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Effectiveness of a neutral electrolysed oxidising water (NEOW) device in reducing *Legionella pneumophila* in a water distribution system: a comparison between culture, qPCR and PMA-qPCR detection methods.

Sara Bonetta[a1*], Cristina Pignata[a1], Silvia Bonetta[a], Lorenza Meucci [b], Donatella Giacosa[b], Elena Marino[b], Ilaria Gorrasi[a], Giorgio Gilli[a] and Elisabetta Carraro[a]

[a]Department of Public Health and Pediatrics, University of Torino, Piazza Polonia 94, 10126 ,Torino, Italy

[b]Società Metropolitana Acque Torino S.p.A., C.so XI Febbraio 14, 10152, Torino, Italy

***Corresponding author:** Sara Bonetta, Department of Public Health and Pediatrics, University of Torino, Piazza Polonia 94, 10126 , Torino, Italy; tel +39 11 6708192; e-mail: sara.bonetta@unito.it

[1]These authors equally contributed to the work

Abstract

Disinfection of hot water systems is critical for reducing Legionnaires' disease in high-risk buildings. The use of neutral electrolysed oxidising water (NEOW) is a promising method for the control of microorganisms in hot water systems. However, full-scale evaluations of the efficacy of NEOW devices to control *Legionella pneumophila* are currently lacking. The aim of this study was to assess the effectiveness of a NEOW device in reducing *L. pneumophila* in a hotel water network.

Water samples (n=67) were collected from different sites of a hotel distribution system before and after the installation of the NEOW device at the 1st, 4th, 8th and 12th week. Detection of *L. pneumophila* was performed comparing culture, qPCR and PMA-qPCR methods. Total bacterial counts (22°C and 37°C), *Pseudomonas* spp. and physico-chemical parameters were also monitored.

The NEOW treatment resulted in a reduction of the amount of *L. pneumophila* positive samples (-32%) and of the number of heavily contaminated points (> 10^4 CFU/L and > 10^3 CFU/L) (-100% and -96%, respectively). Treatment maintained *L. pneumophila* at low levels (< 10^2 CFU/L), which do not require specific intervention measures. The effectiveness of the disinfection system was also confirmed by PMA-qPCR ($p < 0.001$). The use of PMA resulted in a signal decrease in almost all samples upon the disinfection treatment.

The NEOW disinfection device appears to be a promising approach to reduce the colonisation of hot water systems by *L. pneumophila*; however, further investigations are needed to ascertain its efficiency over longer time periods.

Keywords: *Legionella pneumophila*, hot water, neutral electrolysed oxidising water, propidium monoazide, qPCR

1. Introduction

Legionella spp. are ubiquitous microorganisms that are widely distributed in aquatic environments. From these natural reservoirs, this opportunistic pathogen can spread to and colonise artificial aquatic environments (e.g. building water systems, cooling towers). *Legionella pneumophila* is most frequently associated with human disease (Cunha and Cunha, 2017) and in Europe, 6,573 confirmed cases of *L. pneumophila*-associated Legionnaires' Disease were reported in 30 countries in 2015. Four countries (France, Germany, Italy and Spain) accounted for 69% of all notified cases. As in previous years, most cases (69%) were community-acquired, while 21% (1,141 cases) were travel-associated infections (ECDC, 2017).

Many studies have demonstrated the widespread presence of *L. pneumophila* in water systems of tourist reception and spa facilities (Coetzee et al., 2012; Mouchtouri et al., 2015). For this reason, it is important to adopt measures to prevent and control the dissemination of *Legionella* through careful risk assessment and management. A range of physical and chemical disinfection methods have been proposed with the aim of controlling *L. pneumophila* contamination; however, to date, the most effective procedures have not been defined (Li et al., 2017; Lin et al., 2011; Marchesi et al., 2016). Therefore, alternative disinfection methods that are effective in controlling the proliferation of *L. pneumophila* could be useful tools to reduce the risk of the spread of Legionnaires' disease.

Electrolysed oxidising water (EOW) is a technology (Thorn et al., 2012) based on the electrodialysis of a sodium chloride solution in an electrolysis chamber with an anode (acid EOW) and a cathode (alkaline EOW) separated by a membrane. The mixture of these two solutions forms the neutral EOW (NEOW). NEOW has proved to be effective in the reduction of many waterborne pathogenic microorganisms in laboratory settings (Issa-Zacharia et al., 2010; Park et al., 2004, Cossali et al. 2016) and in the food industry (Rahman et al., 2016). However, full-scale evaluations

of the efficacy of NEOW devices to control *Legionella pneumophila* in hot water systems are currently lacking.

To date, the most commonly employed method for *L. pneumophila* detection in water samples is the standard culture technique. Despite being essential for identifying and typing *Legionella* strains, the culture method has several limitations, including the inability to detect viable but non-culturable bacteria (VBNC) that may represent a public health hazard, especially when a reclamation treatment is performed (Casini et al., 2014). Quantitative polymerase chain reaction (qPCR) may overcome many of the disadvantages of traditional culture methods; however, qPCR does not allow for viable cells to be distinguished from dead cells. A promising approach for detecting viable cells involves the use of qPCR along with photoactivatable DNA intercalators such as propidium or etidium monoazide (PMA or EMA), which can penetrate the membranes of compromised cells and block PCR amplification (Delgado-Viscogliosi et al. 2009; Mansi et al., 2014, Scaturro et al., 2016), with PMA having been shown to be more specific for dead cells compared to EMA (Scaturro et al., 2016). At this time, some studies using the PMA-qPCR method to detect *Legionella* in water samples have already been published (Ditommaso et al., 2016, 2014; Yanez et al., 2011), all of which dealing with water reclaimed with traditional disinfection strategies.

The aim of this study was to assess the effectiveness of a NEOW device in reducing both culturable and nonculturable *Legionella pneumophila* in a hotel water network. The standard culture method was used with qPCR alone and in combination with PMA (PMA-qPCR).

2. Materials and methods

2.1. Site description

The study was conducted in the hot water distribution system of a hotel located in a mountainous area in the province of Turin. The building was constructed in the 1930's and renovated and opened

as a hotel in the 1980's. It consists of four floors with 73 rooms and a spa. The water used in the hotel is collected from a private well located near the building and treated with a UV lamp prior to distribution.

Since 2014, sampling plans have been implemented to assess *Legionella* spp. contamination in the water distribution system. Some samples collected in the hotel before the NEOW device installation were primarily contaminated by *Legionella pneumophila* serogroups 2-14 at concentrations $>10^4$ CFU/L.

2.2. Installation of the neutral electrolysed oxidising water (NEOW) device

The NEOW device was installed in the hot water distribution system in addition to the UV treatment. Specifically, it was installed after the boiler that heats the water coming into the building (operating temperature of 50°C).

NEOW was generated from the electrolysis of a saturated solution of NaCl in a Danolyte Just in time 200 (DJIT) commercial NEOW generator (DueDi s.r.l., Carmagnola, Torino, Italy). NEOW is a product that contains hypochlorous acid, with a neutral pH (~7.0), an active chlorine concentration of 500 mg/L and characterized by an oxidation reduction potential (ORP) of ~850 mV. The dosage unit includes a multifunction volumetric pump with a Cl_2 analyser at the boiler inlet. The chlorine concentration at the injection point was set at 0.8 mg/L. The device was also connected to an operations centre to allow for remote control.

2.3 Sample collection

From September to December of 2015, water samples (n = 67) were collected from the distribution system of the hotel. Detailed information about the sampling sites are reported in Table 1. Samples

were collected before (sampling 1) and after the installation of the NEOW device at the 1st, 4th, 8th and 12th week (samplings 2-5).

At each sampling point, water samples were collected in three sterile glass bottles (1 litre) (sodium thiosulfate 0.1g/L) and used for culture, qPCR and PMA-qPCR determinations within 24 h. Water was flushed and samples were taken mid-stream.

2.4 Plate culture method

Isolation of *Legionella* from water samples was performed by culture method according to the modified ISO 11731-2 (2004). Confirmed colonies were identified (*L. pneumophila* serogroup 1, 2-14 or *Legionella* spp.) using the agglutination test (Legionella latex test; Oxoid). Results were expressed as CFU/L, and the theoretical detection limit of the procedure for 1 L of sample was 1 CFU/L.

2.5 qPCR and PMA-qPCR

The remaining two litres of samples were concentrated by filtration on two 0.22-µm pore size polycarbonate membranes (Isopore, Millipore). The first filter was directly added to the lysis solution for DNA extraction using a commercially available kit (Aquadien^{TM} Kit, BioRad, Milan, Italy) according to the manufacturer's instructions. The second filter was first overlaid with 1 mL of PMA (25 µM) in 60 mm Petri dishes and incubated in the dark for 5 min, followed by a 5 min exposure to a 500 W halogen light source on ice. After irradiation, the filter was added to the lysis solution for DNA extraction using experimental conditions that were optimised in a previous study (Bonetta et al., 2017).

Quantitative PCR was performed with a Chromo4^{TM} (BioRad) and a iQ-Check Quanti *L. pneumophila* kit (BioRad) according to the manufacturer's instructions. Each sample was assayed

in duplicate. Results are expressed as the number of genome units (GUs) per litre. The detection limit of this qPCR method was 5 genome units (GU) per well; the theoretical detection limit of the total method (DNA extraction + qPCR) was 80 GU/L. The quantification limit was 15 GU/5 µL, corresponding to 500 GU/L.

2.6 Physical, chemical, and microbiological analyses

Water temperature (Radiometer TIM870, Hach Lange S.r.l., Lainate, Milan, Italy) was determined at the time of sample collection. Conductivity, pH, turbidity, ammonia, and the residual chlorine concentrations in the samples were also analysed (Rice et al., 2012). *Pseudomonas* spp. and total bacterial counts (TBC) at 22°C and 37°C were determined in all samples (ISO 16266:2006; ISO 6222:1999).

2.7 Statistical analysis

All qPCR data were analysed by the Opticon Monitor Analysis Software version 3.4 (Biorad). Statistical analysis was conducted with the statistical package IBM SPSS Statistics 24.0 (IBM Corporation, Armork, NY, USA). Significant differences between the concentrations of *Legionella pneumophila* (using culture, qPCR and PMA-qPCR) in the five sampling campaigns were assessed by ANOVA and Tukey's multiple comparison test. Significance was evaluated within 95% confidence intervals ($p \leq 0.05$). Pearson's test was used to evaluate the correlation between *Legionella pneumophila* and the total bacterial counts.

3. Results and discussion

3.1 Quantification of Legionella pneumophila using culture and molecular methods

Figure 1 shows the observed *Legionella pneumophila* contamination at the inlet of the hotel water system and in the water distribution system before (Figure 1a) and after (Figure 1b-e) the NEOW treatment using three different methods. In the examined building, only *L. pneumophila* serogroups 2–14 were isolated.

Before treatment, all water distribution system samples (13/13) were positive according to culture method (Table 2). Five out of the 13 samples exceeded 10^4 CFU/L and four out of the remaining samples exceeded 10^3 CFU/L. The highest *L. pneumophila* concentrations were observed in certain rooms (R1 and R7), in the return loop (RL) and in dressing room (D1 and D2) samples (Figure 1a). Some studies showed that the return loop is one of the most frequently contaminated sites in the water systems of different hotels (Bonetta et al. 2010; Cuhna et al., 2016). The high level observed in some of the rooms and dressing rooms is probably related to the intermittent use of hot water, which can promote water stagnation and *Legionella* proliferation (Bargellini et al., 2011; Cuhna et al., 2016). Also the qPCR method (with or without PMA treatment) showed *L. pneumophila* contamination in almost all the sampling sites of the water distribution system (12/13 and 13/13 respectively). The concentration of *L. pneumophila* (over a range of 3-5 Log GU/L) was generally higher than that estimated as CFU/L reported in other studies (Ditommaso et al., 2014; Yanez et al. 2011). The low differences between qPCR and PMA-qPCR observed in our samples confirmed that only a minimal fraction of non viable cells was present, probably because of a lack of a chemical disinfection system (Scaturro et al., 2016). In contrast, the water sample collected at the inlet of the water system (I) was found contaminated using the culture method, although no contamination was observed by qPCR or PMA-qPCR (Figure 1a). Considering the low contamination of the inlet water entering the structure, these results indicated a phenomenon of bacterial growth within the hotel water distribution system.

During the second sampling campaign, after the application of the NEOW treatment (Table 2), a reduction in the proportion of *L. pneumophila*-positive samples was observed (70%) using the

culture method. In particular, the percentage of heavily contaminated points (*L. pneumophila* loads > 10^4 CFU/L and > 10^3 CFU/L) was reduced to 0% and 10% respectively. In different sampling sites (B, R1, R3 and R6 R4, R5, R7, W, T) (sampling 2, Figure 1b) *L. pneumophila* contamination was revealed using both culture and molecular methods. In particular, a PMA-induced signal reduction of genomic units compared to qPCR was observed, suggesting the presence of a certain proportion of membrane-compromised cells (Ditommaso et al., 2014; Yanez et al., 2011) likely related to the NEOW treatment, as reported with other disinfection systems (Mansi et al. 2014; Marchesi et al., 2016). The effectiveness of the NEOW treatment was also confirmed in samples B, R1, R3, R6 and RL, where a complete reduction of genomic units was observed using PMA-qPCR.

During the following sampling campaigns, the number of positive samples did not change substantially over time (Table 2). However, the percentage of heavily contaminated points (*L. pneumophila* loads > 10^4 CFU/L and > 10^3 CFU/L) dropped to 0% already after 1 week and 2 months of treatment, respectively. A general reduction of *L. pneumophila* contamination (< 2 Log CFU/L or UG/L) was observed during samplings 3, 4 and 5 (Figure 1 c,d,e), but the highest *L. pneumophila* concentrations were observed in the wellness area (samples W and T) in sampling 3 (Figure 1c). This could represent a possible risk due to the formation of aerosol, which can promote the spread of *Legionella* (Ahmed et al., 2013; Cuhna et al., 2016; Euser et al., 2010). Some samples (B, R1, R2, R6, sampling 3, Figure 1c), (R2, R5, R7, W, T, sampling 4, Figure 1d), (RL, D1, sampling 5, Figure 1e) did not show any *L. pneumophila* contamination using culture method and PMA-q-PCR, confirming the effectiveness of the disinfection system over time. However, some samples were positive using the culture method (Figure 1 c, d, e) but PMA caused a complete loss of signal. The same trend was also observed in other studies (Lizana et al., 2017) and was probably due to the lower detection limit of the culture method (1CFU/L) with respect to the molecular one (80G/L). This discrepancy could indicate a limit for the usefulness of PMA-qPCR for the evaluation of *L. pneumophila* contamination in a disinfected water distribution system; however, the difference

was observed only in some cases and only when the contamination levels were very low, below the intervention threshold values.

Overall, the NEOW device, which continuously produces and dispenses hypochlorous acid, allowed a reduction of *L. pneumophila* contamination. ANOVA test confirmed a significant difference in *L. pneumophila* measured by culture method and PMA-qPCR (log CFU/L or log GU/L) among the five sampling campaigns ($F = 20.936$; $p \leq 0.001$ and $F = 29.318$; $p \leq 0.001$). In particular, the post hoc Tukey's test highlighted a statistically significant difference between *L. pneumophila* before (sampling 1) and after (sampling 2-5) the NEOW application ($p \leq 0.001$ for both methods). Treatment maintained contamination at low levels during the three-month observation period, leading to contamination levels $\leq 10^2$ CFU/L that do not require specific intervention measures, according to the Italian guidelines (Ministero della Salute 2015), even if the concentration of *L. pneumophila* was not reduced below the limit of detection in all the sampled points.

In the study of Marchesi et al. (2011), which compared the effectiveness of different disinfection methods, chlorine dioxide (ClO_2) turned out to be more efficient than shock superheating and shock hyperchlorination in reducing *L. pneumophila*. ClO_2 treatment maintained *L. pneumophila* contamination at low levels during the observation period, but did not eradicate it from the system. This trend was similar to what was observed in our study, although ClO_2, with respect to NEOW, generally requires a prolonged time to reach significant reductions of *L. pneumophila* and a strict control of chlorine injection to prevent malfunctioning. Other studies have shown that the use of monochloramine seemed to be an alternative effective approach; however very high levels of monochloramine (> 3 mg/L) were necessary to obtain *Legionella* spp. reduction $<10^2$ CFU/L (Mancini et al., 2015; Marchesi et al., 2016); another disadvantage includes its complicated on-site generation (Lin et al., 2011). In contrast, NEOW can be easily produced in situ and corrosive phenomena are not known to occur at the concentrations used in water distribution systems (Thorn et al., 2012). Furthermore, other disinfection systems were less efficient in inducing a stable reduction of *L. pneumophila* compared to the NEOW treatment. In most cases, superheating and

hyperchlorination were associated with an initial reduction of contamination, after which values returned to pretreatment levels (Marchesi et al., 2011; Lin et al., 2011). This trend was probably related to the VBNC legionellae generated upon treatment (Marchesi et al., 2016). It would be interesting to investigate the effect of the NEOW treatment on the regrowth of VBNC *Legionella pneumophila* after long periods of time (> 3 months).

3.2 Microbiological indicators, physical and chemical analyses

The results of the TBC at 22 and 37°C are shown in Figure 1. *Pseudomonas* spp. were not found in any of the samples analysed. A positive and statistically significant correlation (Pearson test) was observed between the *L. pneumophila* concentration and TBC at 22°C ($r = 0.501$; $p \leq 0.01$) or TBC at 37°C ($r = 0.586$; $p \leq 0.01$) as reported in other studies (Bargellini et al., 2011). In accordance to what we observed for *L. pneumophila*, TBCs were affected by the NEOW treatment. ANOVA test confirmed a significant difference among the concentrations of TBCs in the five samplings ($F = 4{,}017$; $p \leq 0.01$ for TBC 22°C and $F = 7{,}647$; $p \leq 0{,}001$ for TBC 37°C). The post hoc Tukey's test highlighted a statistically significant difference between the TBC at 22°C before the NEOW treatment and the fifth sampling campaign ($p \leq 0.01$), and between the TBC at 37°C before the NEOW application and the third (after 1 month) and fifth (after 3 months) sampling campaigns ($p \leq 0.05$ and $p \leq 0.001$ respectively).

Table 3 shows the physicochemical characteristics of the water samples before and after the NEOW treatment. No relationship was observed between physicochemical parameters and *L. pneumophila* concentration.

4. Conclusions

This is the first study to identify the effect of an in situ NEOW treatment on the reduction of *L. pneumophila* contamination in a hotel water network.

Our study demonstrated the effectiveness of continuous chemical disinfection in the reduction of *L. pneumophila* concentration. The use of the NEOW device appears to be a promising approach to reduce colonisation by *L. pneumophila* and has the advantage of a low annual cost of production (0.02 € for 1 litre of NEOW with an active chlorine concentration of 500 mg/L) and the maintenance of a device (approximately 2000 €), which can be remotely controlled for pH and residual chlorine. However, further investigations are needed to ascertain its efficiency over extended time periods since a complete eradication from the water distribution system has not been achieved. Moreover, the results obtained confirmed that PMA-qPCR offers some advantages compared to conventional qPCR also in water samples disinfected with the NEOW device, as it allows to gather information regarding the viability of *Legionella* cells and the presence of VBNC cells, improving the knowledge about the exposure risk to *L. pneumophila*.

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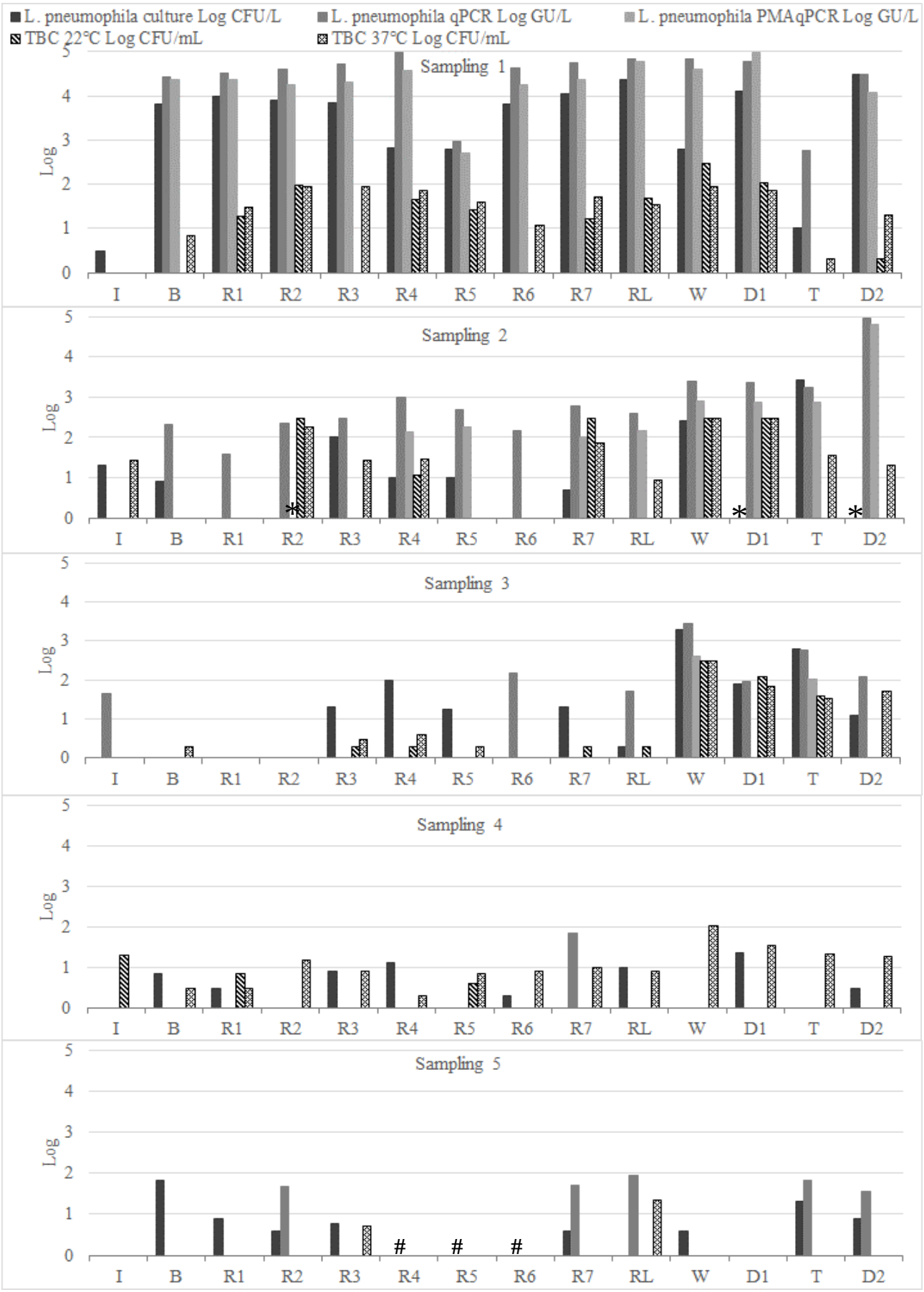
Figure 1. Results of *Legionella pneumophila* contamination (culture, qPCR and PMA-qPCR) and TBC at 22 °C and 37 °C in all sites during the five sampling campaigns.



∗ sample not detected for overgrowth of interfering colonies; # sample not collected

Table 1. Description of sampling sites.

Sampling sites	Water	Samples	Sampling point
Inlet of water system*	cold	Inlet before the water treatment (I)	tap
Boiler	hot	Boiler (B)	tap
Return loop	hot	Return loop (RL)	tap
Distal outlets - Rooms	hot	Room (floor 0) (R1)	tap
		Room (floor 1) (R2)	shower
		Room (floor 2) (R3)	tap
		Room (floor 3) (R4)	shower
		Room (floor 3) (R5)	shower
		Room (floor 4) (R6)	tap
		Room (floor -1) (R7)	tap
Wellness area	hot	Whirlpool (W)	tap
		Dressing room (D1)	shower
		Turkish bath (T)	shower
		Dressing room (D2)	tap

* This sampling site was located outside the building but belonging to mains. In this point water was not treated with chlorine and it was not affected by the NEOW treatment.

Table 2. Percentage of sampling sites contaminated by *L. pneumophila* and the level of contamination in the water distribution system before and after the NEOW device installation.

	Before	After NEOW			
		1 week	1 month	2 month	3 month
L. pneumophila positive, n (%)	13/13* (100%)	7/10 (70%)	9/13 (69%)	8/13 (62%)	8/11 (73%)
Mean CFU/L	9.050	299	219	5	11
$>10^4$ CFU/L, n (%)	5/13 (38%)	0/10 (0%)	0/13 (0%)	0/13 (0%)	0/11 (0%)
$>10^3$ CFU/L, n** (%)	9/13 (69%)	1/10 (10%)	1/13 (7.7%)	0/13 (0%)	0/11 (0%)

* The water sample collected at the inlet of the water system (I) was not considered because it was not affected by the NEOW treatment

* * The number includes also samples with CFU/L $>10^4$

Table 3. Physicochemical parameters (mean ± SD) measured at the inlet of the hotel water system and in the water distribution system before and after the NEOW device installation.

	Temp °C	pH	Conductivity µS/cm	Turbidity NTU	Ammonium mg/L	Free residual chlorine mg/L
Inlet (I)	15.24±2.05	8.10±0.00	157.75±1.5	0.44±0.22	0.01±0.01	< 0.03*
Sampling 1	22.16±2.41	8.10±0.04	157.84±1.28	0.83±0.59	0.02±0.01	< 0.03*
Sampling 2	38.47±5.73	8.06±0.05	286.23±41.63	0.90±0.61	0.02±0.01	0.15±0.04
Sampling 3	37.71±4.78	8.01±0.04	181.85±13.25	0.86±0.42	0.01±0.01	0.13±0.06
Sampling 4	37.44±0.90	8.11±0.09	220.77±10.88	0.60±0.37	0.01±0.01	0.17±0.05
Sampling 5	38.71±1.44	ND	ND	0.67±0.33	0.01±0.01	0.10±0.02

*Before the NEOW device installation, water at the inlet and in the distribution system was not treated with chlorine.
ND: not detected.