Application of PCR to the detection of pathogenic bacteria in biofilms from pipes and reservoirs of the EPAL water distribution system

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Abstract
The increasing importance of pathogenic bacteria is due to their capacity to adhere to the internal surfaces of structures from water distribution systems, forming biofilms which allow their growth and proliferation, that could affect water quality and present a public health risk. Searching for indicator microorganisms of water contamination is generally performed by classic methods of culture. However, for many harmful pathogens it's necessary to employ more specific, sensitive and rapid detection methods arising from molecular techniques. Application of Polymerase Chain Reaction (PCR) techniques to water and biofilm samples provided promising results in the detection of potential pathogens.

The present work focused on the detection of pathogens such as *Aeromonas hydrophila*, *Campylobacter jejuni*, *Mycobacterium* spp., *Legionella pneumophila* by PCR and *Legionella* spp. by Seminested PCR, for a total of 100 biofilm samples of which only 9 had *Legionella* spp.. By artificial contamination of some samples, the validation of the extraction, the protocols and the presence of PCR inhibitors were examined. It was verified that the extractive process was adequate and the *L. pneumophila* protocol needed optimisation or search of new primers. Furthermore, the effect of different volumes of inoculum added and the matrix support of the biofilms were briefly analysed.

The work described herein emphasizes the importance of PCR for pathogen detection from biofilms and studies water quality on formation of these ecosystems and their capacity to aggregate microorganisms as well as on the adequacy of pipes and reservoirs materials.

Introduction
Water is essential to life and human activities. It can be a vehicle of transmission of various pathogenic microorganisms, either naturally occurring in aquatic environments or introduced to water by hosts.

The transmission of pathogenic microorganisms can occur via diverse pathways, namely, oral-faecal paths, ingestion, contact during bathing or washing and even by inhalation of water droplets [6, 7]. However, waterborne diseases are more commonly transmitted by human and animal excretions [7].

Important pathogens that can be transmitted by water are viruses, bacteria, protozoa and helminths [2]. The persistence of these microorganisms in water is affected by several factors including the water characteristics, the residence time and stagnation of water within the distribution system, the effect of sunlight, organic load and temperature [5].

The presence of biofilms in water distribution systems presents an adequate environment for pathogenic microorganism survival and proliferation. Biofilms have been defined as immobilized microorganism communities that are adherent to biotic or abiotic surfaces, which could be formed by populations from one or more species in a matrix of extracellular polymeric substances [1]. Biofilm formation depends on the quality and concentration of available nutrients, ions and other substances, which influence bacteria and promote biofilm growth. Pipe geometry and composition also play an important role in biofilm formation [3]. These structures have been associated with increased antimicrobial resistance, namely to disinfectants, dehydration and predators [1].

Water quality is monitored through routine methods to guarantee quality and detect occasional or systematic anomalies. Since the detection of all microorganisms is currently unobtainable, one group of microorganisms is used to predict water contamination by pathogens, the faecal indicator microorganisms. Their principal characteristics are based on the fact that
they are not human pathogens; they are easily and rapidly detected; universally present in high numbers in human and warm-blooded animal excreta; always present when pathogens exist and their persistence and degree of removal following water treatment may be similar to pathogens, whose density can be correlated with health hazard [4, 6, 7]. Cultural methods are insensitive to the presence of some microorganisms, limited to pathogens with known growth characteristics, provide poor discrimination between microorganisms with similar behavioural characteristics, require long detection times and moreover, many microorganisms are not culturable [2]. Therefore, molecular methods such as those based on PCR detection are being developed to allow the direct detection of microorganisms from water and biofilm samples. Other molecular techniques include Restriction Fragment Length Polymorphism analysis, Fluorescent In-Situ Hybridization and DNA microarrays. Previously unrecognizable pathogenic bacteria or bacteria rarely identified by routine methods can be addressed by PCR technique, which allows simultaneous analysis of high number of samples and the identification of the origin of contamination.

The purpose of this work was to verify the adequacy of PCR protocols to detect *Aeromonas hydrophila*, *Campylobacter jejuni*, *Legionella* spp., *Legionella pneumophila* and *Mycobacterium* spp. in biofilm samples recovered from pipes and reservoirs of the EPAL water distribution system. These microorganisms are responsible for many outbreaks causing high number of death on a global scale. They are emerging pathogens, undetectable by methods based on the detection of indicator microorganisms of faecal origin.

The correlation between drinking water quality and pathogen release from biofilms has been established. Biofilms can maintain large quantities of viable pathogens that can be gradually released into the water. The PCR technique, allied to microorganism detection on biofilms also allows the gathering of information on the treatment efficiency, the adequacy of pipe and reservoir materials and to evaluate the success of preventive measures.

**Materials and Methods**

**Samples:** A total of 100 biofilm samples from pipes and reservoirs of the EPAL water distribution system were analysed by PCR in order to detect pathogens. Samples differed in sampling points and in material surfaces from which they were recovered. Biofilm samples were recovered from iron, ductile iron, fibrocement and concrete pipes.

**DNA extraction:** DNA from biofilm samples was extracted using the commercial kits, FastKit DNA for Soil and UltraClean Soil DNA Isolation Kit, both from Roche. DNA from cultured microorganisms was prepared using the High Pure PCR Template Preparation Kit, (Roche).

**Primers**

*Aeromonas hydrophila* primers. The two oligonucleotide primers were complementary to the genes that code for 16S rRNA of that species. The sequences were: 16S1 rRNA, 5’-GAA AGG TTG ATG CCT AAT ACG TA-3’ and 16S2 rRNA, 5’-CGT GCT GGC AAC AAA GGA CAG 3’. The PCR performed with these primers amplified a fragment of 685 bp.

*Campylobacter jejuni* primers. CF03 and CF04 were the oligonucleotide primers chosen to amplify a fragment with an estimated size of 340 bp. The sequences were: CF03, 5’-GCT CAA AGT GGT TCT TAT GCN ATG G-3’ and CF04, 5’-GCT GCG GAG TTC ATT CTA AGA CC-3’ which were from the *flaA* and *flaB* sequences of *Campylobacter jejuni*.

*Legionella* spp. primers. Leg225, Leg 858 and Leg448 were the three oligonucleotide primers used in the *Seminested* PCR assay. Leg225, 5’-AAG AGT AGC CTG CCT CGG AT-3’ and Leg 858, 5’-GTC AAC TTA TCG CGT TTG CT-3’ were used to amplify a 654 bp fragment of the 16S rRNA gene. Leg448, 5’-GAG GGT GGT TAG GTT AAC AGC-3’ and Leg858 were used for the second-step PCR to produce a 430 bp fragment of the 16S rRNA gene.
**Legionella pneumophila primers.** Two protocols were used to identify this microorganism. Three oligonucleotide primers, PT69, 5’-GCA TTG GTG CCG ATT TGG-3’; PT70, 5’-GCT TTG CCA TCA AAT CTT TCT GAA-3’ and PT181, 5’-GTT TTG CCA TCA AAT CTT TTT GAA-3’ were used to amplify a 168 bp fragment of the *mip* gene. The other protocol used only two primers, LmipL920, 5’ -GCT ACA GAC AAG GAT AAG TTG-3` and LmipR1548, 5’-GTT TTG TAT GAC TTT AAT TCA-3` to amplify a fragment of 650 bp of the *mip* gene.

**Mycobacterium spp. primers.** The two oligonucleotide primers, Tb11, 5 `-ACC AAC GAT GGT GTG TCC AT-3` and Tb12, 5’-CTT GTC GAA CCG CAT ACC CT-3’ were complementary to the genes that code for *hsp*, heat shock protein, specific for nontuberculous mycobacteria, and were used to amplify a 383 bp fragment.

**PCR conditions.** PCR amplification of the target sequence was performed with a PCR Core Kit (Roche) and a DNA thermal cycler, GeneAmp PCR System 9700 (Applied Biosystems).

**Aeromonas hydrophila.** The PCR mixture contained 1×PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl; 2 mM MgCl₂; 0.2 mM (each) deoxynucleoside triphosphate; 0.25 μM for each primer and 25 U/ml of Taq polymerase.

A total volume of 25 μl was initially denatured at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 67°C for 45 s. After the last cycle the samples were kept at 72°C for 7 min to complete synthesis of all strands.

**Campylobacter jejuni.** The PCR mixture was similar to that used for *Aeromonas hydrophila* except that the concentration of Taq polymerase was 20 U/ml and 40 μg/ml of BSA was added. PCR was performed on a total volume of 25 μl.

The following conditions were used: the mixture was initially denatured at 94°C for 4 min and then subjected to 40 cycles of heat denaturation at 95°C for 5 s, annealed at 53°C for 30 s, followed by DNA extension at 72°C for 40 s. The final extension was performed at 72°C for 10 min.

**Legionella spp..** The reaction mixture used for both PCR steps contained 1×PCR buffer (10 mM Tris-HCl [pH 8.3]; 50 mM KCl; 1,5 mM MgCl₂); 0,2 mM for each deoxynucleoside triphosphate; 0,28; 0,24 and 0,26 μM respectively for Leg225, Leg858 and Leg448, 8 μg/ml of BSA and 50 U/ml of Taq polymerase.

For the first-step PCR, a total volume of 25 μl was denatured at 95°C for 90 s followed by 35 cycles of denaturation at 95°C for 10 s, annealing at 62°C for 1 min and extension at 74°C for 1 min. After that the samples were kept at 72°C for 7 min.

The second-step PCR had the same total volume but 1μl of the first step PCR products was added to 24 μl of the PCR mixture. The initial denaturation step was the same as the first step followed by 20 cycles of denaturation at 95°C for 30 s, annealing at 66°C for 1min and extension at 74°C for 1 min. The final extension was at 72°C for 7 min.

**Legionella pneumophila.** Two protocols were tested for *Legionella pneumophila* detection. The first protocol included a PCR mixture of 1×PCR buffer (10 mM Tris-HCl [pH 8.3]; 50 mM KCl; 1,5 mM MgCl₂); 0,2 mM for each deoxynucleoside triphosphate; 0,8 μM for each primer and 20 U/ml of Taq polymerase.

The total volume of 25 μl was subjected to an initial denaturation at 96°C for 3 min followed by 30 cycles of heat denaturation at 95°C for 45 s, annealing at 61°C for 45 s and extension at 72°C for 45 s. The final extension was performed at 72°C for 7 min.

The second protocol, used for real samples, had 10×PCR buffer (100 mM Tris-HCl [pH 8,3]; 50 mM KCl; 15 mM MgCl₂); 0,2 mM (each) deoxynucleoside triphosphate; 0,35 μM for each primer and 20 U/ml of Taq polymerase.

A total volume of 25 μl was initially denatured at 96°C for 3 min followed by 40 cycles of heat denaturation at 95°C for 30 s, annealing at 50°C for 1 min and extension at 72°C for 1 min. After the last cycle, the samples were subjected to a final extension at 72°C for 7 min.
**Mycobacterium spp.** The PCR mixture contained 1×PCR buffer (10 mM Tris-HCl [pH 8.3]; 50 mM KCl; 1.5 mM MgCl2); 0.2 mM (each) deoxynucleoside triphosphate; 0.5 μM for each primer and 25 U/ml of Taq polymerase.

PCR was performed on a total volume of 25 μl that was initially denatured at 95ºC for 5 min followed by 45 cycles of denaturation at 95ºC for 1 min, annealing at 56ºC for 1 min and extension at 72ºC for 1 min. The final extension was at 72ºC for 5 min.

For all microorganisms the total volume was composed of 15 μl of the PCR mixture and 10 μl of each sample and all PCR assays included both the samples, positive and negative controls. Positive control treatment was as for the samples but instead of adding 10 μl of the sample, it was added 1 μl of the total DNA solution of the microorganism of interest, except for *Legionella pneumophila*. For this bacterium, the first protocol required 10 μl of DNA solution, whereas for the second protocol, used for real samples, only 4 μl was necessary. The volume was adjusted with MilliQ water.

A negative control was also performed, in which 10 μl of MilliQ water was added instead of sample.

**Gel electrophoresis:** Gel electrophoresis of PCR products (12 μl of the product + 3 μl stop solution) was performed on 1.3% agarose (Roche) with 1×TBE buffer (BIORAD) using a molecular weight marker IX 72-1353 bp (Roche). It was visualized by ethidium bromide (Merck) staining for 1h30min at 80V. Images were acquired using GelDoc 2000 (BIORAD).

**Results**

**Protocols application and optimization.** Firstly, the adequacy of protocol for each microorganism in the study was analysed. The specific protocol conditions for *Aeromonas hydrophila*, *Campylobacter jejuni*, *Legionella spp.* and *Mycobacterium spp.* detection were all successfully reproduced. This means that in the positive control of every microorganism, fragments of the predicted size corresponding to each amplification product were observed (i.e. 685 bp, 340 bp, 430 bp and 383 bp, respectively).

The *Legionella pneumophila* protocol, in which primers PT69, PT70 and PT181 were used, was successfully realized and a band corresponding to 168 bp DNA was visible. However, for a clear visualization of the fragment, it was necessary to add large volumes of DNA solution, 10 μl, compared to 1 μl for the other protocols. Furthermore, PCR amplification of positive samples from interlaboratorial tests weren’t detected by this protocol. This indicates the need to establish conditions to optimise detection. Therefore, in order to analyse real samples for *L. pneumophila*, two primers, LmipL920 and LmipR1548, were used. This modification to the protocol allowed the appearance of a visible 650 bp DNA band with much lower volumes of DNA solution, 4 μl.

**Analysis of samples from interlaboratorial assays.** Due to the limitations of the first *L. pneumophila* protocol, positive interlaboratorial samples were tested with the new protocol. Except for the positive control, all samples failed to give a positive response. Protocol optimization was also attempted but this led to unviable and ineffective results.

**Analysis of contaminated real samples.** Sample contamination was performed before and/or after extraction, with inoculum and/or addition of the total DNA solution of each microorganism, respectively. The selection of samples to contaminate took into account the available sample quantity, which comprised several types of materials.
The contamination with inoculum was carried out before extraction to samples 33 (iron), 34 (concrete), 36 (ductile iron), 38A and B (fibrocement) and 34A (pipe transporting water from the treatment plant to the distribution system of Lisbon). The addition of 100 \( \mu l \) inoculum of each microorganism was performed except for \textit{Mycobacterium} spp. because inoculum was not available. After PCR on each microorganism, it was verified that all samples produced positive results in the presence of \textit{Legionella} spp. since all of them showed a 430 bp band.

Data generated with \textit{Aeromonas hydrophila} and \textit{Campylobacter jejuni} were more complex. The former failed to produce bands in samples 34 and 34A, while for the latter microorganism, the unsuccessful samples were 36 and 38A.

In order to analyse the influence of sample volume for a particular matrix and microorganism, the same procedure was followed but adding 400 \( \mu l \) of inoculum of \textit{Aeromonas hydrophila} and \textit{Campylobacter jejuni}. Previously negative samples for \textit{Campylobacter jejuni} had already given positive results. Conversely, samples inoculated with \textit{Aeromonas hydrophila} generated unsuccessful results. Nevertheless, 4 \( \mu l \) of the total DNA solution of \textit{Aeromonas hydrophila} was added to samples 34 and 34A but the expected band was still not observed.

Contamination by adding 5 \( \mu l \) of the total DNA solution of each microorganism was only carried out on samples from pipe transporting water from the treatment plant to the distribution system of Lisbon, 34A (90º) and 67 (270º), because these samples existed in larger quantity.

DNA of \textit{Aeromonas hydrophila} added to samples previously contaminated with inoculum did not produce bands but samples contaminated only with DNA solution showed amplification products of 685 bp. This kind of contamination was successfully detected for \textit{Legionella} spp. and \textit{Campylobacter jejuni}. Amplification products were not detected for \textit{Mycobacterium} spp. and \textit{Legionella pneumophila}.

On the other hand, the detection of \textit{Mycobacterium} spp. was successful in samples of other materials, 37B (fibrocement), 11 0B (ductile iron) and 27 (iron), contaminated with their own genomic DNA.

**Analysis of real samples.** From all of the analysed samples, the vast majority gave a clear indication of the presence of \textit{Aeromonas hydrophila}. Only one sample gave an uncertain result for this microorganism. On one hand, after PCR amplification of sample 1A 180°, one band with weak intensity and length near to the amplification product (685 bp) was observed but, on the other hand, the shape of the band was non-linear.

For \textit{Campylobacter jejuni}, amplification of the target gene which characterizes this species did not result in the detection of any band relating to the amplification of the product, 340 bp.

The amplification of 16S rRNA gene sequences characteristic of \textit{Legionella} spp. became evident in the presence of positive samples of that bacterium, namely, samples 13A and 13B; 34A, for which recovery of the biofilm was done at a 270° angle, as for all the reservoirs. Besides the 430 bp DNA band relating to the amplification product of the second step PCR, two more bands of higher lengths were obtained in all positive samples including in the positive control.
Figure 1 – Electrophoresis in agarose gel 1.3% (p/v) of the PCR product from *Legionella* spp. rRNA 16S gene in biofilm real samples. 1 – Negative control; 2 – S. Jerónimo reservoir 1 (RSSJ1); 3 – S. Jerónimo reservoir 2 (RSSJ2); 4 – Telheiras reservoir (RST); 5 – Campo de Ourique reservoir (RSCO); 6 – Monsanto reservoir (RSMS); 7 – Vale Escuro reservoir (RSVE); 8 – 34A (270˚); 9 – Positive control (1 μl of DNA); M – Molecular weight marker IX (72-1353 pb); 1` – 13A (iron); 2` – 13B (iron); 3` – 14A (ductile iron); 4` – 14B (ductile iron); 5` – 16A (unknown material); 6` – 18 (iron); 7` – Positive control (1 μl of DNA).

The detection of *L. pneumophila* only makes sense to positive samples of the *Legionella* genus. PCR was carried out with the conditions of the second protocol described for that microorganism and the results were negative for the presence of that species.

Following the appearance of *Legionella* spp., another study was performed which consisted of detection of that bacterium in previously analysed reservoir samples but with samples extracted from water samples rather than from biofilms. The gel electrophoresis did not reveal any amplification fragment (430 bp) relating to that microorganism, except the positive control band.

The PCR assay for *Mycobacterium* spp. did not detect the presence of a 383 bp DNA band corresponding to the amplification product. It should be noted that because of insufficient quantities of extracted samples, only 96 of the 100 samples were analysed for that microorganism.

With the exception of *Legionella* spp. and *Mycobacterium* spp., the reservoir samples, RSCO and RSRM, showed fragments with very high molecular weight in all protocols. These fragments were localized before the start of the DNA ladder IX (above 1353 bp).

**Discussion**

It has been demonstrated that the majority of the protocols described in this study offered efficiency, sensitivity and good resolution for specific gene detection of each microorganism.

PCR assays for *Aeromonas hydrophila*, *Campylobacter jejuni*, *Legionella* spp. and *Mycobacterium* spp. were successful in the sense that the expected amplification products, related to the microorganism of interest, were obtained.

Studies carried out with *L. pneumophila* gave bands from the positive control, although the need for large volumes of total DNA solution, 10 μl, and the absence of bands resulting from amplification of the product of positive interlaboratorial samples, indicates the need for optimization steps or the development of new detection conditions. Even after several optimization attempts, the bands of positive interlaboratorial samples were absent suggesting the unsuitability of the protocol, especially when there were only small quantities of DNA. As there wasn’t information about DNA concentration for that microorganism in the positive control sample, and it wasn’t determined, it was impossible to compare this protocol with the others. Therefore, no conclusions were made about protocol sensitivity. This was despite the large volume of samples, which could only contain few microorganisms, and even the lack of the target microorganism, which could result in the absence of the amplification fragment from those bacteria.
The development of a new *L. pneumophila* protocol was assumed to be important and after several optimization steps new PCR conditions were selected, in which primers LmipL920 and LmipR1548 were used. The modified protocol also involved lower volumes of total DNA solution, 4 μl, which were sufficient to make the band from positive control visible. As the sample was the same used on the previous protocol, both protocols could be compared. Thus, it was verified that this optimized protocol had greater sensitivity for the detection of *L. pneumophila*.

The appearance, in agarose gel 1.3%, of a migration corresponding to various molecular weights in a wide area of the gel was possibly due to DNA degradation. Weaker bands were also seen below the amplification products, which may be explained by the excess of primers added during the preparation of the PCR mixture.

Because of the need for new PCR conditions to detect *L. pneumophila*, before real sample analysis, a PCR assay was performed with positive interlaboratorial samples to test the efficiency and adequacy of that protocol. The negative results obtained may suggest that samples have a concentration below the detection limit of the PCR technique. However, these results were not very credible in the sense that, as positive samples, they had the purpose to be identified by PCR. The hypothesis of sample DNA damage during the months of storage may need to be considered. On the other hand, because positive control was only seen with about 4 μl of DNA, it could be assumed that poor sensitivity of the method made it unsuitable for samples with small concentrations of the bacterium. So, as in the case of presence of the bacterium, that was not supposed to be in high quantities, to improve protocol sensitivity it seems necessary and more adequate to investigate other optimization steps or the search for an alternative protocol. It is suggested that the selection of other primers, for example, primers for other specific genes such as dnaJ, may offer improvement.

The comparison between protocols of other microorganisms was not possible since the total DNA volumes added in positive controls could not be compared. The concentration of each microorganism present in each positive control sample should be determined, for example, by measuring the cellular density.

Afterwards, the contamination of samples was carried out. The purpose of the study was to validate the extraction process and verify the occurrence of the amplification reaction and, at the same time, the presence of reaction inhibitors in excessive amounts. This study was performed on diverse samples which differ from each other in terms of the sampling point and the sample matrix.

Samples were contaminated in two ways: adding 100 μl of inoculum not extracted from each bacterium (except for *Mycobacterium* spp.) to samples 33, 34, 36, 38A or B, 34A and 67 before extraction and adding 5 μl of the total DNA solution after sample extraction, for the first and second purpose, respectively.

In this study, inocula and the total DNA volumes were also analysed instead of microorganism concentration. As a result, for some microorganisms, for example, in 100 μl of inoculum, large numbers of bacteria could exist, while for others, even with large volumes the number of microorganisms present could be lower or even zero.

By adding 100 μl of inoculum, it was observed that only with *Legionella* spp. did the fragment corresponding to the amplification product appear. The same was not observed for *Aeromonas hydrophila* and *Campylobacter jejuni* but with different samples, i.e., for the first, amplification did not occur on samples 34 and 34A neither on samples 36 and 38A but related to the second microorganism.

As the sample material and quantity of inoculum may influence the amplification reaction, depending on the microorganism, extraction was repeated with 400 μl of inoculum. The outcome was successful for *Campylobacter jejuni* but not for
Aeromonas hydrophila. It may be inferred that for 36 and 38A sample matrices, from ductile iron and fibrocement respectively, the amplification reaction only occurs or it was only visible with high volumes of inoculum.

For those two microorganisms the cause of high inoculum volumes was not related to the number of microorganisms present. This is due to other samples whose results were well successful, where lower volumes of the same inoculum were added.

In the study of Aeromonas, for both inocula, it was not possible to make conclusions about the efficacy of the extraction because samples extracted at the same time gave positive results, and the poor results can not be due to reaction inhibitors because the analysis of the same sample generated successful results for different microorganisms.

Generally, the extraction process may be validated and it was found to be efficient in spite of some unexpected results. The fact that sample extraction was performed together and some were successful, suggests that if a limitation of the method exists, it wasn't due to the extraction process but with something before or after.

Sample contamination, after sample extraction, with 5 μl of the total DNA solution was carried out on all microorganisms but only to samples 34A (90°) and 67 (270°), because they had higher sample amounts.

An explanation for the negative results for the Aeromonas species was not found in contaminated samples where inoculum was previously added. However, for the same samples but without added inoculum, it was possible to view bands characteristic to their presence.

Both Legionella spp. and Campylobacter jejuni had revealed fragments that corresponded to their presence, which indicates that the amplification reaction functioned well and the absence of reaction inhibitors, or at least a concentration of inhibitors which did not interfere in the reaction. As expected, bands relating to the amplification of Legionella pneumophila were not verified, which places some doubt on the performance and adequacy of the chosen protocol.

Finally, as in other cases, Mycobacterium spp. also had negative results. Although, to made evidence of protocol suitability and make sure of results veracity, once that microorganism was only tested to pipes transporting water from the treatment plant to the distribution system of Lisbon (samples 34A and 67), it was proceeded the contamination with other samples from other locals and materials (37B, fibrocement; 1.20B, ductile iron and 27, iron) in order to verify matrix influence. It was established that samples 34A, 67 and 37B were not successful while the remainder gave positive results. It is worth pointing out that these results are not completely reliable and conclusive due to the low quantities which led to different sample analysis compared to the other microorganisms, except for pipes transporting water to the distribution system of Lisbon (34A and 67). So it was not possible to make a conclusion regarding sample 37B relating to the presence of reaction inhibitors as well as for samples 34A and 67, which had given positive results for some of the other microorganisms.

Furthermore, any observation made did not have a sound basis. In order to make appropriate and definitive conclusions, it would be necessary to carry out extensive studies relating to the influence of DNA amounts added and the dependency of the material type on the amplification reaction.

Real biofilm samples can be obtained from pipes of the distribution system in programmed repairing (i.e. whilst under repair) as well as reservoirs (after emptying and immediately before cleaning) or from pipes transporting water from the treatment plant to the distribution system of Lisbon. It was verified that Campylobacter jejuni and Mycobacterium spp. weren’t present in the studied samples.

Aeromonas hydrophila amplification showed one band of irregular shape and length slightly below the fragment of the amplification product, although the small difference between lengths must be due to a slight gel inclination. The presence of
this microorganism in sample 1A 180º could only be confirmed by further analysis, which was impossible due to the lack of sample. Alternatively, the band of interest could be isolated from the gel and sequenced to confirm the presence of the species. Another sample collected from the same environment and at the same angle could also be analysed, although it would be impossible to reproduce exactly the conditions of the older sample.

All reservoirs samples, 34A (270º), 13A and 13B samples were positive to *Legionella* spp.. Favourable conditions for biofilm development were extremely important to the appearance of microorganisms in the samples. The presence of microorganisms in reservoirs was expected due to the likelihood of water stagnation, temperature, presence of organic matter and so on, which are determinant factors for biofilm establishment on such structures. The positive outcome on samples 13A and 13B, which were obtained from an iron pipe, reveals the importance and the influence of the metal on bacterial growth.

Despite the presence of *Legionella* spp. in sample 34A (270º) and its absence on the same pipe but at a 90º angle, it didn't necessarily mean that the latter sample had no bacteria. The result may be due to lower concentrations of the microorganism, which didn't allow detection at different angles, or to the absence of the microorganism in the precise location of the sampling, because of the small quantities of the recovered samples.

In fact, samples from the same locality, differing only in the recovery angle should not give distinct results because almost all the pipe walls were parallel and not possess curves or bends. However there was no information about walls disposal of the pipe of interest.

It should be noted that the detection of positive samples did not mean that the bacteria was in a viable state, as one of the principal PCR limitations was the inability to prove the viability of the microorganisms detected. An association with culture methods was not useful because it did not detect viable but non-culturable bacteria, a state acquired by some pathogens, like *Legionella*.

The visualizations of those samples on agarose gel still allowed verification of the presence of other bands which were not from the amplification product. The fragment with slightly superior length, 654 bp, relatively to amplified product, 430 bp, was simple to interpret as it corresponded to the first-step PCR amplification product. The other band, higher than 654 bp, appeared not only in samples but also in positive controls and may be due to non specific hybridization from other areas of the genome of the target microorganism rather than from interfering microorganisms, because it was also visible in the positive control.

Verification of the species level from samples found to be positive for *Legionella* genus did not detect any band from amplification, although it was not possible to make conclusions about their absence until optimization or development of the study protocol is carried out.

Reservoir samples, RSCO and RSRM, except for *Legionella* spp. and *Mycobacterium* spp., demonstrated bands with high molecular weight dimensions, above 1353 bp, which may be derived from contamination during extraction or before amplification. The appearance of those bands in three protocols for three different bacteria, in which primers were not the same for each microorganism, suggests that the area involved in amplification wasn't specific enough. In order to make conclusions about the origin of these bands, ideally the fragment would have been cut from the gel and sequenced.

For positive samples of *Legionella* spp. another study was carried out relating to their presence in some samples extracted from the same location, namely, reservoirs RSCO, RSVE, RSMS and RST. Instead of biofilm samples, the samples were directly obtained from the water recovered from these structures. For all samples, the absence of that microorganism was verified which may lead to conclusions relating to the capacity and/or the kinetics of bacterial detachment from the biofilms.
The kinetics appeared to be slow, releasing quantities of microorganisms that this method is incapable of detecting that would not provide concern to public health. However, direct comparisons could not be made because samples were not recovered at the same time (time of sampling differed by several months), so conditions of the water and surrounding environment were not the same and may not have even been similar.

Conclusions
The results obtained suggest that PCR is a promising method to detect pathogens originating from biofilms. All microorganisms, except *Legionella pneumophila*, could be detected by these PCR protocols. For *Legionella pneumophila* further work is necessary.

Our results indicate that the drinking water supplied by EPAL is of good quality since no *Aeromonas hydrophila*, *Campylobacter jejuni* and *Mycobacterium* spp. could be detected in biofilm samples from the pipes and reservoirs analysed. The exception was for *Legionella* spp. whose presence was noted in biofilm samples from three pipes and in all reservoir samples. Although, the bacteria were present in biofilms, it was not detected in water reservoirs.

In addition, it was also possible to indirectly and briefly conclude that the efficiency of water treatment is good, and that materials used in pipes and reservoirs materials in the EPAL water distribution system are adequate. Although, *Legionella* spp. could be detected by PCR, because the cultivation of the microorganism was not carried out, it was unknown if bacteria was in a viable state. To overcome this limitation, it may be interesting to adopt molecular techniques based on PCR detection, such as Reverse Transcriptase PCR and Real-Time PCR to confirm viability and to quantify the results obtained.

From a future perspective, it would be interesting to estimate biofilm detachment capacity associated with the release of microorganisms, and the effect on human drinking water. It would also be useful to study biofilm formation on different materials and their capacity to aggregate microorganisms, as well as to study the relationship between the pipes and reservoirs materials and the microbial species that establish as a biofilm.

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