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DOI

[10.1038/s41545-020-0065-7](https://doi.org/10.1038/s41545-020-0065-7)

Publication date

2020

Document Version

Final published version

Published in

npj Clean Water

Citation (APA)

Farhat, N., Kim, L. H., & Vrouwenvelder, J. S. (2020). Online characterization of bacterial processes in drinking water systems. *npj Clean Water*, 3(1), Article 16. <https://doi.org/10.1038/s41545-020-0065-7>

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ARTICLE OPEN



Online characterization of bacterial processes in drinking water systems

Nadia Farhat^{1,3}, Lan Hee Kim^{1,3}  and Johannes S. Vrouwenvelder^{1,2}

The use of traditional drinking water microbial quality monitoring methods, including heterotrophic plate counts (HPCs) and total coliform counts, are not only laborious and time-consuming but also do not readily allow identification of risk areas in the network. Furthermore, if areas of concern are identified, and mitigation measures are taken, it takes days before the effectiveness of these measures is known. This study identified flow cytometry (FCM) as an online sensor technology for bacterial water quality monitoring in the distribution network. We monitored the total bacterial cell numbers and biodiversity in a drinking water distribution system (DWDS) using an online FCM. Two parallel online FCM monitoring systems were installed on two different locations at a drinking water treatment plant (DWTP; Saudi Arabia) supplying chlorinated water to the distribution and in the network 3.6 km away from the DWTP. The FCMs were operated at the same time in parallel to assess the biological stability in DWDSs. The flow cytometric data was compared 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 the quantification of changes in the drinking water both with time and during distribution. Results demonstrate the value of FCM as a tool for compliance monitoring and risk assessment of DWDSs.

npj Clean Water (2020)3:16; <https://doi.org/10.1038/s41545-020-0065-7>

INTRODUCTION

Safe water supply is essential to public health and plays a critical role in human well-being as well as the success of our society and economy. Drinking water quality guidelines protect public health by applying limits on concentrations of contaminants in drinking water. Bacterial growth in drinking water distribution systems (DWDSs) can lead to failure in meeting water quality guidelines, deterioration of the aesthetics of the water, and might be linked with an increased risk of diseases¹. The microbial water quality in DWDSs can deteriorate if a suitable growth environment exists². An increase in bacterial cell number or the amount of active biomass in water is caused by the degradation of biodegradable nutrients originally present in the water leaving the treatment plant and/or nutrients leaching from materials in contact with water such as pipes and reservoirs in the distribution network³. Note that the presence of bacteria on its own is not a health risk indicator, e.g., numbers of bacteria (10^3 – 10^6 cells/mL) in drinking water does not link to human health⁴. However, with increasing bacterial cell numbers in the drinking water during distribution, (i) more biofilm on the inside of the pipes^{5,6}, and (ii) a larger chance of elevated numbers of higher organisms such as aquatic sow bugs (e.g., Asellidae, 1–12 mm) and oligochaete worms⁶, a (iii) higher risk of occurrence of *Aeromonas*⁵, total coliforms⁷, *Escherichia coli*⁸, and opportunistic pathogens such as *Pseudomonas* sp.⁹ and *NTB mycobacteria*¹⁰, as well as *Legionella pneumophila*¹¹ will occur in the network. Moreover, there is higher risk for consumer complaints (e.g., brown water, smell) and higher costs (e.g., corrosion, misreading of water meters). In other words, pursuing the distribution of biological stable water “with no or limited microbial growth” will reduce or prevent microbial problems in the network and reduce consumer complaints and maintenance cost.

Bacterial growth in the DWDSs is attributed to numerous aspects, including the amount of nutrient present for growth (e.g., biodegradable dissolved organic carbon), oxidant residuals, presence of predators, presence of corrosion, and operational conditions (e.g., feed water temperature and flow regimes)^{4,12}. Bacterial growth in DWDS can be controlled by adding a disinfectant such as chlorine, chlorine dioxide, and monochloramine before distribution of drinking water in concentrations that would maintain a disinfectant residual in DWDS or by limiting growth-promoting nutrients in the water^{4,13}. Despite disinfection, bacteria can still grow in DWDSs systems with low-nutrient environment and residual disinfectant¹⁴; therefore, monitoring of microbial quality is required.

To monitor microbial density in drinking water, many analytical methods have been introduced and utilized, including (i) cultivation-dependent heterotrophic plate count (HPC, selective media) and (ii) cultivation-independent cell counting such as flow cytometry (FCM) and microscopic counts and (iii) molecular methods such as quantitative polymerase chain reaction (qPCR), viable qPCR and droplet digital PCR (ddPCR)¹⁵. Cultivation-dependent quantification methods and parameters to measure bacterial presence and growth remain the primary implemented compliance parameters for drinking water monitoring in mostly all legislation around the world¹⁶. Plate counting (i.e., HPC) and coliforms being the most applied methods that were proposed over 100 years ago^{15,17,18}. However, the World Health Organization (WHO) acknowledges that HPC does not link to the health risk in people who are already healthy^{19,20}, but HPC bacteria may pose a higher risk for the immunocompromised²¹. HPC only detects a fraction of total bacteria that can grow on the medium (<1% of bacteria) not including viable, but non-culturable bacteria (VBNC)^{22,23}. Also, the HPC and total coliform quantification requires 24–48 h to obtain the results, thus preventing early detection of the risks and timely implementation of

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corrective measures before water distribution²⁴. Increased insights on bacterial presence and growth can be gained through a combination of several cultivation-independent methods^{25–27}, including direct (label-free) biosensors such as optical (e.g., optical interferometry, surface plasmon resonance)²⁷, bioluminescent (e.g., adenosine triphosphate (ATP) detection)^{13,28–30}, piezoelectric²⁵, electric impedance sensors²⁵, indirect (labeled) biosensors such as fluorescent labels (e.g., FCM)^{31–33} and microbial metabolism based electrochemical sensors²⁵.

Online microbial quality monitoring is important for an early warning system, enabling potential risk identification to enforce measures protecting the end-user²⁴. The automated systems for online monitoring of bacterial density such as FCM^{34–36}, ATP analyzer³⁰, and optical sensors based on three-dimensional (3D) image recognition²³ have been employed for drinking water. FCM is a bacterial cell-counting tool that incorporates deoxyribonucleic acid (DNA) staining protocols for the assessment and evaluation of bacterial water quality. The FCM has been widely used to characterize aquatic microbial ecosystems (e.g., seawater, freshwater, wastewater, drinking water) by measuring (i) total cell numbers of both prokaryotic and eukaryotic cells, (ii) cell viability, and (iii) microbial metabolic activity^{36,37}.

Online FCM with an automatic system for sampling, staining, and cleaning has quickly gained attention and consideration throughout the water sector over the past years to quantify both total and intact bacterial cell counts^{22,36,38–40}. The real-time monitoring of bacterial cell numbers through online FCM can be helpful to indicate locations of concern throughout the distribution network. Detection of bacterial growth with FCM measurement at locations with a depleted residual disinfectant can reveal a location of possible concern. Besides, the flow cytometric results from the online FCM can be used to rapidly determine the biodiversity using flow cytometric fingerprinting method^{41–44}. By applying additional gates on the green fluorescence histogram produced by FCM, low and high nucleic acid content bacterial communities (LNA, HNA) can be distinguished, and changes in this bacterial fingerprint can be monitored⁴⁵, providing further information on the microbial growth. Rapid and accurate identification of locations of concern and changes in biodiversity is essential for risk assessment evaluation to achieve the biological stability of drinking water during distribution. Therefore, this study aimed to evaluate the online FCM as a technique that would enable the identification of areas of concern in the distribution network.

RESULTS AND DISCUSSION

Comparison of conventional biological stability monitoring techniques and flow cytometric analysis

Evaluation of the biological parameters at two locations was performed together with chlorine and pH measurements. Table 1 shows the total and free chlorine concentration and the microbial

water quality at the two locations tested. A low bacterial cell concentration of $2.3 \times 10^2 (\pm 3.5 \times 10^1)$ cells/mL was detected at the location at drinking water treatment plant (DWTP) directly after chlorination feeding the network while HPCs and total coliforms were not detected (<1 colony-forming unit (CFU)/1 mL and <1 CFU/100 mL). At the network location, the concentrations of HPCs and total coliforms were still below the detection limit, while the bacterial cell concentrations from the FCM measurements were a factor 10 times higher compared to the water feeding the network. The analysis of HPC and total coliforms in drinking water has been utilized to determine biological stability in drinking water¹⁹. 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^{19,22,36,46}. 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 methods²⁶. The FCM enabled early detection of changes in microbial water quality with the advantages of short analytical time (10–30 min), bacteria-specific detection, and high sensitivity and accuracy to detect (low) bacterial cell numbers²⁶.

Rapid and accurate detection of changes in water bacterial cell numbers using FCM

Figure 1 shows the changes in total bacterial cell numbers and the ratio between high nucleic acid (HNA) and low nucleic acid (LNA) content cells for water samples taken with high frequency over a 5 day period. The location at the treatment plant directly after chlorination had a stable bacterial cell number (below 5×10^2 cells/mL) (Fig. 1a, Supplementary Fig. 1), while higher bacterial cell numbers with higher variations in time were observed at the network location (Fig. 1b). The online monitoring allowed detection of gradual fluctuations in the bacterial cell numbers as well as rapid incidences that would have gone unnoticed with less frequent measurements. The decrease in bacterial cell concentration at the network location during the period of 25–26 August 2019 could be attributed to higher water consumption in the network. Other studies reported that higher variations in total cell numbers in the network can be affected by environmental factors such as water temperature, water consumption, residual chlorine concentration, and stagnation time^{1,4,13}. The regrowth of bacterial cells and biofilms in DWDSs can cause higher total cell numbers in the network due to the reduced residual chlorine concentration through the distribution pipelines. To prevent the regrowth of bacteria, a minimum of 0.3 mg/L of free chlorine should remain in the water⁴⁷. In this study, the free chlorine concentration was reduced from an initial concentration of 0.4 mg/L down to 0.06 mg/L through the distribution pipelines. This loss of

Table 1. Water quality at the DWTP after chlorination and in the network.

| Analytical parameters | Chlorination point at DWTP | Network |
|--|---|---|
| Chlorine concentration | | |
| Free chlorine (mg/L) ($n = 2$) | 0.44 (± 0.01) | 0.06 (± 0.00) |
| Total chlorine (mg/L) ($n = 2$) | 0.46 (± 0.01) | 0.07 (± 0.00) |
| Microbial water quality | | |
| Total cell numbers (cells/mL) ($n = 5$) | $2.3 \times 10^2 (\pm 3.5 \times 10^1)$ | $2.0 \times 10^3 (\pm 3.8 \times 10^2)$ |
| HPC 35 °C (CFU/mL) ($n = 5$) | <1 | <1 |
| Total coliforms (CFU/100 mL) ($n = 3$) | <1 | <1 |
| <i>Escherichia coli</i> (<i>E.coli</i>) (CFU/100 mL) ($n = 3$) | <1 | <1 |

DWTP drinking water treatment plant, HPC heterotrophic plate count, CFU colony-forming unit.

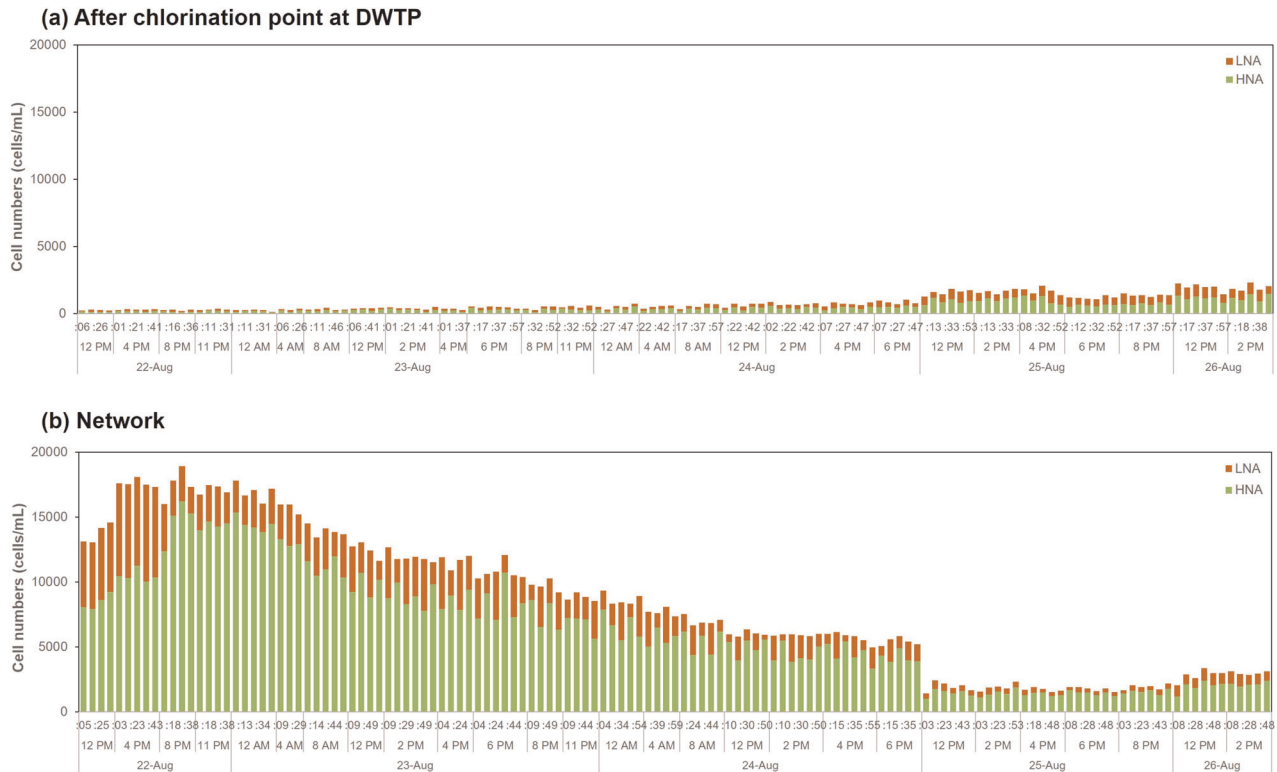


Fig. 1 Online detection of changes in bacterial cell numbers. Total cell numbers at the (a) drinking water treatment plant (DWTP) after chlorination supplying the network and in the (b) network. Total cell numbers are obtained by the sum of HNA and LNA bacteria. Bacterial cells were monitored for 5 days in the last week of August 2019. The sampling date, hours, minutes are shown along the x-axis (high nucleic acid content cells; HNA, low nucleic acid content cells; LNA).

residual chlorine in DWDSs can be caused by external factors such as temperature, total organic carbon (TOC), corrosion, and age of pipes^{48–50}. The residual chlorine may also indirectly enhance bacterial growth by increasing the assimilable organic carbon (AOC) concentration through the breakdown of higher molecular weight organics⁵¹.

Figure 2 shows the percentages of HNA and LNA bacteria. The averaged percentage of HNA and LNA bacteria was 57.5 and 42.5% at the DWTP after chlorination, while the percentage of HNA content bacteria increased to 77.1% at the network location. This observation indicates that the conditions in the network selected for stronger growth of HNA bacteria. Previous studies showed that the activity of HNA bacteria is correlated to total cell activity. In contrast, LNA bacteria have been reported as inactive or dead⁵². However, other studies showed that LNA could be grown as well as HNA bacteria at the oligotrophic environmental condition (<1 mg/L of TOC) and predominant in the end branches of DWDSs were nutrients or residual disinfectant are mostly depleted^{45,47}. Therefore, under oligotrophic conditions, both HNA and LNA bacterial communities should be measured to characterize the total bacterial community and its growth. Understanding the distribution of HNA and LNA bacteria and their proportional change is DWDSs aids in evaluating disinfection regimes and mitigating the associated risk of bacterial regrowth in DWDSs. Ramseier et al.⁵³ evaluated in their study the effect of increasing concentrations of different disinfectants on membrane damage of HNA and LNA bacteria. The study concluded that some disinfectants (e.g., chlorine dioxide and permanganate) were more effective in causing damage to HNA bacteria, while LNA bacteria were more sensitive to other disinfectant types (e.g., ozone).

Rapid assessment of bacterial community shift using a flow cytometer

The changes in the bacterial community were analyzed by a plot of flow cytometric histogram image comparison (flowCHIC) analysis based on the flow cytometric data (Fig. 3).

Flow cytometric fingerprinting is a promising and powerful technology to determine the changes in the structure of microbial communities⁵⁴. Koch et al.⁴² developed the flowCHIC method by processing the flow cytometric density plots. The flowCHIC plot enables to analyze the changes in bacterial community rapidly (10–30 min) and compared to next-generation sequencing (NGS). As a high-throughput DNA sequencing technique, the NGS analysis takes approximately 10 days with several experimental steps, including DNA extraction, library preparation, and automated sequencing^{54,55}. Note that flowCHIC analysis cannot determine taxonomic changes of the bacterial community. However, the changes in bacterial community structure point toward changes in environmental conditions or bacterial intrusion⁵⁴.

The flowCHIC plot (Fig. 3) shows the samples at the DWTP feeding the network clustered closely together, signifying their similarity, while the samples in the network are more scattered, an indication of regrowth of a much more diverse bacterial community. Bacteria are present in the water and the biofilms on the inside of distribution pipe walls. The random detachment of bacterial cells from the pipe walls may (partly) explain the more diverse bacterial community in the network. Also, reduced residual chlorine concentrations from the initial concentration at the treatment plant (0.43 mg/L) down to 0.06 mg/L in the network could decrease dominant antimicrobial-resistant bacterial communities after chlorination, and enhance regrowth of other bacterial species in the network⁵⁶. Unlike conventional HPC

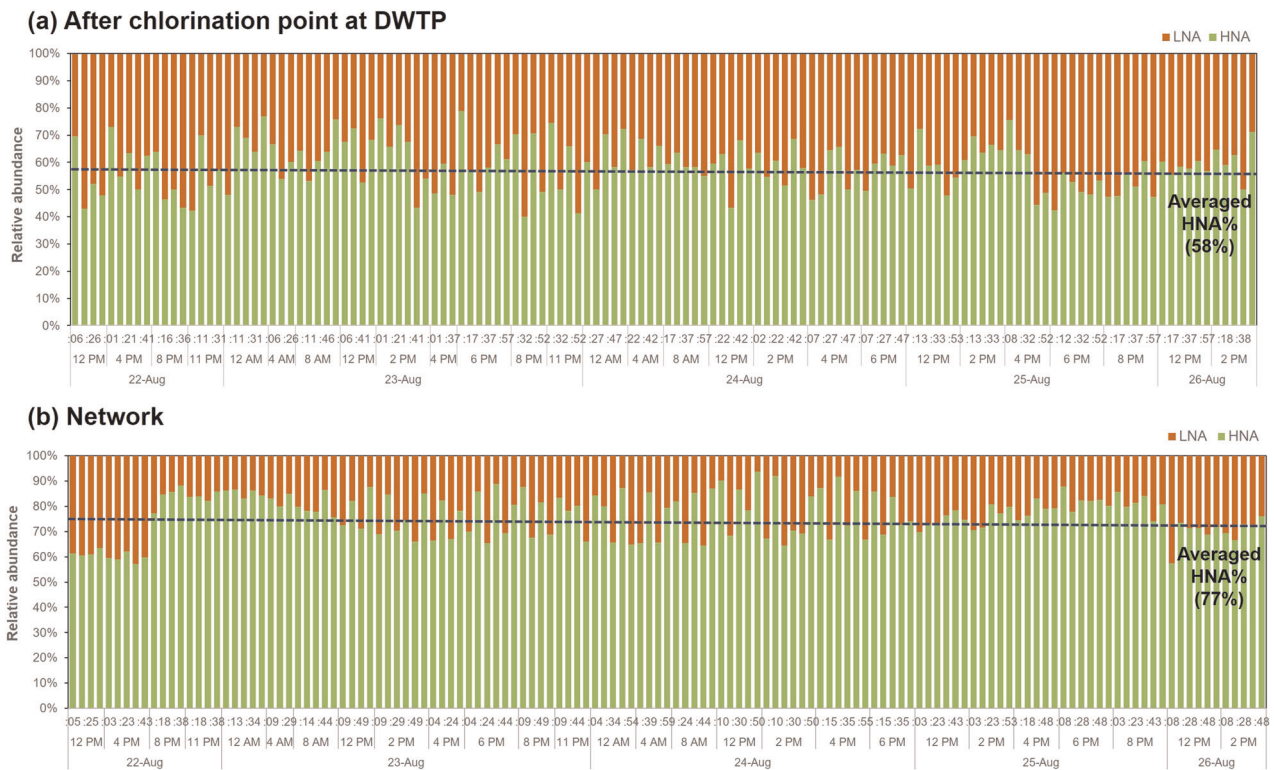


Fig. 2 Online profiling of high and low nucleic acid bacteria ratio. The relative abundance of HNA and LNA at the (a) drinking water treatment plant (DWTP) after chlorination feeding the network and in the (b) network illustrating a higher ratio of active cells in the network compared to the water feeding the network. The dotted line shows the average HNA bacterial cell percentage, 58 and 77% at the chlorinated point and network, respectively (high nucleic acid content cells; HNA, low nucleic acid content cells; LNA).

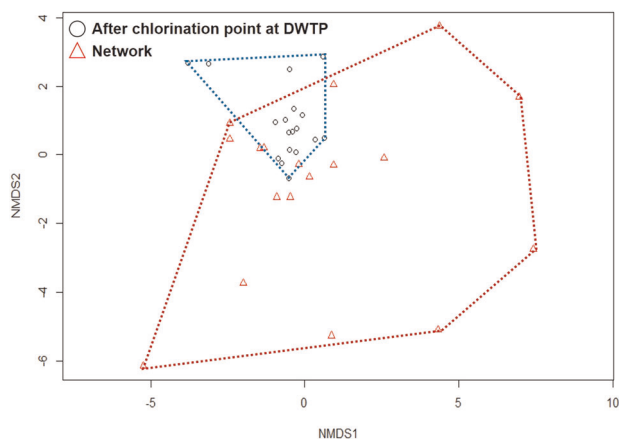


Fig. 3 Shifts in bacterial community structure. Bacterial community clusters analyzed by flowCHIC analysis at two locations: DWTP after chlorination feeding the network and in the network, respectively, demonstrating dissimilar microbial clusters between two locations and higher diversity in the network.

results, online FCM is rapid and accurate and, therefore a better tool to assess and understand the dynamics of bacterial cell numbers and predict bacterial community changes during water storage and transport.

Further outlook for online FCM monitoring in DWDSs

Results from this study with an on-line flow cytometer revealed: (1) an increase in bacterial cell numbers in the network water compared to the location feeding the network, (2) variations of

bacterial cell numbers as a function of time, and (3) changes in bacterial community structure.

The increase in total bacterial cell numbers in the network indicates regrowth of bacteria and possible the sloughing off of cells from biofilms on pipes in the DWDS. The fluctuation of bacterial cell numbers over time at the DWTP and in the network indicates that drinking water feeding the network is not biostable, as bacterial growth occurs. This study clearly showed the potential of online FCM to be used as an efficient online monitoring tool and early warning technique to quickly and accurately assess changes in drinking water microbial communities. Application of water quality monitoring, with such high frequency as in this study, would become more practical once operators are capable of automatically implementing necessary response actions to observed water quality changes (e.g., increase disinfectant concentration). Nevertheless, such monitoring aids in identification of higher risk locations in a distribution network that can be addressed with manual changes to the treatment process.

As well as monitoring the biostability of water quality, early detection of growth of microorganisms of concern is also important, since it is directly related to human health. Online monitoring of such specific bacteria presence and growth in DWDSs can be an alternative strategy to overcome culture-dependent specific bacteria detection methods. A challenge for the FCM will be the very low numbers of such organisms of concern in the water, possibly requiring a concentration step. The development of advanced specific bacteria monitoring tools with online FCM combined with bacteria-specific fluorescent probes such as an antibody⁵⁷, nucleic acid probes⁵⁸, and aptamers^{59,60} could be suggested in further studies.

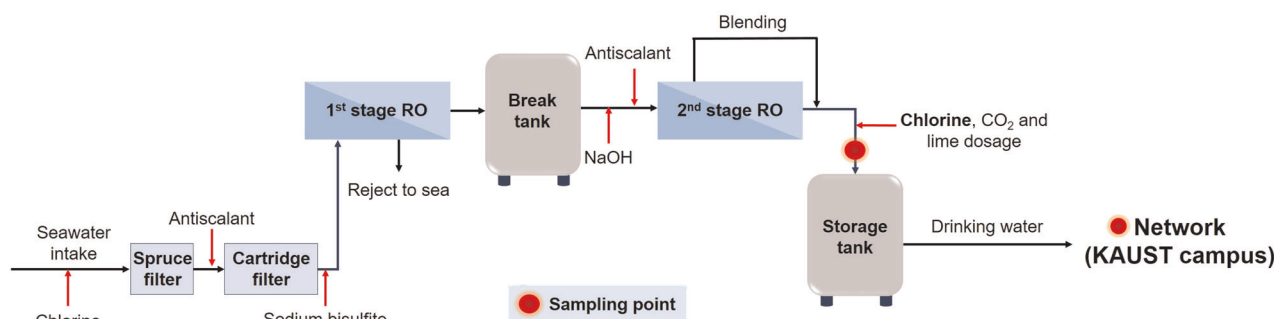


Fig. 4 Water sampling to characterize bacterial processes in drinking water distribution systems (DWDSs). Schematic diagram of the drinking water treatment process at the seawater desalination plant with the two sampling locations: after the chlorination at the drinking water treatment plant (DWTP) and in the network at King Abdullah University of Science and Technology (KAUST) campus, Thuwal, Saudi Arabia. The estimated distance of water pipes between the two sampling locations is 3.6 km.

METHODS

DWTP and sampling points

To analyze the water quality, water samples were collected in 2019 from two locations: (i) chlorinated water (0.43 mg/L chlorine) leaving the DWTP (King Abdullah University of Science and Technology (KAUST), Saudi Arabia) and (ii) one location in the network (Fig. 4). The DWTP produces the drinking water by desalination of seawater using reverse osmosis (RO) membranes. Seawater from the Red Sea is first disinfected with chlorine and pre-treated using spruce and cartridge filters. The residual chlorine in the filtered seawater is afterward removed by the addition of sodium bisulfite before the water is pumped towards the RO pressure vessels. The RO produced water (first stage RO) is stored in a break tank and then blended with the permeate water of the second stage RO. The blended RO produced water is subsequently dosed with chlorine, CO₂, and lime and stored in a storage tank, then distributed to the KAUST water distribution network, consisting of polyvinyl chloride (PVC) pipes, all of the same age (10 years) of placement in the network. The second stage RO produced water showed low ion concentrations that meet the WHO drinking water guidelines⁶¹ (Supplementary Table 1).

HPC and total coliforms

HPC was determined according to the 9215 HPC standard method (American Water Works Association, 2017)⁶² using a plate count agar (BD, USA). Hundred microliters of water sample was spread on agar plates ($n = 5$ per water sample) and incubated at 35 °C (HPC 35 °C) for 48 h⁶². The quantification of total coliforms was determined using a Colilert test kit (IDEXX, USA). A 100 mL of water sample was mixed with one packet of Colilert defined nutrient in a sterile vessel and shaken until dissolved. The experiment was done in triplicate ($n = 3$). The mixture of a water sample with reagent was poured into a Quanti-Tray and sealed in an IDEXX Quanti-Tray Sealer (IDEXX, USA). The sealed tray was put in a 35 ± 0.5 °C incubator for 24 h. Positive wells with dark color or turbidity are counted, and a most probable number (MPN) (equivalent to CFU)⁶³ is obtained from the provided MPN table.

FCM analysis

FCM was used to determine the total bacterial cell concentration, as described previously⁴⁶. 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 incubated for 10 min at 37 °C in the dark before analysis. The total cell numbers of water samples were 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.³⁶. 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 HNA

and LNA were analyzed at the same time every 10 min for 5 days at the DWTP after chlorination and in the network.

Data analysis

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 analysis was performed using R packages flowCHIC^{54,55}. The non-metric multidimensional scale (NMDS) plot was made using R software (version 3.5.2).

DATA AVAILABILITY

The authors declare that the data supporting the findings of this study are available within the paper.

Received: 1 December 2019; Accepted: 12 March 2020;

Published online: 27 March 2020

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ACKNOWLEDGEMENTS

The research reported in this publication was supported by funding from King Abdullah University of Science and Technology (KAUST).

AUTHOR CONTRIBUTIONS

N.M.F., L.H.K., and J.S.V. designed the experiment. N.M.F. and L.H.K. wrote the manuscript. N.M.F., L.H.K., and J.S.V. interpreted the data and revised the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information is available for this paper at <https://doi.org/10.1038/s41545-020-0065-7>.

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