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Bacterial community dynamics and disinfection impact in cooling water systems

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ABSTRACT

Understanding the bacterial dynamics in cooling towers is imperative for the assessment of disinfection efficiency and management of microbial risks linked to aerosol formation. The objective of this study was to evaluate the impact of feed water on the cooling water bacterial microbiome and investigate the survival ability of its members when exposed to continuous chlorine disinfection. Water from an industrial cooling water system (2600 m^3/h) was collected over a 5-month period at 3 locations along the feed water line and 3 locations in the cooling tower. ATP measurements suggested that the average ATP-per-cell in the cooling tower evolved independently from the average ATP-per-cell in the feed water. Flow cytometry and 16S rRNA gene amplicon sequencing were then combined to quantify the bacterial dynamics in the whole system. A mass balance based equation was established to determine net growth and net decay of the cooling tower bacterial communities in order to evaluate the impact of continuous chlorination (0.35–0.41 mg Cl_2/L residual chlorine). The results indicated that cooling tower main community members were determined by the input feed water microbiome and the bacterial community structure was further shaped by varying decay rates of the microorganisms. Notably, the order *Obscuribacterales* showed to be growing in the cooling tower in the presence of residual chlorine up to 0.4 mg Cl_2/L, with a recurrent net growth of 260 ± 95%, taking into account the impact of the concentration factor. This conclusion was only possible thanks to the systematic analysis described in this paper and generates discussion about the resistance of *Obscuribacterales* to residual chlorine. The described mass balance approach provides a high level of understanding on bacterial dynamics and should be considered for future characterization studies of cooling towers in which accurate investigation of microbiome changes is essential.

1. Introduction

The use of evaporative cooling towers is crucial to cool down full-scale industrial processes. In the Netherlands alone, the number of evaporative cooling towers is estimated to be around 4000 (RIVM, 2018). Cooling water constitutes the highest water withdrawal in several European countries and represent a major source for Legionnaires' disease (European Commission, 2018; Llewellyn et al., 2017).

In order to avoid the proliferation of harmful species such as *Legionella pneumophila* and to reduce the risk of biofilm formation and microbial corrosion in the system, the daily operation of cooling towers usually includes continuous or shock dosage of biocide. The most common biocide, chlorine, is often added as sodium hypochlorite (NaOCl) or chlorine dioxide (ClO_2).

Despite continuous chlorine dosage, prior research has shown that active bacteria can still be present in cooling tower systems (Chien et al., 2013; Helmi et al., 2018) and outbreaks keep occurring (ECDC, 2019), bringing into question the efficacy of this method in managing risks linked to *Legionella*. Chlorine efficiency can decline by a low-quality feed water or side-reaction with organic compounds concentrating in the cooling tower (Hua et al., 2015). Maintenance and investigation of an appropriate chemical

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combination for scaling and corrosion inhibition are crucial. A few case studies have reported the identification of bacterial communities and microbial interactions in chlorinated cooling towers (Di Gregorio et al., 2017; Paranjape et al., 2019; Pereira et al., 2017; Tsao et al., 2019). Feed water has been considered as a major factor affecting cooling water microbial communities based on results from 18 cooling towers from 6 geographic locations and using local water sources (Paranjape et al., 2019). Another paper pointed out the recurrence of biofilm-forming taxa in cooling tower basins (Tsao et al., 2019). However, most studies simply report the observed communities without quantifying the contribution of the feed water microbiome or evaluating the impact of biocides on decay or resistance of microorganisms in the cooling water.

The complexity of evaporative cooling systems makes the study of their microbiome at laboratory-scale and the collection of representative results challenging (Chien et al., 2013). Therefore, the available knowledge on the microbial dynamics in cooling towers is still limited. Investigating the behaviour of the bacterial populations in long-term operated full-scale systems is a necessary step in identifying the factors shaping the community compositions and understanding how biocidal treatments impact the cooling tower microbial communities.

In this study we combined flow cytometry, adenosine triphosphate (ATP) and 16S rRNA gene amplicon sequencing analyses of water samples from an industrial full-scale cooling water system (2600 m^3/h). Data collected from the feed line (3 locations) and from the cooling tower (3 locations) over a 5-month period was used to: (i) characterize the microbiome along the system, (ii) evaluate the impact of feed water on cooling water bacterial dynamics, (iii) detect bacteria potentially resistant to continuous chlorine dosage. The approach provides qualitative and quantitative information on the temporal and spatial bacterial changes in the cooling water system and on the disinfection efficiency by chlorine dosage.

2. Materials and methods

2.1. Full-scale system

The studied system is located in Zeeland, the south-west region of the Netherlands. As shown in Fig. 1, it consists in two main parts: the feed water line and the open recirculating cooling tower. The pre-treatment of the feed water is performed at the Braakman water treatment plant of Evides Industriewater before being distributed to the induced draft cooling tower situated on the site of an industrial gases company. The freshwater originates from two main sources, Belgian polder water and surface water from the Biesbosch. Pre-treatment involves the dosage of an iron-based coagulant, then use of bandfilter, microstrainer, rapid sand filter for removal of suspended solids and dosage of sodium hypochlorite as a biocide. The pipe distance of the distribution line connecting both sites is approximately 8 km. The cooling tower has a capacity of 25 MW and is maintained at a concentration factor (i.e. ratio between basin and feed water conductivities) of around 5 based on online conductivity measurements. When the conductivity in the basin exceeds 3500 μS/cm, the discharge valve opens and part of the concentrated water is replaced by new feed water. The system recirculates on average 2600 m^3/h of cooling water and operates at a temperature difference of 6–8 °C between the inlet and outlet of the heat exchanger line, location points D and E respectively (Fig. 1). The chemical dosages include mild steel and copper corrosion inhibitors (containing respectively phosphoric acid and sodium tolyltriazole), $CaCO_3$ scale inhibitor (phosphinosuccinic oligomer) and dispersant (high stress polymer). Online monitoring of the water parameters is achieved by sensors located in the cooling tower and allows adjustment of the dosages by an automatic controller. pH is stabilised at 7.5 with sulphuric acid and oxidation-reduction potential (ORP) is maintained between 550 and 600 mV with sodium hypochlorite dosage. More precisely, sodium hypochlorite was dosed at two locations in the entire studied system. The first location was before distribution of the feed water to the cooling tower, before sampling point B (Fig. 1). The second location was in the basin of the cooling tower, point D. The full-scale cooling tower was continuously operated under stable conditions since the last cleaning, approximately 2 years before the sampling campaign was executed. No biofilm growth was visually observed in the cooling tower basin and packing material.

2.2. Water sampling

Water samples from 3 locations along the feed water line and 3 locations in the cooling tower were studied by monthly analyses over a period of 5 months. The labels A to F indicate the sampling points (Fig. 1): pre-treatment inlet (A), pre-treatment outlet (B),

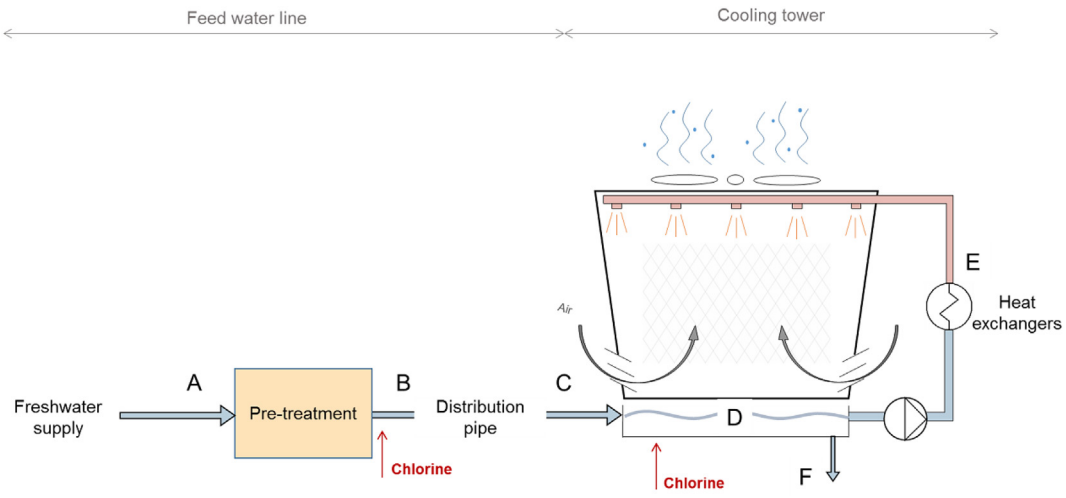

Fig. 1. Simplified scheme of the cooling water system. Capital letters indicate the sampling locations: pre-treatment inlet (A), pre-treatment outlet (B), cooling tower feed (C), cooling tower basin (D), cooling tower heat-exchanger outlet (E) and cooling tower discharge (F). Cooling tower cold and warm waters are represented by blue and red backgrounds. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cooling tower feed (C), cooling tower basin (D), cooling tower heat-exchanger outlet (E) and cooling tower discharge (F). The samples were collected in 1 L sterile glass bottles containing a solution of 10 mM (1% v/v) sodium thiosulfate for ATP, flow cytometry and microbial community analysis to neutralize residual chlorine before transportation. ATP and flow cytometry measurements for total and intact bacterial cell numbers were performed within 4 h after sampling. 400 mL of water was filtered through 0.22 μm pore size and 33 mm diameter sterile PVDF syringe filter (Merck, Germany) and the filter membrane was frozen at −20 °C for later DNA extraction. Samples for trace metal analyses were collected in 15 mL metal-free centrifuge tubes (VWR, USA), filtered through 0.45 μm pore size and 33 mm diameter sterile PVDF syringe filters (Merck, Germany), diluted 10 times with ultrapure water and treated with nitric acid (1% v/v) prior to the ICP-MS analyses. All samples were kept on ice between sampling and processing.

2.3. Water quality analyses

The following water quality parameters were measured (Table 1): pH (C6010, Consort, Belgium), conductivity (C3010, Consort, Belgium), temperature (PT100 temperature sensor, Testo BV, Nederland) and residual chlorine (Controller Dulcometer, Prominent, Germany and Lovibond PC22/GB, Tintometer, Germany) at the dates of the sampling. Total organic carbon (TOC) was analysed after filtration through 0.45 μm pore size sterile PVDF syringe filters (TOC analyser model TOC-L CSH, Shimadzu Corporation, Japan). The range of ammonia concentration was reported by the feed water supplier. Nitrate, sulphate and phosphate concentrations (Table S1) were measured by spectrophotometry (DR 3900, Hach, USA).

2.4. Experimental analyses

2.4.1. ATP measurements

ATP measurements were carried out to quantify the intracellular and extracellular ATP concentration in the water samples. The intracellular ATP measurements were performed according to the supplier's instructions. 20 mL of water sample were filtered through a 0.22 μm sterile nylon syringe filter. 5 mL of lysis reagent were processed through the filter. 100 μL of the lysis solution containing ATP was mixed to 100 μL of reconstituted Water-Glo reagent (Water-Glo Substrate mixed with Water-Glo Reconstitution Buffer). After 40 s waiting time, light intensity was measured with 2 s integration time by a Glomax 20/20 luminometer (Promega Benelux BV, Netherlands). A provided ATP standard in lysis buffer (1000 pg ATP/mL) was used to convert light units into ATP concentration. For extracellular ATP measurements, 100 μL of the filtered water sample was mixed to 100 μL of reconstituted Water-Glo reagent and analysed identically as intracellular ATP. A calibration curve was initially obtained from successive dilutions of ATP standard in ultrapure water to convert light units into ATP concentration. All samples were analysed in duplicate.

2.4.2. Flow cytometry measurements

All collected samples were diluted by a factor 10 in ultrapure water prior to the flow cytometry analyses. The staining protocol and flow cytometry analyses were based on previous papers (Hammes et al., 2008; Prest et al., 2013). Two staining solutions were prepared from stock solutions of SYBR Green I (10000 x conc., Invitrogen, USA) and propidium iodide (30 mM in DMSO, Invitrogen, USA). A working solution of 10 μL SYBR Green I in 1 mL of 10 mM Tris buffer pH 8 (solution SG) was used to stain the bacterial cells. For the intact cells, 10 μL of SYBR Green I stock solution was mixed with 20 μL of propidium iodide stock solution in 1 mL of 10 mM Tris buffer pH 8 (solution SGPI). 5 μL of SG for total cell count (TCC), or SGPI for intact cell count (ICC) was added to 495 μL sample. The final concentration of the stains in the samples were 1.96 μM for SYBR Green I and 6 μM for propidium iodide. After stain addition, the samples were mixed for 3 s, incubated in the dark at 37 °C for 10 min and mixed again. The measurements were performed with a BD Accuri C6 flow cytometer (BD Biosciences, Belgium) equipped with a 50 mW laser emitting at a fixed wavelength of 488 nm. Fluorescence intensity was collected at Fl1 = 533 ± 30 nm, Fl3 > 670 nm. Analyses were performed at a flow rate of 66 mL/min on 50 μL of sample volume with a threshold value of 700 on FL1. Gating strategy and data collection were performed as previously described (Prest et al., 2013) with a BD Accuri C6 software.

2.4.3. DNA extraction and 16S rRNA gene amplicon sequencing

After filtration of 400 mL of sample, the 0.22 μm pore size and 33 mm diameter membrane filters were recovered from the syringe filter units (Merck, Germany) and were preserved at −20 °C until processing. The genomic DNA was extracted using the DNeasy UltraClean Microbial Kit (Qiagen, Netherlands). Extraction was done following company's standard protocol with addition of an alternative lysis step. This included a combination of 5 min of heat (65 °C) followed by 5 min of bead-beating on the filters for cell disruption on a Mini-Beadbeater-24 (Biospec, USA). The quality of the extracted DNA was evaluated by (i) performing gel electrophoresis to control that the DNA was not degraded and that the molecular weight was superior to 1 kb, and (ii) checking that the target size of 465 bp was obtained after qPCR amplification. Samples were sent to Novogene Ltd. (Hongkong, China) for amplicon sequencing of the V3-4 region of the 16S-rRNA gene (position 341–806) on an Illumina paired-end platform. The raw sequences were quality filtered, checked for chimera and operational taxonomic units (OTUs) were generated based on 97% similarities using the software Mothur. The alignment and taxonomic classifications were performed using the SILVA database.

The abundances of potentially pathogenic genera, obtained from the drinking water quality guidelines (WHO, 2011), have been calculated and added in supplementary materials (Table S3).

2.4.4. Alpha and beta diversity analyses

Diversity analyses were performed on the OTUs generated after

Table 1
Main water parameters along the cooling water system (Fig. 1) **over the 5-month period.** Square brackets [ ] indicate the range of values containing the data from all months. Abbreviation n.d. indicates that the value was not determined at this specific location.

Parameter	A	B	C	D	E	F
pH	[7.8–8.2]	[7.9–8.4]	[7.7–8.4]	[7.6–7.8]	[7.5–7.7]	[7.5–7.8]
Conductivity (uS/cm)	[600–750]	[600–750]	[600–750]	[3000–3500]	[3000–3500]	[3000–3500]
Temperature (°C)	[5.2–20.2]	n.d.	n.d.	[15–20]	[20–25]	[15–20]
Residual chlorine (mg/L)	n.d.	[0.32–0.42]	[0.02–0.20]	[0.35–0.41]	n.d.	n.d.
Total organic carbon (mg/L)	[4.1–11.5]	[4.8–11.5]	[4.8–11.1]	[34.1–53.6]	[35.7–55.4]	[36.1–52.2]
NH_4 (mg/L)	n.d.	n.d.	[0.005–0.05]	n.d.	n.d.	n.d.

the sequencing and their relative abundances in the samples. Alpha diversity indices Shannon and Chao were calculated in Mothur and are available in supplementary materials (Table S2). Shared and non-shared OTUs as well as the corresponding number of sequences were obtained from the venn command in Mothur and Venn diagrams were constructed using the software Venn Diagram Plotter. Beta diversity measurement of the samples was assessed with principal coordinates analysis (PCoA) in Mothur using unweighted UniFrac distance matrix. Analysis was performed with subsampling parameter of 30276 sequences, corresponding to the sample with the lowest number of sequences. PCoA results provided the phylogenetic relatedness between the bacterial communities retrieved from the different sampling locations and at different sampling times.

2.4.5. ICP-MS analyses

Potassium (K39) was used to evaluate the water flows in the system as it is assumed that no loss of the element would occur in evaporation or precipitation. Moreover, the chemicals added to the cooling tower do not contain potassium. In one of the 5 months (November), analysis of K39 was not available. The concentration of strontium (Sr88) was used as a substitute element for the mass balance calculation for identical reasons as stated for potassium. Water samples were filtered with 0.45 μm pore size and 33 mm diameter sterile PVDF syringe filters (Merck, Germany), diluted 10 times with ultrapure water and treated with nitric acid (1% v/v) prior to the analyses. The element was analysed by inductively coupled plasma mass spectrometry (ICP-MS PlasmaQuant, Analytik Jena, Germany). In the analysis method, an Ar flow of 9.0 L/min was used for the plasma, with an auxiliary flow of 1.4 L/min and nebulizer flow of 1.1 L/min. The R/F was set to 1.3 kW. Measurements were performed in no gas mode.

In the calculation described in section 2.5., the concentration factor was based on the ratio of K39 (or Sr88) concentration in the cooling tower water by the concentration in the feed water for each sampling date (Table 2).

2.5. Mass balance-based equation for assessment of net decay or growth of taxonomic orders

Taking into account the addition of bacterial cells by the feed water and the concentration factor estimated by the potassium (or strontium) measurements, and supposing that all cells remain intact in the cooling tower, a theoretical concentration can be obtained for each taxonomic order in the cooling water:

$$[X]_{CT.Th} = [X]_F * C = \%X_F * TCC_F * C \quad (1)$$

with $[X]_{CT/F}$: concentration of organism X in the cooling tower (theoretical) or in the feed; C: concentration factor; $\%X_F$: relative abundance of organism X in the feed; TCC_F: total cell count in the feed.

An approximation of the real concentration can be determined separately:

$$[X]_{CT} = \%X_{CT} * TCC_{CT} \quad (2)$$

with $\%X_{CT}$: relative abundance of organism X in the cooling tower; TCC_{CT}: total cell count in the cooling tower.

The ratio of both numbers calculated using equations (1) and (2) is used to determine growth or decay of the bacterial order:

$$R = \frac{[X]_{CT}}{[X]_{CT.Th}} \quad (3)$$

R > 100%: growth.
R < 100%: decay.
R = 100%: cell retention (no net decay nor growth).

3. Results

Water samples were collected from 3 locations in the feed line (A, B and C) and 3 locations in the cooling tower (D, E and F) (Fig. 1) over a 5-month period. Cell counts, ATP and next generation sequencing (NGS) analysis was performed on all samples to investigate the spatial and temporal changes in bacterial microbiome. A closer look was taken at the cooling tower feed (C) and the cooling tower samples (D, E and F) to assess the impact of chlorine dosage on the main bacterial communities composing the cooling tower water (Fig. 1). The net decay or net growth of specific orders of bacteria were estimated from a mass balance based equation taking into account the concentration factor.

3.1. Water composition

Water parameters collected by online monitoring or with handheld measurement devices are reported in Table 1. Despite some small variations in pH, conductivity and temperature in the feed line, the cooling tower parameters were kept stable. The samples had a pH between 7.7 and 8.4 in the feed water line (A, B and C). In the cooling tower (D, E and F) the pH was between 7.5 and 7.8 due to the dosage of sulphuric acid to limit scaling and improve sodium hypochlorite disinfection efficiency (Fig. 1). The conductivity in the feed line varied between 600 and 750 μS/cm over time while the conductivity in the cooling tower was maintained between 3000 and 3500 μS/cm. Total organic carbon concentrations reached between 34.1 and 55.4 mg/L in the cooling tower due to the concentration factor induced by the evaporation. Residual chlorine concentrations were the highest in location B and D reaching up to 0.42 and 0.41 mg Cl_2/L respectively due to the dosage of sodium hypochlorite at these locations. Lower values in C indicate a decay in residual chlorine along the distribution system. Chlorine concentrations in E and F were not quantified but were expected to contain similar concentration of residual chlorine as D, as a result of the high water recirculation rate and continuous dosage. Ammonia concentration was not analysed in the cooling tower but the feed water supplier reported a low concentration in the incoming water, between 0.005 and 0.05 mg/L. Formation of monochloramine in the cooling water cannot be excluded but is assumed to have minor impact on the residual chlorine concentration measured. The

Table 2
ICP-MS analyses from the feed and the cooling tower water for each month. The second values (±) represent the standard deviation of three measurements of the same water sample. Potassium K39 (or strontium Sr88 in November) was used to calculate the concentration factor in the cooling tower.

	September	October	November	December
Reference element	K39	K39	Sr88	K39
Concentration in feed water (mg/L)	8.98 ± 0.05	6.58 ± 0.02	0.204 ± 0.001 -Sr88-	8.6 ± 0.2
Concentration in cooling water (mg/L)	50.4 ± 0.6	43.5 ± 0.4	0.94 ± 0.01 -Sr88-	30.1 ± 0.3
Concentration factor C	**5.6 ± 0.1**	**6.6 ± 0.2**	**4.6 ± 0.1**	**3.5 ± 0.1**

temperature was highly variable in the feed water, gradually decreasing from 20.2 to 5.2 °C during the sampling campaign from August to December 2017. On the contrary, the cooling water temperature was kept between 15 and 25 °C due to the continuous water recirculation through the heat exchangers.

The results from the ICP-MS analyses, with the corresponding concentration factor, are shown in Table 2. The concentration factor varied between 3.5 ± 0.1 and 6.6 ± 0.2.

3.2. Bacterial cell number and ATP measurements

3.2.1. Spatial changes in the system

Flow cytometry was applied to measure the total and intact cell counts and ATP measurements were performed to quantify the bacterial intracellular and extracellular ATP concentrations in the water samples. Fig. 2 shows the results of the analyses performed in September. The results from the other months revealed the same trends despite some variations in absolute values. The corresponding figures for all monthly samples are available in the supplementary materials (Figs. S1 and S2).

Sodium hypochlorite was dosed at two locations in the system. The first location was before distribution of the feed water to the cooling tower, before sampling point B (Fig. 1). The second location was in the basin of the cooling tower, point D. The intact cell counts and intracellular ATP at the inlet of the feed line (A) were the highest from all samples, with [0.4–1.5] x 10^6 cells/mL (Fig. 2a, Figs. S1 and S2) and [80–700] pg ATP/mL respectively (Fig. 2b, Figs. S1 and S2). The intact cell counts then showed a strong decrease in the concentration range between sampling points A and B, where the pre-treatment steps and first NaOCl dosage were applied. The reduction varied between 79 and 98% in intact cell count depending on the sampling month. The intracellular ATP measurements indicated a similar trend, with a decrease of 85–98% between sampling points A and B.

In August and September, an increase of [6–7] x 10^4 in intact cells per mL was measured along the distribution pipe between points B and C. Higher feed water temperature in this period of the year could have caused regrowth in the distribution line. The opposite trend was observed in November and December, with a loss of intact cells and intracellular ATP.

No strong difference in the intact cell count and intracellular ATP was noticeable between the feed water in point C and the cooling water in points D, E and F despite continuous sodium hypochlorite dosage in the basin of the cooling tower. The chlorine dosage did not neutralize all cells but it helped to prevent further increase in cell concentration considering the concentration factor (Table 2) and the water retention time of around 5 days in the system. The 3 samples collected in the cooling tower (D, E and F) showed comparable results for each sampling date although point E always exhibited a slightly higher microbial contents of [1–2] x 10^4 intact cells/mL.

3.2.2. Evolution of ATP-per-cell in time

The ATP data and intact cell counts were used to identify the spatial and temporal evolution in average ATP-per-cell value. The amount of intracellular ATP per intact cell count was calculated for each sample and the average values for feed and cooling water samples were plotted in Fig. 3, together with the feed water temperature on each sampling day. The feed water samples showed a strong decrease in ATP per cell between August and November from 4.1 × 10^{-16} to 6.2 × 10^{-17} g ATP/cell and stabilised between November and December. The feed water temperatures also decreased in this period from 20.2 to 5.2 °C. The amount of ATP per intact cell inside the cooling tower was more stable over the observation period (between 2.7 × 10^{-16} and 6.8 × 10^{-16} g ATP/cell). The results suggest that (i) the average ATP-per-cell in the cooling tower evolved independently from the average ATP-per-cell

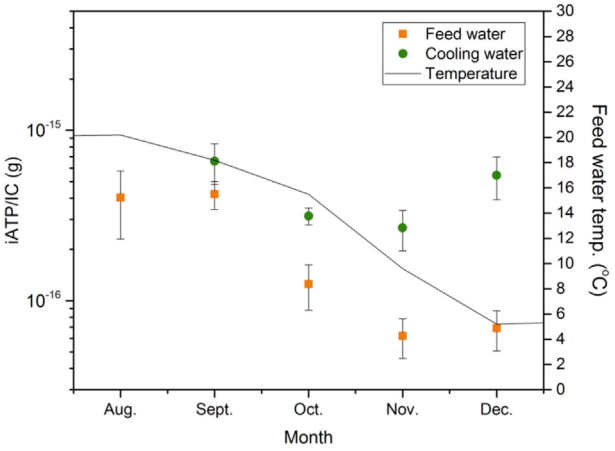

Fig. 3. Ratio of intracellular ATP (iATP) per unit of intact cells (IC) as a measure of average ATP-per-cell over the 5-month period. The black line indicate the variation of temperature in the feed water line over time. The error bars of the data points for the feed and cooling water represent the standard deviation on the 3 feed samples (A, B and C) and the 3 cooling water samples (D, E and F).

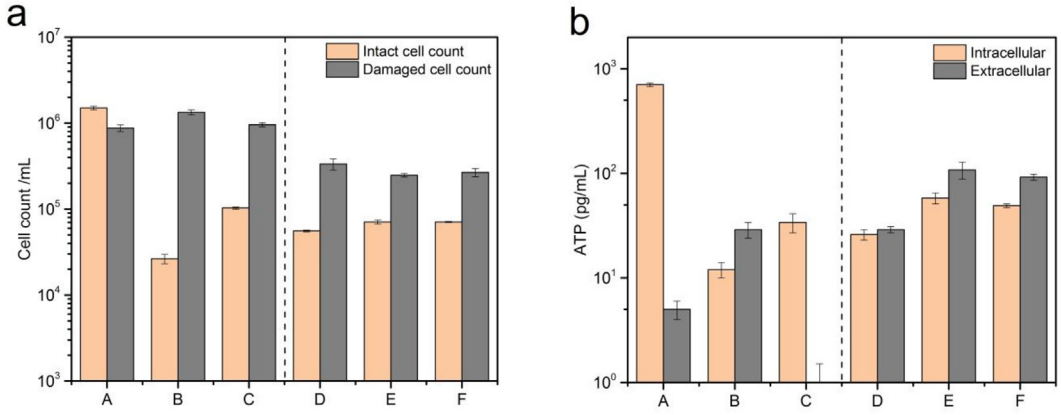

Fig. 2. Intact and damaged cell counts (a) and ATP measurements (b) in September 2017. The letters A to F correspond to the coded sampling locations shown in Fig. 1. Results from the other months are available in supplementary material (Figs. S1 and S2). The error bars indicate the standard deviation on duplicate measurements.

in the feed water; (ii) the feed water is more affected by the changes in environmental conditions than the cooling water.

The divergence between the intracellular ATP per cell in the feed water and in the cooling tower water could be explained by the better controlled conditions in the cooling tower or by different bacterial communities populating the two sections of the system.

3.3. Taxonomic diversity and relative abundances

The changes in bacterial composition in the feed and cooling water was monitored by 16S rRNA gene amplicon sequencing. The impact of the cooling tower conditions and the continuous chlorine dosage on the microbiome were evaluated. The relative abundances of potentially pathogenic genera are available in the supplementary materials (Table S3). The *Legionella* genus represented only 0.28% of the total number of reads from all samples and did not show an increase in relative abundance between the feed and the cooling tower. The bacterial community in the feed water was dominated by *SAR_clade* (37 ± 19%) and *Frankiales* (16 ± 8%) orders (Fig. 4a and Figs. S3–S5), from the phyla *Proteobacteria* and *Actinobacteria* respectively. Only the sample from October diverged by a strong increase in *Sphingomonadales* after the pre-treatment steps (B) of 20% (Fig. S4). A less abundant but recurrent bacterial order was *Betaproteobacteriales* (6 ± 3%). *SAR_clade* and *Frankiales* abundances dramatically dropped in the cooling tower (2 ± 2%). The main taxonomic orders common to all cooling tower samples (locations D, E and F) were *Caulobacterales* (28 ± 12%) and *Obscuribacterales* (18 ± 8%), followed by *Rhizobiales* (6 ± 2%), *Spingomonadales* (6 ± 5%), and *Betaproteobacteriales* (5 ± 2%). The venn diagrams shown on Fig. 4b and Figs. S3–S5 indicate that the taxa shared between the feed water (C) and the basin of the cooling tower (D) accounted for 85–94% of the total sequences depending on the month. In addition, the cooling tower shows less diversity than the feed water with a lower number of taxa. Hence despite the strong switch observed in relative abundance of microorganisms, the cooling tower main bacterial members were already present in the feed. When comparing all collected samples, only 0.2% of the total number of OTUs (33 OTUs) were shared between all locations over the 5 months. However, the 33 shared OTUs accounted for 46% of the total sequence reads. These common taxa representing a

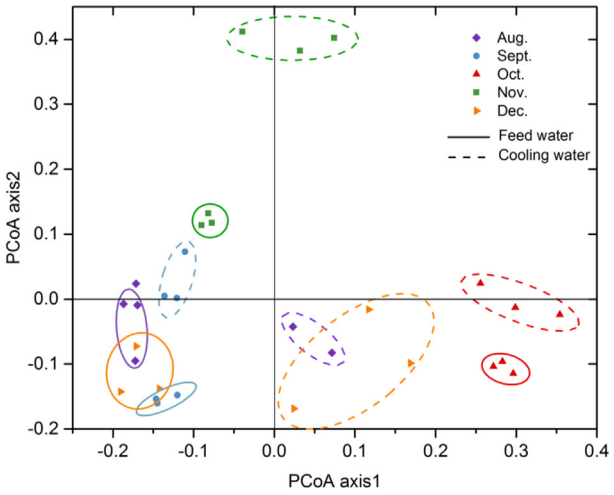

Fig. 5. Principal coordinates analysis (PCoA) representing relatedness between bacterial communities of the cooling water system based on phylogenetic tree. The plain circles and dotted circles reveal the clusters respectively formed by the feed water samples (locations A, B and C) and by the cooling water samples (location C, D and E).

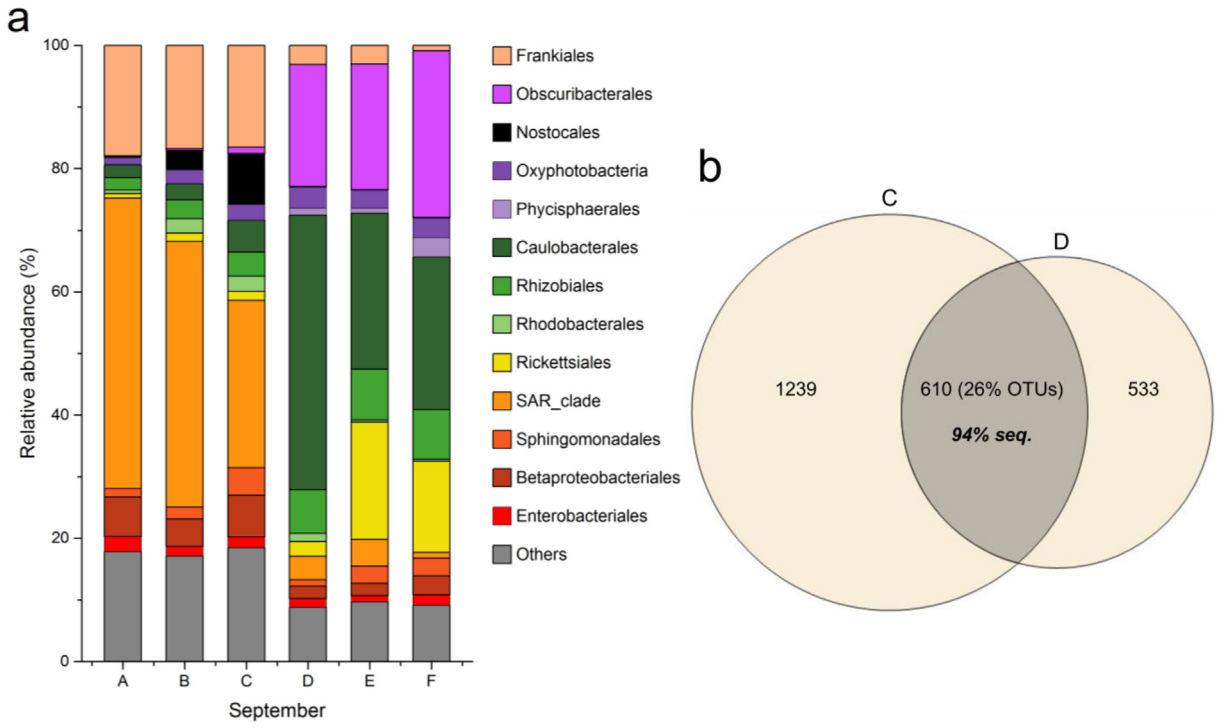

Fig. 4. Relative abundance of bacterial order in samples of the cooling water system in September 2017 (a) and Venn diagram of cooling tower feed water C and basin water D (b). Relative abundance was generated for each OTU but displayed at the order taxonomic rank to improve visualization of the changes. "Others" contains orders of less than 1% relative abundance. The DOI of the raw sequencing data is indicated in supplementary materials. b indicates the shared OTUs between samples C and D (Fig. 1), respectively cooling tower feed water and basin water, and the percentage of total sequences (bold) included in the shared OTUs. The data from the other months are available in supplementary material (Fig. S3, S4 and S5).

large fraction of the total sequences constitutes the core microbiome of our system.

Principal coordinates analysis (Fig. 5) shows the phylogenetic relatedness between communities of all samples based on the generated OTUs, illustrating the changes in bacterial community structures in time and at the different locations. The clusters represented with plain and dotted circles reveal structural divergence between the feed and the cooling water samples for each month. Greater distances are observable between the feed water and cooling tower samples than between the 3 samples from each section. In addition, the feed water samples clustered more closely than cooling water samples. The outlier observed in August and corresponding to the basin sample (location D) shows strong similarities with the feed water structure, suggesting that the feed water valve was open at the time of the sampling. In the same figure, the monthly clusters formed by the feed water samples do not seem to converge suggesting temporal variations in bacterial community structure in the water source, upstream from the studied system. The chart with letters corresponding to each location is available in supplementary materials (Fig. S6).

To summarize, the sequencing results suggest that the cooling tower bacterial composition is continuously determined by the feed water microbiome but is further shaped by the process conditions.

3.4. Growth and decay of bacterial orders in the cooling tower

The strong change in relative abundance of the main bacterial members between the feed water and the cooling tower may be due to differential growth or differential decay of the feed water community members when accessing the basin of the cooling system. The sequencing data refers to all intracellular DNA, both from viable and non-viable bacteria. To estimate which community members show a net growth or decay, the relative abundances were combined with the total cell counts in a mass balance based equation (described in section 2.5). A similar approach has been implemented in the past with flocculent sludge and anaerobic digestion systems (Mei et al., 2016; Saunders et al., 2015; Winkler et al., 2012). The taxonomic rank order was chosen for visualization of the changes in microbiome and identify trends over the sampling period, as no specific functionalities were investigated.

The results presented in Fig. 6 show the growth and decay of taxonomic orders in relative (Fig. 6a) and absolute numbers (Fig. 6b). The data indicates that the majority of the bacterial groups are decaying with less than 10% remaining cells (less than 2% for the *Frankiales* and *SAR11_clade*). Among the dominating organisms from the feed water and the cooling water, only the taxonomic order *Obscuribacterales* showed considerable and recurrent increase in the cooling tower microbiome (260 ± 95%) (Fig. 6a) corresponding to an absolute increase of 10^4 to 10^5 cells/mL (Fig. 6b) depending on the month. The strong increase in 2 main OTUs within the *Obscuribacterales* order were causing this rise. *Caulobacterales* (48 ± 30%) showed a slower decay than *Rhizobiales* (9 ± 3%) and *Sphingomonadales* (8 ± 7%). A further look at the most abundant OTUs of these main taxonomic orders revealed that all main genera contributed to the decay of the orders. The DOI of the raw sequencing data is indicated in supplementary materials.

Quantification of the changes in microbial structures suggest different decay rates and adaptability of individual bacterial groups under continuous chlorine dosage in the recirculating cooling water (from 0.35 to 0.41 mg Cl_2/L, Table 2). Only the *Obscuribacterales* order was able to significantly increase in cell number in the cooling tower despite the high residual chlorine content of the water. The absolute increase in cells, taking into account the impact of the concentration factor, could only be determined by the use of our mass balance-based approach combining flow cytometry and sequencing data.

4. Discussion

4.1. Commonly applied residual chlorine concentration does not allow complete inactivation of cooling tower bacteria

The use of surface water as feed water for cooling tower operation requires a powerful biofouling inhibitor to avoid pathogen outbreaks and biofilm formation. The high bactericidal efficiency and competitive cost of sodium hypochlorite makes it the most common inhibitor in cooling processes (Frayne, 1999). Chlorine dosage in the studied cooling water system:

(i) reduced by up to 98% the active bacterial biomass concentration in water when dosed in the feed line (Fig. 2), with a negligible impact on the relative abundance of the microbial community members in the surface water feeding the cooling tower (Figs. 4a and 5);
(ii) limited the bacterial growth in the cooling tower water caused by the increase in nutrient content (Table S1) and 5-day retention time (Fig. 2).

Continuous dosage of biocide prevented the growth of pathogenic bacteria, including *Legionella*. However, it was not effective to entirely inactivate the bacterial cells in the water even at residual chlorine concentrations up to 0.4 mg Cl_2/L. Resistance of some specific organisms to relatively high residual chlorine concentration (0.6 mg Cl_2/L) has been recently described (Song et al., 2019; Sun et al., 2013) and previous studies on cooling tower reported that a continuous free chlorine residual of 1 mg Cl_2/L was optimal to achieve recommended biological control criteria (10^4 CFU/mL) with NaOCl (Chien et al., 2013; Helmi et al., 2018). However, concentrations higher than 0.5 mg Cl_2/L is usually not a feasible option due to the corrosiveness of the chemical, side-reaction with other inhibitors and formation of disinfection by-products (Boulay and Edwards, 2001; Hsieh et al., 2010; Padhi et al., 2019). The high total organic carbon concentration in the cooling tower, up to 55 mg/L, may have led to the reaction of organic matter with the sodium hypochlorite dosed in the basin.

As an additional point, the quantification of intact cells at different locations in the cooling tower indicated a higher concentration of viable cells after the heat exchanger, suggesting cell growth or release from biofilms in that section of the process. The sampling location is therefore of importance for evaluating the microbiome of a cooling tower. In the studied cooling tower, 16S results showed low abundance of potentially-pathogenic genus (Table S3). However, the observation that the chlorine dosage does not completely prevent microbial growth in the heat exchanger could be a concern for other cooling towers since the water from the outlet of the heat exchanger line is sprayed on top of the column. This is also the point where pathogens might be spread in the surroundings. To reduce the risks of outbreak, chlorine dosage could be performed right before the spray nozzles instead of inside the cooling tower basin.

4.2. Main cooling tower bacterial members originated from the feed water microbiome but the process conditions further influenced the bacterial community structure

A substantial core microbiome was shared between all collected samples (46% of the total sequence reads was contained in 33 OTUs). The shared taxa between the feed water and the cooling tower basin water on each month accounted for 85–94% of the

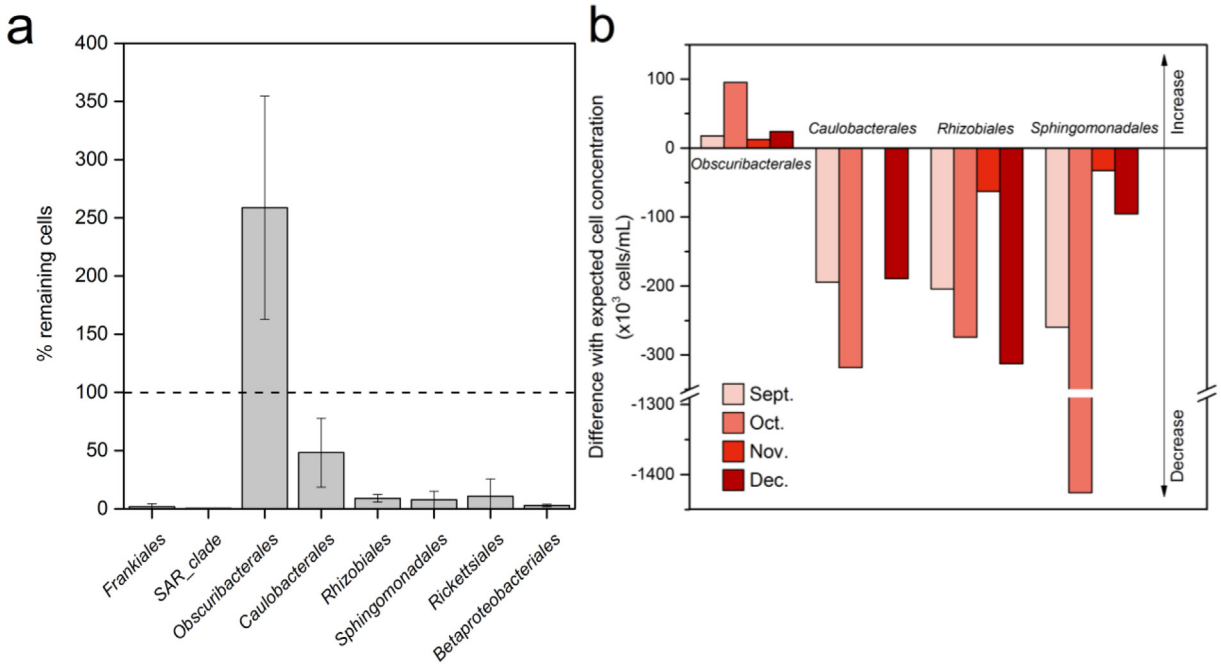

Fig. 6. Changes in bacterial orders in the cooling water system, expressed in percentage (a) and absolute numbers (b). a shows the percentage of total remaining cells for each main taxonomic orders in the cooling tower compared to the input from the feed water, calculated according to section 2.5. Values above 100% indicate growth. The error bars represent the standard deviation from the monthly samples. b represents the absolute deviation to the expected cell concentration for each month. Positive values indicate growth, negative values indicate decay. The 4 more recurrent taxonomic orders from the cooling water samples are represented in the graph b.

sequences (Fig. 4b) supporting the idea that the planktonic population present in the cooling tower originated mainly from the feed water. The remaining fraction of microorganisms could have originated from different sources such as the ambient air and particles, precipitations or biofilm growth. Rain and cloud waters can contain up to 10^6 bacterial cells/mL (Hu et al., 2018) and the air to water ratio can reach up to 1.5 m^3/L in a cooling tower (Keshtkar, 2017), potentially affecting the water community composition of these open recirculating systems.

Although the main cooling tower bacterial members originated from the feed water, a strong switch in relative abundance of the microbial community members was observed including a change of the dominant microorganisms (Figs. 4a and 5). Main taxonomic orders for the feed water, *LD12* subclade of *SAR_11* (*Alphaproteobacteria*) and *Hgcl* subclade of *Frankiales* (*Actinobacteria*), are common groups described in freshwater reservoirs and brackish waters (Henson et al., 2018; Llirós et al., 2014; Salcher et al., 2011) but not recurrent in cooling towers. Cooling water bacterial communities, on the contrary, were dominated by the recurrent orders *Obscuribacterales* (*Melainabacteria*) and *Caulobacterales* (*Alphaproteobacteria*) followed by *Sphingomonadales* and *Rhizobiales* (*Alphaproteobacteria*). A decrease of *Actinobacteria* and rise of *Proteobacteria* have been previously observed in cooling water (Paranjape et al., 2019), corresponding to our findings with the strong decline in *Frankiales* and suggesting a high sensitivity of these organisms to the changes in environmental conditions.

This shift in relative abundance can be linked to several environmental factors, the main one being continuous disinfection. Continuous chlorine dosage affects bacterial community structure depending on the tolerance of the bacterial species to the biocide or their presence in protective biofilms. Even though no deposit was visible in the cooling tower basin, *Caulobacterales*, *Sphingomonadales* and *Rhizobiales* are bacterial orders frequently encountered in biofilms (Farenhorst et al., 2017; Pang and Liu, 2007) and post disinfection samples (Gomez-Alvarez et al., 2012; Shaw et al., 2015) of drinking water distribution systems. The observed *Caulobacterales* family *Hyphomonadaceae* is known as prosthecate bacteria producing polysaccharide-based adhesin, called holdfast, allowing surface colonization (Abraham and Rohde, 2014; Dang and Lovell, 2015; Nagaraj et al., 2017). Some *Sphingomonas* species have also been linked to biofilm initiation through production of water-soluble polysaccharides and surface-adhesion proteins (Bereschenko et al., 2011; Pollock and Armentrout, 1999) or have shown higher chlorine tolerance (Sun et al., 2013; Wang et al., 2019), both leading to deteriorations of membrane processes (Bereschenko et al., 2010; de Vries et al., 2019). To a lesser extent, chlorine-resistance properties of the *Rhizobiales* order have been discussed in a drinking water distribution system (Wang et al., 2018). The biofilms located on the inner surfaces of these systems constitutes the main reservoirs for bacterial regrowth (Berry et al., 2006). A recent study also reported the presence of these community members in cooling tower biofilms (Di Gregorio et al., 2017), suggesting that detached biofilm fragments likely influence cooling water bacterial composition.

Chlorine disinfection can also explain the lower diversity in OTUs encountered in the cooling tower water compared to the feed water (Fig. 4b and Figs. S3–S5).

To summarize, most cooling tower community members originated from the feed water microbiome but the process conditions further influenced the bacterial community structure.

4.3. Cooling tower community structure is mainly shaped by differences in decay rate of populations caused by residual chlorine

Since 16S rRNA gene amplicon sequencing does not differentiate between live, dormant and damaged cells (Li et al., 2017), the association of relative abundance with total cell count can help quantify the abundance of bacterial groups and identify growth or decay of specific taxonomic orders. Combination of flow cytometry with 16S rRNA gene amplicon sequencing has previously been

investigated for drinking water monitoring (Prest et al., 2014) and showed to be a promising method for quantification of bacterial communities in water systems. We applied the same method in this study, combined with a mass balance, to assess the impact of chlorine dosage on each bacterial order composing the cooling tower (section 2.5.). To our knowledge, this is the first paper that describes net growth and decay of cooling tower microorganisms.

Based on our results, all dominant feed and cooling water microorganisms showed strong net decay with exception of the *Obscuribacterales* order. In the case of *Caulobacterales*, *Sphingomonadales* and *Rhizobiales*, the high relative abundance in the cooling tower resulted from a slower decay compared to the other microbial community members. Importantly, the potentially-pathogenic bacterial group *Legionella* showed negligible abundance in all samples (Table S3) and demonstrated decay in the studied cooling tower, indicating that risks of outbreak were prevented. A control study without disinfection would be able to confirm that chlorine is causing these microbial community changes but operation of a full-scale cooling tower without biocide dosage is not feasible due to the threat of pathogenic bacteria.

We can conclude from our study that the decay rate of the different bacterial orders is the main factor shaping the cooling water microbiome, not bacterial growth potential. This finding implies that the feed water acts as a continuous inoculant, highly impacting the cooling tower populations, when chlorine is used for disinfection. The importance of the feed water composition has been observed by studying the similarities in bacterial members between multiple cooling towers based on their geographic locations (Paranjape et al., 2019) but has never been thoroughly investigated.

4.4. The bacterial group Obscuribacterales succeeded in growing in the presence of residual chlorine

As indicated previously, the only microorganism group showing a net growth in the cooling tower was the order *Obscuribacterales*. *Obscuribacterales* belong to the phylum *Melainabacteria*, a recently discovered phylum closely related to the *Cyanobacteria* but consisting of non-photosynthetic members (Bruno et al., 2018; Di Rienzi et al., 2013). Although described as a strictly fermentative bacterial phylum, a newly identified species has been shown to possess the ability for fermentation as well as aerobic and anaerobic respiration with complete respiratory chain (Soo et al., 2014). To our knowledge, the order *Obscuribacterales* has been rarely observed in engineered water systems and never described in biofilms. It has however been identified in other cooling tower studies by (Pereira et al., 2017) and (Paranjape et al., 2019). In the latter, *Obscuribacterales* was present in 7 out of 18 evaporative cooling towers and represented a substantive portion of the total bacterial community (between 10 and 30% relative abundance) in 2 of them. Daily and continuous disinfections were applied and residual chlorine concentrations of 0.32 and 0.27 mg Cl_2/L were reached in these 2 systems. Those results are in line with our finding and would suggest that dominance of *Obscuribacterales* is favoured by the presence of continuous high residual dosage. However, the explanation for the chlorine resistance is so far unknown, due to the lack of information available on the microorganisms and biocide's mechanism. Chlorine disinfection does not require specific target sites to exert a cytotoxic effect. Higher resistance of cells to chlorine is believed to result from reduced cell membrane permeability, or formation of a mucopolysaccharide outer layer (Bower and Daeschel, 1999). Further characterization would therefore be necessary to understand the metabolism and physiology of the bacteria. In order to avoid chlorine-resistant organisms to develop, a complementary disinfection method could be considered depending on the resistance mechanism, such as UV or quaternary ammonium compounds (Phe et al., 2009; Sun et al., 2013; Rahmani et al., 2016). In addition to cell resistance, the formation of cell aggregates can also provide protection against external stress factors including biocides (Mir et al., 1997). Aggregates have not been observed during the study but their presence should not be excluded.

4.5. The described mass balance approach allows better characterization of cooling water bacterial changes

The use of a mass balance in combination with flow cytometry and sequencing data provided a higher level of understanding of the bacterial dynamics. Full-scale studies often omit to describe the feed water bacterial composition, which is essential information when discussing bacterial changes in chlorinated cooling water systems. Ignoring the feed water microbiome can lead to data misinterpretation, e.g. by assuming that all main cooling water taxa became accustomed to the process conditions and disinfection. In this study we included the feed water composition to identify the factors shaping the bacterial communities in the cooling tower. The studied cooling tower did not show an increase of potentially pathogenic bacterial groups (Table S3). However, the method would be able to detect an outbreak. Specifically, if *Legionella* continuously shows net growth, this would indicate the necessity of implementing alternative disinfection methods.

In addition, approximation of the net growth or net decay of microorganisms is highly valuable to detect chlorine resistant microorganisms. With this approach we could prove that the increase in *Obscuribacterales* was not only relative to the other communities nor due to the effect of concentration in the cooling tower, but resulted from the absolute growth of this microorganism group in the system containing residual chlorine. Application of this method to a large cooling tower dataset can identify recurrent and growing bacterial groups, and help further optimisation and evaluation of disinfection technique.

Limitations are however present when quantifying absolute abundances based on genomic analyses (Kleiner et al., 2017), specifically regarding the limited resolution (Poretsky et al., 2014) of the method and taxonomic profiling (Sczyrba et al., 2017). The real net growth and net decay can therefore show some deviations from the calculated values. Improvements can be made in the future by (i) taking into account copy numbers in the mass balance when the organisms are better identified and (ii) sampling and analysing the cooling tower surface to identify attached microbial communities and assess the potential impact on the microbial dynamics.

5. Conclusions

The microbiome characterization of a full-scale industrial cooling tower (2600 m^3/h) - with residual chlorine between 0.35 and 0.41 mg-Cl_2/L and concentration factor between 3.5 and 6.6 - and comparison of bacterial community structures between the water feeding the cooling tower and the cooling tower water led to the following conclusions:

- The bacterial community composition in the cooling tower is driven by the feed water community composition. However, the structure of the bacterial community is governed by differences in decay rate of the bacterial groups in presence of residual chlorine. The characterization of feed water bacterial composition is therefore of great importance when studying microbiome dynamics in cooling towers.
- The taxonomic order *Obscuribacterales* was able to grow in the cooling system despite the presence of up to 0.41 mg-Cl_2/L

residual chlorine, suggesting potential chlorine-resistance ability of this bacterial group. This taxonomic order is not commonly found in biofilms - as opposed to the other dominant organisms found in the cooling water - but has been previously observed in cooling tower studies in presence of residual chlorine.
- The proposed approach based on mass balance combining flow cytometry and 16S rRNA gene amplicon sequencing is a useful tool to determine net growth and decay of microorganisms and to assess biocide impacts on cooling tower communities.

This study points out the importance of full-scale data for identification of organisms with higher ability to survive disinfection. The establishment of a database from industrial scale cooling towers would contribute to determine recurrence of specific organisms in these processes. A better understanding of the biological processes and microbiome changes could be used to predict and develop strategies to control pathogen outbreaks.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2020.115505.

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