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Spatio-temporal survey of opportunistic premise plumbing pathogens in the Paris drinking water distribution system

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ABSTRACT

Opportunistic premise plumbing pathogens present in drinking water are linked to a significant number of infections for health compromised patients. However, their monitoring is not required in current water potability standards and they have been poorly studied in a full-scale network. In this study, we quantified, by qPCR, three opportunistic pathogens, *Mycobacterium* spp., *Legionella pneumophila*, *Pseudomonas aeruginosa* throughout the Paris drinking water network over a one-year sampling campaign. While Mycobacteria spp. seemed ubiquitous whatever the distribution system and the time of the year, the occurrence of *L. pneumophila* and *P. aeruginosa* showed seasonal variations. Unlike *L. pneumophila* and *P. aeruginosa*, the concentration (copies number/L) of *Mycobacterium* spp. varied between sampling sites. The variation in microbial numbers did not demonstrate any correlations with temperature, pH, chlorine, conductivity, orthophosphate or nitrate levels. In conclusion, *Mycobacterium* spp. are common inhabitants of the Paris network while *L. pneumophila* and *P. aeruginosa* presence fluctuate over space and time. Such qPCR approach would help to better understand the behaviour of opportunistic premise plumbing pathogens.

1. Introduction

Water treatment is essential to limit the presence of pathogens and substances that may be harmful to human health in drinking water (Ashbolt et al., 2001). Despite disinfection treatments (UV, ozonation, chlorination …), drinking water contains noticeable bacterial flora. These bacteria come from the treated water, but also from the network itself where they can be found within biofilms. The important diversity of bacteria within the network could be considered as the water network microbiota. To avoid pathogen of gastrointestinal origin the focus of water potability standards is to detect faecal contamination, through the detection of enterococci and Escherichia coli by culture methods. Therefore, they allow to detect problems in the water network (rupture of pipe, disinfectant defect …) leading to the potential presence of pathogenic bacteria in the network microbiota.

Apart from classical waterborne human pathogens (e.g., *Vibrio cholerae*), it is possible to find environmental organisms presenting a sanitary risk called opportunistic premise plumbing pathogens (OPPPs). OPPPs are ubiquitous bacteria in soil and water environments but are also “regular” inhabitants of drinking water distribution systems (DWDSs) (Ashbolt, 2015). Three of the most frequently encountered OPPPs are *Mycobacterium* spp., *Legionella pneumophila* and *Pseudomonas aeruginosa* (Falkinham et al., 2015). Non-tuberculous mycobacteria (NTMs) are described as colonizers of drinking water distribution systems since they are found in distribution networks that vary greatly in their design and the treatments applied to them (Falkinham, 2016). Among all NTMs, somes species are responsible for the majority of human infections, specially the Mycobacterium avium complex, but other species are also involved (Hatzenbuehler et al., 2017; Astagneau et al., 2001; Falkinham, 1996). The diseases are mainly pulmonary since they correspond to 90% of cases of NTMs (Kasperbauer and Huitt, 2013) but also cause skin infections (Aubry et al., 2017; Yu et al., 2013) and other hospital-related infections such as catheter related bloodstream infections superficial, deep surgical site infections, infections associated with laparoscopy, infections of inplanted devices like pace makers or heartvlelves (Tagashira et al., 2015; Baker et al., 2017;

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Cooksey et al., 2008). *L. pneumophila* is responsible for legionellosis or "Legionnaire's disease”, in which bacteria reach the pulmonary alveoli and infect macrophages (Fields et al., 2002). The prevalence of the disease seems to increase year over year. Between 2011 and 2015, the number of cases rose from 0.97 to 1.30 per 100,000 on average in 29 European countries (Beauté and The European Legionnaires' Disease Surveillance Network, 2017). *P. aeruginosa* causes various pathologies depending on the location of the infection (Falkinham et al., 2015). It can thus cause infections in wounds and burns, as well as acute and chronic respiratory infections, particularly in patients with cystic fibrosis (Ratjen and Döring, 2003); and nosocomial infections in ICUs and neonatal care facilities. In some rare cases, pneumonia can also be contracted from exposure to recreational water (Hlavsa et al., 2015).

Currently, even if measurement of *L. pneumophila*. and *P. aeruginosa*. could be achived by international standardised methods (NF EN ISO 11731–2 and NF EN ISO 16266, respectively) any regulation requires this measurement on drinking water at a regular basis. NTMs detection is not standardised, though some researchers have tried to compare available methods (Radomski et al., 2013, 2010). Intensive utilisation of culture methods for OPPPs survey in a drinking water distribution networks is limited by several parameters. In particular, viable OPPPs in drinking water may be not cultivable, which makes them difficult to detect and count (Bédard et al., 2014; Bej et al., 1991). In contrast to the conventional microbiological methods, the use of quantitative PCR-based methods (qPCR) allows for the detection and quantification of these OPPPs in a specific and sensitive way (Mao et al., 2018; Qin et al., 2017; Räsänen et al., 2013; Whiley et al., 2014). However, these molecular tools do not differentiate between viable cells and free DNA or even dead cell DNA, thus leading to an overestimatation of the OPPPs quantity. Thus, they provide estimates on the occurrence and quantity of OPPPs and thus allow the microbial quality of drinking water to be assessed.

Previous studies on the detection of OPPPs in the DWDSs of Paris have reported the occurrence of NTM in various sections of the distribution network (Dubrou et al., 2013). However, no information on temporal evolution of NTM concentration was available. Also, *L. pneumophila* and *P. aeruginosa* have not been routinely measured and thus no information is available on their spatial distribution in the drinking water network of the city of Paris. In the context of microbiological monitoring of a DWDS, detecting and quantifying these three OPPPs in a large-scale sampling campaign would make it possible to estimate the potential risk of infection caused by the presence of these bacteria for susceptible persons.

The objective of this study was to examine occurrence and density of three OPPPs, *Mycobacterium* spp., *L. pneumophila* and *P. aeruginosa* in a large-scale drinking water network over one year. Temporal monitoring of OPPPs would help to gain a better knowledge of the dynamics these bacteria. Also, the potential impact of physico-chemical parameters was assessed to understand their relationship with OPPPs. Understanding the ecology of these OPPPs could help to mitigate the associated risks in drinking water.

2. Material and methods

2.1. Site locations and sampling procedure

The city of Paris is supplied with drinking water by four separate DWDSs (Fig. 1). The Ménilmontant DWDS is supplied with treated surface water from the Marne river, the Montsouris and the Saint-Cloud DWDSs are supplied with treated groundwater from multiple sources around Paris and the L’Haÿ-les-Roses DWDS is supplied with a mix of treated groundwater and surface water from the Seine river. Groundwater is treated with granular activated charcoal filtration followed by ultrafiltration (around 10 nm pore size). Surface water is treated by settling, sand filtration (slow or regular, depending of the plant), followed by ozonation (Ct between, 2–4 mg.min/L) and filtration through granulated activated charcoal, and ultraviolet disinfection (at 40 mJ/cm2) and finally chlorination (0.4 ppm in reservoir and 0.2 ppm in the network). After treatment, the drinking water from each DWDS is transported to a specific reservoir.

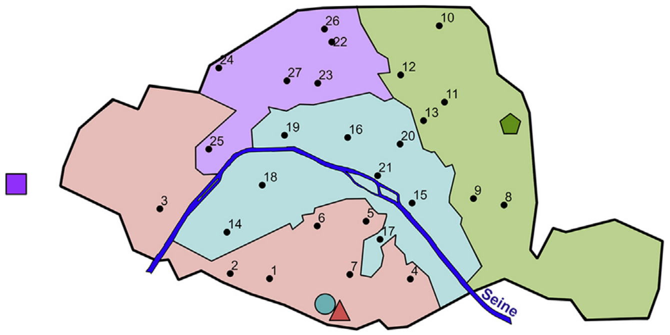

Fig. 1. Map of the four DWDSs of Paris and all sampling sites. . L’Haÿ-les-Roses 🟥, Ménilmontant 🟩, Montsouris🟦 and Saint-Cloud 🟪 distribution networks are supplied by l’Haÿ-les-Roses▲, Ménilmontant ⬟, Montsouris ● and Saint-Cloud ■ reservoirs, respectively.

For each DWDS, one sample from the reservoir, containing the treated drinking water before entering within the distribution network, was collected monthly. “Samples were collected monthly from seven sites for the Montsouris, L’Haÿ-les-Roses, and Saint-Cloud DWDSs and six sites for Ménilmontant DWDS were collected monthly from pipes of the distribution network (Fig. 1). A total of 368 1 L-drinking water samples were collected from the four DWDSs and their respective reservoirs from March 2016 to February 2017. Each water sample was transported to the laboratory at 4 °C and processed within 24 h. For each sample, physico-chemical parameters (temperature, pH, free chlorine, conductivity, and orthophosphate and nitrate concentration) were measured according to the European regulation on separated samples.

2.2. DNA extraction

One-liter water samples were filtered through 0.2 µm cellulose acetate membranes. Total DNA was then extracted from the entire membrane using the PowerWater DNA Isolation Kit (Qiagen germany), as described by the manufacturer. After extraction, DNA was stored at − 20 °C.

2.3. Quantitative polymerase chain reaction (qPCR)

In order to detect and quantify *Mycobacterium* spp., *L. pneumophila* and *P. aeruginosa*, a gene specific to each bacterial target was amplified by qPCR. Primers and probe were designed according to three studies that optimized qPCR for *Mycobacterium* spp. with the *atpE* gene (Radomski et al., 2013), *L. pneumophila* with the *mip* gene (Joly et al., 2006) and for *P. aeruginosa* with the *regA* gene (Shannon et al., 2007). Quantitative PCR was carried out using a TaqMan probe as indicated in the reference publications and with a Viia7 real-time PCR system (Life Technologies, ThermoFisher, Waltham, MA). The 10 µL reaction mixture contained 0.5 mM of specific primers, 0.25 mM of TaqMan® probe, 1X TaqMan® Fast Virus 1-Step Master Mix and, 4.25 µL of water, 2 µL of DNA template. Thermal cycling conditions were established as described by the manufacturer of TaqMan® Fast Virus 1-Step Master Mix (Thermo Fisher Scientific) without the reverse transcription step. Negative controls (template DNA replaced by nuclease-free water) and 10-fold serial dilution of standard plasmid (from 10^1 to 10^5 copies/µL) were included in triplicate in each qPCR run. The limit of quantification was established according to the method proposed by Forootan et al., (2017). The most diluted point of the standard curve must have a coefficient of variation less than 35% (Forootan et al., 2017). Thus, samples with a quantification of less than 20 copies per reaction (10 copies/μL of DNA template) were considered as negative. In order to quantify the specific genes of each OPPP, standard curves were obtained with serial dilutions of plasmids with the amplicon of interest as an insert.

2.4. Plasmids construction

Amplicons of the fragments of interest were obtained by PCR using the enzyme Hot Diamond Taq (Eurogentec, Liège, Belgium), as described by the manufacturer. PCR products were purified using the Nucleospin Gel and PCR Clean up (Macherey Nagel, GmBH & Co. KG, Düren, Germany) kit, according to the supplier's recommendations. The plasmids were constructed with the pGEM-T Easy Vector Systems kit (Promega, Madison, WI), as described by the manufacturer, and cloned in a chemically competent strain of *Escherichia coli* TOP10 F' (ThermoFisher Scientific). Plasmid extractions were performed using the NucleoBond Xtra Midi kit (Macherey Nagel), according to the supplier's recommendations.

2.5. Statistical analyses

All statistical tests were performed with R software 3.3.3 (R Core Team, 2017). The normality of data sets was assessed using the Shapiro-Wilk tests. The occurrence of the targeted bacteria was analyzed with Pearson's Chi2 tests. Two by two comparisons of bacterial quantification were performed using Mann-Whitney tests. Multiple comparisons were made using a Kruskal-Wallis test followed by Dunn's post-hoc tests. To evaluate the impact of temporality on variations of OPPPs, multiple comparisons with repeated measures were made using a Friedman test followed by Dunn's post hoc tests. Canonical Correspondence Analysis (CCA) was performed using the Vegan package (Oksanen et al., 2017). Significance of all statistical tests was set at a Pvalue ≤ 0.05.

3. Results and discussion

3.1. Uniform occurrence of OPPPs in the four DWDSs

In order to better understand the dynamic of OPPPs in a large scale water network, *Mycobacterium* spp., *L. pneumophila* and *P. aeruginosa* were quantified within the four different DWDSs of Paris, through a spatio-temporal sampling campaign (Fig. 1). To our knowledge, this sampling campaign (n = 368) is one of the most thorough important described so far for such a study with regard to the number of samples. The objective was to determine the spatial dispersion and the temporal evolution of these OPPPs within the network and to detect potential variation in the microbiological quality of drinking water. To this aim, we chose to quantify the three OPPPs by targeting specific genes by qPCR. All sampling sites were positive at least once for these three OPPPs. Moreover, each was frequently detected in drinking water samples across the four DWDSs (Table 1). The highest occurrence (95%) was obtained for *Mycobacterium* spp., demonstrating its quasi-omnipresence in the network. *L. pneumophila* and *P. aeruginosa* also showed occurrence rates of 50% and 52% in the water samples, respectively. The detection rates of *Mycobacterium* spp., *L. pneumophila* and *P. aeruginosa* were similar between the four DWDSs (Pearson's Chi2; Pvalue = 0.19, 0.72 and 0.46, respectively). The presence of *Mycobacterium* spp. was consistently high over the year with detections in 75–100% of samples (Fig. 2). This high occurrence was expected since itwas assumed that the genus *Mycobacterium* spp. would be ubiquitous in DWDSs (Falkinham, 2016). In addition, our results show that *Mycobacterium* spp. presence was uniform over the sampling period. However, it should be understood that the results could be the summary of the contributions to a variety of different Mycobacterium species. In contrast, the monthly occurrence of *L. pneumophila* and *P. aeruginosa* was highly variable, ranging from 0 to 100% of samples. These two OPPPs also present a similar pattern since their occurrence was very high and rather stable during the same period from March 2016 to May 2016 and then decreased and fluctuated over the rest of the sampling period (Fig. 2). These values are in the high range of those reported in similar studies since *L. pneumophila* was detected in 4%–37% of the samples (Ghanizadeh et al., 2016; King et al., 2016; Rakic et al., 2013; Wang et al., 2012) and *P. aeruginosa* in 1%–46% of samples (Lu et al., 2015b, 2015a; Wang et al., 2012). These results demonstrate that these persist in DWDSs and show different patterns over the sampling period.

Table 1
Occurrence rate (%) of OPPPs *Mycobacterium* spp., *L. pneumophila* and *P. aeruginosa* in drinking water samples of the four DWDSs. HLR: L'Haÿ-les-Roses; MNM: Ménilmontant; MTS: Montsouris; STC: Saint-Cloud.

Targeted organisms	Samples (n = 368)				
	HLR (n = 96)	MNM (n = 83)	MTS (n = 95)	STC (n = 87)	Mean
Mycobacterium spp.	94.62%	95.18%	97.,89%	92.55%	95.11%
L. pneumophila	47.31%	48.19%	51.58%	54.84%	50.14%
P. aeruginosa	47.31%	50.60%	52.63%	59.57%	52.17%

3.2. Differences in Mycobacterium spp concentrations in the distribution systems Mycobacterium

We assessed the concentration of *Mycobacterium* spp. at different sampling sites within thefour DWDSs. The *atpE* gene copy numbers from all collected sample is presented in Fig. 3. This spatial assessment show that *Mycobacterium* spp. was quite abundant in all samples with a mean copy number (± standard error) *atpE* of $1.3 \times 10^5 \pm 3.7 \times 10^4$ copies/L (Fig. 3A). The observed concentration is similar with other published results, with gene copy numbers *Mycobacterium*between 10^3 and 10^5 gene copies per liter (Lu et al., 2015a, b; Wang et al., 2012), and in accordance with Next Generation Sequencing (NGS) methods indicating the importance of mycobacteria in distribution system-colonization (Liu et al., 2016; Perrin et al., 2018).

As is the case, the standard errors are very high, thus showing the high variability in the concentration of *Mycobacterium* spp. in DWDS. L'Haÿ-les-Roses DWDS showed significantly higher concentrations of *Mycobacterium* spp. when compared to Ménilmontant and Montsouris DWDSs but not Saint-Cloud DWDS (Fig. 3A). More specifically, concentrations of *Mycobacterium* spp. varied within each DWDS. Specifically, within the L'Haÿ-les-Roses DWDS, each sampling site showed different concentrations of *Mycobacterium* spp. than at least one other sampling site (Friedman's test, Pvalue < 0.05). In contrast, *L. pneumophila* and *P. aeruginosa* were shown to be present at a lower concentration than *Mycobacterium* spp., with an average copy number of $3.0 \times 10^3 \pm 1.0 \times 10^3$ and $2.5 \times 10^3 \pm 4.3 \times 10^2$ copies/L respectively (Fig. 3B and C). Also, *L. pneumophila* and *P. aeruginosa* concentrations were rather constant in all DWDS (Friedman's test, Pvalue > 0.05), and no significant difference were seen between the four DWDSs for *L. pneumophila* and *P. aeruginosa* (Kruskal Wallis; Pvalue = 0.82 and 0.66, respectively).

Although utilisation of qPCR allows a good reliability of the quantification, this method can lead to an overestimation of bacteria because it also allows detection of dead cells or free DNA, (Carini et al., 2016; Inkinen et al., 2016). Moreover, our data only represents quantification of planktonic bacterial communities while biofilms could contain important concentration of bacteria including OPPPs. Nevertheless, our results showed that the three targeted OPPPs might be present at noticeable concentrations in the drinking distribution system despite the water treatments, the chlorine disinfectant residual and a low TOC. The concentration of the three OPPPs was also examined according to the location of the samples (reservoir or distribution system) (Fig. 3).

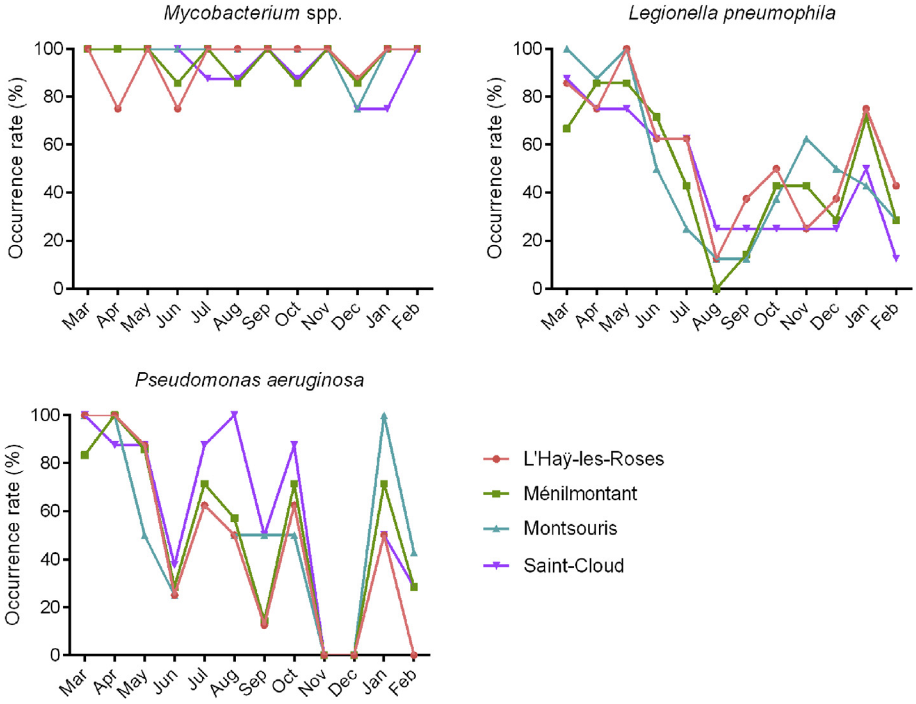

Fig. 2. Occurrence of *Mycobacterium* spp., *L. pneumophila* and *P. aeruginosa* in the four DWDSs from March 2016 to February 2017 for each DWDS (n = 6 to 8).

L'Haÿ-les-Roses DWDS had a significantly higher quantity of *Mycobacterium* spp. within its reservoir compared to its distribution network (Mann-Whitney; Pvalue = 8.2×10^{-5}). The other three DWDSs did not show significant difference between reservoirs and drinking water distribution network for *L. pneumophila* and *Mycobacterium* spp. (Mann-Whitney; Pvalue > 0.05). To better evaluate the variations in OPPPs between the reservoir and its drinking water distribution network, we correlated the mean concentration of each OPPPs was compared to the mean distance between each sampling point and the supplying reservoir. Only variations of *Mycobacterium* spp. within L'Haÿ-les-Roses DWDS showed a weak positive correlation with distance (Pearson's R = 0.29, Pvalue = 0.008).

Decrease of *Mycobacterium* spp. concentrations in one DWDS compared to reservoir (in the L'Hay les Roses DWDS), or even the absence of variation between the reservoir and the distribution network for other OPPPs was surprising since in general, growth occurs in the distribution system (Falkinham et al., 2001; Ling et al., 2018; Torvinen et al., 2004). Dubrou et al. have suggested that competition or exclusion niches existing between mycobacterial species might explain variations of occurrence between production, storage, and distribution locations (Dubrou et al., 2013).

3.3. OPPP concentrations showed seasonal variation

As OPPP numbers were collected throughout the year of collection, it was possible to demonstrate seasonal changes (Fig. 4). The concentration of *Mycobacterium* spp. showed no significant differences throughout the year in Ménilmontant, Montsouris and Saint-Cloud DWDSs (Friedman's test, Pvalue = 0.31 and 0.32, respectively). In Saint-Cloud DWDS, significant higher quantities of *Mycobacterium* spp. were recorded in October 2016 compared to December 2016 (Friedman's test, Pvalue = 0.01). In L'Haÿ-les-Roses DWDS, significant lower *Mycobacterium* concentrations were recorded in April and June2016 when compared to September and October 2016 (Friedman's test, Pvalue = 2×10^{-4}). *L. pneumophila* appeared to show a similar pattern in all four DWDSs with an increase of concentration during spring months (March to May 2016) and a stabilization from June 2016 to February 2017. However, the concentrations during the month of March 2016 were higher than the other months in Montsouris DWDS and during May 2016 for the Saint-Cloud DWDS (Friedman's test; Pvalue < 1.10^{-4} and Pvalue = 6.10^{-4}, respectively). The concentrations of *P. aeruginosa* showed a pattern similar to *L. pneumophila*. For this OPPP, the quantities were significantly higher in March and April 2016 for the four DWDSs (Friedman's test, Pvalue < 1.10–4). These concentration results correlate with the results on occurrence, showing that *Mycobacterium* population was rather stable over the year while *L. pneumophila* and *P. aeruginosa* population were higher from March to April 2016.

3.4. Absence of correlations of OPPPs concentrations and physico-chemical parameters

Concentrations of each of the three OPPPs were analyzed regarding several physico-chemical parameters (total chlorine, electrical conductivity, pH, temperature, orthophosphate and nitrate concentrations) that were measured for each drinking water sample. Impact of physico-chemical parameters on the quantity of the three OPPPs was first investigated using CCA. However, the constrained inertia values were very low, representing 6.9% of the total inertia (Fig. 5). These results were unexpected especially for the temperature since OPPPs are usually described as temperature dependent (Inkinen et al., 2016). Only variations of *P. aeruginosa* within the Montsouris DWDS showed a negative correlation with temperature (Pearson's R = −0.37, Pvalue = 1.8.10–4). All the other correlations were not significant (Pvalue > 0.05). Thus, it seems that the measured physico-chemical parameters of drinking water had very little impact on the concentration of the three OPPPs studied.

Variations in concentrations of the OPPP did not correlate to the physio-chemical parameters leading is to hypothesize that the large variation of results on the same point, in particular for *Mycobacterium* spp., could indicate than an other, yet to be discovered, could hide the physico-chemical influence. Additionally, the low variation observed for some parameter such as free chlorine and orthophosphate could also explain the difficulty to observe bacterial fluctuation in relation with water physico-chemical parameter change. It would be likely that these variations are only occasional and are the consequence, for example, of

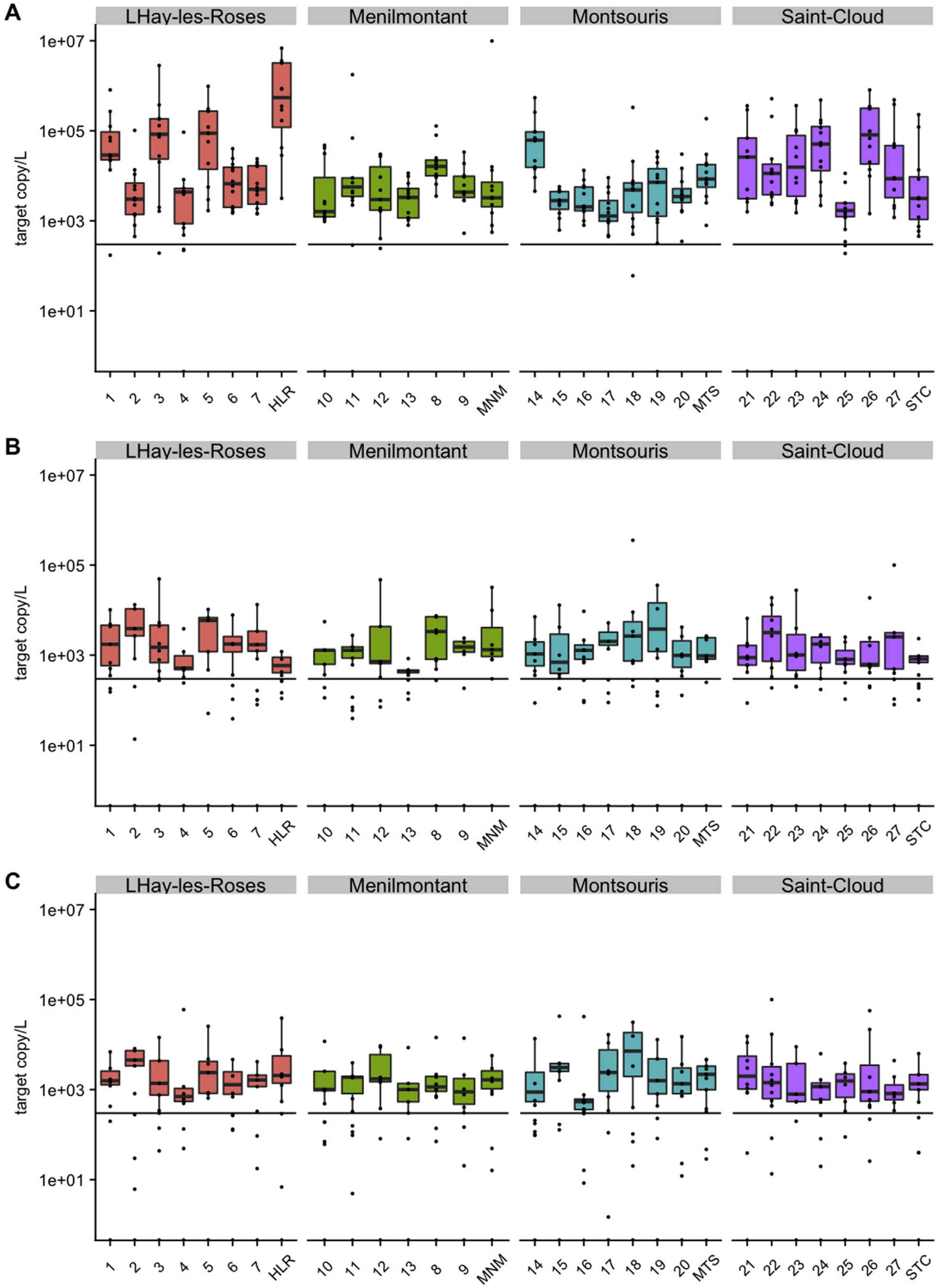

Fig. 3. Spatial study of the concentration of OPPPs (A) *Mycobacterium* spp., (B) *L. pneumophila* and (C) *P. aeruginosa* in the four DWDSs according to the sample's location. Numbers from 1 to 27 represent distribution network sites. HLR, MNM, MTS and STC represent the reservoirs. The line corresponds to the limit of quantification. The values below the limit of quantification are not considered for the boxplot representation (n = 11 to 12).

a local detachment of biofilm within the distribution network, resulting from a change in water flow, damage to infrastructure, or a random event (Douterelo et al., 2016; Lehtola et al., 2006). The ability of OPPPs to form or persist in biofilms (Wingender and Flemming, 2011), to interact with amoebae (Bouyer et al., 2006; Delafont et al., 2013; Gebert et al., 2018) could facilitate their persistence in DWDSs. Moreover, numerous publications have shown that *Mycobacterium* spp. (Gomez-Alvarez et al., 2012; Lee et al., 2010), *L. pneumophila* (Alleron et al., 2008; Williams and Braun-Howland, 2003), several species of mycobacteria and *P. aeruginosa* (Behnke et al., 2011; Grobe et al., 2001) are

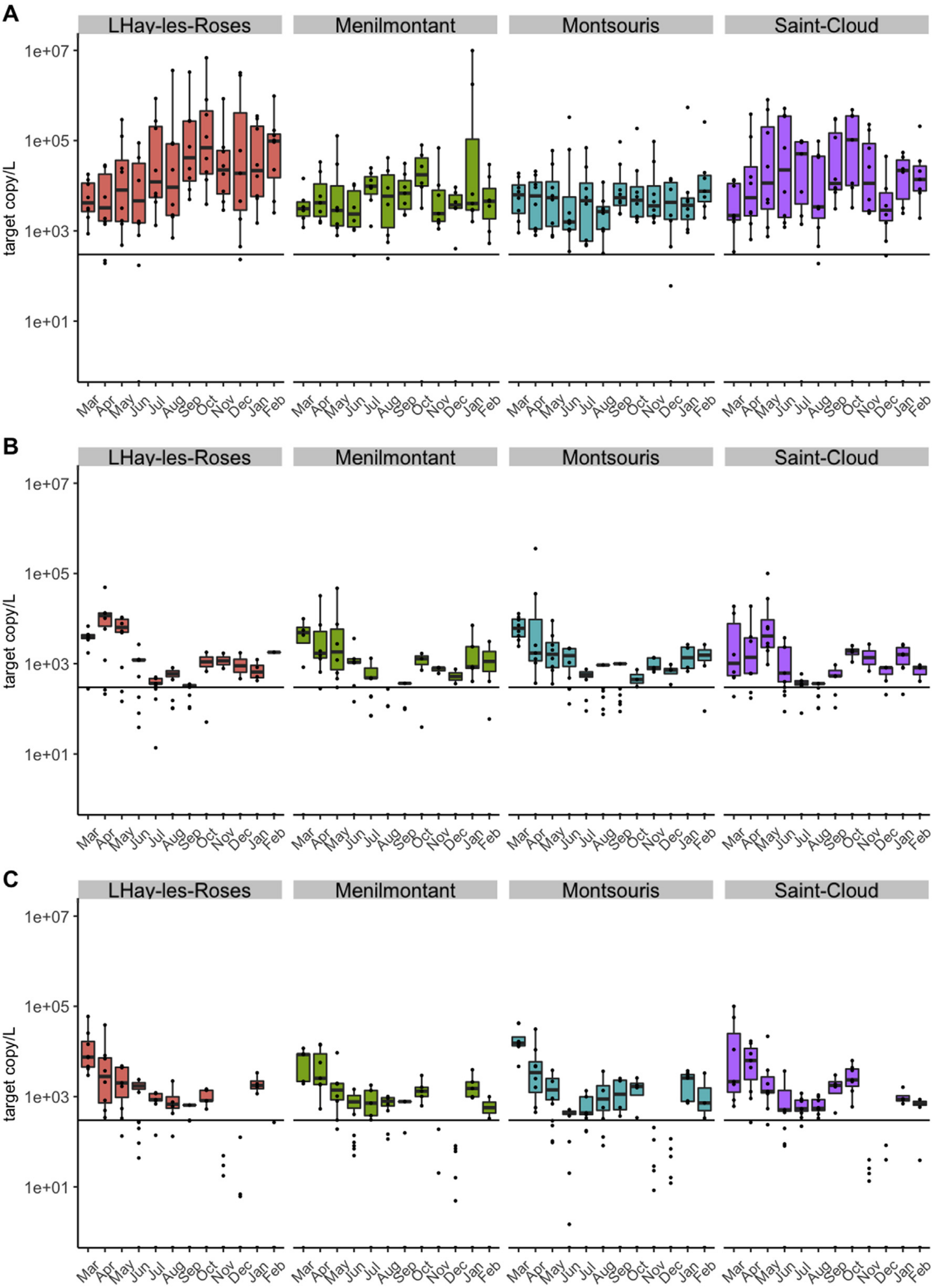

Fig. 4. Temporal study of the concentration of OPPPs (A) *Mycobacterium* spp., (B) *L. pneumophila* and (C) *P. aeruginosa* in the four DWDSs from March 2016 to February 2017 for each DWDS. The line corresponds to the limit of quantification per liter. The values below the limit of quantification are not considered for the boxplot representation (n = 6 to 8).

tolerant to residual chlorine or monochloramine applied in some DWDS and, their tolerance can be increased when these organisms form biofilms (Steed and Falkinham, 2006).

4. Conclusions

This report is the first study focusing on the occurrence and quantification of OPPPs such as *Mycobacterium* spp., *L. pneumophila*, *P. aeruginosa* in a large DWDS through a large-scale (both spatial and

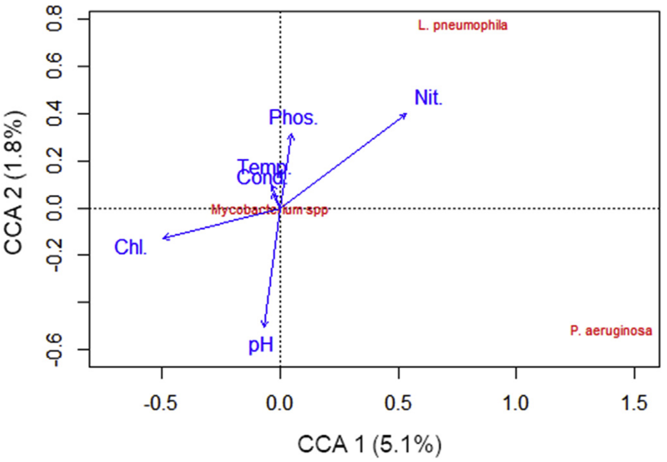

Fig. 5. Influence of physico-chemical parameters on the concentration of the three OPPPs *Mycobacterium* spp., *L. pneumophila* and *P. aeruginosa*, as represented by canonical correspondence analyses (CCA). Chl. = Chlorine concentration, Cond. = Conductivity, Nit. = Nitrate concentration, Phos. = Phosphate concentration, Temp. = Temperature.

temporal) sampling campaign. The results showed that *Mycobacterium* spp. occurrence was very high and constant, underlying that this bacterium is a common inhabitant of this drinking network. Also, the concentration of *Mycobacterium* spp. was rather stable throughout the year while some variations were reported between the sampling sites. *L. pneumophila* and *P. aeruginosa* occurrence and concentration were lower than those of *Mycobacterium* spp. interestingly, they presented a similar pattern since their occurrence and concentration was higher from March to May 2016. We demonstrated that this kind of study is a valuable tool to evaluate the presence of OPPPs and follow water quality from a bacterial point of view. Finally, it would be interesting to implement viable qPCR to get a better estimation of the possible impact of these OPPPs on public health. This would estimate the potential reservoir role of DWDS in the risk of transmission of OPPPs.

Declarations of interest

None.

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