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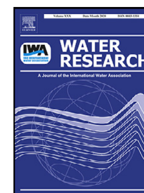

Efficient cooling tower operation at alkaline pH for the control of *Legionella pneumophila* and other pathogenic genera

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ABSTRACT

Efficient control of pathogenic bacteria, specifically *Legionella pneumophila*, is one of the main concerns when operating industrial cooling towers. Common practices to limit proliferation involves use of disinfectants, leading to formation of disinfection by-product and increase in water corrosiveness. A disinfectant-free *Legionella* control method would make the industry more environmentally friendly. A pilot-scale cooling tower (1 m^3/h) operated with demineralized water was used to investigate the potential of high-pH conditioning as a disinfectant-free alternative for control of *L. pneumophila* and other pathogens. One control experiment was performed under standard full-scale operation involving sodium hypochlorite dosage. Thereafter 3 alkaline pHs of the cooling water were tested: 9.0, 9.4 and 9.6. The tests lasted between 25 and 35 days. The cooling water from the basins were analysed for total cell count by flow cytometry, *L. pneumophila* concentration by plate count and occasional qPCR analyses targeting the mip-gene, bacterial and eukaryotic community analyses with 16S and 18S rRNA gene amplicon sequencing, relative abundance of eukaryotic to prokaryotic DNA by qPCR of the 16S and 18S rRNA gene. The *L. pneumophila* analyses showed considerable growth at pH 9.0 and pH 9.4 but was maintained below detection limit ($<$ 100 CFU/L) at pH 9.6 without disinfection. Interestingly, the results correlated with the overall abundance of protozoa in the water samples but not directly with the relative abundance of specific reported protozoan hosts of *Legionella*. The pathogenicity based on 16S rRNA gene amplicon sequencing of the cooling water DNA decreased with increasing pH with a strong decline between pH 9.0 and pH 9.4, from 7.1% to 1.6% of relative abundance of pathogenic genera respectively. A strong shift in microbiome was observed between each tested pH and reproducibility of the experiment at pH 9.6 was confirmed with a duplicate test lasting 80 days. High-pH conditioning $\geq$ 9.6 is therefore considered as an efficient disinfectant-free cooling tower operation for control of pathogenicity, including *L. pneumophila*.

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1. Introduction

Evaporative cooling towers are prone to the growth of *Legionella pneumophila*, a bacterial pathogen which induces a severe form of pneumonia, named Legionellosis, when inhaled (Prussin II et al. 2017; Ricketts et al., 2012). Together with other artificial water systems such as shower plumbing, swimming pools or wastewater treatment plants, they represent important sources of outbreaks due to the formation of aerosols spreading to the surrounding environment (Bartram et al., 2007). Evaporative cooling towers are the most often confirmed sources of Legionellosis outbreaks and can contaminate large numbers of people as their aerosols can spread over several kilometres (Paschke et al., 2019). Number of cases of Legionellosis in the European Union has increased over the last decade, causing at least 450 yearly death (Beauté, 2017), and is expected to worsen due to increasing number of cooling towers and climate change (Paschke et al., 2019).

Current methods to control pathogen growth and ensure safety of people consists in continuous or shock dosage of disinfectants, often oxidative, such as sodium hypochlorite, chlorine dioxide, or bromine (Bartram et al., 2007). Despite the biocides, outbreaks of *L. pneumophila* still occur due to an inappropriate balance in the

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complex chemical conditioning and delayed response caused by sparse and indirect plate count monitoring (Bentham, 2000). In the Netherlands, *L. pneumophila* plate count is the standard monitoring method, with a detection limit of 100 CFU/L. While the Drinking Water Act requires response actions to be taken when the concentration exceeds 100 CFU/L, the Environmental Protection Act that applies to evaporative cooling towers does not specify any threshold (National Academies of Sciences, 2020). The industrial areas can therefore implement their own risk management plan, often using a 10^4 CFU/L threshold according to technical guidelines (ESCMID, 2017).

Growth of *L. pneumophila* in water systems has been linked to presence of protist organisms. Past studies have shown that this pathogenic species performs intracellular replication within the vacuoles of amoebas and ciliated protozoan cells, where it obtains required nutrients for growth, and later evades the hosts via host cell lysis (Lau and Ashbolt, 2009; Wadowsky et al., 1988). Protozoan species such as *Vermamoeba*, *Acanthamoeba* and *Naegleria* have been reported to constitute reservoirs allowing proliferation of *L. pneumophila* in nutrient poor waters, such as process water or drinking water (Wadowsky et al., 1988). In addition, this interaction provides protection of the pathogen towards sudden changes in environmental conditions and chlorine due the ability of the host to switch to a cyst form (Kilvington and Price, 1990).

Past research has been focusing on complementary or alternative methods to disinfection for cooling tower operation by altering system conditions in order to prevent pathogen growth and also prevent formation of disinfection by-products that are discharged in the environment. As *L. pneumophila* grows at temperature between 20 and 45 °C, Kusnetsov et al. (1997) showed that a recirculating water temperature below 20 °C allowed to maintain *L. pneumophila* concentration below 1000 CFU/L. However, operating with a cooling water temperature kept below 20 °C is unlikely in most industrial sites. Alternatively, application of alkaline pH has been suggested in past studies (Brouse et al., 2017; States et al., 1987) and applied on some industrial sites but no thorough assessment of the effect of alkaline pH conditioning on the overall cooling tower microbiome and pathogen presence has been previously described. It has been reported from laboratory studies that *L. pneumophila* can grow in a pH range of 5.5 to 9.2 (Wadowsky et al., 1988) but there is still a lack of literature available above that range. In general, a strong knowledge gap remains on the survival ability of *L. pneumophila* and other pathogens at alkaline pH, i.e. above 9.0.

In this pilot cooling tower study, demineralized water at elevated pH was used as cooling water with the aim of controlling growth of *L. pneumophila* and other potential pathogens, without involving use of disinfectants. More precisely, the goals of this investigation were to (i) identify an optimal pH above which *L. pneumophila* growth is prevented, (ii) characterize the changes in overall microbiome caused by the increase in alkalinity, with focus on potential pathogenic genera, and (iii) discuss advantages and disadvantages of this approach in term of industrial applicability. The use of a pilot-scale facility was preferred to a laboratory-scale setup as it allowed to accurately control operational parameters without excluding the impact of environmental conditions linked to external temperatures, precipitations and air quality, which are crucial factors in the microbiome dynamics.

2. Materials and methods

2.1. Cooling tower installation

2.1.1. Pilot description

One pilot-scale evaporative cooling system was built in a mobile container located in Terneuzen, Netherlands, at the time of the study. Suspended materials were initially removed from the inlet water by a candle filter. The packing fill of the cooling tower was composed of HDPE pall rings. The system was operated with a basin volume of 1 m^3 and a recirculation flow of 1 m^3/h. An automatic valve allowed the refill of the basin. Heating was performed by a secondary water loop warmed up by a water boiler and recirculating through 6 steel-based heat exchangers mounted in series. Cooling efficiency was controlled by automatic adjustment of the blower speed. The water temperatures were maintained at 25 °C in the cold section of the cooling system and simultaneously at 35 °C in the hot section of the same system. When maximal conductivity was reached, a valve triggered the discharge of concentrated cooling water.

2.1.2. Online monitoring

pH (Orbisint CPS11D, Endress+Hauser, Switzerland), conductivity (Condumax CLS21D, Endress+Hauser, Switzerland), temperatures (Thermophant T TTR31, Endress+Hauser, Switzerland) and free chlorine concentration (Analyzer AMI Codes-II CC, Swan, Switzerland) were monitored online.

2.1.3. Conditionings

Conditionings and corresponding physical parameters are described in Table 1. Conditions were tested consecutively. The control condition (C) simulated the conventional full-scale operation of cooling tower, with a conductivity of 3500 µS/cm corresponding to a cycle of concentration of 5 of the feed surface water. Dosage of antiscalant and corrosion inhibitors were applied manually. Sulphuric acid for pH control at 7.5 and sodium hypochlorite for disinfection (Brenntag, Switzerland) were added automatically. The conductivity was maintained via discharge of concentrated cooling water and automatic refill with fresh surface water. In all alkaline conditions (pH 9.0, pH 9.4, pH 9.6(a) and pH 9.6(b)), the high purity of the demineralized feed water resulted in a negligible increase in conductivity. No discharge was thus applied. In the first tested alkaline condition, pH 9.0 was reached naturally and sodium silicate was dosed as corrosion inhibitor. In the other tested conditions, pH was increased by manual addition of sodium hydroxide (Brenntag, Switzerland) to pH 9.4 or sodium hydroxide combined to sodium bicarbonate (Brenntag, Switzerland) to pH 9.6. In pH 9.6(b), 120 g of sodium chloride (Esco, Germany) was added to the basin of 1 m^3 to approach the composition of reverse osmosis permeate. The comparison of pH 9.6(a) and pH 9.6(b) showed negligible impact of sodium chloride on the growth of *L. pneumophila* and overall microbiology. pH 9.6(a) and pH 9.6(b) were therefore considered as duplicate tests in this study. In general, conditions were tested for 25 to 35 days. pH 9.0 was prematurely stopped after 21 days due to the high *L. pneumophila* cell count. pH 9.6(b) was operated for 80 days as a long-term validation experiment.

The cooling system was emptied and a subsequent two-step chemical cleaning was performed between each experiment. The first cleaning step involved the recirculation of demineralized water and sodium hypochlorite at a concentration of 30 ppm active chlorine for one hour, which was then discharged. The second cleaning step involved the recirculation of demineralized water with sulfuric acid dosage to pH 2 for one hour. Chemical solutions were flushed away with fresh demineralized water before the next experiment.

2.2. Experimental analyses

2.2.1. Sampling

Water samples from the cold channel of the recirculation line were collected in sterile glass bottles and kept at 4 °C until analyses. Flow cytometry, plate count and qPCR measurements were performed within 24 h from the sampling time. For *L. pneumophila*

Table 1
Description of the main operational parameters and chemical dosages for each of the tested conditions.

Code	Feed water	Final conductivity	Final pH	Duration (days)	Chemicals	Dosages
C	Surface water	3400–3600	7.5	27	Sodium tolyltriazole 40-60 % Phosphoric acid, dispersant and phosphinosuccinic oligomer Polypropylene glycol 20-30 % Sulphuric acid Sodium hypochlorite	2 mg/L 90 mg/L 2 mg/L to pH 7.5 2 mg-Cl_2/L
pH 9.0	Demineralized water	280 - 300	9.0	21	Sodium silicate	60 mg-Si/L
pH 9.4	Demineralized water	1050 - 1200	9.42	35	Sodium hydroxide Film-forming corrosion inhibitor	280 mg/L 1 g/L
pH 9.6(a)	Demineralized water	2700 - 2800	9.57	34	Sodium hydroxide Sodium bicarbonate Film-forming corrosion inhibitor	200 mg/L 2.8 g/L 1 g/L
pH 9.6(b)	Demineralized water	2450 - 2600	9.59	80	Sodium hydroxide Sodium bicarbonate Film-forming corrosion inhibitor Sodium chloride	200 mg/L 2.8 g/L 1 g/L 0.12 g/L

quantification, Aqualab Zuid laboratory followed the standard NEN-EN-ISO/IEC 17025:2017. For 16S and 18S rRNA sequencing analyses, 400 mL of water was filtered through 0.22 µm pore size and 33 mm diameter sterile PVDF syringe filters (Merck, Germany) and the filter membranes containing the biomass were kept at −20 °C for later DNA extraction. Biological duplicates were analysed at the end of each testing period.

2.2.2. Total cell count

Samples were diluted by a factor 10 (control C) or 100 (pH 9.0, pH 9.4, pH 9.6(a) and pH 9.6(b)) with ultrapure water prior to the flow cytometry measurements. Preparation of the stock solution of SYBR Green I and staining protocol are described elsewhere (Pinel et al., 2020). Analyses were performed on a BD Accuri C6 flow cytometer (BD Biosciences, Belgium) with a flow rate of 66 µL/min on 50 µL of sample. The fluorescence intensity was collected at Fl1 = 533 ± 30 nm and Fl3 > 670 nm with a threshold of 700 on FL1. The applied gating strategy was based on a previous paper (Prest et al., 2013).

2.2.3. *Legionella pneumophila* quantification

L. pneumophila was quantified by both indirect and direct methods with plate count and quantitative polymerase chain reaction (qPCR). Water samples were analysed by Aqualab Zuid laboratory, Werkendam, Netherlands.

2.2.3.1. L. pneumophila plate count. Enumeration of colony-forming units of *Legionella* was based on the protocol described in the standard ISO 11731:2017. In short, 1 mL of the water samples was spread on GVPC (glycine vancomycin polymyxin cycloheximide) agar plates. The plates were subsequently incubated at 36 °C. After 10 days of incubation, the plates were examined for colonies matching the *Legionella* species colonies. *L. pneumophila* colonies were then identified by MALDITOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) analysis, according to the formerly developed method (Dilger et al., 2016) used at Aqualab Zuid. Measurements were performed every 3 days in average.

2.2.3.2. Quantitative PCR. qPCR quantification of the number of gene copies of *L. pneumophila* was performed following the method described in NEN 6254:2012, targeting the mip gene (macrophage infectivity potentiator surface protein). The forward primer LpneuF 5′- CCGATGCCACATCATTAGC −3′, reverse primer LpneuR 5′- CCAATTGAGCGCCACTCATAG −3′ and probe LpneuP 5′-6-carboxyfluorescein [FAM]TGCCTTTAGCCATTGCTTCCG-BHQ1 −3′ were used for the analysis. Taq polymerase was activated at 95 °C for 3 min, and was followed by 43 cycles of denaturation at 95 °C for 20 s and annealing/extension at 60 °C for 60 s.

2.2.4. DNA extraction and sequencing

The membrane filters containing the biomass collected from the water samples were recovered from the syringe filter units (Merck, Germany) for further processing. The genomic DNA was extracted using the DNeasy UltraClean Microbial Kit (Qiagen, Netherlands). The company's standard method was followed during the extraction, with an additional lysis step. The lysis step consisted of 5 min of heating at 65 °C and 5 min of bead-beating on a Mini-Beadbeater-24 (Biospec, USA) for cell disruption of the collected biomass. The quality of the extracted DNA was assessed by gel electrophoresis and size check (465 bp) after qPCR amplification. Samples were sent to Novogene Ltd. (Hongkong, China) for amplicon sequencing of the V4 region (position 528–706) of the 18S-rRNA gene and the V3–4 region (position 341–806) of the 16S-rRNA gene on an Illumina paired-end platform. The raw 18S-rRNA gene sequences were processed by Novogene Ltd. (Hongkong, China) using QIIME software. Chimeric sequences were detected with UCHIME and removed. OTUs were generated based on 97% similarities between the representative sequences using UPARSE. Potential protozoan hosts for *L. pneumophila* were identified based on a previous study (Boamah et al., 2017). The raw 16S-rRNA gene sequences were processed with the software Mothur v.1.40.5 for quality filtering, alignment, chimera check and taxonomic classification. Generation of operational taxonomic units (OTUs) were performed based on 97% similarities between the representative sequences after removal of singletons. The alignment and taxonomic classifications were performed using the SILVA database. Potentially pathogenic bacterial genera were identified based on the list available in the drinking water quality guidelines (WHO 2011). The main representative OTU sequences were compared to the RefSeq NCBI database using the Basic Local Alignment Search Tool (BLAST) for species identification. Results from the duplicate samples are shown in Figures S1, S2 and S3. The DOI of the raw sequencing data is available in supplementary material.

2.2.5. Beta diversity analysis

Diversity analyses were performed on the bacterial OTUs generated after the 16S-rRNA gene amplicon sequencing and their relative abundances in the samples. Beta diversity measurement was assessed with principal component analysis (PCA) in Mothur v.1.40.5 using the thetaYC distance matrix. The spatial separations

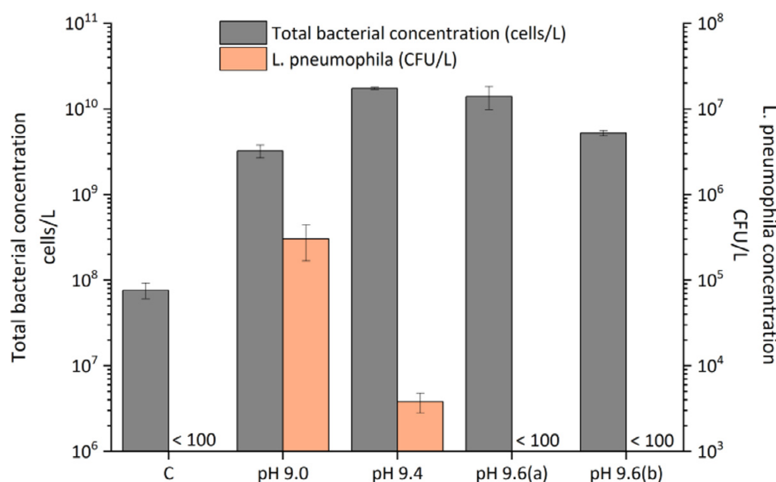

Fig. 1. Bacterial cell counts reached in the cooling waters at the end of the experiments (Table 1) and concentrations of *Legionella pneumophila* in cell forming units over the 5 last days of each experiment. The control experiment C and alkaline conditioning at pH 9.6 had a *L. pneumophila* concentration below detection limit (100 CFU/L).

visualized in the PCA provide information on the similarity between samples.

2.2.6. Abundance of prokaryotes and eukaryotes

qPCR was performed to determine the absolute abundance of prokaryotes and eukaryotes by quantifying the number of copies of the 16S and 18S genes respectively. The 16S DNA standards were commercially obtained. A product template for the 18S DNA was initially created by PCR to be used as standard. The template was based on the DNA extracted from the alkaline test pH 9.6(b). The DreamTaq Green PCR Master Mix (ThermoScientific, USA) was combined to the forward primer Euk1F 5′- ACC-TGG-TTG-ATC–CTG-CCA-GT −3′, reverse primer Euk563R 5′- ACC-AGA-CTT-GCC–CTC–C −3′ and pH 9.6(b) DNA extract according to manufacturer's specifications. PCR was performed with the following settings: 30 cycles of 30 s at 95 °C, 40 s at 55 °C and 40 s at 72 °C. The size of the amplified DNA fragments was assessed by agarose gel electrophoresis. Amplified DNA was purified following the Monarch DNA Gel Extraction Kit (BioLabs, UK). A final DNA concentration of 10.7 ng/L was measured with a Qubit fluorometer (Invitrogen, USA).

Serial dilution from 10^{-3} ng DNA/μL to 10^{-8} ng DNA/μL were used to generate the standard curves. qPCR reaction mix were composed of 0.2 μL of each primer at a concentration of 50 μM, 10 µL of IQTM SYBR® Green Supermix (Bio-Rad, USA), 7.6 μL of nuclease-free water (Sigma-Aldrich, UK) and 2 μL of the extracted DNA from the cooling water samples. The primer sequences, thermal profiles and qPCR efficiencies are described in Table S1.

3. Results

3.1. Total cell counts

Total bacterial cell concentration in the cooling water was measured by flow cytometry throughout the testing periods. Fig. 1 shows the cell number reached at the end of each experiment, averaged over the last 5 days. The control C, subjected to chlorine disinfection, had the lowest bacterial cell number with 6.6 (± 1.5) × 10^7 cells/L. Chlorine disinfection applied in the control experiment was efficient in maintaining a low cell count. The alkaline conditions, without biocide dosage, reached 3.2 (± 0.5) × 10^9 cells/L at pH 9.0, 1.7 (± 0.1) × 10^{10} cells/L at pH 9.4, 1.4 (± 0.4) × 10^{10} cells/L and 5.2 (± 0.4) × 10^9 cells/L at pH 9.6 (a) and (b) respectively.

3.2. Impact of increasing pH on L. pneumophila *growth*

The *L. pneumophila* plate count results for the 5 pilot tests are also shown in Fig. 1. The control test (C) performed under the conventional full-scale operating conditions, at pH 7.5 with sodium hypochlorite dosage, maintained a *L. pneumophila* concentration below the detection limit of 100 CFU/L during the entire 27 days of operation. Tests at pH 9.0 and pH 9.4 exceeded the tolerated limit of 10^3 CFU/L set on the industrial site, with 3.0 (± 1.4) × 10^5 and 3.8 (± 1.0) × 10^3 CFU/L respectively at the end of the experiments. qPCR measurements confirmed the presence of *L. pneumophila* with values reaching up to 4.4 × 10^5 and 950 gene copies/L at pH 9.0 and pH 9.4 respectively. Demineralized water at pH 9.0 seems to constitute a suitable environment for the growth of *L. pneumophila* in cooling towers. The ability of the pathogen to also grow at pH 9.4 was unexpected and therefore indicates that pH 9.4 is not alkaline enough to prevent proliferation. The duplicates at pH 9.6 both maintained *L. pneumophila* at very low or undetectable levels during the duration of the experiments, i.e. 34 and 80 days respectively. pH 9.6(a) reached 200 CFU/L on day 19 and 100 CFU/L on day 21 while pH 9.6(b) reached 100 CFU/L on days 14 and 35. In the other plate count analyses (87%), the concentration was below quantification limit (<100 CFU/L) while all qPCR measurements were below quantification (<375 gene copies/L). The results demonstrate that a pH of or above 9.6 constitutes an efficient conditioning for the control of *L. pneumophila* in open recirculating cooling tower, alternatively to biocide dosage.

3.3. Relative abundance of 18S (eukaryotic) to 16S (prokaryotic) gene copies

The absolute quantification of the 18S and 16S gene copies by qPCR allowed an estimation of the proportion of eukaryotic DNA to prokaryotic DNA in the samples. The ratio of 18S to 16S gene copies is displayed in Fig. 2. All samples contained a substantially lower amount of eukaryotic gene copies compared to prokaryotic gene copies, between 1 log and 3 log fewer. Control C at pH 7.5 and tests at pH 9.4 and pH 9.6 (a) and (b) display a ratio of 18S:16S gene copies of 1.7 (± 0.3) × 10^{-3}, 0.9 (± 0.2) × 10^{-3}, 1.4 (± 0.5) × 10^{-3} and 3.5 (± 2.2) × 10^{-3} respectively. At pH 9.0, however, the ratio strongly diverges from the other samples with 5.7 (± 3.5) × 10^{-2} indicating a relatively higher concentration of eukaryotic DNA per prokaryotic DNA with more than 1 log difference when compared to the other tested conditions. The greater relative fraction of eukaryotic DNA at pH 9.0 may suggest a higher avail-

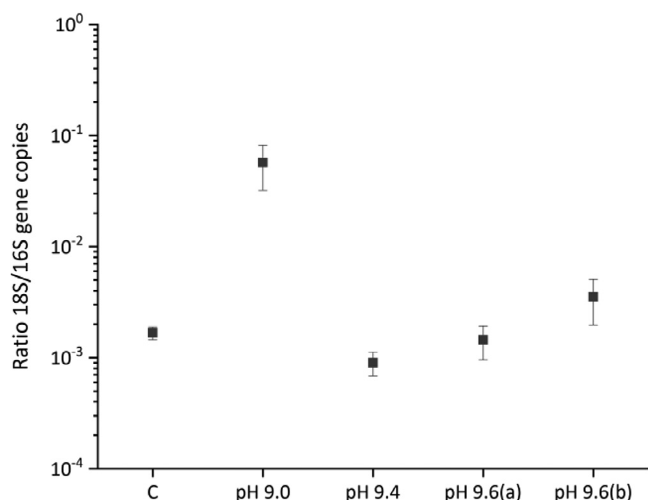

Fig. 2. Ratio of the number of gene copies of 18S to 16S in the cooling water samples illustrating a higher relative abundance of eukaryotic DNA to prokaryotic DNA at pH 9.0 compared to the other tested conditions.

ability of potential hosts for *L. pneumophila* to multiply intracellularly.

3.4. Presence of potential protozoan hosts of L. pneumophila

Identification of the eukaryotic community members was performed with 18S rRNA gene sequencing of the DNA extracted from the cooling water samples to assess the presence of potential hosts. Fig. 3A shows the relative abundances of the main eukaryotic kingdoms. Protista and Chromista kingdoms accounted for the majority of the reads in the control C and at pH 9.0 and pH 9.4 with 43 (± 10)%, 62 (± 5)% and 58 (± 8)% respectively. The duplicate tests at pH 9.6 show only 2 (± 1) and 3 (± 1)% of Protista and Chromista abundances, with dominance of the kingdom Fungi. The combination of the results from Figs. 2 and 3A therefore suggests a limited availability of hosts for *L. pneumophila* at pH 9.6 due to the low occurrence of protists. The composition and structure of the protist communities diverged strongly (Fig. 3B) between the tested conditions. The test at pH 9.0, with the highest *L. pneumophila* concentration and eukaryotic to prokaryotic DNA ratio, was largely dominated by the genera *Spumella* from the phylum Ochrophyta and *Vorticella* from the phylum Ciliophora. These genera were also identified in the other tested conditions in lower abundances. The duplicate tests at pH 9.6 show high similarity with each other regarding the protist community structures, dominated by *Spumella*, *Phytophthora* and *Halophytophthora*. *Halophytophthora* is a reclassified genus previously assigned to *Phytophthora*, but regrouping marine species (Cooke et al., 2000). As the only difference in the operation of the duplicate tests was the dosage of NaCl, the higher salt concentration in pH 9.6(b) likely justifies the increase in relative abundance of *Halophytophthora*.

The protist organisms present in the water samples were then compared to experimentally defined hosts and potential hosts of *L. pneumophila* (Boamah et al., 2017). Their presence among the protist community of each test can be visualized in Table 2. The control condition showed a negligible abundance of known hosts representing only 0.1% of the total eukaryotic reads. A similarly low number was obtained at pH 9.0 with 0.2% despite the high concentration of *L. pneumophila* measured, suggesting the presence of non-identified *Legionella* hosts. The two commonly found *L. pneumophila* hosts *Echinamoeba exudans* and *Vermamoeba vermiformis* added up to 59% of the protist community and 33% of the whole eukaryotic community at pH 9.4. They also constituted an important fraction of the protists at pH 9.6, 5% and 6% respectively, but representing only 0.1% and 0.2% of the eukaryotic communities due to the low abundance of protists at this pH.

3.5. Presence of potentially pathogenic bacteria under different alkaline pH

The abundance of potentially pathogenic bacterial genera, based on the drinking water quality guideline (WHO, 2011), are listed in Fig. 4. The control C at pH 7.5 and test at pH 9.0 show a greatly

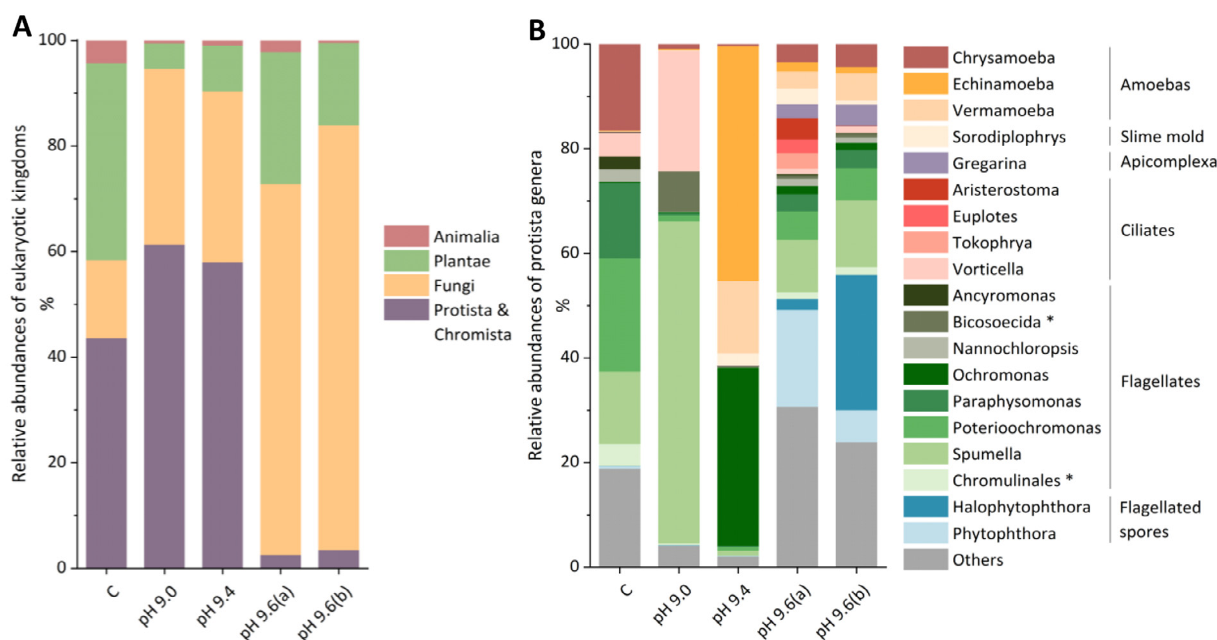

Fig. 3. Relative abundances of eukaryotic kingdoms (A) and composition of the kingdoms Protista and Chromista at the genus taxonomic level (B) in the cooling water samples analysed with 18S rRNA gene amplicon sequencing. The category "Others" accounts for the operational taxonomic units of relative abundance lower than 2%. The symbol * indicates the order taxonomic level when the genus was not identified. Duplicate data are shown in supplementary material (Figs. S1 and S2).

Table 2
Relative abundances of potential protozoan hosts in the water samples based on the 18S rRNA gene amplicon sequencing results.

Potential hosts (% of protist reads)	C	pH 9.0	pH 9.4	pH 9.6(a)	pH 9.6(b)
Acanthamoeba_unidentified *	0.01%	–	–	–	–
Echinamoeba exudans *	0.14%	0.23%	44.93%	1.73%	1.20%
Vermamoeba vermiformis *	0.15%	0.17%	13.81%	3.29%	5.20%
Aspidisca_unidentified	0.02%	–	–	0.02%	0.02%
Colpoda_unidentified	–	0.01%	0.02%	0.15%	0.26%
Euglypha rotunda	–	0.01%	0.01%	0.04%	0.01%
% of total protist reads	0.3%	0.4%	58.8%	5.2%	6.7%
% of total eukaryotic reads	0.1%	0.2%	32.8%	0.1%	0.2%

*Experimentally defined host.

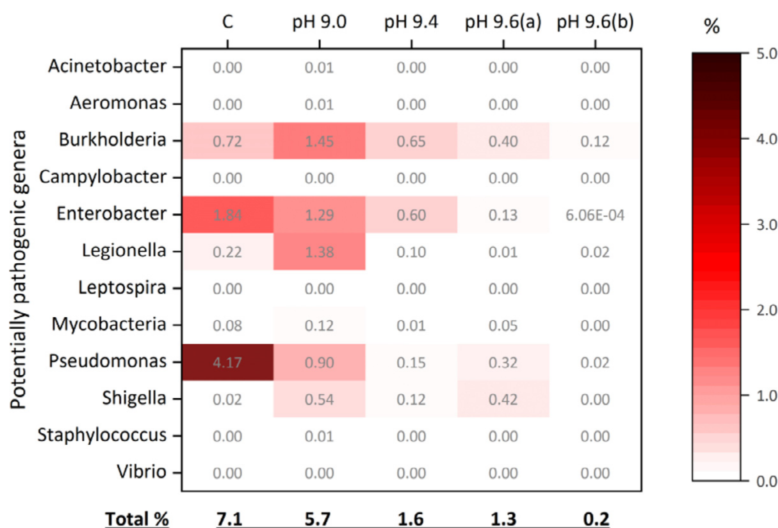

Fig. 4. Heatmap of the potentially pathogenic genera obtained from 16S rRNA gene amplicon sequencing of the cooling water DNA samples. The concentration is expressed in% of the bacterial relative abundance.

higher total relative abundance of potential pathogens (7.1 ± 0.9% and 5.7 ± 0.3% of the total reads) compared to the tests at pH 9.4 and pH 9.6 (1.6 ± 0.1%, 1.3 ± 0.1% and 0.2 ± 0.1%). *Pseudomonas* and *Enterobacter* are the most abundant in the control test, representing 4.2 ± 0.1% and 1.8 ± 0.3% respectively, while *Bulkhoderia* and *Legionella* are dominant at pH 9.0, with 1.5 ± 0.6% and 1.4 ± 0.1%, with also considerable abundance of *Enterobacter* (1.3 ± 0.8%). The increase from pH 9.0 to pH 9.4 therefore considerably reduced the potential pathogenicity of the cooling water based on the results from the 16S rRNA gene amplicon sequencing.

3.6. Overall bacterial community analyses

Fig. 5 shows the relative abundance of the main bacterial genera in the cooling water samples. Variations in microbial community structure can be observed between the different tests with a switch in the main bacterial members. At the phylum level, the main difference originated from the promotion of Bacteroidetes at alkaline pH. While it accounted for 3 ± 1% of the relative abundance in control C, it reached 38 ± 6% and 62 ± 1% at pH 9.6. The remaining abundance was mainly represented by Alpha and Gammaproteobacteria. At the genus level, *Sphingobium* was dominating in the control C at 44 ± 8% but was present in negligible abundance at alkaline pH with less than 0.5%. pH 9.0 and pH 9.4 show a more distributed relative abundance among genera, with the main members being *Sediminibacterium* at pH 9.0 (22 ± 4%) and *Sphingopyxis* and *Flavobacterium* at pH 9.4 (28 ± 6% and 16 ± 6% respectively). The duplicates at pH 9.6, however, display similarity in main community members. The genus *Mongoliitalea* from the family *Cyclobacteriaceae* dominated both cooling waters with 35 ± 5% and 60 ± 1% although (a) contained also large abundances of *Phenylobacterium* (21 ± 6%) and *Aliihoeflea* (13 ± 3%) genera. The blast of the OTU representative sequence corresponding to the *Mongoliitalea* genus resulted in 99.56% similarity in identity with the species *Mongoliitalea lutea*, a facultative alkaliphilic and halotolerant bacterial species collected from haloalkaline lakes. The other 26 main OTUs whose genera are displayed in Fig. 5 were also compared to the database. Among those, the representative sequences of the members labelled with the symbol * showed over 99% similarity with sequences of known alkaliphilic or alkalitolerant species i.e. with optimum pH $\geq$ 9.0 or optimum pH $<$ 9.0 but able to grow at higher values. The sum of the relative abundances of these OTUs was calculated for each experimental test. While confirmed alkalitolerant organisms accounted for only 9% of the relative abundance of the main members in control C, their abundance increased with increasing pH: 28% at pH 9.0, 56% at pH 9.4, 69% and 78% at pH 9.6 for (a) and (b) respectively. As expected, the higher fraction of identified alkaliphilic bacteria at pH 9.4 and 9.6 implies that neutrophilic bacteria were outcompeted.

The principal component analysis in Fig. 6 represents the similarities between samples taking into account the members of the communities and their relative abundances. The bacterial communities at alkaline pH all diverge from the control C at pH 7.5. Among the tested conditions, samples from pH 9.6(a) and pH 9.6(b) form a close cluster on the chart validating the reproducibility of the result for the conditioning at pH 9.6.

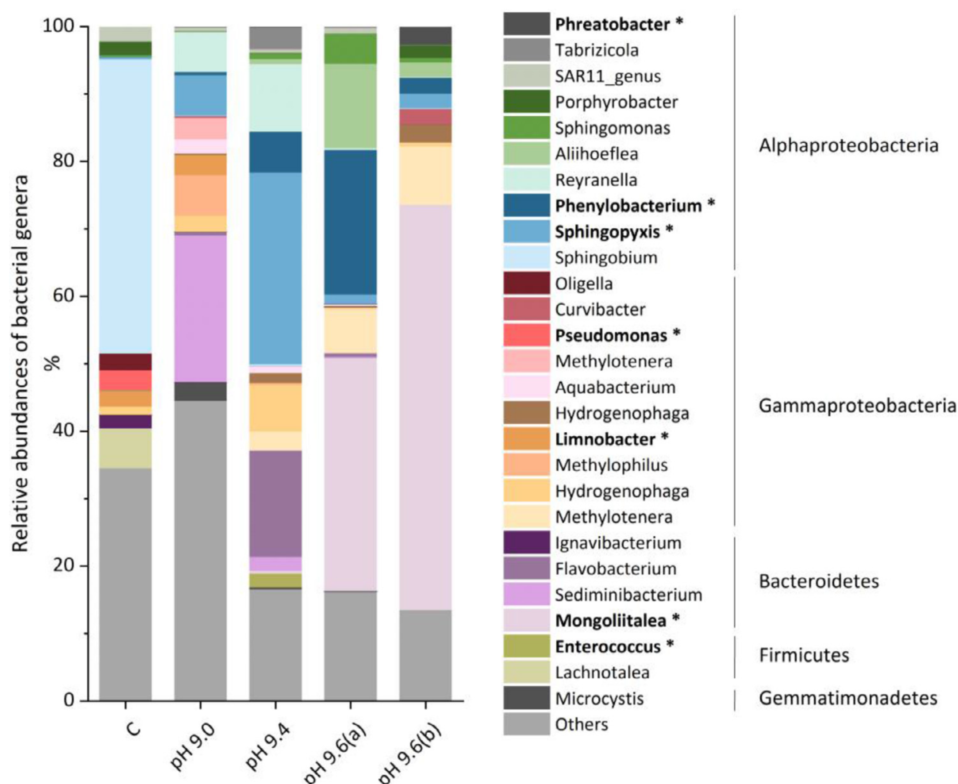

Fig. 5. Composition of the bacterial communities at the genus taxonomic level obtained from 16S rRNA amplicon sequencing of the cooling water DNA samples. The category "Others" accounts for the operational taxonomic units of relative abundance lower than 2%. The symbol * indicates alkalitolerant and alkaliphilic microorganisms i.e. whose representative sequence corresponds - with more than 99% similarity in identity - to a species able to grow at pH above 9. Duplicate data are shown in supplementary material (Fig. S3).

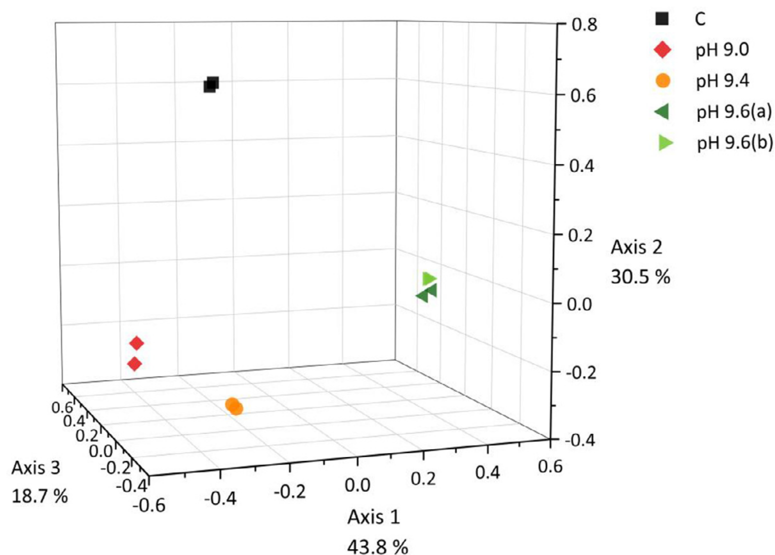

Fig. 6. Principal component analysis (PCA) of bacterial community structures of the cooling water samples based on operational taxonomic units. The bigger the distance between data points, the stronger the dissimilarity in community structures. Duplicate samples are shown for each condition.

4. Discussion

4.1. Cooling tower operation at pH ≥ 9.6 efficiently prevents growth of L. pneumophila

Previous research based on a survey of 40 cooling towers operated in a pH ranging from 6.9 to 9.1 reported higher *L. pneumophila* densities at pH between 8.4 and 9.1 (Yamamoto et al., 1992). An earlier laboratory-controlled pH batch experiment on cooling water samples showed, however, a better correlation of the pathogen growth with neutral pH values (States et al., 1987). These disparities in results point out the significant impact of operational conditions, water sources and surrounding environment on the growth of *L. pneumophila* in full-scale cooling systems. According to the available literature, *L. pneumophila* has been shown to grow at pH up to 9.2 but was not able to multiply from pH 10

(Brouse et al., 2017; Ohno et al., 2003; Wadowsky et al., 1988). An important lack of information remained about the survival of the pathogen within the pH range from 9.2 to 10. Our study explored this gap and revealed that *L. pneumophila* can survive and grow at pH 9.4 although at a much lower concentration than at pH 9.0. Most importantly, *L. pneumophila* was unable to grow at pH 9.6.

Multiplication of *Legionella* in engineered water systems requires presence of host protozoan cells (Lau and Ashbolt 2009; Wadowsky et al., 1988). The amount and nature of nutrients in cooling water is generally too limited for *L. pneumophila* multiplication as a free-living organism since it requires a complex medium to grow, including compounds like amino acids as carbon and energy source (Sauer et al., 2005; Tesh and Miller, 1981; Tesh et al., 1983) or fatty acids (Fonseca and Swanson, 2014). The intracellular components of some amoebas such as *Acanthamoeba*, *Vermamoeba* and *Naegleria* sp. (Jjemba et al., 2015; Lau and Ashbolt, 2009; Molofsky and Swanson, 2004) and ciliated protozoa (Caicedo et al., 2018; Fields et al., 1984) therefore constitute an appropriate reservoir for the growth of the pathogen. When encountering extreme conditions such as chlorine disinfection, protozoa go through a mechanism of encystment to protect themselves from the hostile environment (Kilvington and Price, 1990). Contrarily to their vegetative trophozoite form, cysts of protozoa are in a dormant state and do not proliferate but their reinforced cell wall constitutes a strong protection for the intracellular pathogens.

Our study reflects a positive correlation between the abundance in protozoa (Figs. 2 and 3A) and *L. pneumophila* counts (Fig. 1), as was also reported in the cooling tower survey by Yamamoto et al. (1992). The dominance of protozoa at pH 9.0 and 9.4 and the higher eukaryotic to prokaryotic DNA ratio at pH 9.0 suggest the presence of unicellular trophozoite reservoirs to sustain the observed *L. pneumophila* growth in these two tested conditions. In the duplicate test runs at pH 9.6, a poor abundance of protists was observed concomitant with a good control of *L. pneumophila* growth. The relative abundance of potential hosts (Fig. 3b) in our samples - based on a suggested list derived from laboratory experiments and environmental analyses (Boamah et al., 2017) – did, however, not show a clear correlation. At pH 9.0, the *L. pneumophila* counts were substantial but previously reported hosts were present only in minor abundance contrarily to pH 9.4 (Table 2). *Spumella* (flagellate) and *Vorticella* (ciliate), the main genera at pH 9.0, have not been reported as hosts but the limited knowledge available on the specificity of *L. pneumophila* for intracellular replication (Guerrieri et al., 2005) as well as the non-exhaustive list of cultivated natural hosts cannot exclude their involvement. In the experiments at pH 9.6, the fraction of the protist abundance of reported hosts (5.2 and 6.6%) represented a negligible fraction of the total abundance of eukaryotes. In fact, the eukaryotic communities were mainly represented by fungi. Our study revealed a substantial impact of the pH increase from 9.4 to 9.6 on the limitation of protists and a direct correlation with the inhibition of *L. pneumophila* growth.

4.2. Increased pH leads to a strong bacterial community shift with lower pathogenicity

A strong shift in bacterial community was observed between the control experiment at pH 7.5 with chlorine disinfection and each of the tested alkaline pH conditions with no disinfection. Proteobacteria dominated the control C with *Sphingobium* sp., a recurrent organism in drinking water and cooling systems subjected to chlorination due to their oligotrophic nature and ability to grow in biofilm mode (Shaw et al., 2015; Vaz-Moreira et al., 2011; Paranjape et al., 2020). *Pseudomonas* is also a common genus encountered in cooling systems under disinfection (Paranjape et al., 2020). Bacterial community composition and relatively low total cell count in the control experiment therefore reproduced the environment of a full-scale system under standard operation. Regarding alkaline conditions, the abundance of Bacteroidetes was previously shown to positively correlate with increasing pH (Ganzert et al., 2014; Lauber et al., 2009) as it is the case in our study. *Mongoliitalea Lutea* (*Cyclobacteriaceae*, Bacteroidete) is a facultative alkaliphile able to develop at pH between 6.5 and 12.0 with an optimum at pH 9.0 (Yang et al., 2012). However, it was in our study only present in negligible abundance at pH 9.4 while it was the dominant bacterium at pH 9.6. Our data suggests that the pH increase from 7.5 (C) to 9.6 gradually limited the growth of neutrophiles and promoted alkalitolerant and alkaliphilic species such as *Mongoliitalea Lutea*. Driving pH away from neutral value requires bacteria to adjust their proton motive force to maintain a cytoplasmic pH in the neutral range (Krulwich et al., 2011). In too alkaline conditions, the proton concentration in the surrounding water is not sufficient and causes the cell to decay while alkaliphile organisms rely on alternative membrane processes such as additional antiporters and coupled membrane transporters using sodium motive force (Padan et al., 2005). Most pathogenic bacteria show optimum growth at physiological pH which allows them to infect and proliferate within the body (Kim and Ndegwa, 2018) but will decay when facing extreme conditions. The decrease in relative abundance of potentially pathogenic bacteria that we observed correlated with this assumption, with a strong decline from 7.1% to 1.6% between pH 7.5 and pH 9.4. Limitations remain regarding the use of amplicon sequencing for the assessment of pathogenicity, related to the limited resolution of the technique, the varying gene copy number per organism, and the lack of data available on pathogenic microorganisms. To accurately assess the levels of pathogens, alternative methods should be applied such as qPCR analyses of the known species which will provide absolute concentrations for the comparison of tested conditions. Yet, the observed loss of overall potential pathogenicity of the water at elevated pH, based on abundance of genera, suggests an extra advantage of alkaline cooling tower conditioning.

4.3. Implications for industry

In the presented study, the pilot cooling tower was operated at different alkaline pHs and demineralized water was selected as feed water. The suggested conditioning does not require use of disinfectant and maintains a simpler chemistry compared to standard practice, with the use of less chemicals while controlling the pathogenicity of the water. It also anticipates a potential increase in regulations which could be expected in the coming years regarding the discharge of disinfection by-products to the environment.

The use of demineralized water has several advantages. The very low content in minerals prevents scaling formation on the surface of the cooling unit thus no dosage of antiscalant – often contributing to a higher nutrient content of the water - is required. The limited concentration in organic compounds considerably restricts the formation of biofilm and therefore the proliferation of protozoa and associated bacteria. Last but not least, the high water quality allows to operate the cooling tower with almost zero discharge, thus maintaining a stable cooling water chemistry which ensue limited dosage of base (NaOH) for alkaline pH control. However, important parts of the process i.e. pumps, spray nozzles and heat exchangers will be prone to corrosion due to the nature of the water. Corrosion is a major drawback shortening the lifetime of the exchangers, leading to system deficiencies and providing space for microbial communities to grow. This phenomenon needs to addressed with alternative material choices or addition of inhibitors effective at high pH and not contributing to the nutrient and mineral content of the water.

For cooling towers already operating with other water sources than demineralized water such as surface water or ground water, the choice for demineralized water is not always possible. Economic feasibility needs to be assessed for each specific case, considering that demineralized water can cost between 0.5 and 1 euros higher per m^3 than surface water. If the original water source is kept, removal of divalent ions (calcium, magnesium) from the feed water is necessary to lower the hardness and switch to alkaline pH without a substantial increase in scaling potential (Hensley, 1985; Matson and Harris, 1979). Additionally, their removal might minimize biofilm formation due to the essential role Ca^{2+} and Mg^{2+} play in biofilm integrity e.g. with cross-linkage (Das et al., 2014; Guvensen and Demir, 2012). Corrosiveness is also a major challenge with softened water, especially due to the high chloride concentration, and needs to be addressed to avoid deterioration of the heat exchangers. Finally, attention should to be paid to the pre-existence of biofilm in the system and a deep cleaning would be required to avoid contamination when converting to alkaline conditioning.

4.4. Recommendations for further research

Past studies suggested that the infection of protozoa by *Legionella* mainly occurs in biofilms (Abdel-Nour et al., 2013; Murga et al., 2001) which provides a more stable environment to multiply e.g. on the wall of the cooling tower basins or packing fill surface. Although a biofilm is not expected to have formed in significant amount during the experimental periods and with the high water quality used, this phenomena can contribute to *L. pneumophila* proliferation. Further research investigating biofilm development and composition in terms of bacterial and eukaryotic communities at alkaline pH would help understand the biological interactions, the biofouling potential and potential for *Legionella* to thrive inside an alkaline biofilm.

Overall, the operation of cooling towers at pH equal or above 9.6 without disinfectant dosage represents an attractive alternative to the conventional practices. The comparison of these results to the microbiome of full-scale cooling towers operating at high pH on the long-term is important to validate the industrial applicability.

5. Conclusions

The investigation of alkaline conditioning in a pilot-scale cooling tower (1 m^3/h) - operated with demineralized water as feed water and without addition of disinfectant - for the control of *Legionella pneumophila* and other potentially pathogenic genera showed that:

- Growth of *L. pneumophila* is prevented in cooling towers maintaining a pH $\geq$ 9.6 without biocide dosage. *L. pneumophila* is, however, still able to grow at pH up to 9.4.
- The overall suspected pathogenicity of the cooling water microbial community declines with increasing alkalinity. Divergence of the alkaline operating pH from physiological pH and dominance of the bacterial community by alkaliphilic microorganisms suggests reduced health risks to the operators and surrounding population, when in contact with aerosols.
- Operating at pH $\geq$ 9.6 is a more sustainable and simpler cooling tower conditioning than dosing disinfection chemicals. It anticipates potential future regulations, further restricting discharge of disinfection by-products. Nonetheless, this alternative pathogen control method requires attention in regard to scaling and corrosion potential and choice of materials.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2021.117047.

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