LUND UNIVERSITY

Processes governing the drinking water microbiome

Chan, Sandy

2018

Document Version:
Publisher's PDF, also known as Version of record

Link to publication

Citation for published version (APA):
Chan, S. (2018). *Processes governing the drinking water microbiome*. Department of Chemistry, Lund University.

Total number of authors:
1

General rights
Unless other specific re-use rights are stated the following general rights apply:
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.
• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: https://creativecommons.org/licenses/

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

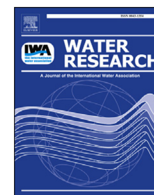
PO Box 117
221 00 Lund
+46 46-222 00 00

Water Research 138 (2018) 27–36

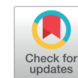
Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres



Monitoring biofilm function in new and matured full-scale slow sand filters using flow cytometric histogram image comparison (CHIC)



Sandy Chan [a, b, c], Kristjan Pullerits [a, b, c], Janine Riechelmann [a], Kenneth M. Persson [b, c, d], Peter Rådström [a], Catherine J. Paul [a, d, *]

[a] Applied Microbiology, Department of Chemistry, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden
[b] Sweden Water Research AB, Ideon Science Park, Scheelevägen 15, SE-223 70 Lund, Sweden
[c] Sydvatten AB, Hyllie Stationstorg 21, SE-215 32 Malmö, Sweden
[d] Water Resources Engineering, Department of Building and Environmental Technology, Lund University, P.O. Box 118, SE-221 00 Lund, Sweden

ARTICLE INFO

Article history:
Available online 13 March 2018

Keywords:
Slow sand filters
Drinking water
Flow cytometry
Schmutzdecke
Cytometric histogram image comparison (CHIC)
Biofilm

ABSTRACT

While slow sand filters (SSFs) have produced drinking water for more than a hundred years, understanding of their associated microbial communities is limited. In this study, bacteria in influent and effluent water from full-scale SSFs were explored using flow cytometry (FCM) with cytometric histogram image comparison (CHIC) analysis; and routine microbial counts for heterotrophs, total coliforms and *Escherichia coli*. To assess if FCM can monitor biofilm function, SSFs differing in age and sand composition were compared. FCM profiles from two established filters were indistinguishable. To examine biofilm in the deep sand bed, SSFs were monitored during a scraping event, when the top layer of sand and the *schmutzdecke* are removed to restore flow through the filter. The performance of an established SSF was stable: total organic carbon (TOC), pH, numbers of heterotrophs, coliforms, *E. coli*, and FCM bacterial profile were unaffected by scraping. However, the performance of two newly-built SSFs containing new and mixed sand was compromised: breakthrough of both microbial indicators and TOC occurred following scraping. The compromised performance of the new SSFs was reflected in distinct effluent bacterial communities; and, the presence of microbial indicators correlated to influent bacterial communities. This demonstrated that FCM can monitor SSF performance. Removal of the top layer of sand did not alter the effluent water from the established SSF, but did affect that of the SSFs containing new sand. This suggests that the impact of the surface biofilm on effluent water is greater when the deep sand bed biofilm is not established.

© 2018 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

One of the oldest technologies for the treatment of drinking water is the use of slow sand filters (SSFs) (Huisman and Wood, 1974). These filters combine multiple cleaning mechanisms including mechanical filtration and sedimentation but are primarily considered as biological filters where a microbial ecosystem develops as biofilm on the sand particles and contributes to the cleaning process (Haig et al., 2015b). As SSFs remove a broad range of microbial contaminants including *Escherichia coli*, *Clostridium* spp., *Cryptosporidium* spp., viral pathogens and toxins (Bourne et al., 2006; Elliott et al., 2008; Hijnen et al., 2007), as well as total organic carbon (TOC) (Wotton, 2002), monitoring the performance of these filters is crucial for the drinking water producer. This type of monitoring however, is complicated by limitations in both knowledge regarding the microbial diversity in these filters; and the analytical methods that are able to follow this diversity in real-time, or near real-time, resolution. Understanding these human-built aquatic ecosystems would facilitate both routine monitoring for quality control as well as optimised design for SSFs. These are both required to produce safe drinking water in a future with climate-related changes such as altered natural organic matter, water temperatures, and pathogen contamination in source water; at a time when urbanization will increase demand for treated water (Ritson et al., 2014; Sterk et al., 2013; van Leeuwen, 2013).

* Corresponding author. Applied Microbiology, Department of Chemistry, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden.

E-mail addresses: sandy.chan@tmb.lth.se (S. Chan), kristjan.pullerits@tmb.lth.se (K. Pullerits), janine.riechelmann@gmx.de (J. Riechelmann), kenneth_m.persson@tvrl.lth.se (K.M. Persson), peter.radstrom@tmb.lth.se (P. Rådström), catherine.paul@tmb.lth.se (C.J. Paul).

https://doi.org/10.1016/j.watres.2018.03.032
0043-1354/© 2018 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Knowledge about the dynamics of bacterial communities in SSFs is limited by the ability of the current routine analyses to describe the microbial processes occurring in the biofilm and water phases with respect to both diversity and time. Heterotrophic plate counts (HPC), and counts of coliforms and *E. coli,* can analyse microbes passing through the SSFs and satisfy traditions of common usage and regulations, however these methods only capture a small fraction of the total microbial population (Allen et al., 2004), and at least 24 h incubation time is required. Studies using molecular techniques have described laboratory and pilot-plant scale SSF systems (Bourne et al., 2006; Calvo-Bado et al., 2003; Wakelin et al., 2011) or focused on elements of the filter, such as the uppermost biofilm, or *schmutzdecke* (Unger and Collins, 2008; Wakelin et al., 2011). Other studies have focused on microbial contaminant removal by SSFs (Bauer et al., 2011; Elliott et al., 2008; Hijnen et al., 2004). Several metagenomic DNA sequencing studies of the microbial community in full-scale SSFs in operating drinking water treatment plants have shown that a highly diverse community dominated by bacteria is living in these biological filters (Bai et al., 2013; Haig et al., 2014, 2015b; Oh et al., 2018). The presence of bacteriophage, protozoa and fungi and their role in SSF ecology has also been examined (Haig et al., 2015a; Prenafeta-Boldú et al., 2017). These studies are invaluable for providing a deep understanding of the microbial ecosystems in SSFs however, the methods used are expensive, with time-consuming laboratory work and demanding data analysis. This currently prevents their use for online routine monitoring.

Flow cytometry (FCM) with DNA staining is used to study the microbial communities of numerous aquatic systems (Berney et al., 2008; Boi et al., 2016; De Corte et al., 2016) including microbial dynamics in both treatment and distribution of drinking water (Besmer et al., 2014; El-Chakhtoura et al., 2015; Lautenschlager et al., 2014). Total cell count (TCC) has been proposed for monitoring drinking water treatment processes (Van Nevel et al., 2017b) and online measurement has been demonstrated (Besmer and Hammes, 2016). Additional quantitative FCM parameters describe the bacteria in a water sample, including the number of intact cells, and a fluorescent fingerprint describing the distribution of DNA content in the bacterial community (Prest et al., 2013).

During SSF operation, the bacteria in the sand consume organic matter and multiply, and over a period of months or years (depending on season and source water) the filter becomes clogged with biomass. To restore the water flow, the top layer of the SSF is removed by mechanical scraping (Huisman and Wood, 1974). This procedure may disturb the filter function, and effluent water from the disturbed SSFs is not used until water quality parameters comply with regulations. The ability to follow SSF function in real, or near-real, time would minimise the time filters are offline to both ensure maximum supply of treated water and reduce costs. This is particularly relevant in Sweden, where SSFs require scraping 2–3 times per year. In this study, three SSFs differing in microbial community maturation and sand composition were followed over a period of several weeks during summer, before and after a scraping procedure. Water quality of influent and effluent were assessed using FCM and conventional microbial and chemical parameters. FCM parameters together with Cytometric Histogram Image Comparison (CHIC) analysis were analysed to assess if this method could resolve dynamic changes in the bacterial communities of the effluent water. In order to examine if this method could be used to monitor the function of the biofilm in SSFs, these profiles were correlated to different traditional microbial water quality indicators. In addition, by observing the different SSFs before and after the removal of the top layer of sand, including *schmutzdecke,* the specific contribution of the deep sand bed biofilms to SSF function could be observed.

2. Materials and methods

2.1. Description of SSFs and sampling

The full-scale SSFs in this study are located in Sweden, at Ringsjö Waterworks, Stehag, Sweden, and operated by Sydvatten AB (Hyllie Stationstorg 21, Malmö, Sweden). During the study, the treatment plant produced 1300 L/sec of drinking water from surface water (Lake Bolmen, Småland, Sweden), supplemented with a small fraction of groundwater. The plant receives the source water through an 82 km tunnel and treated using flocculation with ferric chloride, lamellar sedimentation, rapid sand filtration, slow sand filtration and disinfection with hypochlorite before distribution (Sydvatten AB, 2016). Each SSF at the treatment plant is scraped 2–3 times per year, usually in the summer, when the resistance of flow through the filter is unacceptable. The SSFs are scraped to remove the top layer of sand, including the *schmutzdecke* and then refilled with water from below the sand bed.

In winter 2015, two new SSFs were built at Ringsjö Waterworks. One was constructed using only purchased virgin sand (Sibelco Nordic AB, Baskarp, Sweden), (NEW) while the second SSF (MIX) was constructed with first a layer of virgin sand, topped with a layer of washed sand collected during previous scraping of established SSFs. A third SSF, a well-established working SSF (EST) in the same production line as the newly constructed filters and used for drinking water production over 20 years (built 1995), was included in sampling as a control (Persson, 2013). Water samples were collected during July and August 2015 from above the sand beds, using a telescopic sampler; and after filtration, from continuously running taps. Samples were collected using sterilized borosilicate bottles one day before, and for up to three weeks after, the scraping of each SSF. As scraping for each filter was not carried out on the same calendar day, data and comparisons are presented relative to the day of scraping, with day 1 being the day before scraping, day 2 being the day of the scraping activity and so forth. All three SSFs were scraped within the same three week period of stable ambient temperatures (data not shown).

In summer 2016 (April–August), EST and a second mature SSF from the same treatment line (EST2) were sampled in the same way as describe above except that water samples were collected directly into 50 mL Falcon tubes and not transferred from the borosilicate bottles.

2.2. Water quality measurements

Water samples for conventional microbial parameters were processed by the treatment plant staff according to a routine schedule and coincided in time with the flow cytometry analysis. Heterotrophic plate counts (HPC) were determined by mixing 1 mL of water with R2A agar, with incubation at 22 °C for 72 h (Bartram et al., 2003). Concentration of coliforms and *E. coli* were determined with the Colilert method from IDEXX laboratories, using the Quanti-Tray/2000® and sealed with Quanti-Tray sealer ® according to the manufacturer's instructions (Idexx Laboratories, Westbrook, USA).

Chemical water quality parameters were determined by VA SYD, Malmö, Sweden using standard methods, (Table S1). The temperature of the water over the studied period was measured online in the bulk water at the outlet of the treatment plant.

2.3. Flow cytometry

Samples of 50 mL water for flow cytometry were transferred and stored in sterile 50 mL Falcon tubes on ice and analyzed within 7 h of sampling. Water samples were stained in triplicate for measurement of total cell count and fingerprints according to Prest et al. (2013). Briefly, 5 μL mL^{-1} of SYBR Green I at 100 X diluted with DMSO (stock concentration 10 000 X, Invitrogen AG, Switzerland) was added to samples at room temperature, to a final concentration of 1 X SYBR Green I, before incubation in the dark at 37 °C for 15 min. For intact cell measurements, a working solution of SYBR Green I (100 X) and propidium iodide (PI) (1 mg/mL, Sigma-Aldrich, Germany) was prepared with final concentrations of 1 X SYBR Green I and 3 μM PI in the sample and incubated as above (Gillespie et al., 2014). Live and ethanol-killed *E. coli* were used as controls for examination of cells with intact membranes, referred to hereafter as intact cells, as well as MilliQ water as a control for background fluorescence were used in every run. All measurements were performed using a BD Accuri C6 flow cytometer (Becton Dickinson, Belgium) with a 50 mW laser with an emission wavelength at 488 nm. A quality control of the flow cytometer using Spherotech 8-peak and 6-peak validation beads (BD Biosciences) was conducted each day measurements were taken in order to allow samples from different days to be compared. 50 μL of 500 μL samples were measured in triplicate for each sample at a flow rate of 35 μL min^{-1} and a threshold of 500 arbitrary units on the green fluorescence channel.

2.4. Data analysis

Data processing and gating were performed with FlowJo software (Tree Star Inc, USA). Signals were collected and analyzed by gating on the dot plot with green fluorescence (FL1, 533 ± 30 nm) and red fluorescence (FL3, >670 nm). Gating was done following the gating strategy described in (Prest et al., 2013) and identical gating was applied on all samples. The gated data visualized by the green fluorescence histogram plot is referred to as the fluorescent fingerprint. Percentage of bacteria with low nucleic acid content (LNA) and bacteria with high nucleic acid content (HNA) were determined as described in Prest et al. (2013). Statistical analysis was performed on all data (TCC, ICC and HNA concentration) using one-way ANOVA, followed by Tukey test in R (R Development Core Team, 2017).

Cytometric histogram image comparison (CHIC) analysis on dot plots was performed using R packages flowCHIC and flowCore (Ellis et al., 2016), and visualized by ggplot2 (Wickham, 2009) according to Schumann et al. (2015) and Koch et al. (2013). Gated populations of the flow cytometric dot plots (green fluorescence at x-axis and red fluorescence at y-axis) were converted into 300 × 300 pixel images with 64-channel gray scale resolution for image comparison and to generate values describing the differences between water samples. A nonmetric multidimensional scaling (NMDS) plot based on Bray-Curtis dissimilarity was created from the results and analysis of similarities (ANOSIM) was performed with the formed clusters. All statistical calculations were performed in R (R Development Core Team, 2017). The correlation between the nonmetric multidimensional scaling (NMDS) of the FCM data and the conventional microbial parameters (HPC, coliforms and *E. coli*) was determined using the R function *envfit*, vegan package (Oksanen et al., 2017). Only parameters from plate counts with a significant effect (P-value < 0.05) are presented. After the sampling and FCM analysis were completed, plate count data collected as part of the routine monitoring schedule were obtained from the laboratory at Ringsjö Waterworks. Only FCM profiles and plate counts obtained on the same sampling day were used for the correlation analysis.

3. Results

3.1. Conventional water quality assessments

Water quality parameters, including specific microbial indicators, were measured during a routine scraping event and subsequent operation period for three SSFs (Fig. 1). Influent water showed variations in the concentration of microbial indicators (i.e. HPC, coliforms and *E. coli*), between filters and over time, despite the fact that all SSFs received water from the same process line at the treatment plant.

Effluent water samples from the three SSFs contained heterotrophs during the entire sampling period. The established SSF, hereafter referred to as EST, had the lowest mean value of heterotrophs calculated over time of the three filters, at 1.8 ± 1.6 CFU/mL. Counts for coliforms and *E. coli* above zero were detected in effluent water from the established filter only once during the 35 days of sampling, at 1 CFU per 100 mL of breakthrough coliforms. The SSF with mixed sand (MIX), both coliforms and *E. coli* were detected in the effluent water following the scraping event with a steady decrease of indicators detected over operation time and no detection of *E. coli* after day 8. The SSF containing all new sand (NEW) showed frequent breakthroughs of coliforms and *E. coli* in effluent water during the entire sampling period.

Chemical parameters for water quality were measured in the influent and effluent water for each filter (Table S1). The three SSFs showed similar values for parameters such as nitrite, total phosphorus and conductivity but differed with respect to TOC and pH. TOC and pH of the effluent water of EST were lower and more stable than effluent from MIX and NEW. TOC across EST was reduced 0.6 mg/L ± 0.2 mg/L, from a mean value of 2.9 mg/L ± 0.09 mg/L in the influent water to 2.2 mg/L ± 0.09 mg/L in effluent. This was in contrast to that observed for the new SSFs, where there was less reduction of TOC. MIX gave a reduction in TOC of 0.2 mg/L ± 0.0 mg/L (mean value of TOC in influent 2.9 mg/L ± 0.1 mg/L to effluent 2.7 ± 0.1 mg/L mg/L) and TOC was reduced 0.1 mg/L ± 0.0 mg/L for NEW (mean value of TOC in influent 2.8 mg/L ± 0.05 mg/L to effluent 2.7 mg/L ± 0.05 mg/L).

In EST, pH was lowered from a mean value of 7.8 to 7.4, while the pH of the influent and effluent water of both MIX and NEW were unaffected by filtration and remained with a mean value of 7.7.

3.2. Flow cytometric bacterial counts

Total cell counts (TCC) were determined by FCM to assess changes in the number of bacterial cells in the influent and effluent water of the three SSFs during the routine scraping event (Fig. 2). TCCs of the influent and effluent water of EST peaked one day after scraping (sampling day 3), with $6 \times 10^5 \pm 1.5 \times 10^4$ cells mL^{-1} in the influent and $3.9 \times 10^5 \pm 1.9 \times 10^3$ cells mL^{-1} in the effluent water. This was the highest TCC observed in this study. TCC values in effluent from the newly built filters were in the same order of magnitude, with an average of $2.7 \times 10^5 \pm 5.3 \times 10^4$ cells mL^{-1} in effluent water across the three filters.

After scraping (sampling days 7–10 for EST and MIX, sampling days 6–11 for NEW) the average reduction in TCC performed by EST was 16% ± 1%, compared to MIX, at 25% ± 3%, and NEW, at 30% ± 1%. TCC in the effluent water from EST reached a steady-state level at 5 days after the scraping event (sampling day 7), with almost no change in bacterial numbers over the following sampling days. This was not observed for the two newly constructed filters. Calculating the slope values from linear regression of TCC/time showed the stability of the effluent TCC from EST, giving a rate of change for EST TCC over 10 times lower than that observed for either of the newly built SSFs (EST:1090; MIX: -13100; NEW: -17800).

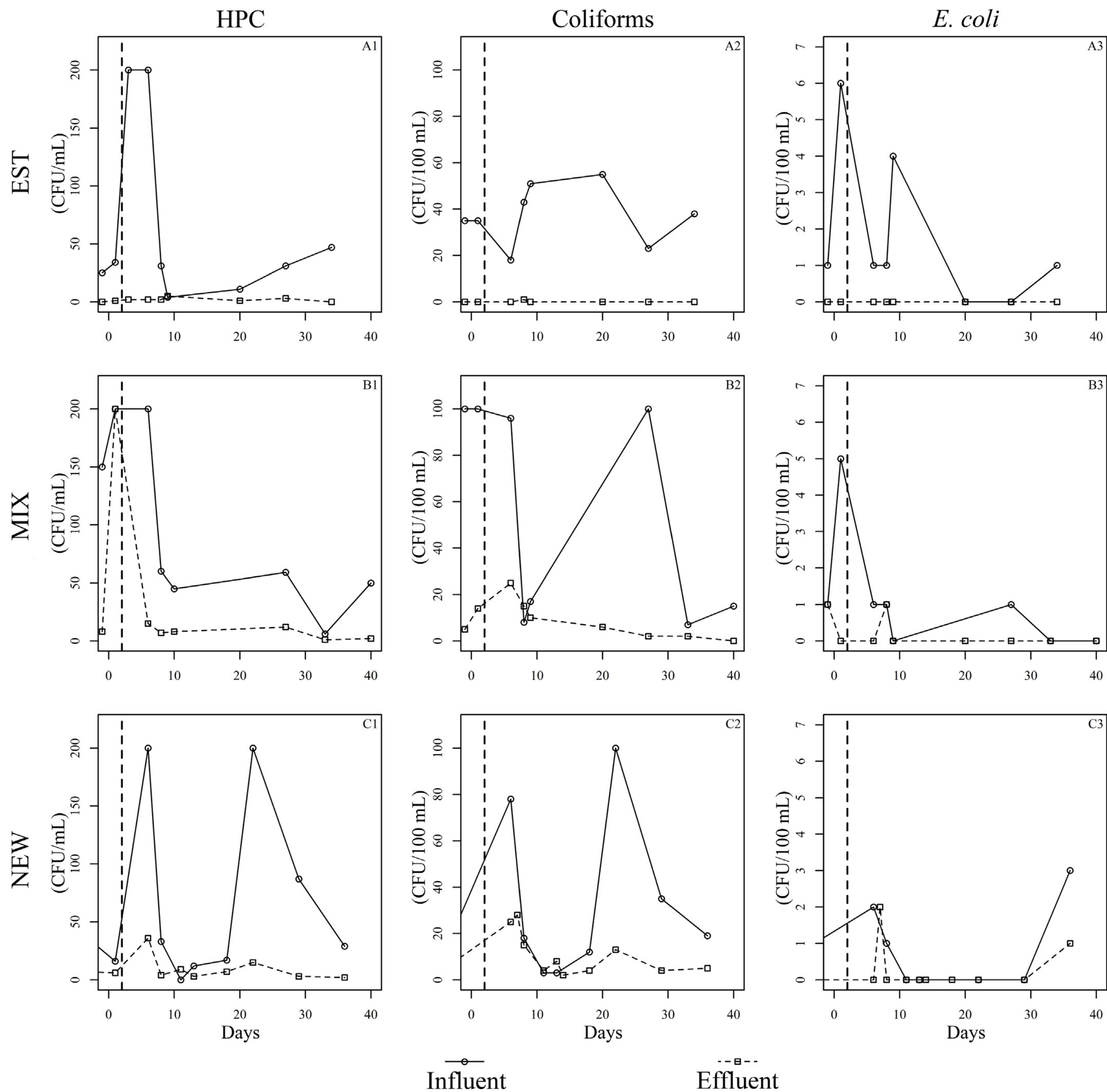


Fig. 1. Conventional microbial water quality assessment Measured microbial parameters, from left to right: heterotrophic plate count (HPC), coliforms and *E. coli* in influent (○ solid line) and effluent (□ dashed line) water of EST (A1-3), MIX (B1-3) and NEW (C1-3). The days on the x-axis correspond to the scraping of the filters that occurred in day 2 (vertical dashed line).

Intact cell counts showed similar trends as TCC (data not shown). Effluent water from EST had on average 80% intact cells, with a statistically significantly higher P-value < 0.05 than both MIX and NEW (averages of 73% and 76% intact cells respectively). The percentage of intact cells in effluent waters from MIX and NEW were not statistically different from each other (Fig. 3). Effluent water from all filters contained, on average, more intact cells than influent, with one exception from MIX before the scraping event (sampling day 1), where the influent had a higher ICC.

3.3. *Profiling bacterial communities by flow cytometric measurements*

Fluorescence distribution histograms from FCM were used to compare DNA-stained bacterial cells in the SSF influent and effluent waters. Each histogram image represents a cumulative fluorescent profile of the individual cells in the bacterial community of a water samples (Prest et al., 2013). Histograms were visualised by FCM as fluorescence fingerprints. Fingerprints obtained from 64 influent

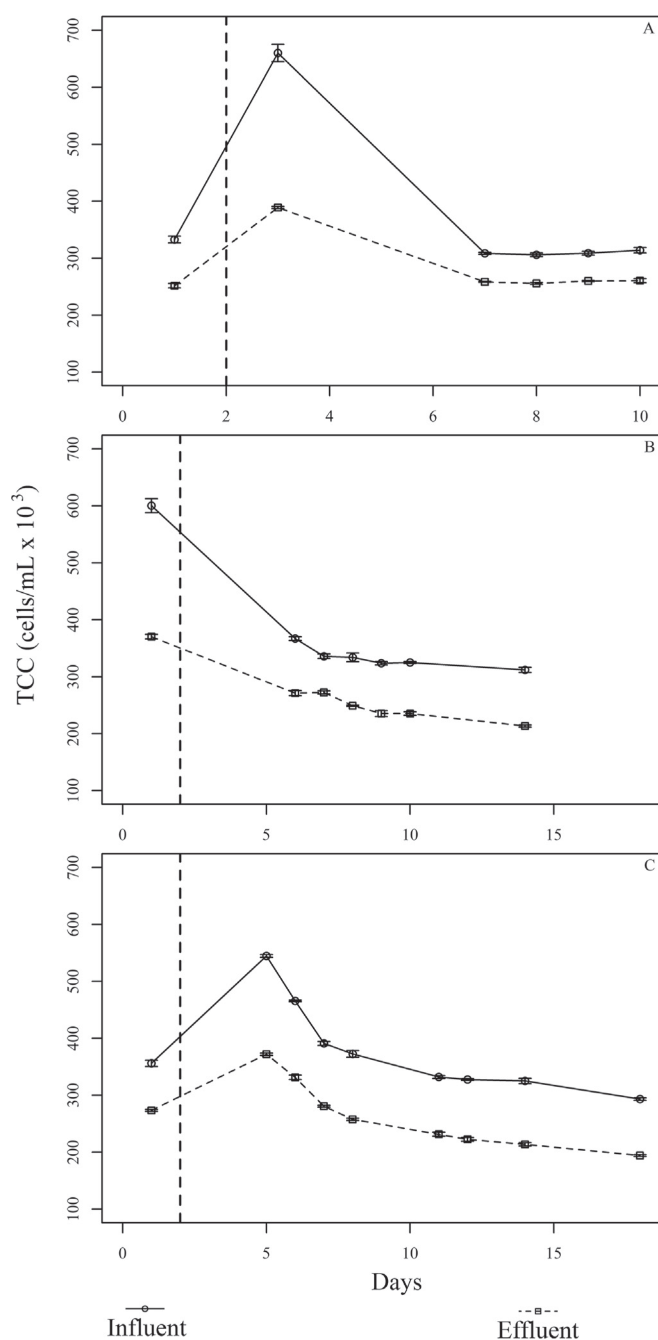


Fig. 2. Cell concentrations in water from slow sand filters. Total cell concentrations (TCC) of influent (○ solid line) and effluent (□ dashed line) water of EST (A), MIX (B) and NEW (C) measured by flow cytometry. X-axis is defined by a scraping event occurring on day 2 (dashed line).

water samples from three SSFs showed similar patterns, indicating comparable bacterial communities with no significant differences in concentration of high nucleic acid bacteria (HNA) (Fig. S1). One exception was influent water for EST, sampled one day after scraping (sample day 3), with an altered fingerprint and HNA concentration of 48.8 ± 0.95%. This anomaly was likely due to cells entering the influent due to disturbance of the SSF biofilm when the SSF was refilled from below after scraping.

Fluorescence fingerprints of effluent from EST and MIX showed a lower concentration of HNA, with a dramatic shift in community composition towards bacteria with low nucleic acid content (LNA) (Fig. 4). The effluent from EST had, on average, 29.6 ± 2.78% HNA bacteria, and MIX effluent had 39.5 ± 3.09% HNA, although only EST maintained a steady-state level of LNA bacteria after the scraping event. In contrast, effluent water from NEW increased in the concentration of HNA bacteria over time and always contained a higher HNA concentration than the other effluent waters, with an average of 46.6 ± 4.43% HNA.

All gated cell dot plots were analyzed using CHIC and presented in an NMDS plot to quantitatively compare the changes and dynamics in the bacterial communities (Fig. 5). CHIC analysis identified four distinct clusters associated with effluent water, and depending on the origin of the SSFs, and one cluster which encompassed all influent water samples. Correlation analysis between traditional plate counts and FCM profiles showed higher levels of indicator bacteria associated with the influent water cluster (In). Analysis of similarities (ANOSIM) confirmed significant separation between all groups (*R*-value = 0.933; *P*-value = 0.001). Data from effluent water samples of EST (E) and MIX (M) were distinct from those of the influent cluster (In), and each other. NEW effluent waters split into two clusters in the NMDS plot. N1 was associated with samples taken before and shortly after the scraping procedure in time. N2 contained samples taken several days after the scraping, and showed water profiles that were most similar to those of the influent (In) water. This division in profile character for NEW effluent water was also observed in the fingerprints over time (Fig. 4).

The effluent water from EST had visually identical fingerprints regardless of whether the samples were taken before or after scraping. This uniformity was also reflected in the compact cluster of group E in the CHIC analysis, and the steady-state behavior of this SSF observed by other parameters (i.e. coliform count). The cluster representing the effluent water of MIX grouped between those of EST and NEW, with day before scraping (sample day 1) and the last sampling points (day 10 and 14) being closer to the EST cluster.

To determine if FCM shows the same bacterial profile for established SSFs at this treatment plant, effluent water was analyzed from EST and a second well-established SSF (EST2), over a five month period. CHIC analysis with all previous SSF effluent water data (Fig. S2) again separated effluent waters from new and established filters, and all histograms describing effluent from established filters clustered together, regardless of sampling date. CHIC analysis of data from only the established filters (EST, 2015, 2016 and EST2 2016), separated into two clusters representing the communities of the influent and effluent water from both filters, and confirmed by ANOSIM (R-value = 0.957; *P*-value = 0.001; data not shown).

4. Discussion

Next generation sequencing (NGS) studies of drinking water biofilters have previously shown that the effluent water community reflects the content of the biofilm (Haig et al., 2015b; Li et al., 2017; Oh et al., 2018; Pinto et al., 2012). Some studies have examined the use of FCM to characterize the influent and effluent communities from biofilters (Lautenschlager et al., 2014; Park et al., 2016). The goal of the current study was to examine if FCM can monitor biofilm status and function in SSFs with sand beds of differing maturity and sand composition, including their response to a scraping event. Using FCM, the bacterial communities in the influent and effluent water from four SSFs were followed through time. CHIC analysis was used for statistical comparison to compare total number of cells and distribution of nucleic acid in these cells. This grouped the influent water separately from the effluent water, and each SSF produced effluent water with a unique bacterial profile. The influent water to each SSF was the same, and

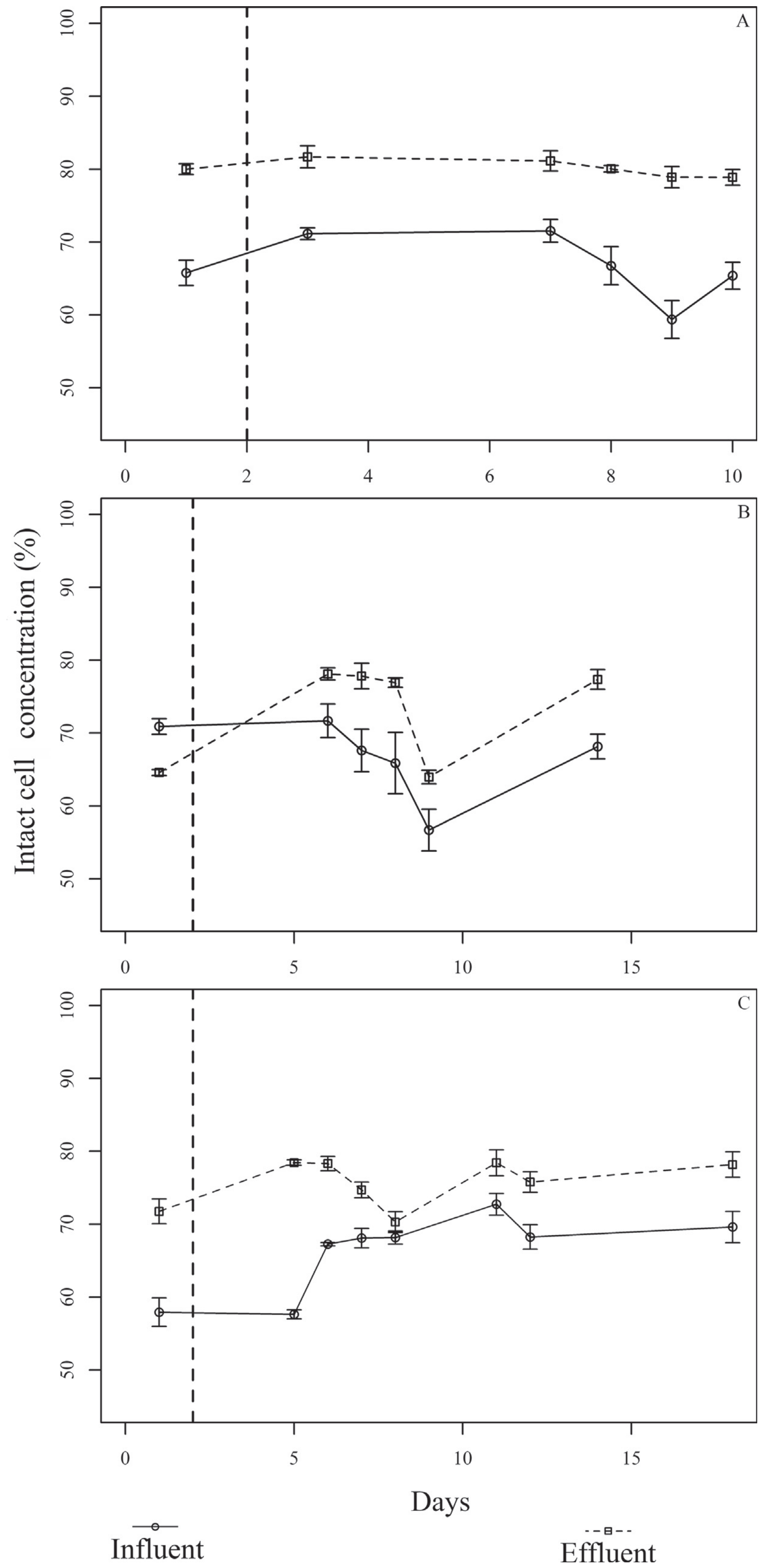


Fig. 3. Intact cell concentrations in water from slow sand filters. Intact cell concentration of influent (○ line) and effluent (□ dashed line) water for EST (A), MIX (B) and NEW (C) measured by flow cytometry during a scraping event occurred in day 2 (dashed line) in the x-axis.

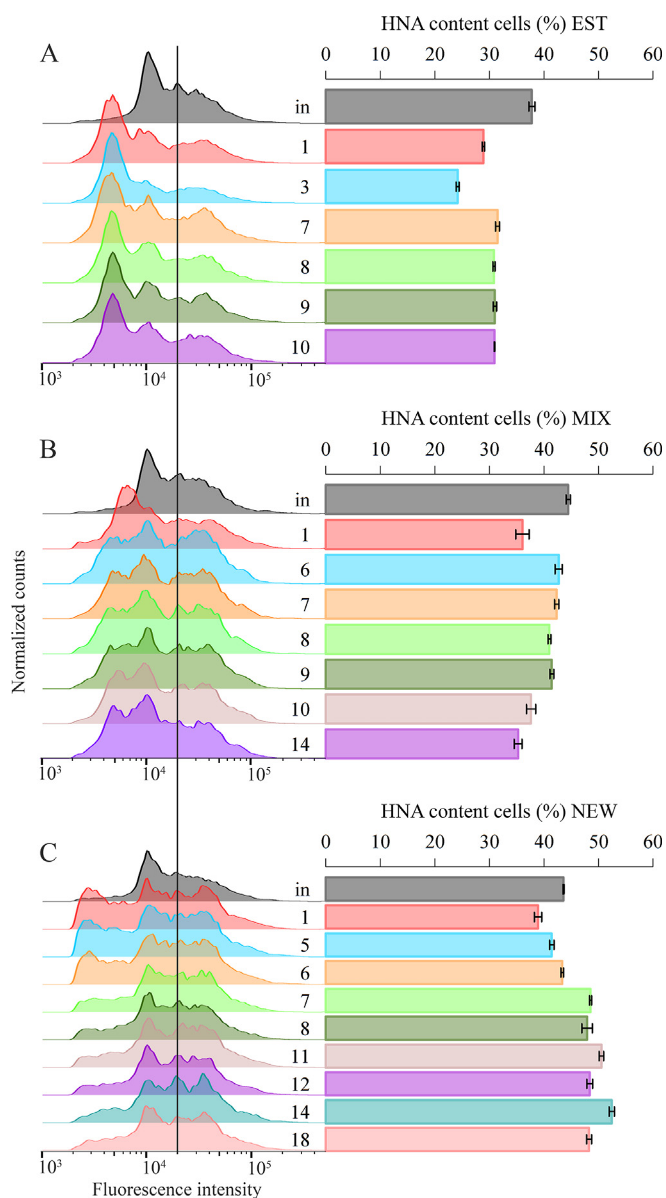


Fig. 4. Fingerprint analysis of effluent water from slow sand filters. Fluorescence distribution histograms from flow cytometry are combined over time for the effluent water of EST (A), MIX (B) and NEW (C) on the left. Percentage of HNA cells separated by a black line adapted from Prest et al., 2013 (around 2×10^4 a.u) are shown on the right as bar plots. The first sample for each filter (in grey) are influent water from sampling day 6. Sampling days corresponding to scraping (occurring in day 2) are shown in the middle of the figure.

differences in the effluents between filters indicated that a distinct microbial biofilm inhabited each of the examined SSFs.

The bacterial profiles in the effluent water of the two established SSFs were similar to each other regardless of sampling year. Closer examination of one of these filters showed consistent chemical and biological transformation of the water quality across the sand bed including: removal of microbial indicators; a lowering of pH; an increase in percentage of intact cells; and decreased HNA content, regardless of fluctuations in the influent water. Importantly, the transformation of the water quality was not dependent on the upper layer of sand: scraping did not result in changes to the FCM bacterial profiles of effluent water, or breakthrough of microbial indicators. This was in contrast to observations in the newly built SSFs, particularly the SSF containing all new sand. In this SSF, removal of the top layer of sand, including the *schmutzdecke,* preceded breakthrough of microbial indicators and FCM profiles showed that the community in the effluent water became more similar to that of the influent. This suggests that the sand bed biofilm in these newly-built filters was not able to transform the influent water to the extent observed for the established filter. The role of the *schmutzdecke* in water purification has long been attributed to the activity of microbes living as biofilm in this region of the SSF (Barrett et al., 1991; Bauer et al., 2011; Huisman and Wood, 1974; Oh et al., 2018). As the function of the mature SSF was not disrupted by scraping, the functional microbial community of this filter resided in the sand bed and not only in the *schmutzdecke*. An NGS study of two full-scale SSFs showed that the bacterial communities between sand samples are highly similar even when sampled from different depths (Haig et al., 2015b) and together with the results in the current study, it seems these core communities contain the essential functionality of SSFs. However, as studies characterizing the ecology of the SSF sand bed have used extracted DNA, without the ability to distinguish between living and dead cells or free DNA, it is difficult to say which mechanisms within the sand bed ecosystem are responsible for effluent water quality. Stable isotope probing showed that removal of *E. coli* from laboratory SSFs was mediated via multiple direct and indirect mechanisms including protozoal grazing, viral killing, reactive oxygen species produced by algae, and mutualistic fungi-algal interactions (Haig et al., 2015a). This study also suggested that ecosystem-wide associations on multiple trophic levels are required for pathogen removal and that the absence of this complexity could explain compromised function, in less diverse filter ecosystems. It is also known that SSF function improves with time; virus removal improved over time in constructed model systems as the *schmutzdecke* and deeper sand biofilm developed (Bauer et al., 2011); and, seven week old freshwater biofilms showed greater enzyme activity for removal of DOC than four week old biofilms (Peter et al., 2011). These observations are supported by the current study as the SSF which had a top layer of washed sand from other SSFs (MIX) was more effective at removing indicator organisms at the end of the study period. CHIC analysis showed the bacterial profile in the effluent from this SSF migrated towards that of the established filters in the days following the scraping event. This suggests that the biofilm community in the mixed SSF may have been approaching that of the established filter biofilm, including acquisition of ecosystem-wide associations required for pathogen removal. Further investigation is required however, to determine if the microbial ecology and/or specific pathogen removal mechanisms differ between the SSFs in this study.

The washed sand used in construction of one SSF (MIX) appears to have inoculated the biofilm with a community preconditioned for SSF function, promoting a more rapid development of a biofilm core community similar to that in the established sand filters. Interestingly, Pagaling et al., (2014) showed that the colonization of a microbial community was predictable, and similar to the original community, when it was introduced to an environment to which it had previously been exposed. The idea that inoculation with preconditioned microbial biomass can lead to rapid establishment of SSF function is supported by laboratory studies by Haig et al. (2014). Lab scale SSF columns constructed using sand from a full-scale SSF differed: non-sterile columns removed indicators after a period of 4–6 weeks, whereas sterile columns required 7–10 weeks to reach the same level of performance.

While the biofilm in the deep sand is essential for shaping the effluent water from well-functioning SSFs, the removal of the top layer of sand and *schmutzdecke* did impact the function of the new SSFs. In the SSF containing all new sand (NEW), CHIC analysis

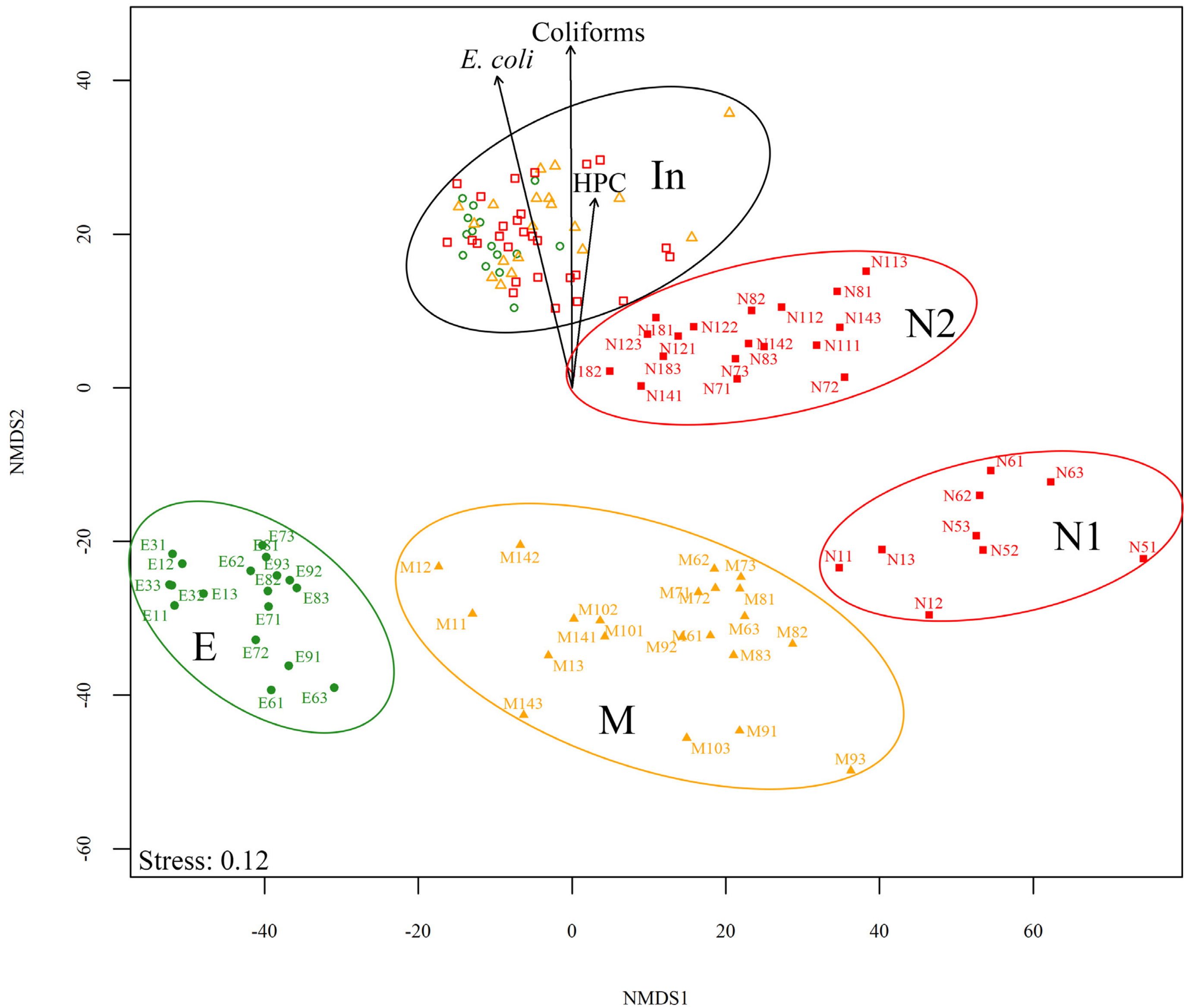


Fig. 5. Non-metric multidimensional scaling (NMDS) ordination plot from CHIC analysis of water samples from different slow sand filters. Profiling bacterial communities by flow cytometric measurements and CHIC analysis grouped water samples into five clusters: a combined cluster of all influent water (black), effluent water from EST (E, green), effluent water from MIX (M, yellow) and two clusters formed by effluent water of NEW (N1, N2, red). The first one or two numbers (if name consist of three numbers) of each effluent sample name indicate days corresponding to the scraping (occurred in day 2), n = 127. Vectors describing the linear relationships between the FCM and plate count data (heterotrophs, coliforms and *E. coli*) are indicated by labelled arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

showed that after the scraping event, the effluent profiles began approaching that of the influent water. This suggests that without the *schmutzdecke*, the deep sand bed in this SSF had minimal impact on the bacterial community in the water. In an NGS study examining response of established full-scale SSFs to scraping, overlap between communities in the influent and effluent water was concomitant with coliform breakthrough (Haig et al., 2015b). Thus, in filters without a well-functioning microbial community in the deep sand bed, the biofilm may not be able to sufficiently impact the effluent water and changes in the effluent water could be more coupled to the status of the *schmutzdecke*. This could explain the emphasis placed on the role of the *schmutzdecke* in water filtration: studies examining its function have largely been conducted on filters that are not performing optimally; or at lab or pilot scale, where a sand bed community has not had years to establish (Haig et al., 2015b; Pfannes et al., 2015; Unger and Collins, 2006). The study showing effective removal of faecal indicators from wastewater identified the *schmutzdecke* as the essential feature of 14 week-old model slow sand filters, but again, the communities in the influent and effluent water were indistinguishable by t-RFLP analysis of bacterial 16S rRNA (Pfannes et al., 2015). The study by Unger and Collins (2006) also showed that the removal of *schmutzdecke* changed filter function although again, these experiments were conducted at lab-scale and over a period of weeks. It is not surprising that the *schmutzdecke* plays an important role in filtration by new SSFs as substrate concentrations and the biomass acting on the substrates are highest at the surface of the sand bed (Bai et al., 2013; Lautenschlager et al., 2014). In filters where for any number of reasons (time, inoculation) the deep sand bed biofilm cannot significantly transform the influent water,

the influence of *schmutzdecke* on filter function may thus be more obvious. As biofilms in both the *schmutzdecke* and sand bed can entrap particles and cells, and support antagonistic microbial interactions (Pfannes et al., 2015; Prenafeta-Boldú et al., 2017), the overall function of the filter is likely a balance between the functions of the biofilm ecosystems in these different regions. A recent metagenomics study predicted that the minimum generation time for a mature SSF sand bed community was shorter than that predicted for the associated *schmutzdecke* metagenome (Oh et al., 2018) suggesting that the degradation of organic material which fuels microbial growth in a mature filter is higher in the sand bed then on the surface. Although the bacterial content in *schmutzdecke* is denser than in the sand bed (10^{11} copies/mL and 10^8-10^9 copies/g respectively) (Pfannes et al., 2015), the total volume of the deep sand is many times greater than that occupied by the *schmutzdecke.* Instead of being dominated by the function in the *schmutzdecke*, the activity of the SSF community in the deeper sand is likely more significant for overall SSF performance than previously thought. Conclusions from lab-based experiments may thus overestimate the impact of the *schmutzdecke,* emphasizing the need for studies conducted at full scale for complete assessment of drinking water treatment by SSFs.

The question still remains: to what extent does the biofilm transform the influent water community to obtain desirable effluent water quality? In this, and other studies (Haig et al., 2015b), an overlap between the bacterial communities of the influent and effluent water were concomitant with indicator breakthrough. It may be a specific and significant transformation of the bacterial community between influent and effluent water that is the signature of a well-functioning SSF. The established filter showed the least reduction of total cells, with an increase in the amount of intact cells, and a decrease in HNA content, suggesting an exchange of communities in the water as it passed this biofilm. An increase in intact cells following SSF has also been reported Lautenschlager et al. (2014). CHIC analysis showed that the bacterial communities from each SSF differed in HNA, suggesting that the distinct biofilms in each individual sand bed altered this aspect of the effluent. HNA and LNA bacteria are thought to be both phylogenetically and physiologically different (Schattenhofer et al., 2011; Wang et al., 2009; Vila-Costa et al., 2012). Changes in the ratio of HNA to LNA bacteria, with LNA bacteria dominating in effluent water, have been observed following biofiltration (Lautenschlager et al., 2014; Vital et al., 2012). The seeding of the treated drinking water with bacteria during biofiltration is thought to be important for the quality of the distributed water (El-Chakhtoura et al., 2015; Lautenschlager et al., 2014; Pinto et al., 2012). The ability of a biofilter to shift the community to include increased numbers of LNA bacteria could be essential to achieve a desirable microbial water quality. The effluent water from the established filter in this study showed this typical change to higher LNA content. In contrast, both new filters had more HNA bacteria in their effluent water compared to influent. The HNA content from the new filter containing mixed sand decreased over the study period, to more closely resemble that of the established filter. The new filter containing new sand, however, had continually increasing HNA content in effluent water. These changes in HNA content appeared to coincide with the ability of the new SSFs to remove indicators. Observing a shift in the distribution of nucleic acid content could provide an alternative way to monitor SSFs, although the relationship between DNA content and SSF function requires more investigation.

The ability of different disinfectants to inflict membrane damage on HNA and LNA cells was examined (Ramseier et al., 2011). This study postulated that HNA bacteria contain higher proportions of, or more accessible, thiol or other non-amine groups in their membrane proteins, and that this difference could increase the sensitivity of HNA cells to chlorine dioxide and permanganate disinfection. When ozonation was examined in more detail, LNA cells were more sensitive to low doses of ozone than HNA cells (Lee et al., 2016). Understanding the origin of the distribution of HNA and LNA bacteria in the SSF effluent may thus impact downstream disinfection as SSFs are often the last biological treatment step with the potential to shape the bacterial community entering the distribution system.

The rapid FCM method used here captured dynamic microbial changes in the SSF biofilm and effluent water. These changes reflected SSF function and could potentially impact downstream disinfection. FCM would thus be useful for process control of SSF in drinking water treatment plants, providing advantages over current methods utilizing routine plating, including cost, speed, and the potential for online monitoring (Besmer et al., 2014; Van Nevel et al., 2017b). FCM has been specifically proposed for monitoring of maintenance in distribution networks (Van Nevel et al., 2017a). Time and water volume lost during maintenance and reconnection of the SSF into the production line could be minimized, reducing the overall cost for water treatment. This would be a particular advantage in countries such as Sweden where scraping of SSFs is required 2–3 times per year. Zonal distributions created by CHIC analysis can establish a baseline profile, with deviations from this profile indicating possible changes in microbial water quality. Understanding how much variation can be expected in the bacterial profile, including the influence of seasonal or operational changes, is required. Given that many factors, including local weather patterns or source water, could impact the bacterial community, the use of FCM with CHIC for process control may require each drinking water producer to establish unique baselines customized for individual treatment plants.

5. Conclusions

- Established SSFs showed consistent performance by FCM bacterial profiling that was not altered by removal of the *schmutzdecke* suggesting that a mature biofilm in the deep sand bed is required for consistent microbial water quality from SSFs.
- Inoculation with sand previously used in SSF at the same treatment plant could explain the more rapidly improved functioning of one new SSF. Improvement in function was not observed for a new SSF constructed only with new sand.
- Alteration of FCM bacterial profiles in effluents from SSFs could indicate compromised function of the filter.
- Using routine CHIC analysis would simplify and reduce bias in assessing microbial water quality, facilitating use of FCM for process control.

Acknowledgements

This study was financially supported by the Swedish Research Council (Grant 621-2013-587), the Swedish Water and Wastewater Association Development Fund, The Crafoord Foundation, Sweden Water Research AB and Sydvatten AB. The authors would like to thank the employees at Ringsjö Waterworks, especially Agneta Jönsson and Marianne Franke for their assistance with sampling and analysis of conventional data.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2018.03.032.

References

Allen, M.J., Edberg, S.C., Reasoner, D.J., 2004. Heterotrophic plate count bacteria—what is their significance in drinking water? Int. J. Food Microbiol. 92 (3), 265–274.

Bai, Y., Liu, R., Liang, J., Qu, J., 2013. Integrated metagenomic and physiochemical analyses to evaluate the potential role of microbes in the sand filter of a drinking water treatment system. PLoS One 8 (4), e61011.

Barrett, J.M., Bryck, J., Collins, M.R., Janonis, B.A., Logsdon, G.S., 1991. Manual of Design for Slow Sand Filtration. AWWA Research Foundation and American Water Works Association, USA.

Bartram, J., Cotruvo, J., Exner, M., 2003. Heterotrophic Plate Counts and Drinking-water Safety: The Significance of HPCs for Water Quality and Human Health. IWA Pub.

Bauer, R., Dizer, H., Graeber, I., Rosenwinkel, K.-H., López-Pila, J.M., 2011. Removal of bacterial fecal indicators, coliphages and enteric adenoviruses from waters with high fecal pollution by slow sand filtration. Water Res. 45 (2), 439–452.

Berney, M., Vital, M., Hulshoff, I., Weilenmann, H.U., Egli, T., Hammes, F., 2008. Rapid, cultivation-independent assessment of microbial viability in drinking water. Water Res. 42 (14), 4010–4018.

Besmer, M.D., Hammes, F., 2016. Short-term microbial dynamics in a drinking water plant treating groundwater with occasional high microbial loads. Water Res. 107, 11–18.

Besmer, M.D., Weissbrodt, D.G., Kratochvil, B.E., Sigrist, J.A., Weyland, M.S., Hammes, F., 2014. The feasibility of automated online flow cytometry for in-situ monitoring of microbial dynamics in aquatic ecosystems. Front. Microbiol. 5, 265.

Boi, P., Amalfitano, S., Manti, A., Semprucci, F., Sisti, D., Rocchi, M.B., Balsamo, M., Papa, S., 2016. Strategies for water quality assessment: a multiparametric analysis of microbiological changes in river waters. River Res. Appl. 32 (3), 490–500.

Bourne, D.G., Blakeley, R.L., Riddles, P., Jones, G.J., 2006. Biodegradation of the cyanobacterial toxin microcystin LR in natural water and biologically active slow sand filters. Water Res. 40 (6), 1294–1302.

Calvo-Bado, L.A., Pettitt, T.R., Parsons, N., Petch, G.M., Morgan, J.A., Whipps, J.M., 2003. Spatial and temporal analysis of the microbial community in slow sand filters used for treating horticultural irrigation water. Appl. Environ. Microbiol. 69 (4), 2116–2125.

De Corte, D., Sintes, E., Yokokawa, T., Lekunberri, I., Herndl, G.J., 2016. Large-scale distribution of microbial and viral populations in the South Atlantic Ocean. Environ. Microbiol. Rep. 8 (2), 305–315.

El-Chakhtoura, J., Prest, E., Saikaly, P., van Loosdrecht, M., Hammes, F., Vrouwenvelder, H., 2015. Dynamics of bacterial communities before and after distribution in a full-scale drinking water network. Water Res. 74, 180–190.

Elliott, M.A., Stauber, C.E., Koksal, F., DiGiano, F.A., Sobsey, M.D., 2008. Reductions of E. coli, echovirus type 12 and bacteriophages in an intermittently operated household-scale slow sand filter. Water Res. 42 (10–11), 2662–2670.

Ellis, B., Haaland, P., Hahne, F., Meur, N.L., Gopalakrishnan, N., Spidlen, J., Jiang, M., 2016. flowCore: basic structures for flow cytometry data. Bioconductor R. package version 1.40.0.

Gillespie, S., Lipphaus, P., Green, J., Parsons, S., Weir, P., Juskowiak, K., Jefferson, B., Jarvis, P., Nocker, A., 2014. Assessing microbiological water quality in drinking water distribution systems with disinfectant residual using flow cytometry. Water Res. 65, 224–234.

Haig, S.-J., Quince, C., Davies, R.L., Dorea, C.C., Collins, G., 2014. Replicating the microbial community and water quality performance of full-scale slow sand filters in laboratory-scale filters. Water Res. 61, 141–151.

Haig, S.-J., Schirmer, M., D'Amore, R., Gibbs, J., Davies, R.L., Collins, G., Quince, C., 2015a. Stable-isotope probing and metagenomics reveal predation by protozoa drives E. coli removal in slow sand filters. ISME J. 9 (4), 797–808.

Haig, S.J., Quince, C., Davies, R.L., Dorea, C.C., Collins, G., 2015b. The relationship between microbial community evenness and function in slow sand filters. MBio 6 (5).

Hijnen, W.A., Schijven, J.F., Bonne, P., Visser, A., Medema, G.J., 2004. Elimination of viruses, bacteria and protozoan oocysts by slow sand filtration. Water Sci. Technol. 50 (1), 147–154.

Hijnen, W.A., Dullemont, Y.J., Schijven, J.F., Hanzens-Brouwer, A.J., Rosielle, M., Medema, G., 2007. Removal and fate of Cryptosporidium parvum, Clostridium perfringens and small-sized centric diatoms (Stephanodiscus hantzschii) in slow sand filters. Water Res. 41 (10), 2151–2162.

Huisman, L., Wood, W.E., 1974. Slow Sand Filtration. World Health Organization, Geneva Switzerland.

Koch, C., Fetzer, I., Harms, H., Müller, S., 2013. CHIC—an automated approach for the detection of dynamic variations in complex microbial communities. Cytom. Part A 83A (6), 561–567.

Lautenschlager, K., Hwang, C., Ling, F., Liu, W.T., Boon, N., Koster, O., Egli, T., Hammes, F., 2014. Abundance and composition of indigenous bacterial communities in a multi-step biofiltration-based drinking water treatment plant. Water Res. 62, 40–52.

Lee, Y., Imminger, S., Czekalski, N., von Gunten, U., Hammes, F., 2016. Inactivation efficiency of Escherichia coli and autochthonous bacteria during ozonation of municipal wastewater effluents quantified with flow cytometry and adenosine tri-phosphate analyses. Water Res. 101, 617–627.

Li, C., Ling, F., Zhang, M., Liu, W.-T., Li, Y., Liu, W., 2017. Characterization of bacterial community dynamics in a full-scale drinking water treatment plant. J. Environ. Sci. 51 (Suppl. C), 21–30.

Oh, S., Hammes, F., Liu, W.-T., 2018. Metagenomic characterization of biofilter microbial communities in a full-scale drinking water treatment plant. Water Res. 128, 278–285.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2017. Vegan: Community Ecology Package.

Pagaling, E., Strathdee, F., Spears, B.M., Cates, M.E., Allen, R.J., Free, A., 2014. Community history affects the predictability of microbial ecosystem development. ISME J. 8 (1), 19–30.

Park, J.W., Kim, H.C., Meyer, A.S., Kim, S., Maeng, S.K., 2016. Influences of NOM composition and bacteriological characteristics on biological stability in a full-scale drinking water treatment plant. Chemosphere 160, 189–198.

Persson, K.M., 2013. Vattnet ska fram!: 50 år med Ringsjöverket. Sydvatten AB.

Peter, H., Ylla, I., Gudasz, C., Romaní, A.M., Sabater, S., Tranvik, L.J., 2011. Multi-functionality and diversity in bacterial biofilms. PLoS One 6 (8), e23225.

Pfannes, K.R., Langenbach, K.M., Pilloni, G., Stuhrmann, T., Euringer, K., Lueders, T., Neu, T.R., Muller, J.A., Kastner, M., Meckenstock, R.U., 2015. Selective elimination of bacterial faecal indicators in the Schmutzdecke of slow sand filtration columns. Appl. Microbiol. Biotechnol. 99 (23), 10323–10332.

Pinto, A.J., Xi, C., Raskin, L., 2012. Bacterial community structure in the drinking water microbiome is governed by filtration processes. Environ. Sci. Technol. 46 (16), 8851–8859.

Prenafeta-Boldú, F.X., Trillas, I., Viñas, M., Guivernau, M., Cáceres, R., Marfà, O., 2017. Effectiveness of a full-scale horizontal slow sand filter for controlling phytopathogens in recirculating hydroponics: from microbial isolation to full microbiome assessment. Sci. Total Environ. 599–600, 780–788.

Prest, E.I., Hammes, F., Kotzsch, S., van Loosdrecht, M.C.M., Vrouwenvelder, J.S., 2013. Monitoring microbiological changes in drinking water systems using a fast and reproducible flow cytometric method. Water Res. 47, 7131–7142.

R Development Core Team, 2017. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Ramseier, M.K., von Gunten, U., Freihofer, P., Hammes, F., 2011. Kinetics of membrane damage to high (HNA) and low (LNA) nucleic acid bacterial clusters in drinking water by ozone, chlorine, chlorine dioxide, monochloramine, ferrate(VI), and permanganate. Water Res. 45 (3), 1490–1500.

Ritson, J.P., Graham, N.J.D., Templeton, M.R., Clark, J.M., Gough, R., Freeman, C., 2014. The impact of climate change on the treatability of dissolved organic matter (DOM) in upland water supplies: a UK perspective. Sci. Total Environ. 473–474, 714–730.

Schattenhofer, M., Wulf, J., Kostadinov, I., Glöckner, F.O., Zubkov, M.V., Fuchs, B.M., 2011. Phylogenetic characterisation of picoplanktonic populations with high and low nucleic acid content in the North Atlantic Ocean. Syst. Appl. Microbiol. 34 (6), 470–475.

Schumann, J., Koch, C., Fetzer, I., Müller, S., 2015. flowCHIC - Analyze flow cytometric data of complex microbial communities based on histogram images. Bioconductor R. package version 1.8.0.

Sterk, A., Schijven, J., de Nijs, T., de Roda Husman, A.M., 2013. Direct and indirect effects of climate change on the risk of infection by water-transmitted pathogens. Environ. Sci. Technol. 47 (22), 12648–12660.

Sydvatten AB, 2016. Ringsjöverket. Sydvatten AB.

Unger, M., Collins, M.R., 2006. In: Gimbel, R., Graham, N., Collins, M.R. (Eds.), Recent progress in Slow Sand and Alternative Biofiltration Processes. IWA Publishing.

Unger, M., Collins, M.R., 2008. Assessing *Eschericia coli* removal in the schmutzdecke of slow-rate biofilters. J. AWWA Am. Water Works Assoc. 100 (12), 60–73.

van Leeuwen, C.J., 2013. City blueprints: baseline assessments of sustainable water management in 11 cities of the future. Water Resour. Manag. 27 (15), 5191–5206.

Van Nevel, S., Buysschaert, B., De Roy, K., De Gusseme, B., Clement, L., Boon, N., 2017a. Flow cytometry for immediate follow-up of drinking water networks after maintenance. Water Res. 111, 66–73.

Van Nevel, S., Koetzsch, S., Proctor, C.R., Besmer, M.D., Prest, E.I., Vrouwenvelder, J.S., Knezev, A., Boon, N., Hammes, F., 2017b. Flow cytometric bacterial cell counts challenge conventional heterotrophic plate counts for routine microbiological drinking water monitoring. Water Res. 113, 191–206.

Vila-Costa, M., Gasol, J.M., Sharma, S., Moran, M.A., 2012. Community analysis of high- and low-nucleic acid-containing bacteria in NW Mediterranean coastal waters using 16S rDNA pyrosequencing. Environ. Microbiol. 14 (6), 1390–1402.

Vital, M., Dignum, M., Magic-Knezev, A., Ross, P., Rietveld, L., Hammes, F., 2012. Flow cytometry and adenosine tri-phosphate analysis: alternative possibilities to evaluate major bacteriological changes in drinking water treatment and distribution systems. Water Res. 46 (15), 4665–4676.

Wakelin, S., Page, D., Dillon, P., Pavelic, P., Abell, G.C.J., Gregg, A.L., Brodie, E., DeSantis, T.Z., Goldfarb, K.C., Anderson, G., 2011. Microbial community structure of a slow sand filter schmutzdecke: a phylogenetic snapshot based on rRNA sequence analysis. Water Sci. Technol. Water Supply 11 (4), 426–436.

Wang, Y., Hammes, F., Boon, N., Chami, M., Egli, T., 2009. Isolation and characterization of low nucleic acid (LNA)-content bacteria. ISME J. 3 (8), 889–902.

Wickham, H., 2009. ggplot2: elegant graphics for data analysis. Springer, New York.

Wotton, R.S., 2002. Water purification using sand. Hydrobiologia 469 (1), 193–201.