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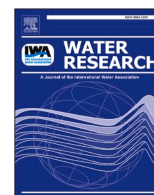

Seawater desalination based drinking water: Microbial characterization during distribution with and without residual chlorine

Nadia Farhat [a,1,*], Lanhee Kim [a,1], Katsuhiko Mineta [b], Mohammed Alarawi [b], Takashi Gojobori [b,c], Pascal Saikaly [a], Johannes Vrouwenvelder [a,d]

[a] Water Desalination and Reuse Center (WDRC), Division of Biological and Environmental Science and Engineering (BESE), King Abdullah University of Science and Technology (KAUST), Thuwal, 23955-6900, Saudi Arabia

[b] Computational Bioscience Research Center (CBRC), Division of Computer, Electrical and Mathematical Sciences and Engineering (CEMSE), King Abdullah University of Science and Technology (KAUST), Thuwal, 23955-6900, Saudi Arabia

[c] Division of Biological and Environmental Science and Engineering (BESE), King Abdullah University of Science and Technology (KAUST), Thuwal, 23955-6900, Saudi Arabia

[d] Faculty of Applied Sciences, Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, Delft 2629 HZ, Netherlands

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ABSTRACT

Monitoring the changes that occur to water during distribution is vital to ensure water safety. In this study, the biological stability of reverse osmosis (RO) produced drinking water, characterized by low cell concentration and low assimilable organic carbon, in combination with chlorine disinfection was investigated. Water quality at several locations throughout the existing distribution network was monitored to investigate whether microbial water quality changes can be identified. Results revealed that the water leaving the plant had an average bacterial cell concentration of 10^3 cells/mL. A 0.5–1.5 log increase in bacterial cell concentration was observed at locations in the network. The residual disinfectant was largely dissipated in the network from 0.5 mg/L at the treatment plant to less than 0.1 mg/L in the network locations. The simulative study involving miniature distribution networks, mimicking the dynamics of a distribution network, fed with the RO produced chlorinated and non-chlorinated drinking water revealed that distributing RO produced water without residual disinfection, especially at high water temperatures (25–30 °C), poses a higher chance for water quality change. Within six months of operation of the miniature network fed with unchlorinated RO produced water, the adenosine triphosphate (ATP) and total cell concentration (TCC) in the pipe biofilm were 4×10^2 pg ATP/cm^2 and 1×10^7 cells/ cm^2. The low bacterial cell concentration and organic carbon concentration in the RO-produced water did not prevent biofilm development inside the network with and without residual chlorine. The bacterial community analysis using 16S ribosomal RNA (rRNA) gene sequencing revealed that mesophilic bacteria with higher temperature tolerance and bacteria associated with oligotrophic, nutrient-poor conditions dominated the biofilm, with no indication of the existence of opportunistic pathogenic species. However, chlorination selected against most bacterial groups and the bacterial community that remained was mainly the bacteria capable of surviving disinfection regimes. Biofilms that developed in the presence of chlorine contained species classified as opportunistic pathogens. These biofilms have an impact on shaping the water quality received at the consumer tap. The presence of these bacteria on its own is not a health risk indicator; viability assessment and qPCRs targeting genes specific to the opportunistic pathogens as well as quantitative microbiological risk assessment (QMRA) should be included to assess the risk.

The results from this study highlight the importance of implementing multiple barriers to ensure water safety. Changes in water quality detected even when high-quality disinfected RO-produced water is distributed highlight microbiological challenges that chlorinated systems endure, especially at high water temperatures.

* Corresponding author.
E-mail address: nadia.farhat@kaust.edu.sa (N. Farhat).

[1] These authors contributed equally to this work.

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1. Introduction

The health and well-being of humans strongly depend on adequate access to microbiologically safe drinking water (Szewzyk et al., 2000; Shannon et al., 2008). Considerable time and money is invested in treating available water resources to remove undesirable microorganisms in drinking water systems (Plappally and Lienhard, 2012). Depending on the water source, water goes through various treatment steps until it becomes adequate for human consumption. After the treated water leaves the treatment plant, it has to flow through the drinking water distribution system (DWDS) and network of pipes before reaching the consumer taps. The DWDS, with its unique environment, can lead to deterioration of the microbial water quality if the appropriate environment for bacterial regrowth is created (LeChevallier et al., 1996; Miettinen et al., 1997; Liu et al., 2002; Schurer et al., 2019). Two primary approaches are often pursued to prevent bacterial regrowth in DWDS: (i) limiting the nutrients available for bacterial growth or (ii) the use of disinfection residuals. In the first approach, water utilities emphasize the removal of readily biodegradable, low-molecular-weight compounds that are considered as food for bacteria to grow (Volk and LeChevallier, 2000). Water utilities put additional efforts to optimize treatment schemes to enable better removal of slowly biodegradable, high-molecular-weight compounds as these compounds degrade at a slower rate leading to bacterial regrowth at farther locations in the distribution network (Hijnen et al., 2018; Schurer et al., 2019). Disinfection is another common approach to prevent bacterial regrowth by using disinfectants such as chlorine, chlorine dioxide, and monochloramine before distributing the water and maintaining a disinfectant residual in DWDS (Propato and Uber, 2004; Liu et al., 2016; Farhat et al., 2018b). Water utilities globally preclude distribution without a disinfectant residual and disinfection is implemented in most countries and usually allows the distribution of biologically stable water. However, one cannot ignore the carcinogenic disinfection by-products that form when disinfectants react with organic compounds present in the water (Garcia-Villanova et al., 1997; Mercier Shanks et al., 2013). Many groups of disinfection by-products are regulated in drinking water guidelines, and water utilities have to limit the production of these by-products, monitor and measure their concentration to make sure they abide by the guidelines (Guilherme and Rodriguez, 2014; Zeng and Mitch, 2016).

The dynamics of nutrient availability in DWDS together with various other aspects such as pipe material, hydrodynamic conditions, water temperature, and presence/absence of disinfection residual, create complex environments in DWDSs that can be hard to predict (Prest et al., 2016). In specific environments and despite all efforts to control bacterial regrowth, bacteria can still grow in DWDS even in distribution systems with low-nutrient conditions and with the presence of residual disinfectant (Potgieter et al., 2018). The water utilities effort to produce high-quality water cannot be complete without expanding these efforts to involve other entities that maintain the DWDS (Vreeburg and Boxall, 2007). Monitoring the microbial quality in the DWDS is essential (Farhat et al., 2020). Most of the existing methods to monitor drinking water depend on the detection of microorganisms in bulk water samples from consumer taps and using cultivation-dependent methods (Douterelo et al., 2018b). Since mostly all legislation around the world still consider the cultivation-dependent quantification methods as the principal applied compliance parameters (Chowdhury, 2012), using these methods underestimates the actual diversity and abundance of microorganisms in the environment. Moreover, attached bacteria inside the distribution pipes are often overlooked, although biofilms can constitute over 95% of the biomass in DWDS (Flemming et al., 2002). Biofilms on the inside of the distribution network pipes have been associated with a higher risk of occurrence of opportunistic pathogens compromising the safety and quality of drinking water (Lehtola et al., 2007; Van der Kooij et al., 2017). Investigating the biofilms developing on pipes is important; and this is mostly excluded in many studies as accessing the surface of pipes within operative networks is difficult (Douterelo et al., 2016). However, it is worth to mention that recently many research groups acknowledged the importance of studying biofilms in DWDS despite the complexity and major efforts to understand biofilms has been reported (Gomez-Smith et al., 2015; Douterelo et al., 2018a; Waak et al., 2018, 2019a, 2019b; van der Kooij et al., 2020; Ahmad et al., 2021; Calero Preciado et al., 2021).

In this study, a combination of approaches to achieve biologically stable water during distribution was investigated. Seawater was desalinated using reverse osmosis (RO) membranes and the produced water was chlorinated prior to distribution to the DWDS under investigation. The RO-produced water is characterized by low bacterial cell concentration and low assimilable organic carbon concentration. The distribution network is unique as all the pipes are of the same age (10 years) and of the same material (PVC) and feeding a confined area (45 km^2) from one source only. First, water quality at several locations throughout the existing distribution network, fed with chlorinated RO-produced drinking water, was monitored to investigate whether microbial water quality changes can be identified. Then a simulative study involving miniature distribution networks, mimicking the dynamics of a DWDS, was conducted. This study investigated the effect of chlorine presence and absence on biological stability of RO produced water using miniature networks to assess whether chlorination was necessary to distribute biostable water. This study uniquely analyzed a combination of bulk water samples feeding and leaving the miniature networks under both chlorinated and non-chlorinated conditions, as well as biofilm samples from the miniature network pipes over six months.

2. Materials and methods

2.1. Analysis of water samples from the plant and distribution network

2.1.1. Site description

The RO membrane desalination plant at King Abdullah University of Science and Technology (Thuwal, Saudi Arabia) produces drinking water through the desalination of seawater from the Red Sea. The plant capacity is 40,000 m^3/d and the treatment process schematic can be found in Farhat et al. (2020) and Fig. S1 in supplementary material. In short, seawater is pretreated through spruce and cartridge filters before reaching the RO modules. The plant has two stages of RO. The permeate water from the first stage is stored in a break tank. Only 20 percent of the first stage RO is treated with a second stage RO. The final drinking water is a blend of first and second-stage RO permeate, subsequently dosed with chlorine, CO_2, and lime and stored in a storage tank. For this study, we had access to the produced water before dosing chlorine (blended first stage and second stage RO permeate with CO_2 and lime) and after chlorine addition. Water quality parameters leaving the treatment plant are shown in Table S1 in supplementary material.

2.1.2. Sampling scheme

Five sampling locations were investigated to analyze the water quality through time. Two locations were examined at the treatment plant: (1) produced water without chlorine; and (2) produced water with chlorine. Three locations were selected at the distribution network with varying proximities from the treatment plant (Fig. 1). Network L1, L2, L3 were 2.4, 2.0, and 1.8 Km away from the treatment plant. The average estimated hydraulic residence times are between 3 and 5 h.

2.1.3. Microbial analysis

Flow cytometry analysis. Flow cytometry (FCM) was used to determine the total bacterial cell concentration (TCC), as described previously (Farhat et al., 2018a). In short, 2 μL aliquot of SYBR Green I (×10,000 concentrate; Molecular Probes, Switzerland) diluted 100 times with deionized water was added to 200 μL of each water sample and

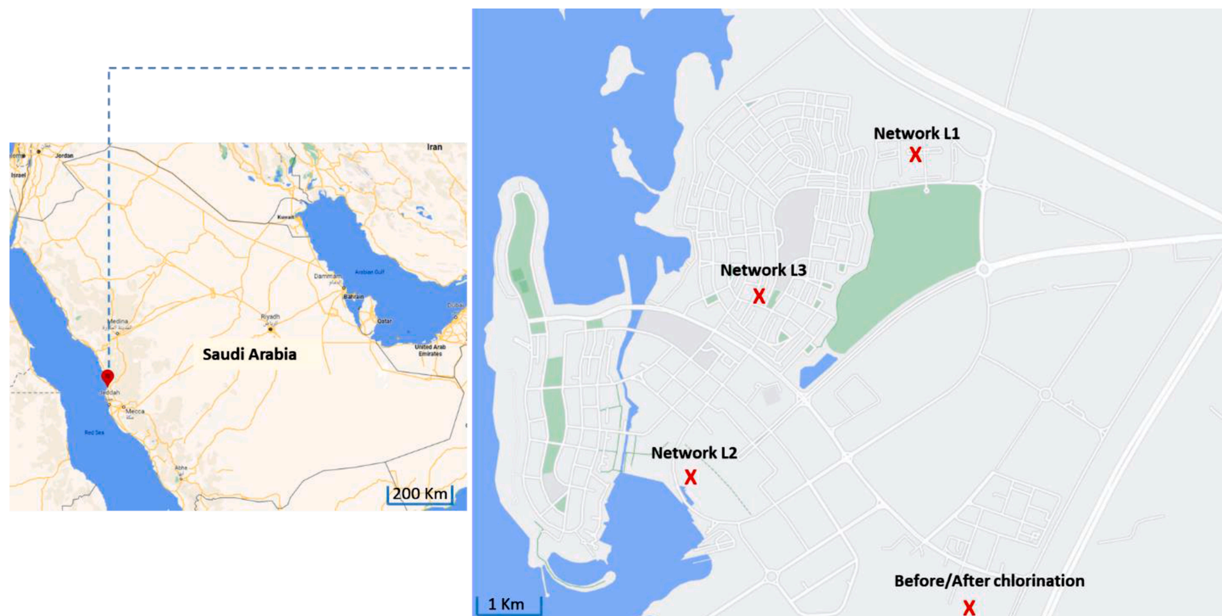

Fig. 1. Locations for water sampling campaign along the distribution network.

incubated for 10 min at 37 °C in the dark before analysis. The TCC of water samples was measured using an Accuri C6 Plus FCM (BD Biosciences, USA).

Online measurements of total cell numbers were done using an automated online monitoring system, OnCyt robot (Oncyt, Switzerland), coupled to an FCM (BD Biosciences, USA), as described by Besmer et al. (2014). Every 10 min, the water sample was taken and stained with SYBR Green I (final concentration: 1×) and subsequently incubated for 10 min at 37 °C inside the online robot. Cleaning procedures with hypochlorite (1% active chlorine; Sigma-Aldrich, USA), sodium thiosulfate solution (100 mM; Sigma-Aldrich, USA), and ultrapure water were automatically conducted after every measurement. The total cell numbers and the ratio between high nucleic acid (HNA) bacteria and low nucleic acid (LNA) bacteria was analyzed at the same time every 10 min. The measurements were conducted for a whole week at the various locations.

The flow cytometric data were analyzed using manufacturer-provided software (cyplot software; Oncyt, Switzerland). The HNA and the LNA bacteria were separated based on green fluorescence intensity (FL1 channel). The flowCHIC (Cytometric Histogram Image Comparison) (Koch et al., 2013; Koch et al., 2014) tool analysis was performed using R packages flowCHIC54,55. The CHIC tool allows a comparison of cytometric datasets without gating decisions and any cytometric pre-experience due to an automated image comparison procedure. The flow cytometric measurements of each sample need to be visualized using Forward scatter (FSC) against fluorescence. The non-metric multidimensional scale (NMDS) plot was made using R software (version 3.5.2).

2.2. Miniature distribution network study

Two miniature water distribution networks were placed at the RO desalination plant to study the effect of chlorine absence and presence on the temporal and spatial dynamics of the microbial community during distribution. The first miniature distribution network was fed with non-chlorinated drinking water, and the second miniature distribution network was fed with chlorinated drinking water of the same quality as the water entering the real distribution network. The miniature distribution networks consisted of nylon tubing, 6 mm in diameter and 270 m length wrapped around a 1 m^2 frame. A water flow velocity similar to the actual distribution system was simulated. The average flow rate inside the miniature networks was maintained at 23 L/h resulting in a flow velocity of 0.2 m/s and a residence time of 25 min. This residence time was shorter that the residence time in the actual network of 3–5 h. A picture of the miniature network system is shown in Fig. S2 in the supplementary material.

The water feeding and leaving the miniature distribution networks was analyzed after 3 and 6 6 months of operation. In addition, biofilm development inside the pipes of the miniature distribution network was investigated.

2.2.1. Adenosine triphosphate (ATP) and total cell concentration (TCC) measurements

The ATP and TCC of the water entering and leaving the miniature network along with ATP and TCC in the biofilm formed on the miniature network pipes were measured. For the pipe biofilm samples, a 4 cm tube length was cut, wiped with 70% ethanol from outside to clean and disinfect the pipe, and placed in a capped tube in 40 mL sterile tap water for ATP analysis and Milli-Q ultrapure water for TCC analysis. The tubes with the pipe sections were mixed on a vortex (few seconds) and sonicated using a sonifier probe (Q700 Qsonica sonicator, USA) for 1–2 min (sample kept on ice) until the liquid was homogenous. ATP was measured using a luminometer (Celsis Advance, Charles River Laboratories, Inc., USA), and TCC was measured according to the protocol explained earlier. Samples were measured in triplicates. Student's *t*-test was used to determine if there is a significant difference between the means at the different conditions (chlorine/no chlorine, inlet/outlet, 3months/6 months). The difference was considered significant when the calculated p values were less than 0.05.

2.2.2. Microbial communities in water and pipe biofilm samples

For the water samples, 20 liters of water was collected and filtered on 0.22 μm mixed cellulose esters membrane (GSWP14250; Millipore, USA) on the same day of sampling. Triplicate 20 L samples were taken after six months of operating the miniature network. Then average of triplicate samples was used to plot taxonomic distribution of bacterial community (relative abundance). For the pipe biofilm samples, 4 pieces of 4 cm pipe section were cut from the miniature distribution network after six months of operating the miniature network. Then average of quadruple samples was used to plot taxonomic distribution of bacterial community (relative abundance) in the biofilm. The filters and pipe sections were stored at −20 °C until processing.

Microbial genomic DNA from the filtration membranes and the pipe sections was extracted using the DNeasy® PowerWater® kit purchased from Qiagen (USA) per the manufacturer protocol. The quantification of DNA concentration was performed using Qubit 4 Fluorometer (Thermo Fisher Scientific, USA) and Qubit dsDNA High Sensitivity (HS) Assay Kit (detection range 0.01–100 ng/μL) (Thermo Fisher Scientific, USA). The total microbial communities in the extracted DNA samples were determined at KAUST Bioscience core laboratory (Saudi Arabia) by performing 16S rRNA gene-based high-throughput sequencing on the Illumina MiSeq platform following the protocol by the manufacturer (Illumina, USA). The input DNA was standardized prior to sequencing. All PCR (Polymerase chain reaction) details were according to the protocol (Illumina, USA). The forward [341F: CCTACGGGNGGCWGCAG] and reverse [805R: GACTACHVGGGTATCTAATCC] primers were selected to amplify the V3-V4 region of the 16S rRNA gene (Herlemann et al., 2011).

Bacterial community analysis was performed using ChunLab in-house EzBioCloud 16S-based microbiome taxonomic profiling (MTP) (Yoon et al., 2017). The reference 16S rRNA gene sequences were maintained as described in Kim et al. (2012). The quality of sequencing for PCR-derived sequences was checked manually by secondary-structure-aware alignment using the EzEditor program (Jeon et al., 2014). Maximum-likelihood phylogenetic trees of each taxonomic group, such as phyla, classes, orders or families, were generated from computationally aligned 16S rRNA gene sequences. All 16S rRNA gene sequences were assigned taxonomically (operational taxonomic unit, OTU-based assignment) to the species level as a part of the complete taxonomic hierarchy, which consisted of phylum, class, order, family, genus and species. 97% 16S similarity is used as the cutoff for species level identification. The average number of valid reads obtained from all the samples was 68,000 ± 22,100. Details can be found in Table S2 in supplementary material.

The bacterial alpha diversity was calculated using the Shannon–Weaver diversity index, H', with the following equation:

$$H' = -\sum_{n=1}^{n} pi^{*} \ln pi$$

where p_i is the proportion of each species in the sample.

The distribution-free statistical similarity test applied was the Wilcoxon rank-sum test also referred to as the Mann–Whitney U test; a nonparametric test of the null hypothesis that, for randomly selected values X and Y from two populations, the probability of X being greater than Y is equal to the probability of Y being greater than X (Yuan et al., 2006). The significance value was set at $P = 0.05$.

The sequences were compared for their Bray-Curtis similarities and represented graphically for spatial distribution in a multivariate statistics plot called Principal Coordinate Analysis (PCoA). Permutational Multivariate Analysis of Variance (PERMANOVA) was used to measure effect size and significance on beta diversity for the different water and biofilm (chlorinated/unchlorinated) sample groups. Beta diversity and Phylogenetic diversity measures are included in the PERMANOVA analysis. The significance is obtained by a permutation test. The level of significance was set at $p < 0.05$.

Submission of sequence reads for this study to DDBJ/ENA/GenBank nucleotide database is in progress.

3. Results

3.1. Microbial water quality monitoring from treatment to distribution

Microbial water quality was monitored using online FCM at five locations: two at the treatment plant and three locations throughout the existing distribution network. At the treatment plant, produced water before chlorination had a higher bacterial cell concentration than after chlorination (Fig. 2). Fig. 2A shows the flow cytometric fingerprint by plotting bacterial cell concentrations versus percentage of high nucleic acid (HNA) bacteria as previous studies showed that the activity of HNA bacteria is correlated to total cell activity. The HNA percentage together with the total cell concentration increased in the network locations. Before chlorination, the average bacterial cell concentration was 3.5×10^3 cell/mL, while chlorination reduced the concentration to below 10^3 cell/mL, the limit of detection (LOD) (Fig. 2B). Moreover, the box plot in Fig. 2B shows that the water bacterial cell concentration values leaving the treatment plant (after chlorine) were the most dispersed (25−75% range the widest). 50% of the concentration values were below the FCM detection limit, possibly resulting in the higher dispersion of values. A 0.5–1.5 log increase in bacterial cell concentration was observed at the network locations compared to the plant location directly after chlorination reaching a concentration of 4×10^4 cells/mL at network L3. The

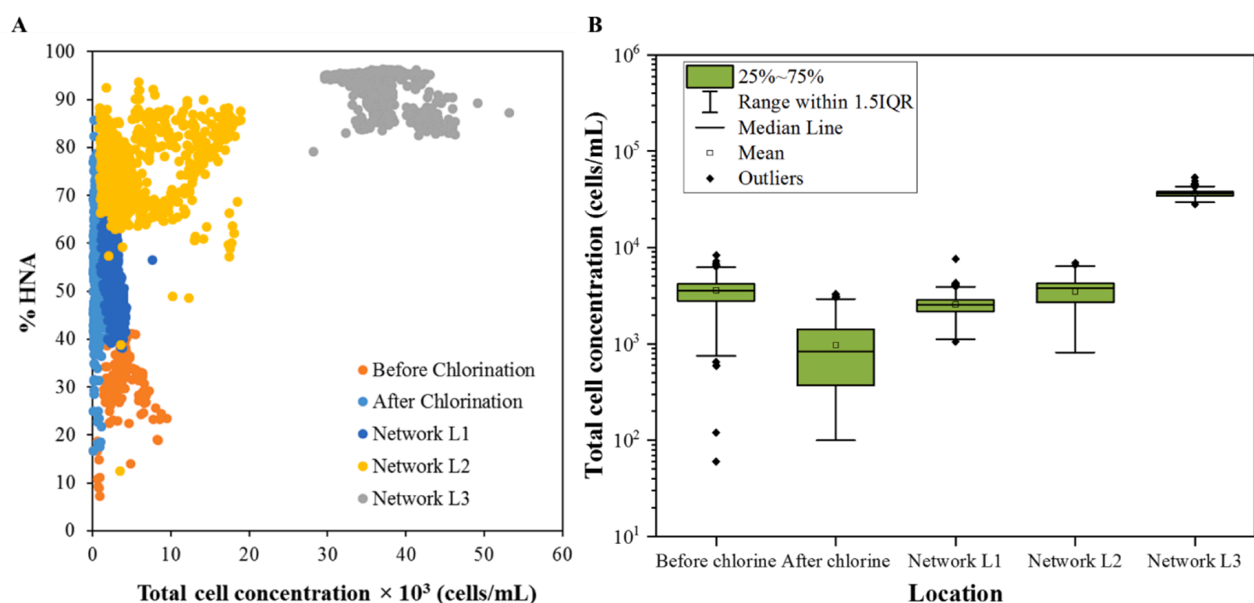

Fig. 2. Online monitoring of bacterial cell concentration for 1 week at five locations: before and after chlorination at the treatment plant and 3 locations in the distribution network. (A) Flow cytometric fingerprint: bacterial cell concentrations and percentage of high nucleic acid (HNA) bacteria (B) Box plot showing the distribution of the bacterial cell concentrations.

residual disinfectant was largely dissipated (Table S3).

The NMDS plot based on the flowCHIC analysis (Fig. 3) shows the samples after chlorination clustered closely together, signifying their similarity. The samples before chlorination appear as a completely different and wider cluster with higher variability. At the three network locations, three bacterial clusters can be distinguished. These clusters at the network locations appear closer to the after-chlorination samples signaling the similarity. However, the samples from the three network locations are more scattered than the after chlorination samples at the treatment plant, which indicates shifts in bacterial characteristics (size, DNA content) and occurrence of a much more diverse bacterial community (wider clusters). These data show that the presence of disinfectant residual did not prevent bacterial changes during distribution (Fig. 3) and as seen in Fig. 2 bacterial regrowth occurred.

3.2. Miniature network studies

Using the FCM monitoring of water from treatment to distribution we could identify changes in the microbial water quality (Figs. 2 and 3). However, assessing the surface of pipes within the operative distribution network to investigate the existing biofilm was difficult, raising the interest in conducting simulative network distribution studies. Moreover, since we could only evaluate the microbial dynamics with the presence of chlorine, the miniature distribution networks were initiated to understand what would be the impact of chlorine presence and absence on the temporal and spatial dynamics of the microbial water quality and community.

3.2.1. Microbial water quality monitoring

For six months, two miniature networks were fed with unchlorinated and chlorinated water produced by RO desalination. Water quality, using ATP, and TCC was assessed at the miniature network inlet and outlet locations (Fig. 4). The miniature network fed with unchlorinated water showed an increase in ATP and TCC in the outlet water after three and six months of operation (Fig. 4A, B). The TCC concentration in the outlet water, after three months of operation, was two times higher the concentration at the inlet (1.2×10^4 cells/mL, inlet and 2.5×10^4 cells/mL in the outlet) (*t*-test, $p = 0.021$) (Fig. 4A). A similar trend was observed for the ATP concentration, which was 2.3 times higher at the outlet (1.5 pg/mL) compared to the inlet (0.7 pg/mL) (*t*-test, $p = 0.035$) (Fig. 4B). At six months of operation, the outlet cell concentration (4.5×10^4 cells/mL) was one log higher than that of the inlet (4.4×10^3 cells/mL) (*t*-test, $p = 0.0002$). However, the ATP increase observed at six months, although significant (*t*-test, $p = 0.006$), was not in proportion to the increase in TCC. The ATP in the outlet water (0.8 pg/mL) was only 2.7 times higher than that of the inlet (0.3 pg/mL). This would signal increased biofilm growth inside the network, with less active cells sloughing out of the biofilm and ending up in the outlet water. The decline in TCC and ATP concentration of the feed water observed at six months compared to three months is a seasonal variation (Fig. 4A,B). The study was initiated in April, and the three and six-month sampling occurred in July and October, respectively.

The miniature network fed with chlorinated water did not show any increasing trend (*t*-test, all $P > 0.05$, Table S4) between inlet and outlet or any temporal increase in TCC or ATP (Fig. 4A,B). There was no difference in chlorine concentration between the inlet and outlet, justifying the observed trend.

3.2.2. Biofilm characterization

In addition to microbial water quality, biofilm development in both miniature networks was investigated. Attached bacteria inside distribution pipes are momentous as biofilms on the inside of the distribution network pipes have been associated with a higher risk of opportunistic pathogens (Van der Kooij et al., 2017).

Biofilm development inside the pipes of the miniature network was characterized by TCC and ATP concentration. For the miniature network fed with produced water before chlorination, a significant concentration of bacterial cells (2.3×10^6 cells/cm^2) was found in the biofilm developing at the inlet after three months of operation (Fig. 5A). The biofilm developing at the outlet had less TCC (0.9×10^6 cells/cm^2) than the inlet, but the difference was not significant (*t*-test, $p = 0.085$). The biofilm ATP concentration showed a similar trend to the TCC between the inlet (135 pg/cm^2) and outlet (103 pg/cm^2), however the difference between the two concentrations was significant (*t*-test, $p = 0.001$). Comparing the biofilm after 6 months to the biofilm after 3 months of operation, almost 1 log increase in the biofilm TCC was observed at the

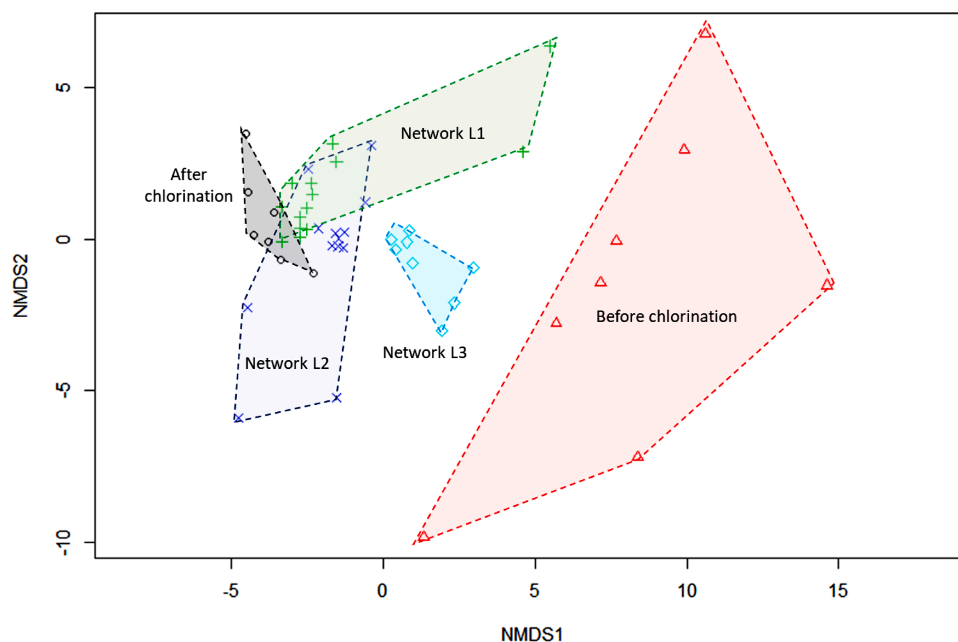

Fig. 3. Nonmetric multidimensional scaling (nMDS) based on flowCHIC image analysis of forward scatter (FSC) versus fluorescence signal plots. Five locations are compared: DWTP before and after chlorination and in the network, respectively, demonstrating mainly dissimilar microbial clusters in samples before and after chlorination and higher diversity before chlorination.

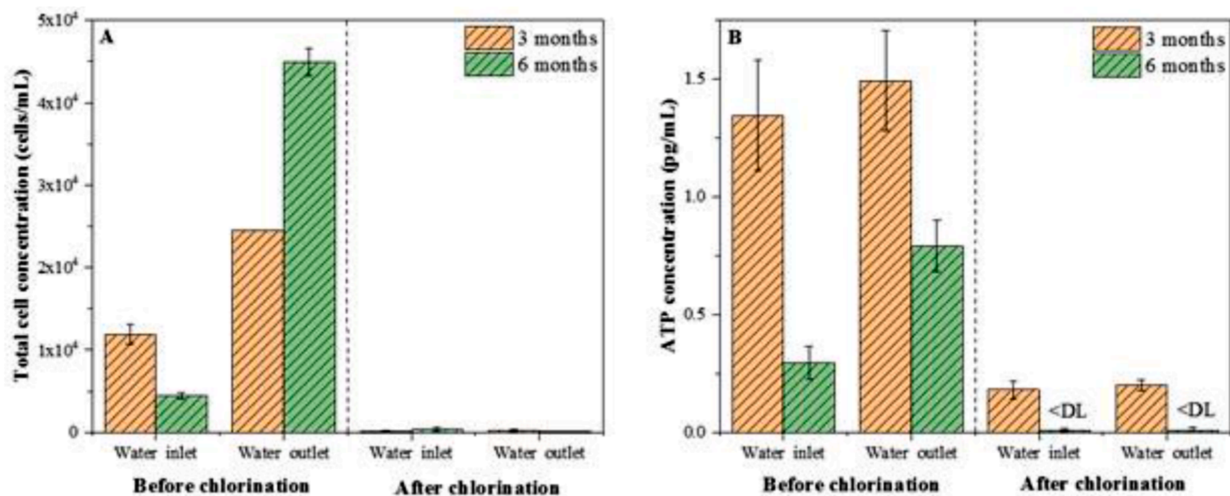

Fig. 4. (A) Total cell concentration (cells/mL) and (B) Adenosine triphosphate (ATP) concentration for the water entering and leaving the miniature networks after 3 and 6 months of operation. Two miniature distribution networks were operated, one before produced water chlorination and one after water chlorination.

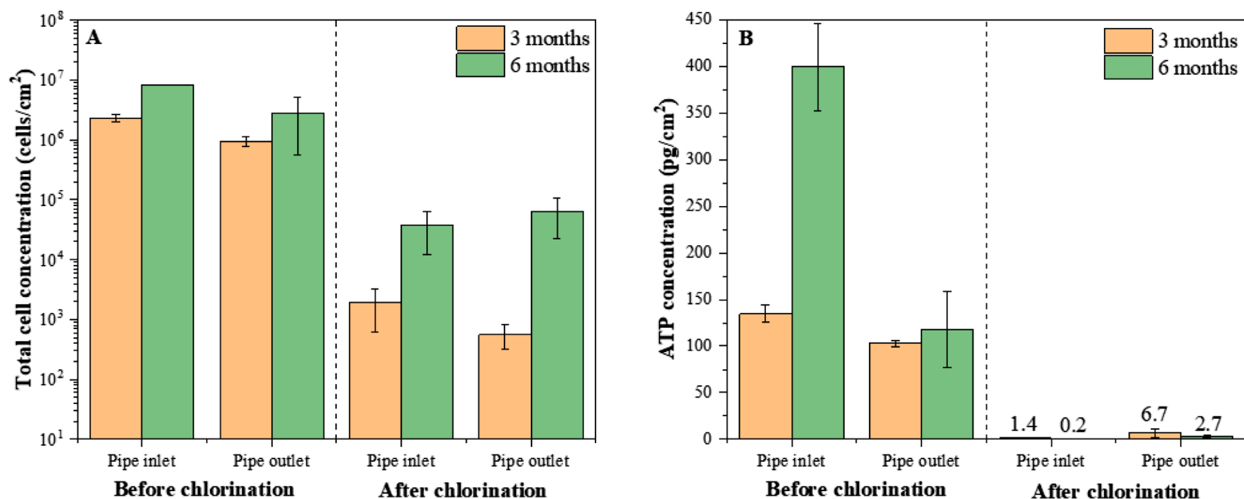

Fig. 5. (A) Total cell concentration (cells/mL) and (B) Adenosine triphosphate (ATP) concentration quantified from 4 cm pipe sections from the inlet and outlet of the miniature networks after 3 and 6 months of operation. Two miniature distribution networks were operated, one before produced water chlorination and one after water chlorination.

inlet (t-test, $p = 0.0096$) with a significant ATP concentration increase (t-test, $p = 0.001$) (Fig. 5A,B). At the outlet, only the biofilm TCC increased in time (6 months compared to 3 months), but the increase was only double (t-test, $p = 0.028$), while ATP concentration remained similar to what it was three months before (t-test, $p = 0.257$). The higher increase in TCC and ATP at the inlet may be explained by higher availability of substrate in the water at the inlet compared to the outlet.

For the miniature network fed with chlorinated water, an increasing TCC in the biofilm was observed in time at both the inlet and outlet of the network (t-test, $p > 0.05$, Table S5). However, the biofilm TCC was 2–3 logs lower than when chlorine was absent (t-test, $p < 0.05$). No distinction could be made between the biofilm at the inlet and outlet as the growth was suppressed by the chlorine presence. The negligible ATP concentration at both the inlet and outlet (Fig. 5B) indicates that the quantified TCC concentration is mainly composed of inactive or dead bacterial cells. This was confirmed with intact cell concentration measurements of the chlorinated water that were all below the LOD (10^3 cells/mL) of the FCM with an average of 100 ± 40 cells/mL.

3.3. Bacterial community composition

Analysis was performed on the DNA extracted from 20 L water samples (in triplicates) at each location and biofilm samples. Water samples were taken at the actual distribution network (location 3) and at the inlet and outlet of the two miniature networks fed with non-chlorinated and chlorinated RO-produced water. DNA was also extracted from the biofilm that developed in both miniature networks.

Principal Coordinate Analysis (PCoA), for measuring beta diversity, is an ordination method that aims to arrange ecological communities along gradients. PCoA was performed on the biofilm and the unchlorinated and chlorinated water in and out of the miniature network and water samples at network location 3. The bacterial community in network L3 was closely clustered to the bacterial community found in the water, leaving the miniature network (PERMANOVA, $p = 0.10$, not significant (N.S.)) (Fig. 6). The chlorinated water feeding the miniature network and the biofilm community that developed inside the miniature network were clustered separately, signifying their differences (PERMANOVA, $p = 0.033$). The water bacterial community leaving the miniature network was clearly impacted by the biofilm developing inside the network. This highlights the importance of the biofilm

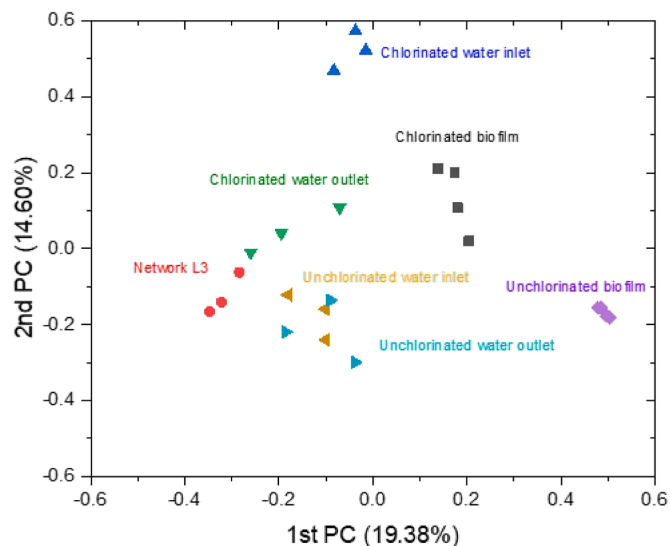

Fig. 6. Principal coordinate analysis (PCoA) based on the Bray-Curtis distance metric. Each point represents the microbial community in a specific sample. The analyzed samples include the unchlorinated and chlorinated water in and out of the miniature network, the biofilm that developed in the unchlorinated and chlorinated miniature network and water samples from the actual distribution network (network L3). Distance between the sample dots signifies similarity; the closer the samples are, the more similar microbial composition they have.

developing in the distribution network in shaping the water received at the tap. As for the unchlorinated water, feeding and leaving the miniature network, the two clusters were quite undistinguishable (very close and overlapping with each other, PERMANOVA, $p = 0.10$, N.S.) signaling their similarity, while the biofilm was farther away, thus, quite different (PERMANOVA, $p = 0.026$). The significance values between the different water and biofilm (chlorinated/unchlorinated) sample groups based on the PERMANOVA analysis are shown in Table S6 in supplementary material.

3.3.1. Chlorinated and non-chlorinated RO-produced drinking water

The RO-produced water feeding the actual distribution network was also used to feed the chlorinated miniature network. The chlorinated RO-produced water was dominated, at the phylum level, by *Proteobacteria* (90.4%), with the presence of *Firmicutes* (2.8%) and *Actinobacteria* (2.5%) (Fig. 7A). At the class level, the chlorinated water had a high abundance of *Alphaproteobacteria* (76.3%), *Gammaproteobacteria* (11.9%), *Betaproteobacteria* (1.8%), and the presence of *Bacilli* (2.3%) and Actinobacteria_c (2.4%) classes. *Blastomonas natatoria*, a mesophilic bacterium, dominated the species present in the chlorinated water (65.1%) (Fig. S3, supplementary material). The second two species in relative abundance were *Pseudomonas stutzeri group* (9.5%) and *Erythrobacter citreus* sp. *(4.5%)*. Compared to the chlorinated RO-produced water feeding the miniature network, both the chlorinated water leaving the miniature network and the water at network location 3 had a lower abundance of *Proteobacteria* (54.5% and 64.5%, respectively) and a higher abundance of *Firmicutes* (39.5% and 29.5%, respectively) (Fig. 7A). At the class level, *Gammaproteobacteria* (47.5%) abundance increased the most at network L3. *Pseudomonas aeruginosa* (29.9%), from the *Gammaproteobacteria* class was found present in the network location 3 (Fig. S3). Comparing the water bacterial community before chlorination with the community after chlorination, the water samples before chlorination, had less *Proteobacteria* (81.3%) and more *Firmicutes* (14.0%) with a low relative abundance of *Actinobacteria* (2.0%) and *Acidobacteria* (1.4%) (Fig. 7A). At the class level, the non-chlorinated water had a lower abundance of *Alphaproteobacteria* (34.1%) compared to 76.2% in the chlorinated water, while classes such as *Gammaproteobacteria* (34.6%) and *Betaproteobacteria* (11.0%) increased in abundance. The most abundant species in the unchlorinated water were the *Sphingopyxis macrogoltabida group* (29.2%), an oligotrophic bacteria and *Reyranella_uc* (12.0%). The following two most abundant species were *Pseudomonas aeruginosa group* (7.1%) and *Anaerobacillus AY642552_s* (5.6%) (Fig. S4). Bacterial community changes were seen in the unchlorinated water leaving the miniature network compared to that feeding the network. At the phylum level, the unchlorinated water leaving the miniature network had less *Proteobacteria* (68.6%) and more *Firmicutes* (24.50%) compared to the water feeding the network (Fig. 7A). At the class level, a higher abundance of *Alphaproteobacteria* (46.9% compared to 34.1%) and *Bacilli* (24.4% compared to 14.0%); while classes such as *Gammaproteobacteria* (14.2%) and *Betaproteobacteria* (7.3%) decreased in abundance (Fig. 7B). Although differences existed between the unchlorinated water feeding and leaving the miniature network, the PCoA (Fig. 6) showed the sample clusters close to each other, signifying their similarities.

3.3.2. Biofilm bacterial community with and without chlorination

The biofilm community was different from that in the water at the phylum level, although *Proteobacteria* dominated all samples. Compared to the unchlorinated water, the relative abundance of *Proteobacteria* decreased in the biofilm developing in the miniature network fed with water before chlorination (from 81.2% to 59.3%) (Fig. 7A). Phylum such as *Saccharibacteria* (13.6%), *Planctomycetes* (11.9%), and *Bacteroidetes* (1.4%) emerged in the biofilm community while phyla such as *Acidobacteria* (5.0%) and *Actinobacteria* (4.6%) increased in abundance. The Shannon phylogenetic alpha diversity index of the biofilm (3.90) was higher than that of the water (3.64), but the difference was not significant (U test, $p = 0.064$). At the class level, *Alphaproteobacteria, Betaproteobacteria* and *Deltaproteobacteria* increased in relative abundance in the biofilm (36.6%, 15.0%, and 2.3%, respectively). In contrast, the abundance of *Gammaproteobacteria* decreased (from 34.6% to 4.2% in the biofilm). In comparison, *Blastocatellia* class had a higher relative abundance in the biofilm compared to the water. *Blastocatellia* was in a very low relative abundance in the feed water and rather emerged in the biofilm.

The biofilm that developed in the miniature network fed with chlorinated water was distinct in the high relative abundance of *Firmicutes* (28.0%) at the phylum level and *Bacilli* (25.7%) at the class level. The Shannon phylogenetic alpha diversity index of the biofilm (4.29) was significantly higher than that of the chlorinated water (1.87) (U test, $p = 0.034$). Biofilms that developed in the two miniature networks were clearly different. More biofilm developed in the network fed with unchlorinated water.

4. Discussion

4.1. Water quality changes for chlorinated RO produced drinking water during distribution

The uniqueness of the water distributed in this network is the extremely low bacterial cell count and substrate concentration at the distribution point (Table S1). The average total bacterial cell concentration leaving the treatment plant was 1×10^3 cell/mL and, in many cases, even lower (Fig. 2). The detection limit for total cell concentration was 1×10^3 cells/mL as determined in another study by us (Putri et al., 2021) and Ziemba et al. (2019). While having a low bacterial cell concentration in the water is foreseen as positive for biological stability, the consequence is that this creates a niche for microorganisms to occupy downstream of the treatment process (Nescerecka et al., 2014) as seen in this distribution network (Figs. 2, 6, 7 and S3). The particularly low cell concentration and chlorine residual (0.5 mg/L) did not prevent bacterial regrowth, reaching 2×10^4 cells/mL in some locations (Fig. 2). Quantifying the assimilable organic carbon concentration (AOC) from the growth potential of the produced water (Hammes and Egli, 2005)

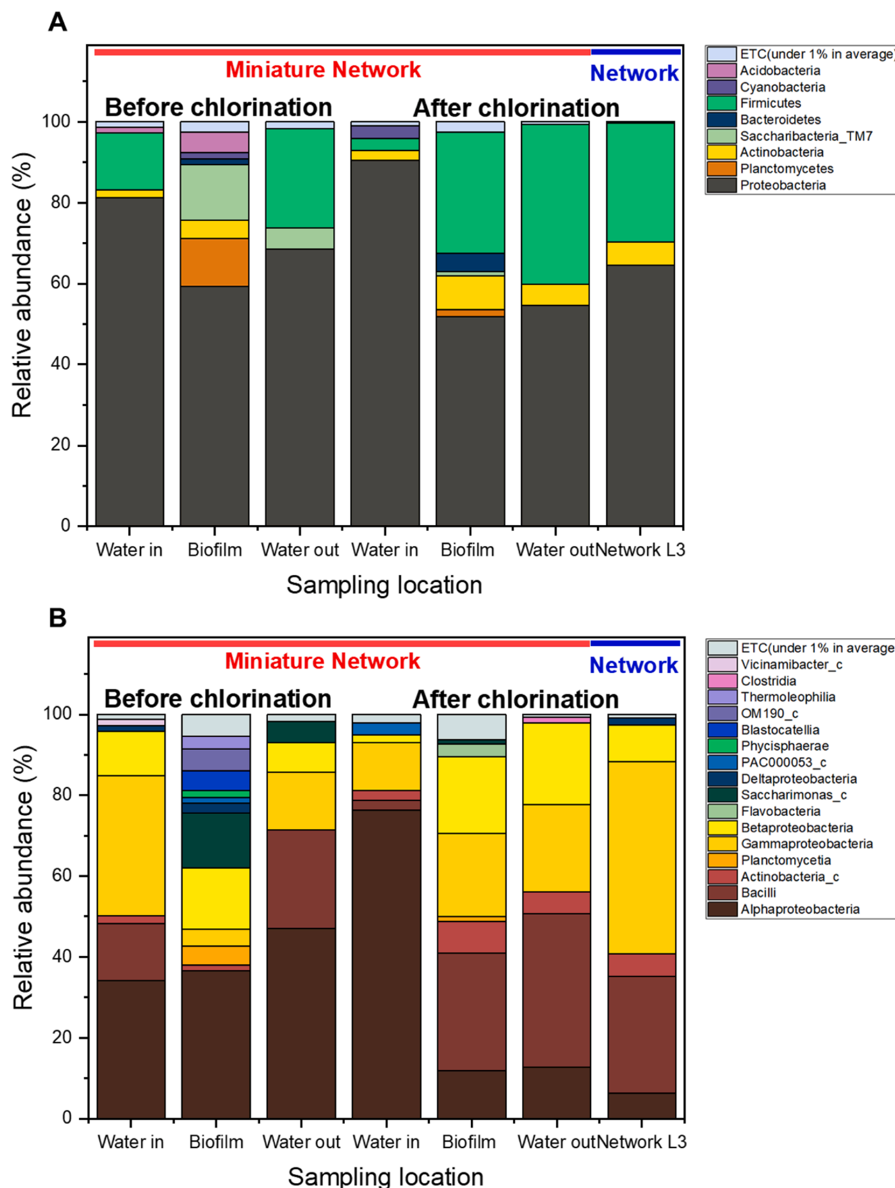

Fig. 7. Taxonomic distribution of bacterial community at the (A) phylum level and (B) class level in the RO produced drinking water before and after chlorination feeding the miniature networks, the biofilm developing in the unchlorinated and chlorinated miniature networks, and water from the actual distribution network (network L3). The y-axis indicates the percentage of total sequences corresponding to relative abundance. "ETC<1″ contains bacterial phyla and class of less than 1% abundance in all samples.

reveals an AOC concentration of 5–10 μg-C/L (Table S1). Such an AOC concentration, derived from a growth potential of 10^4 cells/mL, can cause this amount of biofilm regrowth once chlorine gets reduced or dissipated. Keeping in mind that the cell concentration does not directly link to human health (Prest et al., 2016), and many regions receiving chlorinated water have cell concentrations feeding the network in the range of 10^5 cells/mL (Nescerecka et al., 2014). However, more biofilm on the inside of the pipes is anticipated at higher cell concentrations leading to a higher risk for consumer complaints due to water aesthetics and higher risk of occurrence of opportunistic pathogens (Pavlov et al., 2004; Van der Kooij et al., 2017; Kotlarz et al., 2019). Accessing the health risks of the water should be confirmed using the internationally accepted culture-based approaches (HPC and MPN) and indeed the viability tests (intact cell concentration, q-pcr). However, the limitations of HPC analysis, including the long incubation time required, variation in test results depending on experimental conditions, and detection of only a minute fraction of the total number of viable bacteria have been stated in previous studies (Van Nevel et al., 2017; Cheswick et al., 2019). In a previous study at the same distribution network (Farhat et al., 2020), we compared flow cytometry data with the conventional water quality detection methods (HPC and total coliforms). HPC and total coliforms were constantly below the detection limits, while the FCM provided detectable total cell count data and enabled quantifying changes in the drinking water. Therefore, the direct bacterial quantification method, FCM, has been applied in this study for the drinking water samples to overcome the limitations of conventional bacterial culture-dependent methods (Van Nevel et al., 2013). The inclusion of ATP concentration measurements gives an indication on the activity of the cells measured through FCM.

The variation in the increase in bacterial cell concentration seen in this network resulted from differences in the proximity of sampling locations to the treatment plant, size, and building use. Chlorine decay contributed to bacterial growth and the relatively high water temperature (30 °C) accelerated the chlorine decay (Hua et al., 1999) and bacterial growth compared to DWDS with chlorine at lower water temperature. A decay from 0.5 mg/L to less than 0.05 mg/L was observed in locations over the network. The significant role that temperature plays in these regions cannot be overlooked as higher temperatures facilitate biofilm development and select for certain microorganisms, including pathogens (Delpla et al., 2009; Nescerecka et al., 2014; Bondank et al., 2018). In addition to temperature, building sizes, premise piping lengths, and water usage dictated the chlorine

decay. Samples taken in large buildings with longer premise piping lengths, and more stagnant segments, show lower chlorine concentrations and higher cell concentrations (Bédard et al., 2018; Proctor et al., 2020).

4.2. Chlorination selects against most bacterial groups and creates a niche for microorganisms to occupy downstream

16S rRNA genes sequencing of water and biofilm samples before and after chlorination revealed taxonomically diverse bacterial communities. The diversity of planktonic bacteria declined from 3.64 in the unchlorinated RO-produced water to 1.87 after chlorination (Shannon phylogenetic alpha diversity index). The Shannon alpha diversity index for chlorinated water was lower than values reported in literature for chlorinated water produced by conventional treatment methods (Zhang et al., 2021) and significantly lower than diversity indices reported for non-chlorinated water (Ahmad et al., 2020). This can be attributed to the water production process where Belila et al. (2016) reported that the diversity decreased during the seawater desalination process.

Bacterial community analysis for unchlorinated water and biofilm revealed the abundance of mesophilic bacteria with higher temperature tolerance and bacteria associated with nutrient-poor oligotrophic conditions (Fig. 7, S4,S5). For example, species of the family *Blastocatellaceae* were found to tolerate temperature values of 8–45 °C (optimal growth at 33–40 °C) and exhibit a chemoorganotrophic growth on several substrates with species able to use nitrate as an alternative electron acceptor in the absence of oxygen (Huber et al., 2017; Hu et al., 2019). The relative abundance of *Blastocatellaceae* was shown to be higher in substrate-limited conditions, where Ivanova et al. (2020) showed a negative correlation of relative abundance of *Blastocatellia* with carbon and nitrogen availability. Most of the biofilm bacteria were characterized as non-motile, therefore their strong adherence to surfaces and biofilm-forming ability. The Shannon phylogenetic alpha diversity index of the unchlorinated biofilm was higher than that of the water (3.9), but the difference was not significant ($p = 0.064$). No indication of the occurrence of opportunistic pathogens was observed at the species level (Fig. S6), although the bacterial cell concentration increased and biofilm development inside the miniature network was high ($\approx 10^7$cells/cm^2, $\approx$260 pg-ATP/cm^2). However, opportunistic pathogen occurrence should be confirmed with qPCR targeting opportunistic pathogen specific genes or through culture based techniques (van der Kooij et al., 2018).

The chlorinated water leaving the treatment plant was dominated by *Alphaproteobacteria* (76.5%, Fig. 7B), in agreement with previous findings showing *Alphaproteobacteria* to be the dominant group in treated water samples due to their capacity to survive in low nutrient and chlorinated environments (Chao et al., 2013). Some members of *Alphaproteobacteria*, have been found to commonly contain glutathione biosynthesis genes (Chao et al., 2013). Glutathione has been proven to directly increase bacterial resistance to chlorine compounds (Chesney et al., 1996), therefore possibly explaining the abundance in chlorinated water. Moreover, it has been found that starvation noticeably stimulates glutathione synthesis and subsequently enhances bacterial chlorine resistance (Saby et al., 1999). The RO treated water contains limited nutrient concentrations that might induce starvation conditions (Sousi et al., 2020, 2021). The alpha diversity of chlorinated water was the lowest (1.87) where a single species (*Blastomonas natatoria*, a mesophilic bacterium, known to survive disinfection regimes (Sylvie et al., 2010)) dominated the species present in the chlorinated water (65.1%) (Fig. S3).

As for the water samples leaving the chlorinated miniature network and water samples at network L3 (Fig. 7B), the *Alphaproteobacteria* abundance decreased drastically in favor of *Gammaproteobacteria* and *Betaproteobacteria.* This is consistent with studies showing that *Betaproteobacteria* and *Gammaproteobacteria* were observed more frequently in water with low disinfectant residuals (El-Chakhtoura et al., 2015). The class *Bacilli* class from the phylum *Firmicutes* also increased significantly at these two locations compared to original produced chlorinated water. The PCoA plot (Fig. 6) shows that the water at network L3 and water leaving the chlorinated miniature network is closely clustered and at a middle distance from the chlorinated produced water and from the biofilm. We can see that the water leaving the chlorinated miniature network and at network L3 is impacted by the original bacterial community in the produced chlorinated water and the biofilm community that is developing in the pipes that is distinctly different and more diverse (Shannon phylogenetic alpha diversity index = 4.29, $P = 0.034$). The water flow in the network leads to the detachment of cells from biofilms hence their manifestation in the water phase. In addition, changes in conditions (chlorine concentration, temperature, stagnation, etc.) during distribution induce changes in the bacterial community.

The biofilm that developed in the chlorinated miniature network contained several species identified as opportunistic pathogens (more than 21% of the relative abundance of the species present). These species included *Streptococcus salivarius (7.8%), Staphylococcus aureus (4.2%), Pseudomonas synxantha (2.4%), Streptococcus pneumonia (2.4%), Pseudomonas veronii (2.6%)*, and *Pseudomonas aeruginosa (1.5%)* (Fig. S3). The water bacterial community at network location 3 as well contained several species classified as opportunistic pathogens capable of infecting immunocompromised patients and, in some cases, the immunocompetent population. These opportunistic pathogenic species included *Pseudomonas aeruginosa group* (29.9%) (Diggle and Whiteley, 2020), *Ralstonia insidiosa* (3.4%) (Ryan and Adley, 2013), *Delftia acidovorans* (1.9%) (Bilgin et al., 2015), and *Herbaspirillum huttiense group* (1.5%) (Liu et al., 2019). These results confirm that chlorination limits bacterial abundance in produced water, lowering the bacterial cell count and activity. Chlorination selects against most groups of bacteria, influences some more than others depending on concentration and contact time; what remains are the chlorine resistant strains. Chlorination creates a niche for bacteria to occupy during distribution once the conditions vary. However, opportunistic pathogen occurrence or absence should be confirmed with qPCR targeting opportunistic pathogen specific genes or through culture based techniques (van der Kooij et al., 2018). Without such data, no conclusive evidence of an actual risk can be justified.

4.3. Significance of results from simulative miniature distribution networks

Access to the water before chlorination enabled simulation and evaluation of the consequences of distributing high-quality water without residual disinfection, particularly in regions with elevated water temperatures. The use of miniature network also enabled studying the biofilms developing on the inside of the miniature network pipes and comparing the bacterial community to that in the water samples. Investigating the biofilms developing on pipes is important; and this is mostly excluded in many studies as accessing the surface of pipes within operative networks is difficult. However, it is worth to mention that recently many research groups acknowledged the importance of studying biofilms in DWDS despite the complexity and major efforts to understand biofilms has been reported (Gomez-Smith et al., 2015; Douterelo et al., 2018a; Waak et al., 2018, 2019a, 2019b; van der Kooij et al., 2020; Ahmad et al., 2021; Calero Preciado et al., 2021). The use of a miniature network also enabled the comparison between the bacterial communities in the water, leaving the miniature network with the water in the actual distribution network. Miniature pilot distribution networks have been applied in previous studies (Liu et al., 2013; Álvarez-Arroyo et al., 2015; Abokifa et al., 2016; Brester et al., 2020; Zhou et al., 2020). Liu et al. (2013), for example, evaluated the impact of various treatment schemes on water quality during distribution using miniature networks. The miniature network fed with water before chlorination had more biofilm development, 2–3 log higher TCC and ATP. After six months of operation, the biofilm ATP concentration was 4×10^2 pg/cm^2 (Fig. 5)

and would definitely increase in time. Van der Kooij et al. (2017) determined in their study a threshold concentration of approximately 50 pg ATP/cm^2 for the growth of *Legionella pneumophila* in biofilm. Nevertheless, in this study, the biofilm that developed in the miniature before chlorination, although much more in amount, did not show the presence of opportunistic pathogenic species. Absence of opportunistic pathogens shall be confirmed in future study with cultivation based methods or q-PCR. Excessive bacterial and biofilm growth is not desirable as it can lead to deterioration of the esthetic water quality even if no pathogens exist to pose a human health risk.

Results from this study showed that chlorine limited bacterial growth and biofilm development in the miniature network fed with chlorinated water, minimizing the changes in water quality that could have been observed if unchlorinated water was distributed. On the other hand, chlorine selected against most bacterial groups and the remaining bacterial community were chlorine resistance and opportunistic pathogen species (Figs. 6 and S3). This observation was also seen in the water samples post chlorination at the actual distribution network (Network L3). However, the very low ATP activity and intact bacterial cell concentration signals to no potential risk, which can only be concretely eliminated through pathogen cultivation based methods or q-PCR.

Results from this study indicate that in such conditions, water quality parameters such as (e.g., total cell concentration, HPC) should not be the only risk eliminator, and several checks and barriers such as direct quantification of pathogenic species should be employed to distribute safe water to the end-users.

5. Conclusion

This study evaluated the water quality in a full-scale distribution network supplied with chlorinated RO-produced drinking water. In addition, a simulative study involving miniature distribution networks was conducted to assess whether chlorination was necessary to distribute biostable water. This study distinctively investigated a combination of bulk water samples feeding and leaving the miniature networks under both chlorinated and non-chlorinated conditions, as well as biofilm samples from the miniature network pipes over six months.

The main findings of the study can be summarized by:

- The water leaving the plant had an average cell concentration of 10^3 cells/mL.
- A 0.5–1.5 log increase in bacterial cell concentration was observed at the network locations.
- Chlorine was largely dissipated in the full-scale network from 0.5 mg/L at the treatment plant to less than 0.1 mg/L in the network.
- The low bacterial cell concentration and organic carbon concentration in the RO-produced water did not prevent biofilm development inside the miniature network fed with unchlorinated water.
- The biofilm in the unchlorinated miniature network was dominated by mesophilic bacteria, with higher temperature tolerance and bacteria-associated nutrient-poor conditions.
- Chlorination selected against most bacterial groups and the remaining bacterial community was the one capable of surviving disinfection regimes.
- Biofilms that developed in the presence of chlorine contained species classified as opportunistic pathogens affecting the water bacterial community; the actual risk of this encounter can be only be eliminated through pathogen cultivation based methods or q-PCR.
- High-quality water cannot be employed as the only barrier to ensure water safety, especially in high-temperature regions, therefore, chlorination remains necessary in such circumstances.

Declaration of Competing Interest

None.

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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.2021.117975.

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