability, PCR methods applied in *Legionella* detection may have some hindrances, such as the presence of PCR inhibitors in a sample. Therefore, the employment of internal controls in reactions is recommended [18].

Escherichia coli (*E. coli*) is one of the best known and earliest described human opportunistic pathogenic bacteria. Plenty of methods have been developed for its detection and enumeration, including culture-dependent and culture-independent approaches, such as PCR-based methods [22–24]. Interestingly, the presence of *E. coli* strains was detected in tap water distribution systems all over the world, including low-income as well as high-income countries [25–28]. It is worth mentioning that the potential presence of viable but non-culturable *E. coli* cells in tap water samples leads to underestimations of the actual tap water contamination [29].

Both *L. spp.* and *E. coli* are considered indicator microorganisms of tap water contamination and are included in hot (*L. spp.*) and cold (*E. coli*) tap water routine monitoring in Polish law regulations [30]. Interestingly, the regulations of tap water microbial monitoring focus on

living microorganisms, and the recommended methods are unable to detect dead cells (as they are mostly culture dependent).

The objective of this study was to detect the presence and enumerate pathogenic microorganisms in hot tap water samples collected from six buildings of the university campus. The buildings were selected based on their age, as the approximate dates of their construction are known, namely it was possible to evaluate the influence of the internal installations' age on hot tap water quality. In general, three old and three new buildings were subjected to samples collection. Another objective was to present a fast and accurate method for the detection of microorganisms in tap water which would provide results on the day of sampling. The microorganisms of choice were bacteria from genus *Legionella* (in terms of the entire genus and *L. pneumophila* species) and *Escherichia* (*E. coli*), because they are the best known and characterised examples of pathogenic bacteria dwelling in tap water.

2 Experimental section

2.1 Sample collection

Samples of hot tap water were collected from six buildings located at the campus of the Wrocław University of Science and Technology (Fig. 1) during the summer of 2018. The taps were disinfected by alcohol cleaning and submerging the tap spouts in 96% ethanol (Sigma-Aldrich) for 30 s, and water was flushed for 5 min. Each time, 5 L of hot tap water was taken from each site into sterile polypropylene bottles. The water samples were filtered through a sterile filtration set (Nalgene) with a membrane filter of mixed cellulose esters with a pore size of 0.2 µm (Whatman). Then, each filter was placed separately in a sterile flask, filled with 0.85% physiological saline, and shaken for 3 h on a shaker (Nocturne) at 160 rpm. After this time, the flasks were placed in a bath and sonicated for 30 s (35 kHz). The suspensions were concentrated by centrifugation (4000 g, 10 min), and the pellets were subjected to DNA extraction.

2.2 DNA extraction

To isolate the DNA from the cells, the Isolate II Genomic Kit (Bioline) was used following the manufacturer's instructions with small modifications. The samples were centrifuged for 10 min at 7500 rpm (Eppendorf mini spin), and then the supernatant was removed. 440 µL of lysis buffer and 25 µL proteinase K (10 mg/mL) were added to each sample. The mixture was intensely vortexed and then incubated at a temperature of 50 °C for approximately 20 min for complete cells lysis. Subsequently, 200 µL of binding

Fig. 1 Sample sites—the campus buildings of the Wrocław University of Science and Technology



buffer was added and the sample was transferred to a silica membrane. The mixture was centrifuged for 2 min at 12,000 rpm. For the neutralisation of the impurities present in the sample, 700 μL Wash buffer reagent dissolved in 96% ethanol (Sigma) was added. The samples were centrifuged for 1 min at 12,000 rpm. The procedure was repeated twice. To release bacterial DNA from the column, 50 μL elution buffer was added directly to a membrane, then incubated for 15 min at room temperature (22 $^{\circ}\text{C}$) and centrifuged for 1 min at 8000 rpm. Because small amounts of bacterial DNA were expected (due to the medium of interest, i.e. clean tap water after disinfection), the elution buffer volume was reduced in order to obtain a higher DNA concentration. The concentration and purity of the isolated DNA were measured on NanoPhotometer N60 (Implen).

2.3 qPCR amplification

The presence of *Legionella* spp., *L. pneumophila* and *E. coli* was detected by means of AmpliTest (Amplicon). Each set contained primers replicating a genome fragment of bacteria from genera *Legionella* and *Escherichia*, specific TaqMan probes, and internal control. With the progress of the process and the hydrolysis of the probe, a fluorescent dye FAM was released, recorded by a real-time PCR apparatus, MIC (Bio Molecular Systems). The detection of internal control was possible due to the release of the HEX fluorescent dye. A negative control (NC) with no DNA and a positive control (PC) containing the DNA of the *Legionella* spp.,

Table 1 A temperature profile of all qPCR reactions

Step	Temperature ($^{\circ}\text{C}$)	Time (s)	Number of cycles
Initial denaturation	95	300	1
Denaturation	95	10	45
Amplification ^a	58	25	
Cooling	40	30	1

^afluorescence measurement on FAM and HEX channel

L. pneumophila, and *E. coli*, respectively, were prepared for each set.

All samples were prepared according to the manufacturer's instructions with a final volume of 20 μL , including 8 μL of DNA sample. The qPCR profile is presented in Table 1. Fluorescence measurement was set on FAM (green) and HEX (yellow) channels after completing the amplification step in each cycle. The same reaction profile was performed for every assay applied in this study.

2.4 Standard curve

The quantitative measurement was made by creating a standard curve. Tenfold serial dilutions of genomic DNA extracted from pure strains were prepared for each set. The strains used in the experiment were *Legionella pneumophila* NCTC 11192/ATCC[®] 33152 (BioMaxima) for the standard curves in *Legionella* spp. and *Legionella pneumophila* assays, and *Escherichia coli* NCTC 12241/ATCC[®] 2592 (BioMaxima) for the standard curve in *E. coli* assay.

The strains were provided in the form of lyophilised discs. Each disc was dissolved in 10 ml of the appropriate sterile broth and incubated at 37 °C for 24 h. After that time, DNA was extracted according to the procedure presented in point 2.2. The DNA concentration was measured in triplicate on a NanoPhotometer N60 (Implen), and the mean value of measurements was considered in further steps. The number of genome copies was calculated based on data obtained from GenBank for appropriate strains and calculated in a DNA calculator (<https://cels.uri.edu/gsc/cndna.html>). For *Legionella* spp. and *Legionella pneumophila* curves, genome length of 3.45892 Mb was assumed (NCBI accession number NC_002942.5), and for *E. coli* a genome length of 5.130767 Mb was assumed (NCBI accession number NZ_CP009072.1). The calculated values of copies/ μ L were posted into a qPCR cyler as theoretical amounts. For each standard curve, eight measurement points were prepared, covering the range between 1,780,000 and 0.178 copies/ μ L in the *Legionella* spp. and *Legionella pneumophila* assays, and 1,590,000 and 0.159 copies/ μ L in the *E. coli* assay.

3 Results

3.1 Internal control results

To assess the reliability of obtained results, the internal control reaction measurement was performed on the HEX channel. The results suggest the presence of putative inhibitors in the DNA sample from the H-4 building (Table 2).

3.2 Detection and quantification of *L. spp.* sequences

The efficiency of the assay was 0.8896. The dynamic range of the assay covered given concentrations between 1,780,000 and 178 copies/ μ L. However, all obtained samples' results were in this range. The standard curve equation was $y = -3.618x + 46.36$, where y is Cq and x is \log_{10} of copies/ μ L (Table 3; Fig. 2).

3.3 Detection and quantification of *L. pneumophila* sequences

The efficiency of the assay was 1.0913. The dynamic range of the assay covered given concentrations between 1,780,000 and 1780 copies/ μ L. However, all obtained samples' results were in this range. The standard curve equation was $y = -3.121x + 46.27$, where y is Cq and x is \log_{10} of copies/ μ L (Table 4; Fig. 3).

Table 2 Internal controls for all samples

<i>Legionella</i> spp.		<i>L. pneumophila</i>		<i>E. coli</i>	
Sample	Cq	Sample	Cq	Sample	Cq
D-1	23.03	D-1	22.48	D-1	21.13
D-2	22.67	D-2	23.14	D-2	21.17
C-13	23.08	C-13	22.11	C-13	21.83
H-4	41.50	H-4	27.94	H-4	36.46
SKS	22.07	SKS	22.47	SKS	20.25
C-6	23.09	C-6	21.98	C-6	20.06
NC	22.40	NC	22.47	NC	20.91
PC	21.07	PC	21.48	PC	20.17
Standard 10 ⁰	22.18	Standard 10 ⁰	22.04	Standard 10 ⁰	20.70
Standard 10 ⁻¹	22.04	Standard 10 ⁻¹	20.80	Standard 10 ⁻¹	19.44
Standard 10 ⁻²	23.19	Standard 10 ⁻²	22.25	Standard 10 ⁻²	21.06
Standard 10 ⁻³	22.34	Standard 10 ⁻³	22.58	Standard 10 ⁻³	20.88
Standard 10 ⁻⁴	23.05	Standard 10 ⁻⁴	22.18	Standard 10 ⁻⁴	20.25
Standard 10 ⁻⁵	23.04	Standard 10 ⁻⁵	22.05	Standard 10 ⁻⁵	21.22
Standard 10 ⁻⁶	21.55	Standard 10 ⁻⁶	23.04	Standard 10 ⁻⁶	21.27
Standard 10 ⁻⁷	22.39	Standard 10 ⁻⁷	22.08	Standard 10 ⁻⁷	21.56

3.4 Detection and quantification of *E. coli* sequences

The efficiency of the assay was 0.9665. The dynamic range of the assay covered given concentrations between 1,590,000 and 1.59 copies/ μ L. However, all obtained samples' results were in this range. The standard curve equation was $y = -3.121x + 46.27$, where y is Cq and x is \log_{10} of copies/ μ L (Table 5; Fig. 4).

4 Discussion

The intense development of civilisation carries with it the risk of creating new environmental pollutants of various levels. Their removal may cause some difficulties. New and quick methods are being sought for the detection and identification of pollutants in the aquatic environment, as well as in the air and in the soil [31–33]. Moreover, it is known that the elderly, children, or people with immunocompromise are particularly vulnerable to the presence of pathogenic microorganisms in tap water. It is therefore necessary to use a method with high sensitivity and accuracy. A good example of a fast method suitable for the detection and enumeration of microbial contaminants in water can be the qPCR, as indicated in this study.

The obtained results prove the presence of *L. spp.*, *L. pneumophila*, and *E. coli* in hot tap water installations in the majority of examined buildings at the campus of the Wrocław University of Science and Technology.

Table 3 Standard curve and samples results for *L. spp*

Standard	Cq	Given concentration (copies/ μ L)	Sample	Cq	Calculated concentration (copies/ μ L)	Genomes/mL of hot tap water
10^0	23.94	1,780,000	D-1	28.66	77,930	15.59
10^{-1}	27.09	178,000	D-2	30.52	23,790	4.76
10^{-2}	31.27	17,800	C-13	29.15	56,920	11.38
10^{-3}	34.03	1780	H-4	–	–	–
10^{-4}	38.56	178	SKS	29.75	38,910	7.78
10^{-5}	–	17.8	C-6	30.6	22,620	4.52
10^{-6}	–	1.78	NC	–	–	–
10^{-7}	–	0.178	PC	24.08	1,433,000	–

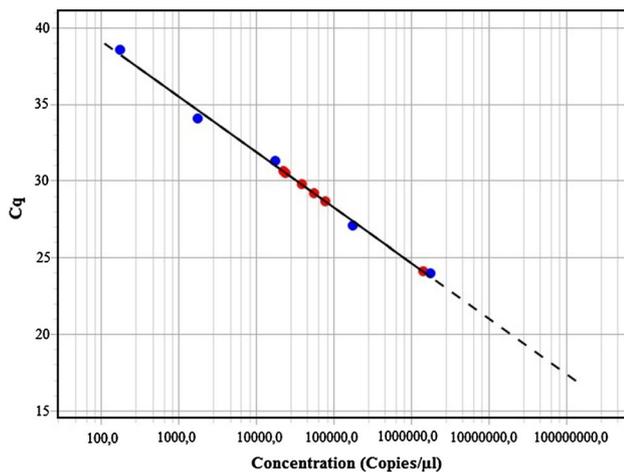


Fig. 2 Standard curve for the *Legionella spp.* assay. The blue points symbolise the standards, and the red points symbolise the samples obtained from buildings (details in Table 3). Adopted from MIC software

Despite the accuracy of the qPCR approach, it is worth emphasising that the method used in this study is not free from limitations, as the environmental samples may contain some qPCR inhibitors. As shown in Table 2, no inhibition was observed, except the DNA sample obtained from

hot tap water from building H-4. The presence of inhibitors in DNA sample H-4 is not surprising, because the DNA measurements revealed impurities in the sample (data not shown). Moreover, the hot tap water sample from building H-4 was contaminated much more than other samples by colour substances, probably iron and manganese. Therefore, the presence of any tested microorganisms' sequence could not be established in this study for the building H-4. The problem of PCR/qPCR inhibition by DNA impurities is frequently encountered in the case of environmental samples [34–36].

According to Inkinen et al., operational conditions may cause to leaching of the pipes, contributing to the changes in tap water microbiome. (Notice that the authors imply that the construction material of the pipes does not greatly affect the forming biomass [14].) Therefore, the poor DNA quality from building H-4 is probably caused by leaching of metals from pipes installed in the building.

The lack of fluorescence signal from any of the NC samples ensures no contamination in any assay performed in this study.

The study results confirm the contamination of the majority of tested hot tap water samples with *Legionella* cells. In all tested samples (except of H-4), the levels of *L. spp.* genome copy number were similar, suggesting

Table 4 Standard curve and samples results for *L. pneumophila*

Standard	Cq	Given concentration (copies/ μ L)	Sample	Cq	Calculated concentration (copies/ μ L)	Genomes/mL of hot tap water
10^0	26.95	1,780,000	D-1	31.36	59,960	11.99
10^{-1}	29.56	178,000	D-2	34.74	4921	0.98
10^{-2}	33.06	17,800	C-13	32.19	32,310	6.46
10^{-3}	36.19	1780	H-4	–	–	–
10^{-4}	–	178	SKS	–	–	< LOD
10^{-5}	–	17.8	C-6	34.39	6382	1.28
10^{-6}	–	1.78	NC	–	–	–
10^{-7}	–	0.178	PC	27.11	1,374,000	–

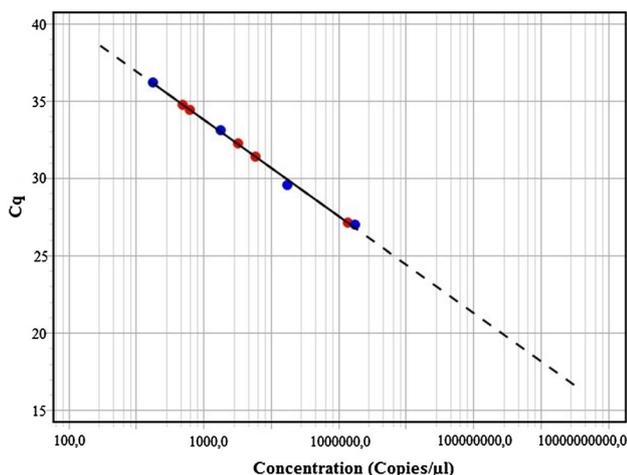


Fig. 3 Standard curve for the *L. pneumophila* assay. The blue points symbolise the standards, and the red points symbolise the samples obtained from buildings (details in Table 4). Adopted from MIC software

comparable hot tap water contamination in all tested buildings. The genomes of *L. spp.* in 1 mL of hot tap water cover a range of 4.52–15.59. The results of *L. pneumophila* detection and enumeration indicate the presence of the species in all the tested buildings except SKS (H-4 excluded from the analysis). Building SKS is the newest of all sampling points tested (built within the last 5 years), so the hot tap water installations are less exploited in comparison with the remaining tested buildings. Hence, the accumulation of *L. pneumophila* bacterial cells in the pipelines might be the lowest. The obtained values seem to be high, indicating the presence of several (up to 12) bacterial cells of *L. pneumophila* in 1 mL of hot water sample.

Another important aspect is the presence of *E. coli* bacteria in hot tap water. It can contribute to the risk of epidemiological disease. *E. coli* sequences were detected in all the tested buildings except D-1 (H-4 excluded from the analysis). The levels of *E. coli* genome copy number

are similar, suggesting comparable hot tap water contamination in all of the tested buildings, like in the case of *Legionella* genus. Building D-1 is one of the oldest buildings among the tested sampling sites, whereas C-13, where the concentration of *E. coli* cells was the lowest, was built in 2007. Therefore, the criterion of age of the building/internal installations seems not to be appropriate into the interpretation of the results in this case. The quantities of *E. coli* are evidently lower than those obtained for *L. spp.* and *L. pneumophila*. It is not surprising, because hot tap water provides conditions more suitable for *Legionella* than *Escherichia*, as the temperature regimes of hot tap water installations encourage *Legionella* growth [18], the fact that was not clearly stated in the literature for *Escherichia*. Bacteria from *Legionella* genus are known to be thermotolerant, with growth-limiting temperature around 50 °C (but some strains were isolated even from hot water systems up to 66 °C) [18]; therefore, their survival abilities in such environments are actually possible. The comparison of the obtained bacterial quantities with the reports of similar research performed in other parts of the world reveals good quality of tap water in Wroclaw. Whiley et al. [37] quantified *L. spp.* and *L. pneumophila* in tap water samples from two water distribution systems in Australia by means of the qPCR method. Despite the values reaching 1238 copies/mL for *L. spp.* and 1981 copies/mL for *L. pneumophila* in the summer season, the results for other seasons obtained by Whiley et al. were comparable (in terms of order of magnitude) with those presented in this study. Because the results of this study concern samples collected in summer in a single sampling campaign, seasonal variability cannot be discussed. In the study by Whiley et al., no *E. coli* cells were detected in two Australian tap water systems, irrespective of the distance from a water treatment plant and the season. It is worth mentioning that Whiley et al. tested the presence of *E. coli* only by means of Colilert™ trays (IDEXX Laboratories), not the qPCR approach, so dead cells of *E. coli* species might have been present in the tested samples [37]. In

Table 5 Standard curve and samples results for *E. coli*

Standard	Cq	Given concentration (copies/μL)	Sample	Cq	Calculated concentration (copies/μL)	Genomes/mL of hot tap water
10 ⁰	16.72	1,590,000	D-1	–	–	< LOD
10 ⁻¹	19.68	159,000	D-2	35.43	4.507	~0.00
10 ⁻²	23.34	15,900	C-13	38.91	0.4278	~0.00
10 ⁻³	26.79	1590	H-4	–	–	–
10 ⁻⁴	30.15	159	SKS	35.39	4.641	~0.00
10 ⁻⁵	33.82	15.9	C-6	36.12	2.832	~0.00
10 ⁻⁶	36.8	1.59	NC	–	–	–
10 ⁻⁷	–	0.159	PC	24.14	9313	–

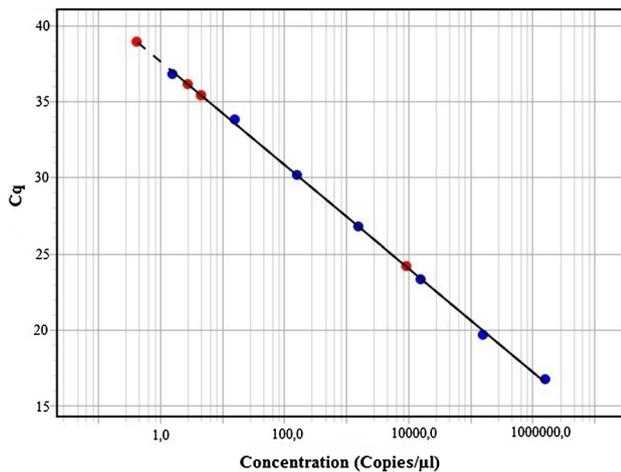


Fig. 4 Standard curve for the *E. coli* assay. The blue points symbolise the standards, and the red points symbolise the samples obtained from buildings (details in Table 5). Adopted from MIC software

another study by Liu et al. [38], the presence of *L. spp.* was detected in all 44 tested tap water samples in northern China, while *L. pneumophila* was detected only in four out of 44 samples. The gene copy number of *L. spp.* ranged from 10.47 to 10,715.19 gene copies/ml. The results presented in this study cover the bottom range or are lower than those obtained from Chinese tap water samples. Nevertheless, the concentrations of *L. pneumophila* gene copies/mL were much lower in the research by Liu et al. [38] than those presented in this study. Chinese tap water was analysed over an entire year (four sampling campaigns in different seasons), increasing the range of detected bacterial cells [38].

It must be emphasised that the differentiation of live and dead cells is not possible in this study, so there is no evidence that any of detected and quantified bacterial cells was live and posed any threat to the consumers' health. The proposed methods for live/dead differentiation might include PMA treatment and light exposure steps during DNA extractions [39]. The usefulness of PMA as the way to differentiate live and dead cells is discussed in the study by Toplitsch et al. [40], pointing to possible difficulties (i.e. the membrane damage is not the only one determinant of cell death, the turbidity of water samples may affect the PMA binding, short amplicons of PCR/qPCR could not be bound with PMA, giving false results) [40, 41]. Nevertheless, the differentiation between live and dead cells may be crucial in terms of evaluating the health risk to tap water consumers. Because bacteria are known to be able to acquire DNA sequences determining the virulence factors in the phenomenon of transformation [42]; however, the presence of pathogenic species, even in the form of

dead cells, should not be neglected. They can provide a reservoir of virulence genes for other living cells.

According to Dietersdorfer et al., viable but non-culturable *Legionella* cells may proliferate in amoebas and affect human health [43]. The report by Dietersdorfer et al. highlights the insufficiency of culture-dependent methods in studies concerning putative pathogens in tap water. Therefore, the combination of culture-dependent and culture-independent approaches may offer a solution. The comparison of culture-dependent methods with qPCR was performed by Toplitsch et al. [40] confirming the usefulness of qPCR in such studies.

To prevent the presence of *Legionella* in tap water, disinfection of water distribution systems is recommended. It has been evidenced that the chloraminated drinking water distribution system in the USA provided water of better quality than the Norwegian system with no residual disinfectant [44]. The crucial role of disinfectant in the prevention of *Legionella* proliferation in hot water systems was also confirmed by a study by Flannery et al. [45]. Although disinfected tap water is not free from disinfection by-products, as confirmed in the study by Pogorzelec and Piekarska [46], often constituting other human health risk factors, the abandonment of disinfection would lead to more serious consequences. Nevertheless, as proven in this study, disinfection is not sufficient to prevent the presence of pathogenic microorganisms in hot tap water. Constant monitoring of such bacteria by means of the qPCR method is advised.

5 Conclusions

Hot tap water samples collected from four out of six buildings of the Wrocław University of Science and Technology campus were found to contain *L. spp.*, *L. pneumophila* and *E. coli* bacterial cells. Because hot tap water is a known reservoir of *Legionella* species, including those responsible for health problems, such as *L. pneumophila*, the presence of bacterial cells of the genus is not surprising. As the presence of any opportunistic pathogen in tap water should not be neglected, these findings may be a source of anxiety. The confirmation of the viability of detected bacteria is crucial in the estimation of the consumers' health risk.

It is worth emphasising that competent living cells dwelling in tap water systems may be able to acquire virulent DNA fragments via the transformation process. Therefore, the presence of bacterial cells of the tested species (particularly *L. pneumophila* and *E. coli*), even if dead, might be regarded as the putative source of virulent DNA for other, living bacteria, therefore contributing (indirectly) to the human health risk.

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Compliance with ethical standards

Conflict of interest The authors have declared that there were no conflicts of interest involved in this work.

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