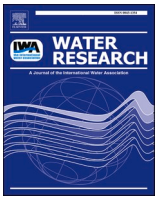
Water Research 277 (2025) 123328

Contents lists available at ScienceDirect

Water Research

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



Impact of environmental and process conditions on the microbial ecology and performance of full-scale slow sand filters in drinking water treatment

Valentina Attiani [a,*], Hauke Smidt [a], Paul W.J.J. van der Wielen [a,b]

[a] Laboratory of Microbiology, Wageningen University & Research, P.O. Box 8033, 6700, EH, Wageningen, The Netherlands
[b] KWR Watercycle Research Institute, P.O. Box 1072, 3430 BB, Nieuwegein, The Netherlands

ARTICLE INFO

Keywords:
Slow sand filtration
Drinking water
Molecular ecology
Biological stability
Schmutzdecke

ABSTRACT

Slow sand filters (SSFs) are commonly used for treating drinking water, effectively removing contaminants such as particles, organic matter, and microorganisms. However, the ecological dynamics of prokaryotic communities within SSFs remain poorly understood. This study investigated the top sand layer, the Schmutzdecke (SCM), along with the influent and effluent water of full-scale SSFs at four drinking water treatment plants (DWTPs) in the Netherlands. These plants use SSFs as the final step in their treatment to produce unchlorinated drinking water. Two DWTPs treat surface water after dune infiltration and do not apply advanced oxidation processes prior the SSF. In contrast, the other two DWTPs treat reservoir-stored surface water and incorporate ozonation or UV and activated carbon filtration as part of their treatment train. All SSFs consistently reduced biomass in the effluent compared to the influent, confirming their role in biomass load reduction. Key biological and chemical parameters showed that pretreatment with dune infiltration produced more biologically stable drinking water compared to reservoir storage. Moreover, while SSFs act as polishing filters when treating dune-infiltrated surface water, they significantly alter the prokaryotic community and biological stability of the water when treating reservoir-stored surface water. Prokaryotic communities in the SCM and water samples showed distinct compositions rather than merely the accumulation of microorganisms in the SCM from the influent water, demonstrating that SSF are active ecosystems different from water. The SCM exhibited a higher relative abundance of the genera *SWB02, Gemmata, Pedomicrobium, Nitrospira*, and *mle1–7*, while in the water samples the genus *Candidatus* Omnitrophus was relatively more abundant. Moreover, each DWTP hosts a unique prokaryotic profiles in both the SCM and water samples. Source water, upstream treatment and/or the biological stability of the influent water are identified as potential causes affecting the prokaryotic communities in SSFs that affect the microbial water quality of the effluent water.

1. Introduction

Several countries in Europe (e.g. Switzerland, the Netherlands, Denmark, and parts of Germany) distribute drinking water without a disinfectant residual. For unchlorinated drinking water to remain safe and biologically stable, it is crucial to maintain a low nutrient load by minimizing levels of biodegradable dissolved organic carbon (BDOC). Excess concentrations of BDOC in the water can lead to growth of opportunistic pathogens that are able to multiply in the biofilm attached to the pipe material within the water distribution system and which can pose a risk for public health (Hammes et al., 2010; Hijnen et al., 2018; Rosario-Ortiz et al., 2016; van der Kooij, 1992; Van der Kooij, 2003; Van der Kooij and Veenendaal, 2014b). In the Netherlands, a guideline for easily assimilable organic carbon (AOC), an important part of the BDOC, has been set at 10 μg/L, in order to maintain biologically stable drinking water throughout the distribution system.

To produce biologically stable drinking water from surface water, drinking water treatment plants (DWTPs) can use slow sand filtration in the treatment train often as the last step, before drinking water is distributed. Slow sand filtration is a sustainable, robust and effective

Abbreviations: SCM, Schmutzdecke; DWTP, Drinking water treatment plant; SSF/s, Slow sand filter/s; DOC, Dissolved organic carbon; BDOC, Biodegradable dissolved organic carbon; AOC, Easily assimilable organic carbon; BPP-W, Biomass production potential for water; MBC7, Maximal biomass concentration during the first seven days of incubation in the BPP-W test; CBP14, Cumulative biomass production during 14 days of incubation in the BPP-W test.

* Corresponding author.
E-mail address: valentina.attiani@wur.nl (V. Attiani).

https://doi.org/10.1016/j.watres.2025.123328
Received 24 July 2024; Received in revised form 15 December 2024; Accepted 18 February 2025
Available online 19 February 2025

treatment to produce microbiologically safe and stable drinking water. The slow sand filters (SSFs) act as biofilters and remove contaminants such as particulate matter, organic carbon, micropollutants and fecal pathogens through an intricate interplay of physical, chemical, and (micro)biological processes (Haig et al., 2011; Huisman and Wood, 1974; Maiyo et al., 2023). Central to the efficacy of the SSFs is the *Schmutzdecke* (SCM), a biologically active layer consisting of biofilm, organic matter and sand that forms on the surface at the top of the sand filter. The SCM harbors a rich biodiversity that can include bacteria, archaea, algae, protozoa, and small invertebrates and the biomass of the SCM increases with SSF age (Huisman and Wood, 1974; Ranjan and Prem, 2018).

The (micro)biological processes within SSFs play a pivotal role in the removal of BDOC (including AOC), highlighting the necessity of exploring microbial communities in these filters to better understand the processes involved in SSF performance for AOC removal and reaching biologically stable drinking water (Campos et al., 2002; Lautenschlager et al., 2014). Others showed that differences in water source and treatment processes affect SSF efficiency in AOC removal resulting in varied levels of biological stability of the drinking water produced (van der Kooij et al., 2017b). These authors observed that water with the highest biological stability was produced at DWTPs that utilized dune filtration and did not employ ozonation in their post-treatment before SSFs. Investigations on the microbial ecology of SSFs have elucidated (i) the general dynamics and distribution of the microbial community in the SSF of a DWTP (Chen et al., 2021; Oh et al., 2018), (ii) the critical role of biological processes in fecal pathogen removal (Haig et al., 2015b), and (iii) how SSF performance and microbial community composition in the SSF and/or water are influenced by operational parameters such as source water type, pretreatment, maintenance processes (e.g. scraping off the SCM), grain size, filter age and, flow rate (Bai et al., 2023; de Souza et al., 2021; Trikannad et al., 2024).

These studies focused on a single DWTP, while Bai et al. (2023) examined different DWTPs. However, their research only investigated the microbiology of sand samples without assessing its impact on the effluent water that is distributed as drinking water to consumers. As a result, these studies provide limited insights into how varying environmental and process conditions across different DWTPs affect the microbial ecology of SSFs and, by extension, the biological stability and quality of the produced drinking water. Moreover, there remains a need for a deeper understanding of how specific source water characteristics and treatment steps shape the microbial community of the SCM and influence the drinking water microbiome. The study aims to address these gaps by evaluating the potential effect of different source waters, treatment trains and operational SSF parameters on the prokaryotic ecology of SSFs and the microbiological water quality, including biological stability of drinking water produced at four DWTPs in the Netherlands.

2. Materials and methods

2.1. Slow sand filter characteristics

For this study, sand from the SCM layer (top 2 cm of the SSF sand bed), and influent and effluent water samples were collected from two SSFs of DWTP Monster, five SSFs of DWTP Scheveningen, one SSF of DWTP De Punt and three SSFs of DWTP Weesperkarspel, all located in the Netherlands (**Fig. S1**). All SSFs are operated indoors as the final step in the drinking water treatment process. The treated water produced by these filters is directly distributed to consumers without undergoing chlorination. The four DWTPs exhibit variations in location, source water, upstream treatments (Table 1) and operational parameters (**Table S1**). Furthermore, it should be noted that the sample collection was partly affected by various technical aspects, such as accessibility to the SSFs and DWTPs, technician availability and logistic constraints during the Covid 19 pandemic.

Table 1
Treatment steps employed at each DWTP. * Biological activated carbon filtration (BACF), ** powdered activated carbon (PAC).

Weesperkarspel	Scheveningen and Monster	De Punt
Seepage water from Bethune Polder	River Meuse	River Drentsche Aa
Coagulation with $FeCl_3$ + sedimentation	Storage in river section + $FeSO_4$ dosing in the river section to remove ortho phosphate	Grid filtration
Reservoir storage in lake	Micro sieves (March – October)	Transportation to the mixing basin
Rapid sand filtration	Rapid sand filtration	Reservoir storage in mixing basin (60 days)
Transportation to the DWTP	Transportation to dune area	Grid filtration
Ozonation	Dune infiltration (60 days)	(optional) pH correction with HCl
Softening with NaOH	Water extraction from the dunes	Transportation to DWTP
BACF*	Softening with NaOH	Coagulation/ flocculation/ lamellar sedimentation
Slow sand filtration	PAC** dosage	Double layer-filtration (anthracite and sand)
Storage tanks	Aeration	BACF*
Distribution	Rapid sand filtration	UV-disinfection (2 × 20 mJ/cm^2 in series)
	Slow sand filtration	Slow sand filtration
	Storage tanks	Cascade for CO_2 removal
	Distribution	(optional) pH correction with NaOH
		Storage tanks
		Distribution

2.2. Sand sampling

Two sampling approaches were used to collect the SCM from the SSFs. The first approach involved lowering the water level in the filters to expose the top layer of the sand bed 2–3 h before filter scraping. Once exposed the SCM was collected with a sterile stainless-steel spoon at various points (A, B, and C) located at increasing distances from the influent inlet. The second approach involved collecting SCM samples directly during filter operation. A sterile 15 ml Falcon tube attached to a sterile stainless-steel stick was used to collect samples without lowering the water level. This second approach was used only for SSFs 3, 6, 7A, and 9A at DWTP Scheveningen, whereas the first approach was used for the other SSFs (**Table S1**). **Fig. S2** shows the dimensions and sampling points of all SSFs investigated in this study. All samples were placed in 15 ml sterile Falcon tubes, transported in Styrofoam boxes equipped with icepacks, and stored at −20 °C until DNA extraction. SCM samples from the different SSFs were sampled on different days as reported in **Table S1**.

2.3. Water sampling and chemical parameters

One liter of water influent and effluent of all SSFs (except SSFs 3, 6, 7A and 9A in Scheveningen) was collected in duplicates by using sterile plastic bottles (Identipack, Netherlands) between 1 and 14 days before the SCM layer was scraped off. The water samples were transported to the laboratory in Styrofoam boxes containing icepacks and filtered within 24 h over a 0.2 μm filter (Isopore TM PC membrane, 47 mm hydrophilic, Merck, Millipore) to collect the microorganisms present. The filters were then stored at –20 °C until DNA isolation. Water influent and effluent samples from the same SSF were collected on the same day which was 1 to 14 days prior to SCM sampling depending on the SSF, as reported in **Table S1**.

Data on the routinely monitored chemical parameters of the treated drinking water (which is the mixed effluent water of the SSFs at the DWTP) were provided by the drinking water companies. These data

included adenosine triphosphate (ATP) concentration, dissolved organic carbon (DOC) concentration, PO_4 concentration, pH and the biomass production potential parameters MBC7 and CBP14 (van der Wielen et al., 2023). The data of these measurements were taken from the same month or year as the sand sampling. When current data were not available, we used historical data from previous studies on the same DWTPs (van der Kooij et al., 2017b; van der Wielen et al., 2023) (**Table S2**).

2.4. DNA isolation and library preparation

DNA was extracted from water samples using the DNeasy PowerBiofilm Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction except for the first step for which the thawed filters were placed directly into the PowerBeads tubes. DNA was isolated from sand samples with the Powersoil Pro kit (QIAGEN, Hilden, Germany) using a range of 0.5–1 g of sand as starting material. The amount of sand used was noted for future reference and normalization. A negative control consisting of one empty PowerBead Pro Tube was included during DNA extraction for quality control. For both water and sand samples, the bead beating was performed using the FastPrep-24 5 G bead beating grinder and lysis system (MP Biomedicals, Irvine, USA), and by applying one cycle at 4.0 m/s for 45 s. After DNA extraction, DNA concentrations were measured fluorometrically (Qubit dsDNA BR assay, Invitrogen) and the DNA was stored at −20 °C.

The hypervariable region V4 (~290 bp) of the bacterial and archaeal 16S rRNA gene was amplified from the extracted DNA with a PCR reaction prepared with 10 μL of 5× Phusion Green HF Buffer (Thermo Scientific, USA), 1 μL each of 10 μM 5′-barcoded primers 515F-n (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R-n (5′-GGACTACNVGGGTWTCTAAT-3′) (Apprill et al., 2015; Parada et al., 2016), 1 μL of 10 mM dNTPs mix (Promega Corporation, USA), 0.5 μL of 2 U/μL Phusion Green Hot Start II HF DNA polymerase (Thermo Scientific), the DNA template (final concentration of ~20 ng/μL DNA) and Nuclease-free water to reach a final volume of 50 μL. Positive controls, non-template controls (only PCR mix) and negative controls (PCR mix and Nuclease-free water instead of the template DNA) were included in the PCR analyses for quality check. The amplification program included an initial denaturation at 98 °C for 30 s, then 28 cycles consisting of denaturation at 98 °C for 10 s, followed by annealing at 50 °C for 10 s and elongation at 72 °C for 10 s, and a final extension at 72 °C for 7 min. Presence and length of PCR products were verified by gel electrophoresis. Subsequently, PCR products were purified using the CleanPCR magnetic beads kit (CleanNA, Netherlands) according to the manufacturer's protocol. Purified products were quantified fluorometrically (Qubit dsDNA BR assay, Invitrogen). Thereafter, purified PCR-products were pooled in equimolar amounts into libraries, including negative and positive controls. After pooling, the mixed libraries were purified and concentrated again using CleanPCR magnetic beads to a concentration between 200 and 250 ng/μL with a final volume of 40 μL. The final purified PCR products including those amplified from SSF samples, positive and negative controls were sequenced on an Illumina Novaseq 6000 platform at Novogene (Cambridge, United Kingdom). Raw 16S rRNA gene sequences with barcode and primer removed and supporting metadata were deposited in the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under accession number PRJEB77612.

2.5. qPCR

Quantitative PCR (qPCR) was used to measure total bacterial 16S rRNA gene copy numbers in the sand and water samples. The DNA concentrations used in the qPCR reactions were adjusted to 1 ng/μL by diluting original extracts in DNase/RNase free water before use as the template in qPCR. The qPCR mix was composed of iQTM SYBR Green Supermix (Bio-Rad Laboratories, USA), universal primers targeting the bacterial 16S rRNA gene (1369F 5′-CGGTGAATACGTTCYCGG-3′ and 1492R 5′-GGWTACCTTGTTACGACTT-3′; 123 bp), 1 μL of DNA template and sterile nuclease-free water in a total volume of 10 μL. Each sample was assayed in technical triplicates by using a C1000 Thermal Cycler (CFX384 Real-Time system, Bio-Rad Laboratories, USA) with the following protocol: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 15 s each; then one cycle of 95 °C for 1 min; and a stepwise increase of temperature from 60 °C to 95 °C (at 0.5 °C per 5 s) to obtain melt curve data. The qPCR data was analyzed using CFX Maestro 2.3 (Bio-Rad) and Microsoft Excel (version 2021).

2.6. 16S rRNA gene amplicon sequence data processing

NG-Tax 2.0 was used for processing of 16S rRNA gene sequence data with default settings (Poncheewin et al., 2020; Ramiro-Garcia et al., 2016). Subsequently, amplicon sequence variants (ASVs) were identified on a per sample basis. Taxonomic assignment of ASVs was performed referring to the SILVA 138.1 16S rRNA gene reference database (Quast et al., 2013). The NG-Tax output was imported in R *(4.3.3)* (R Core Team, 2020), and the phyloseq object was built combining the ASV table with the phylogenetic tree and metadata using the package *phyloseq (1.46.0)* (*McMurdie and Holmes, 2013*). Data pre-processing included filtering by removing ASVs that could not be identified at the Domain level and singletons (ASVs of which the sum of reads is equal to one).

The R packages *microbiome (1.24.0)* (Lahti and Shetty, 2018), *phyloseq* and *microbiomeutilities (1.00.17)* (Shetty, 2024) were used for data handling and visualization. Alpha diversity was computed with Shannon, Chao1 and Pielou indices calculated at ASV level using the *microbiome* package. Before performing alpha diversity analyses the dataset was rarefied using to adjust for differences in library sizes across samples. Beta diversity was assessed with Principal Coordinates Analysis (PCoA) based on weighted UniFrac distance (considering relative abundance and phylogenetic relatedness of ASVs) using the *phyloseq* package after compositional transformation of the data. The 16S rRNA gene read count data were first transformed to microbial relative abundance with the *microbiome* package. The relative abundance of taxa is calculated based on the proportional representation of each taxon within the total community. This is necessary because with the 16S rRNA gene sequencing method, the total number of reads differs between samples, making it impossible to reliably compare absolute abundances of the taxa. The relative abundance is derived from the number of 16S rRNA gene sequences (amplicon reads) assigned to each taxon, normalized by the total number of sequences in a sample. For general data handling, additional packages used included *dplyr (1.1.4)* (Wickham et al., 2023), *speedyseq (0.5.3.9018)* (McLaren, 2023), and *tidyr (1.3.1)* (Wickham, 2024). For data visualization, such as bar plots, scatter plots, and boxplots, were used the packages *ggplot2 (3.5.1)* (Wickham, 2016), *ggpubr (0.6.0)* (Kassambara, 2023), *RColorBrewer (1.1.3)* (Neuwirth, 2022), *ggsignif (0.6.4)* (Ahlmann-Eltze and Patil, 2021), and *microViz (0.12.1)* (Barnett et al., 2021).

2.7. Statistical analyses

The statistical analyses in this study were performed in R with package *vegan* and *stats (4.3.3)*. The significance level was set at $p < 0.05$ to determine statistically significant findings. Descriptive statistics, including measures of central tendency and variability, were calculated to summarize the data. Permutational multivariate analysis of variance (PERMANOVA) was employed to test significant differences between groups (beta diversity) with the number of permutations set at 999. If significant differences were observed, pairwise tests with Benjamini-Hochberg adjustment using the package *pairwiseAdonis (0.4.1)* (Martinez Arbizu, 2017) were performed.

Redundancy analysis (RDA) was performed with the *vegan* package, forward and backward selection was performed using the 'ordistep' function. For RDA visualization were used the packages *microViz* and

ggplot2.

Differential abundance analyses, which identify taxa that are significantly different in their relative abundance between groups or conditions, were performed in R according to the linear models for differential abundance analysis of microbiome compositional data (LinDa) (Zhou et al., 2022) using the packages *MicrobiomeStat (1.2)* (Zhang, 2024) and *vegan*. Mixed-effects models (~fixed_variable+(1|SSF)) were employed, with the exception for the group of samples coming from the same SSF for which we used a fixed effect model. Analysis parameters included a prevalence filter of 0.1, a mean abundance filter of 0.01 (matrix type) and 0.002 (for treatment), and p-values were adjusted using the Benjamini-Hochberg method with an alpha of 0.05. The LinDa analysis was performed on taxa that were aggregated at genus level.

Mann-Whitney-Wilcoxon test was used to test the significance of differences between the mean values of the water effluent chemical parameters between the two different water sources. Spearman correlation was used to test for correlation among the water effluent chemical parameters. This statistics analysis was conducted in R using the *vegan* package.

3. Results

For this study, a total of 40 SCM and 14 water influent and effluent samples were collected from four DWTPs in the Netherlands. These DWTPs employed three different drinking water treatment processes. Quantification of bacterial 16S rRNA gene copies as a proxy for bacterial biomass in these samples was performed using qPCR. Furthermore, prokaryotic community composition was analyzed using 16S rRNA gene amplicon sequencing followed by data interpretation based on the alpha and beta diversity and differential abundance analysis.

3.1. Total bacteria quantification on sand and water

The qPCR analysis revealed that the 0–2 cm sand layer of all SSFs had a total bacterial biomass ranging from 1.0×10^8 to 7.7×10^8 16S rRNA gene copies per gram of wet sand. The highest bacterial 16S rRNA gene copy numbers were observed in the sand from filter 2 of DWTP De Punt and filters 5 and 6 of DWTP Scheveningen, whereas the lowest bacterial 16S rRNA gene copy numbers were observed in filter 7A and 9A at DWTP Scheveningen (Fig. 1A; **Table S3A**).

The qPCR analysis of the influent and effluent water samples consistently showed for all SSFs that the influent water had higher bacterial 16S rRNA gene copy numbers than the effluent water (Fig. 1B; **Table S3B**). The highest 16S rRNA gene copy (gc) numbers in the influent water were observed for filter 12 of DWTP Weesperkarspel (1.4×10^5 gc/ml) and for the effluent water for filter 1 of DWTP Weesperkarspel (5.5×10^4 gc/ml). The lowest 16S rRNA gene copy numbers in both the influent (5.4×10^3 gc/ml) and effluent (2.2×10^3 gc/ml) water were observed for filter 5B of DWTP Monster.

3.2. Prokaryotic community composition of the different matrix types

The alpha diversity, measured using the Shannon index (representing both ASVs richness and evenness), showed variability across different matrix types and DWTPs (**Fig. S3C**). SCM samples displayed consistent values for the Shannon index ranging from 4 to 5 across all filters and DWTPs, with SSFs at Weesperkarspel having the lowest values. In contrast, influent and effluent water samples showed greater variability in the Shannon index both between and within DWTPs. Specifically, influent and effluent samples from the SSF at DWTP De Punt exhibited the lowest values compared to other DWTPs. Comparable trends are showed in the Chao1 index (richness) and Pielou index (evenness) (**Fig. S3A-B**)

When examining the beta diversity using pairwise weighted UniFrac distances, the samples clustered according to their matrix type (PERMANOVA, $p = 0.001$), namely water influent, water effluent and sand from the SCM layer regardless of the DWTP where samples were collected. The pairwise comparisons results confirmed that each pair of matrix types harbored significantly distinct prokaryotic communities ($p_{adj} = 0.001$). The prokaryotic communities of the SCM samples exhibited lower variability than the water samples, indicating greater similarity of the community composition in SCM than in the water across different DWTPs (Fig. 2; **Table S4**).

To identify prokaryotic groups that vary in relative abundance across different matrix types of all SSFs analyzed, we conducted a differential abundance analysis using the LinDa model targeting taxa that exhibit statistically significant alterations ($p_{adj} < 0.05$). A positive log2 fold change indicates a higher relative abundance in a given matrix type compared to other types, whereas a negative log2 fold change signifies a lower relative abundance, with the magnitude of this value indicating its extent. Fig. 3 shows the results of the differential abundance analyses organized by DWTP and matrix type.

At DWTP Monster the main common taxa with increased relative abundance in the SCM compared to both water types were the NS9 marine group, *Anaerolineaceae* family and SBR1031 order. The ones more relatively abundant in both the water types compared to the SCM were the orders *Rokubacteriales, Woesearchaeales,* and *Candidatus* Peribacteria, as well as the genera *Candidatus* Omnitrophus and *Pseudomonas*. After passing through the SSF, the effluent exhibited a higher relative abundance of the *PLTA13* and S085 orders and depleted in *Comamonadaceae* compared to the influent.

At DWTP Scheveningen the common taxa with higher relative abundance in the SCM compared to both water types were the NB1-j phylum, the BD2–11 terrestrial group class, the *Pirellulaceae, Bacteriovoracaceae,* and B1–7BS families, and the genera SWB02 and *Gemmata*.

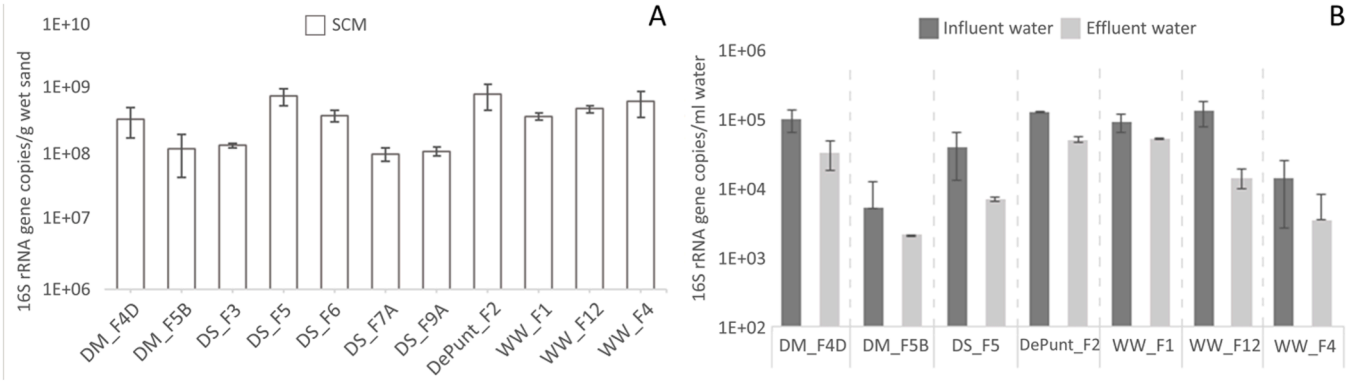


Fig. 1. Total bacterial 16S rRNA gene copies of A) Schmutzdecke (SCM) samples and B) influent and effluent water samples from each DWTP (Monster (DM), Scheveningen (DS), De Punt (DePunt) and Weesperkarspel (WW).

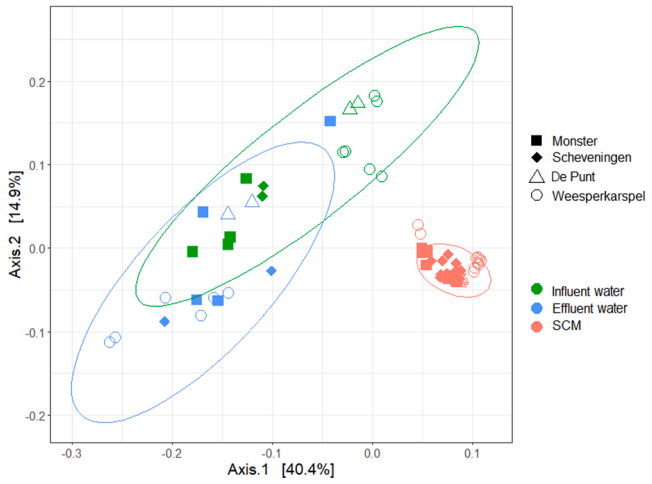


Fig. 2. PCoA plot of the beta diversity, based on pairwise weighted UniFrac distances, for Schmutzdecke (SCM), influent and effluent water samples of the SSFs from DWTPs that use dune-infiltrated surface water (Monster and Scheveningen, closed symbols) or surface water after reservoir storage (Weesperkarspel and De Punt, open symbols).

The ones with higher relative abundance in both the water types compared to the SCM were the *Rokubacteriales* order, the *Vicinamibacteriaceae* family and the *Candidatus* Omnitrophus genus. The effluent compared to the influent showed a higher relative abundance of the RCP2–54 phylum, *Vicinamibacteriales* order and *Gemmataceae* family, whereas the NS9 marine group, *Comamonadaceae* family and *Pedomicrobium* genus were more relatively abundant in the influent.

At DWTP De Punt the relatively more abundant in the SCM compared to both water types were the *Gemmataceae* family and bacteriap25 class. The taxa relatively more abundant in both water types compared to the SCM were *Woesearchaeales* order, Clade III family and hgcl_clade genus. The effluent, compared to the influent, showed a higher relative abundance of *Vicinamibacteriales Woesearchaeales* and *Planctomycetales* orders, *Alphaproteobacteria* class, and the *Vicinamibacteriaceae* family. The influent, compared to the effluent, exhibited a higher relative abundance of the genera *Sediminibacterium* and *Candidatus* Methylopumilus and the *Comamonadaceae* family.

At DWTP Weesperkarspel the common taxa that exhibited a higher relative abundance in the SCM compared to both influent and effluent water were *Dadabacteriales* order, *Blastocatellaceae, Pirellulaceae* and *Gemmataceae* families, and the mle-7 genus within the *Nitrosomonadaceae* family. The taxa relatively more abundant in both the water types compared to the SCM were *Woesearchaeales and Candidatus* Peribacteria orders, SM2D12 and TRA3–20 family and *Aquicella* genus. The effluent compared to the influent showed a higher relative abundance of the *Woesearchaeales, Vicinamibacteriales and Candidatus* Peribacteria orders, bacteriap25 and *Alphaproteobacteria* classes, and *Candidatus* Omnitrophus and *Aquicella* genera. The influent, compared to the effluent, exhibited a higher relative abundance of the genera *Polaromonas, mle-1–7* and *Nitrospira,* the families *Gallionellaceae, Comamonadaceae, Blastocateallaceae* and TRA3–20, and the *Planctomycetales* order.

Interestingly, the *Candidatus* Omnitrophus genus was relatively more abundant in the water samples compared to the SCM for all DWTPs except De Punt. At Weesperkarspel, this genus also exhibited a higher relative abundance in the effluent compared to the influent. Still, these results show that not only matrix determines the differential abundance of taxa between influent, SCM and effluent, but that each DWTP determines more specifically which of the above-mentioned taxa are relatively more abundant in each of the matrix types at a given DWTP.

The beta diversity analysis revealed significant differences in the SCM prokaryotic communities across the DWTPs ($p = 0.001$, PERMANOVA; **Table S5**). Specifically, the analysis indicated that the SCM samples from the DWTPs Monster and Scheveningen, which both treated dune-infiltrated surface water and employed identical upstream treatments, were more closely associated (pairwise comparison **Table S5**). Samples from these two DWTPs were grouped distinctly separate from samples taken at DWTPs De Punt and Weesperkarspel, which both treated reservoir-stored surface water but employed different upstream treatments (Fig. 4A**;** Table 1). Similarly, the DWTP variable had significant effect in shaping the community of the water samples (PERMANOVA, $p = 0.001$). The SCM samples with different ages up to 5.6 years (Table S1) from the same DWTP still clustered together. At Weesperkarspel, however, we observed a clear distinction between the 12 year old SCM samples and the other samples younger than one year (Fig. 4A).

In contrast to the sand results, the water influent and effluent samples from DWTPs Monster, Scheveningen and Weesperkarspel demonstrated close resemblance to each other, and clustered more distantly from those of the DWTP De Punt (Fig. 4B**;** pairwise comparison **Table S5**). Additionally, the influent and effluent samples from either Monster or Scheveningen, treating dune-infiltrated water, clustered closely together (Fig. 4B), thus showing highly similar community composition in influent and effluent. In contrast, the influent and effluent samples from either De Punt or Weesperkarspel, treating reservoir-stored water, clustered further apart, showing more distinct community composition between influent and effluent.

Moreover, a closer examination of only samples from Monster and Scheveningen indicated that, despite their SCM prokaryotic community compositions being more similar to each other than to those from Weesperkarspel and De Punt, significant differences were still observed between the two DWTPs (PERMANOVA, $p = 0.001$; **Table S7,** Fig. 4C). In contrast, the prokaryotic communities of the influent and effluent water samples from Monster and Scheveningen did not exhibit significant differences (PERMANOVA, $p > 0.05$; **Table S7;** Fig. 4D).

Fig.5 presents the outcomes of a differential abundance analysis on the SCM between the combined results from Monster/Scheveningen with Weesperkarspel or De Punt. Notably, both *Pseudomonadales* and *Planctomycetales* orders were relatively more abundant in the SCM from the DWTPs De Punt and Weesperkarspel employing reservoir-stored surface water compared to Monster/Scheveningen. Some common taxa were significantly lower ($p < 0.05$) in the SCM of Monster and Scheveningen compared to both Weesperkarspel and De Punt, such as the *Anaerolineaceae* and BSV26 families, the IMCC26256 and *Rhizobiales* orders, and the *Rhodopirellula* genus. Interestingly, Monster/Scheveningen and Weesperkarspel shared some taxa that were significantly more abundant in their SCM compared to the SCM from De Punt. These taxa included the NS9 marine group and *Blastocatellaceae* families, the PLTA13 and 11–24 orders, and the MB-A2–108 class. However, additional unique taxa exhibited significant differences in abundance between Monster/Scheveningen and De Punt, as well as between Weesperkarspel and De Punt, indicating unique distinctions in their SCM microbial compositions.

3.3. Water quality parameters and their correlation with prokaryotic community composition

As described above, the SCM prokaryotic community composition clustered based on the water source used (Fig. 4A). Therefore, we analyzed whether the pH, phosphate, DOC and ATP concentration, and two biological stability parameters (MBC7 and CPB14, parameters from the biomass production potential test for drinking water (van der Wielen et al., 2023) were different between the DWTPs that used dune-infiltrated surface water and the DWTPs that used reservoir-stored surface water. Statistical evaluation of these effluent water quality parameters from DWTPs employing dune-infiltrated surface and reservoir-stored surface water demonstrated pronounced differences. Specifically, the ATP and DOC concentration, and the MBC7 and CBP14

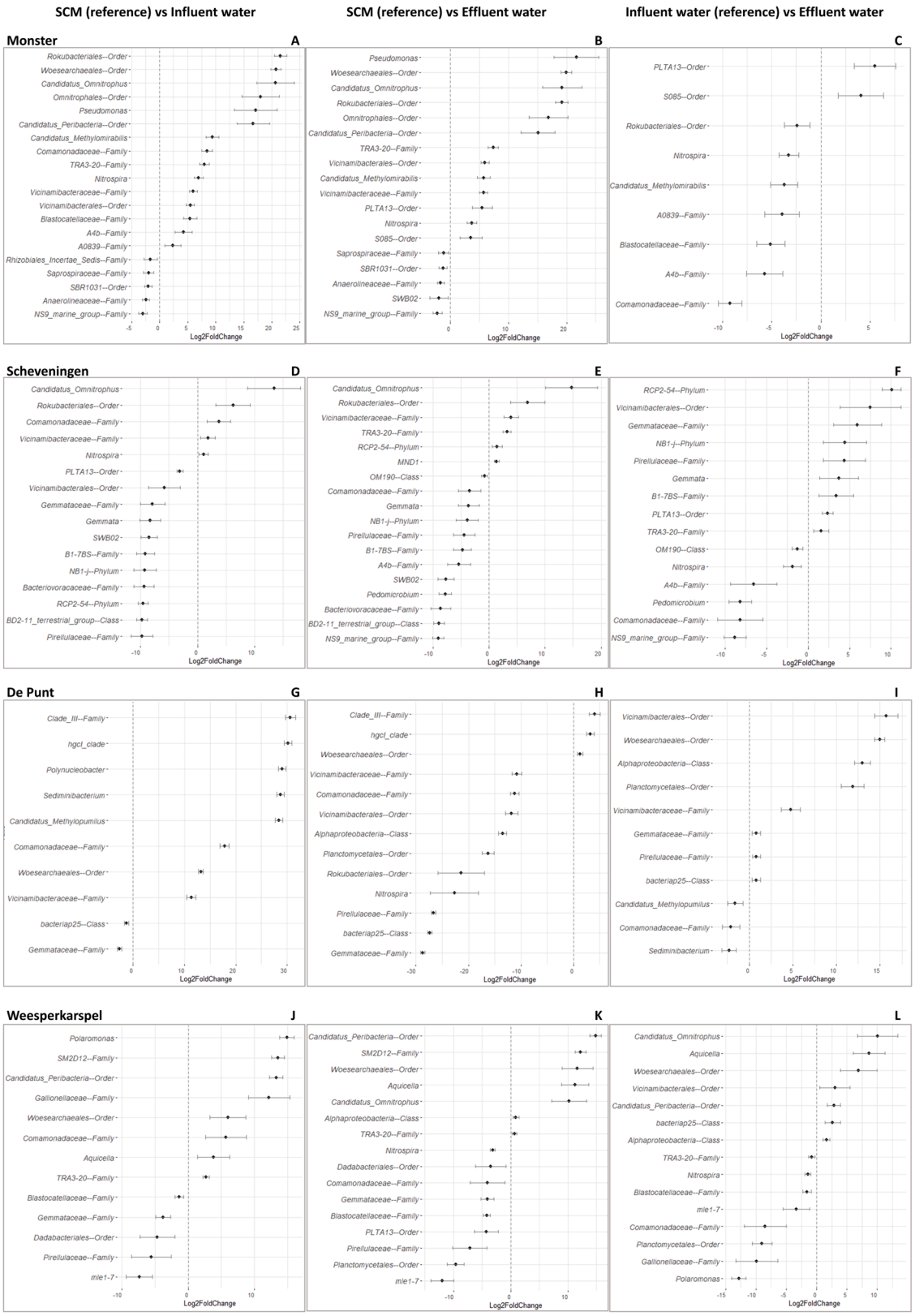


Fig. 3. Differential abundance analysis performed with LinDa on SCM and water samples, and organized by DWTPs. The figures present log2 fold changes derived from a mixed-effects model (with the formula ~Matrix_Type+(1|SSF)) for DWTPs Monster, Scheveningen and Weesperkarspel and fixed-effect model (~Matrixtype) for De Punt, with prevalence of 0.1 and mean abundance threshold of 0.01. A-C) DWTP Monster, D-F) DWTP Scheveningen, G-I) DWTP de Punt, and J-L) DWTP Weesperkarspel . The plots on the left (A, D, G and J) show taxa differentially abundant in water influent compared to SCM. The central plots (B, E, H and K) show taxa differentially abundant in water effluent compared to SCM. The plots on the right (C, F, I and L) show taxa differentially abundant in water effluent compared to water influent.

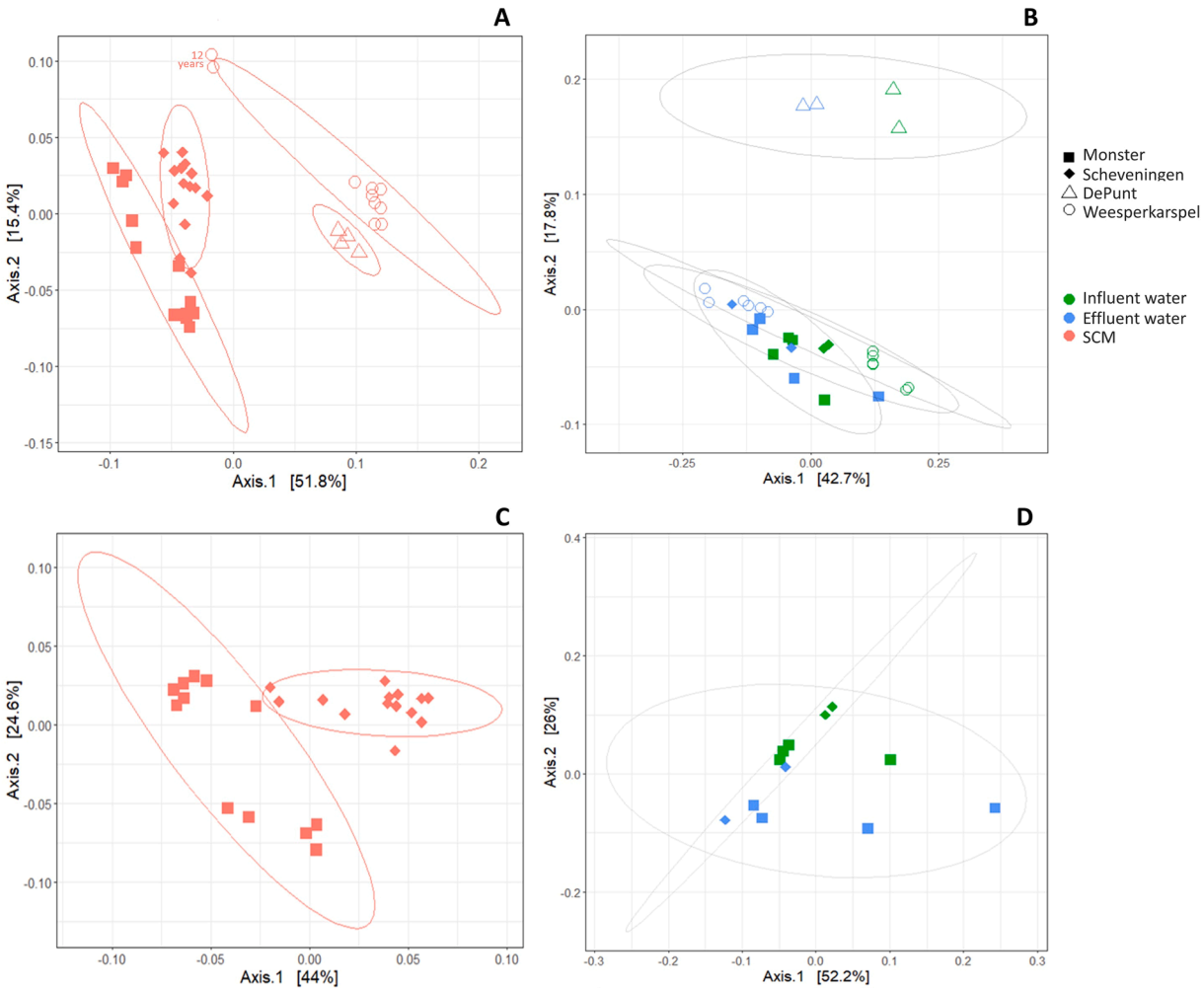


Fig. 4. A-B) PCoA plots of the beta diversity, based on pairwise weighted UniFrac distances, for Schmutzdecke (SCM) samples (A) and influent and effluent water samples (B) of the SSFs from DWTPs that use dune-infiltrated surface water (Monster and Scheveningen, closed symbols) or surface water after reservoir storage (Weesperkarspel and De Punt, open symbols). C-D) PCoA plots of the beta diversity, based on pairwise weighted UniFrac distances, for C Schmutzdecke (SCM), and D) influent and effluent water samples of the SSFs from only the DWTPs that use dune-infiltrated surface water Monster and Scheveningen.

values were significantly higher ($p < 0.001$) in the effluent of the SSFs of DWTPs that treated reservoir-stored surface water compared to the ones that treated dune-infiltrated surface water. Conversely, PO_4 concentration and pH were significantly lower ($p < 0.001$) in the effluent of DWTPs that treated reservoir-stored surface water than those treating dune-infiltrated surface water (Fig. 6A**; Table S8**).

It was also determined whether the different water quality parameters were correlated with each other using Spearman correlation analysis. The results revealed strong and significant positive correlations ($p < 0.05$, $R > 0.9$) between the ATP, DOC, MBC7, and CBP14 parameters (Fig. 6B**; Table S9**). These same parameters were significantly and strongly negatively correlated with PO_4 ($p < 0.05$, $R < -0.8$) and exhibit significant but more moderate negative correlation with pH ($p < 0.05$, R between -0.49 and -0.54). Conversely, PO_4 and pH were significantly and strongly positively correlated ($p < 0.05$, $R > 0.85$).

We also performed a redundancy analysis (RDA) to further determine to which extent the water quality parameters could contribute to explaining the observed variation in prokaryotic SCM community composition in the SSFs and the possible influence of the water source used by the DWTPs. The RDA plot showed a clear clustering of taxa and samples by DWTP and water source (Fig. 6C**; Table S10**). The water quality parameters associated with active biomass (ATP) and biological stability (DOC, MBC7 and CBP14) were positively correlated with the SCM prokaryotic community composition of De Punt and Weesperkarspel, while orthophosphate concentrations (PO_4) and pH correlated more with the SCM prokaryotic community composition of Monster and Scheveningen. ATP, DOC, MBC7 and CBP14 were higher in De Punt/Weesperkarspel than in Monster/Scheveningen, whereas this was the opposite for PO_4 and pH (Fig. 6A). Thus, the results indicated that these six parameters might influence the SCM community composition. However, we also observed that several of these water quality parameters showed high variance inflation factor (VIF) values (**Table S10**), which reflects multicollinearity between parameters. These high VIFs persisted even after forward and backward selection. Consequently, the relationships shown in the RDA plot could have been biased by multicollinearity.

4. Discussion

4.1. SCM and water samples have specific prokaryotic communities

Our findings demonstrated that the SCM, water influent and water effluent samples collected from full-scale SSFs of four different DWTPs have distinct prokaryotic community compositions. These observations align with prior research on a similar full-scale SSF system of a single DWTP in Switzerland where the drinking water was not chlorinated either (Lautenschlager et al., 2014) and on a pilot-scale rapid biofilter that directly treated surface water and was frequently backwashed (Ma

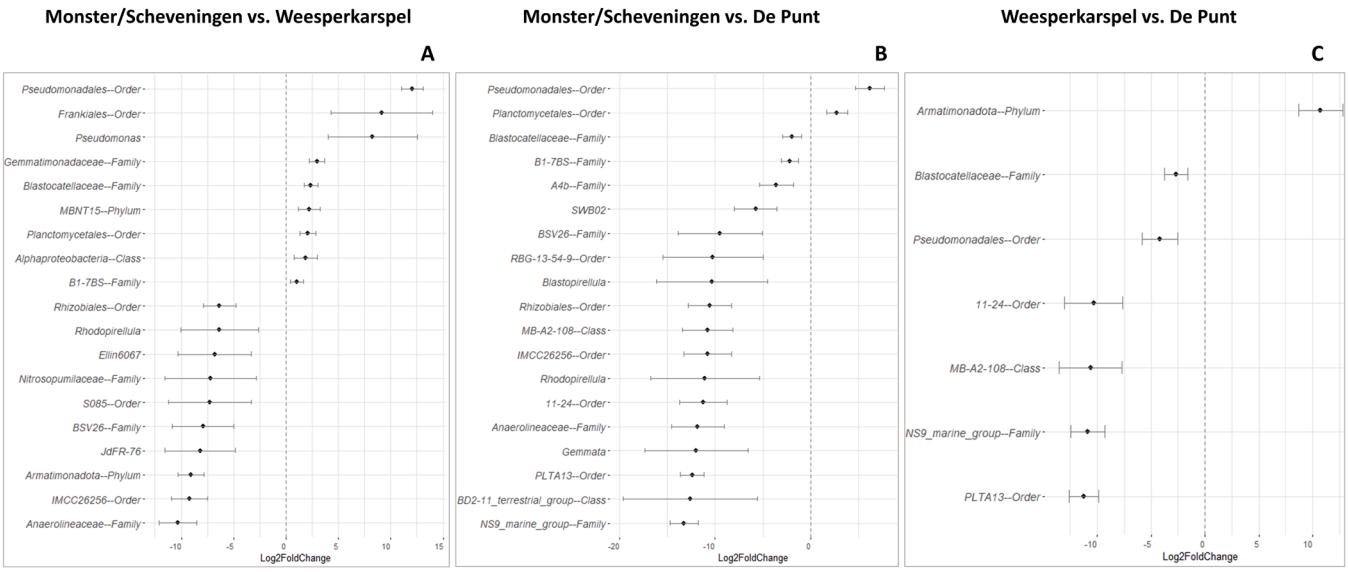


Fig. 5. Differential Abundance Analysis results performed with LinDa on SCM divided by Treatment location. The figures present log2 fold changes derived from a mixed-effects model (with the formula ~Treatment+(1|SSF)), prevalence 0.1 and mean abundance threshold of 0.002. A) Shows taxa differentially abundant in Weesperkarspel samples compared to Monster/Scheveningen SSFs. B) Shows taxa differentially abundant in WBG SSF compared to Monster/Scheveningen SSFs. C) Shows taxa differentially abundant in De Punt SSF compared to Weesperkarspel SSFs.

et al., 2020). Another study on SSFs, which were operated differently (outdoors, uncovered and where the drinking water was chlorinated) from the ones in our research, also showed significant variations in prokaryotic community composition between the SCM, water influent and effluent (Haig et al., 2015a). This implies that the observed patterns reflect a general phenomenon relevant to treatment systems other than the SSFs studied.Furthermore, these observations showed that, despite water passing through the sand bed, not all the microorganisms from the influent water readily colonized and thrive in the SCM, as also observed by (Chen et al., 2021). This indicates that the prokaryotic community in the SCM was highly adapted to the sand bed environment, which is different from the water environment. The presence of specific microbial taxa within the SCM is influenced by physical-chemical processes, such as their capacity to adsorb and attach to sand particles, alongside the straining process (Huisman and Wood, 1974; Weber-Shirk and Dick, 1997). If the process of retaining microbes from the effluent water depended only on these mechanisms the microbial community within the SCM would closely resemble that of the influent water over time, due to the gradual accumulation of microbes. Such observations are not only made in SSFs fed intensively pretreated water, but also by rapid sand/anthracite filters that directly treat surface water after coagulation (Abkar et al., 2023). However, the distinct and specialized prokaryotic community observed in the SCM, significantly different from that in the influent, suggests that active prokaryotic growth and competition are dominant processes in the SCM contributing to the microbial dynamics. This prokaryotic growth in the SCM results in biofilm formation, a key adaptive strategy that allows microbial communities to establish themselves firmly on surfaces, such as sand grains in the SCM (Toyofuku et al., 2016). Besides differences in prokaryotic community composition, SCM and water samples also differed for bacterial biomass content indicated by the 16S rRNA gene copy numbers. The bacterial biomass was higher in the SCM layer than in the influent and effluent water samples. Furthermore, we and (Trikannad et al., 2024) observed that the influent water consistently had a higher total bacterial biomass compared to the effluent water at all DWTPs. This finding underscores the efficacy of the SSF in reducing the bacterial load in the effluent water. We observed that the biomass in the SCM varied between the SSFs of the different DWTPs, which might have been caused by filter age, since it has been observed that older SSFs accumulate biomass in the SCM overtime (Campos et al., 2002; Trikannad et al., 2024).

4.2. *Differentially abundant taxa in the SCM, influent, and effluent water*

Some of the predominant families (*Gemmataceae, Nitrospiraceae* and *Pirellulaceae*) were observed in the SCM of all the SSFs investigated in our study. These families have also been reported by others who studied the prokaryotic communities in SSFs that were operated under similar conditions (Bai et al., 2023; Chen et al., 2021; Lautenschlager et al., 2014), but were not predominant in SSFs where an intensive pretreatment was not present (Haig et al., 2015a). These families, thus, have metabolic capacities that corresponds to the water quality of intensively pretreated surface water. It is known that the SCM is rich in organic material and nutrients, which are trapped in the biofilm and used by the microbes as the water passes through (Campos et al., 2002; Huisman and Wood, 1974; Ranjan and Prem, 2018). This abundance of and diversity in nutrients can support the growth of microbes with various metabolisms. Cultivated representatives of *Gemmata* and *Pedomicrobium*, two genera that were relatively abundant in the SCM samples of DWTP Scheveningen, are aerobic chemoheterotrophs that have been found in a wide range of environments, including aquatic ones (Franzmann and Skerman, 1984; Ivanova et al., 2021; Kulichevskaya et al., 2006, 2020; Seeger et al., 2017; Sly et al., 1988a). *Pedomicrobium* was also found in rapid sand filters used for in drinking water production (Vandermaesen et al., 2017). This could indicate that these genera are involved in BDOC degradation in the SCM layer of the SSFs, although some *Pedomicrobium* species are also capable of iron and/or manganese oxidation (Gounot, 1994; Larsen et al., 1999; Sly et al., 1988a; Trudinger et al., 1980). However, it is unlikely that iron and/or manganese oxidation are dominant microbial processes in the SSFs studied, as reduced iron or manganese is not expected since the redox conditions in the water is (sub)oxic during the whole treatment train. Furthermore, *Gemmata* and *Pedomicrobium* can form robust biofilms when they have a surface to attach to (Kaboré et al., 2019; Sly et al., 1988b)., which might explain their preference in the SCM over the water phase.

The *mle1–7* bacteria, which are part of the *Nitrosomonadaceae* family, and *Nitrospira* were relatively more abundant in the SCM compared to the water samples of the SSFs of DWTPs De Punt and/or Weesperkarspel, and are likely to be involved in nitrification (Daims et al., 2016; Prosser et al., 2014). Compared to ammonia oxidizing archaea (AOA), the *Nitrospira* and members of the *Nitrosomonadaceae* thrive better at higher ammonia concentrations (Di et al., 2009; He et al., 2018; Kowalchuk and

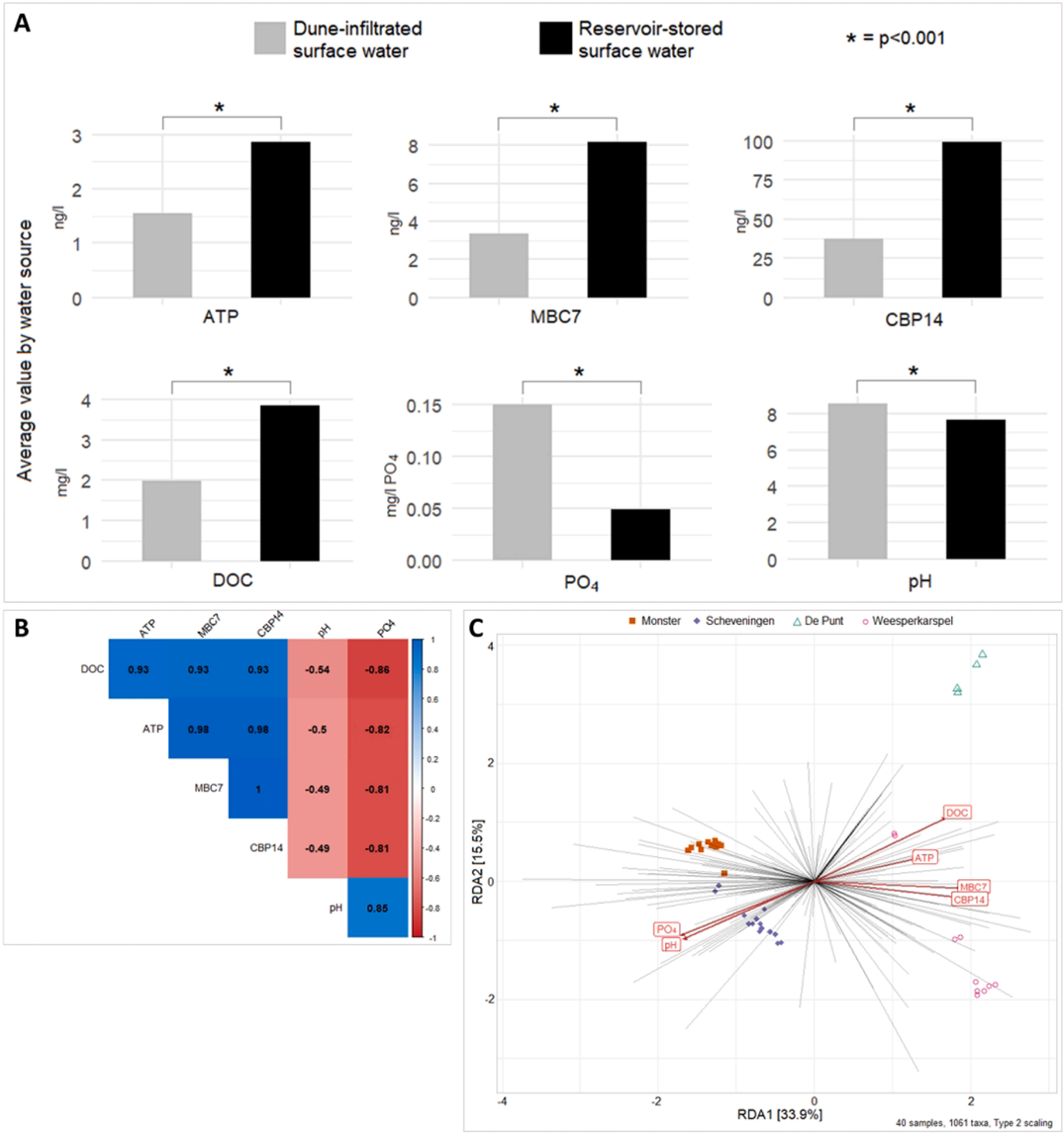


Fig. 6. A) Bar plots indicating average effluent water quality parameter values across DWTPs treating dune-infiltrated surface water (Monster and Scheveningen) and reservoir-stored surface water (Weesperkarspel and De Punt). B) Heatmap of Spearman's correlation analysis of water effluent parameters. Coefficient factor is shown in individual squares. C) RDA of SCM samples based on clr transformed relative abundance data, with forward and backward selection of the water effluent parameters using the 'ordiplot' function. Scaling 2 (clustering based on microbial community composition). All variables shown on the plot have a statistically significant effect on the microbial community composition, regardless of the order of input in the RDA model ($p < 0.001$) tested with ANOVA like permutation test for Redundancy Analysis set on "margin". Full shapes are assigned to the DWTPs treating dune-infiltrated surface water, while empty shapes are used for the DWTPs treating reservoir-stored surface water.

Stephen, 2001; Prosser and Nicol, 2012). This might explain the higher abundance of *mle1–7* and *Nitrospira* in the SCM where, due to higher biological activity and nutrient accumulation, higher ammonia concentrations are expected. Others have also observed that *Nitrospira* members were abundant in SSF-associated biofilms (Chen et al., 2021; Oh et al., 2018). Although specific ammonia measurements in the SCM and effluent are unavailable, the SCM supports higher biomass, which utilizes influent nutrients like ammonia and releases them back through continuous turnover, sustaining microbial activity and nutrient cycling. This is further supported by a previous study where ammonia concentrations were higher in the influent than effluent (9 and 6 μg/l N/L NH_4^+ respectively) in similar SSFs (Trikannad et al., 2024). Moreover, the

ammonia removal was higher in the SCM layer than the deeper layers at those SSFs. While the degraded ammonia appears minimal, it should be considered the constant influent ammonia loading and the significant impact that biodegradable compounds at μg/L-range already have on microbial communities in water systems in the Netherlands (van der Kooij et al., 2017b).

The genus *Candidatus* Omnitrophus was notably more prevalent in water samples than in the SCM across all DWTPs, except for De Punt, suggesting a particular affinity for or resilience in the water matrix. This observation aligns with findings by (Chen et al., 2021), who also reported a higher relative abundance of *Candidatus* Omnitrophus in water samples over sand samples within SSF systems. Similarly, (Learbuch et al., 2022) detected this genus in drinking water sampled from the distribution system of DWTPs in the Netherlands that also use SSFs in their treatment. This indicates that taxa that were present in the influent or effluent of SSFs remain a dominant component in the drinking water distribution system. The *Comamonadaceae* family exhibited a distinct pattern, showing a higher relative abundance in the influent and SCM than in the effluent. This pattern was also found in the SSF systems studied by (Haig et al., 2015a) and suggests that *Comamonadaceae*, introduced into SSFs through the influent, proliferate within the SCM rather than being carried over into the effluent. This could imply a shift in species composition within the *Comamonadaceae* because of specific ecological or metabolic adaptation that allows different *Comamonadaceae* species to thrive in the influent and in the SCM environment. This is likely due to the availability of specific nutrients or favorable physicochemical conditions within the biofilm. Supporting evidence to this hypothesis is further provided by (Bai et al., 2023) who identified *Comamonadaceae* as representatives of the active prokaryotic community of the SCM in SSFs like those analyzed in our research.

Transfers of taxa from water to the SCM were also observed by (Lautenschlager et al., 2014) on SSFs with upstream treatment and unchlorinated water effluent comparable with the SSFs of our research. In addition, they also observed an increase in *Acidobacteria* within the SCM compared to the water samples and a higher relative abundance of the phylum *Patescibacteria* (Ley et al., 2006; Rinke et al., 2013) in the water samples compared to the SCM. These parallel observations are important to highlight as it remains uncertain whether an observation at a single DWTP, as was the case in the study of (Lautenschlager et al., 2014), occurs also at other similar DWTPs. In this case, the parallel observations suggest a consistent behavior among certain taxa in SSFs operated under similar conditions in different countries.

The *Vicinamibacteriales* order was consistently more abundant in the effluent than in the influent across most DWTPs, suggesting an origin from the sand bed, resistance to filtration or proliferation during transport. However, growth within the sand bed and during transportation is negligible due to the short residence time in the SSF and low transport temperatures. Lower gene copy numbers in the effluent confirm bacterial load reduction by filtration, though variations in taxa removal rates cannot be excluded. The exact source of enriched taxa remains unclear without data from deeper sand bed depths.

Overall, inferring the ecological roles of specific taxa based solely on 16S rRNA gene sequencing, can be problematic, particularly when the taxonomic identifications are at the family level or higher. The functions of thesetaxa within SCM ecosystems remain speculative without more comprehensive analyses. To explore the microbial functions in SCM and deeper sand layers, future research should incorporate metagenomic, metatranscriptomic, and/or proteomic approaches, along with advanced cultivation methods.

4.3. The combined effect of source water and upstream treatments in shaping the prokaryotic community of SSF systems

The four DWTPs analyzed are situated in three areas in the Netherlands (**Fig. S1**) and treat surface water that comes from three different sources: river Meuse (Monster and Scheveningen), river Drentsche Aa (De Punt), and seepage water from the Bethune Polder (Weesperkarspel). Additionally, the DWTPs have three different treatments trains (Table 1). It was observed that each DWTP had a DWTP-specific prokaryotic community in the collected sand and water samples (Fig. 4A–B). Still, the water samples were more similar between DWTPs Monster, Scheveningen and Weesperkarspel compared to De Punt.

4.3.1. Water influent and effluent

Upstream treatment steps, such as UV-disinfection, ozonation, dune-infiltration and reservoir storage, selectively shaped the water prokaryotic community by removing or promoting certain microorganisms. For example, at DWTP De Punt, UV-disinfection applied just before the SSF eradicated a large part of the prokaryotic community, potentially favoring UV-resistant taxa (LeChevallier and Au, 2004). This was reflected in the absence of taxa found in the influent water of other DWTPs and lower alpha diversity in influent at De Punt. While UV-disinfection impacted the prokaryotic community of the water, ozonation employed at Weesperkarspel did not reduce species richness in the SSF influent. This is likely due to biologically active carbon (BAC) filtration placed after ozonation and before the SSF, which harbors a diverse prokaryotic community as previous studies have shown (Boon et al., 2011; Knezev, 2015). Thus, the selective pressure of UV-treatment is the expected reason for the distinct clustering of the influent water samples of De Punt from those of Weesperkarspel, Monster and Scheveningen. Moreover, this effect also extended to the effluent community composition, as the effluent samples of De Punt also cluster separately from those of the three other DWTPs, demonstrating that a disinfection treatment step placed directly before SSFs impacts the microbial drinking water composition.

The SSF step had a different impact on the microbial community in the effluent depending on whether pretreated dune-infiltrated or reservoir-stored water was used as influent. At DWTPs using dune-infiltrated water, the SSFs influent and effluent, shared similar common taxa and clustered closely together in the beta diversity analysis, demonstrating a marginal effect of the SSF on the prokaryotic community composition in the effluent. The surface water treated by the DWTPs applying dune-infiltration travels through dune sand for 60 days before it is abstracted and treated in SSFs. Thus, dune-infiltration acts as a natural long sand filtration process, pre-shaping the microbial community to a composition similar to the SSF effluent. In contrast, the SSF influent and effluent at DWTPs using reservoir storage clustered separately in the beta diversity analysis and showed different abundant taxa between influent and effluent water, demonstrating a larger effect of the SSF on the effluent community. The surface water at these two plants resided in the reservoir before being abstracted, treated with ozone or UV and BAC, and then processed through the SSFs. Therefore, the difference in source water and/or pretreatment between Monster/Scheveningen and Weesperkarspel/De Punt is the likely cause for the difference in the microbial community between these locations. It is remarkable, however, that the effluent community composition of the SSFs from the DWTP at Monster, Scheveningen and Weesperkarspel was similar, despite the fact that Weesperkarspel uses different source water and treatment train. In contrast, the effluent at De Punt differed, which can be attributed to the distinct community already present in its influent. Plants using dune-infiltration produce more biologically stable water with low BDOC concentrations than those relying on reservoir storage combined with ozone or UV and BAC (van der Kooij et al., 2017b). We hypothesize that dune-infiltration results in biologically stable SSF influent, reducing the need of subsequent community alterations in the SSF, compared to reservoir-stored water. Thus, we conclude that differences in SSF influent water quality are mainly caused by source water and/or upstream treatments, which directly or indirectly shape the prokaryotic communities in the influent and effluent. At dune-infiltration DWTPs, SSFs act as polishing filters with minimal effluent impact, while at reservoir-storage DWTPs, they actively shape

effluent communities.

When comparing our findings to previous studies, (Ma et al., 2020) observed that environmental and operational parameters shape the bacterial communities within biofilters, but these communities have a relatively minor impact on the microbial composition of the filtered water. Their results suggest that biofilter microbiome enhance water quality through the conversion of contaminants and nutrients rather than through direct alteration of the microbial community in the filtered water. Conversely, (Pinto et al., 2012) concluded that biofilters play a dominant role in shaping the bacterial communities of the filtrate. Our findings align more closely with (Ma et al., 2020) in the case of dune-infiltrated water but show a greater influence of SSFs on microbial composition in UV-disinfected reservoir-stored water, underscoring the importance of source water characteristics and upstream treatments in shaping microbial outcomes.

4.3.2. SCM

Our findings suggest that the biological stability and chemical composition of the influent water contribute to shaping the prokaryotic communities of the SCM. The SCM communities of the SSFs from DWTPs treating reservoir-stored surface water were more similar compared to the SCM communities of the SSFs from DWTPs that treat dune-infiltrated surface water. This study demonstrated that this difference relates to the difference in water quality parameters, which are probably caused by the different pretreatment applied at the DWTPs.

Previous studies showed that SSF effluent water from DWTPs utilizing dune infiltration had a higher biological stability than those from DWTPs that use reservoir storage (van der Kooij et al., 2017b; van der Wielen et al., 2023). Consistent with these findings, we found that DWTPs using reservoir-stored surface water produced SSF effluent with significantly higher DOC and ATP concentrations and increased microbial growth potential, all indicative of a lower biological stability. Conversely, dune-infiltrated water produced effluent with higher pH and PO_4 concentration, emphasizing the influence of source water type on effluent quality. It has been shown that a lower biological stability of the treated water results in enhanced regrowth (i.e. heterotrophic plate and *Aeromonas* counts) in the distribution system (van der Wielen et al., 2023).

The RDA analysis further revealed that chemical water parameters correlated with source water type drive differences in SCM community between SSFs that treat dune-infiltrated water or reservoir-stored and treated water. These findings align with previous studies which linked water quality parameters to microbial community composition in RSFs treating surface water directly after coagulation or ozonation (Abkar et al., 2023; Ma et al., 2020; Pinto et al., 2012), suggesting a broader patterns across diverse water treatments beyond SSFs.

Our observation that influent water from different sources and pretreatment drives differences in the SCM microbial community aligns with findings by Bai et al. (2023), who investigated comparable SSFs. We expanded their work by incorporating analyses of influent and effluent samples, in addition to the SCM, along with source water types and treatment trains. This approach allowed us to investigate the factors driving influent water variations across different DWTPs and how these differences shape SCM communities and what effect both influent and SCM communities have on the prokaryotic community in the effluent that is distributed as drinking water to the consumers.

Although SCM sample comes from SSFs with different ages, age did not appear to be the primary driver of community differences; instead, location, source water and treatment played a more significant role, since community composition of SSFs that varies in age within a plant differ less from each other than community compositions between treatment plants. Only at Weesperkarspel we observed a distinction in community composition between 12-year-old SCM and less than one year old, indicating age may also play a role. However, assessing the precise role of age, was beyond the scope of our study, and requires controlled long-term experiments under similar conditions, as cross-location comparisons are limited by differences in source water, treatment trains, and other operational variables.

Overall, the prokaryotic community dynamics of SSFs in drinking water treatment are shaped by a complex interplay of factors, including source water used, pretreatments applied, chemical water quality and biological stability. Full-scale studies are limited in disentangling the precise contribution of each factor on shaping the SSFs community due to variability in source water, treatment train, influent quality and biological stability across DWTPs. Lab- or pilot-controlled studies, where these parameters can be tested separately, are needed to determine the dominant factors shaping the communities in SSFs and drinking water and how these aspects relate to the biological stability. Furthermore, the distinct microbial ecology of each SSF, even within a DWTP, highlights the need of investigating filter-specific parameters, such as depth and age, to better understand their influence on the microbial community composition and biomass.

4.4. Practical implications

The results from our research also offers valuable insights for optimizing SSF systems. First, our findings revealed higher biological stability (DOC, ATP, MBC7, and CBP14) in drinking water produced by SSFs that treat dune-infiltrated surface water than those treating reservoir-stored surface water. Next, we showed that SSFs at DWTPs that treat dune-infiltrated water, operate as polishing filters, which opens possibilities to refine the operational conditions without sacrificing water quality. Examples of such refinements could be higher flow rates and/or decreased sand bed heights which would increase process capacity and lower operational expenses. Crucially, however, such refinements need to undergo testing at both pilot and full-scale levels, with a keen focus on ensuring the efficacy of SSFs for sufficient fecal pathogen and BDOC removal to ensure the production of safe and biologically stable drinking water.

For SSFs fed with (pre)treated water after reservoir storage other modifications to SSF operations could be beneficial. For instance, interventions like bioaugmentation, or the addition of specific beneficial microorganisms to the SSF, might enhance BDOC removal, which could help achieve water quality with a higher biological stability, comparable to that of DWTPs that use dune-infiltration. As we have shown that the prokaryotic community is DWTP specific, such interventions, however, can be complex as it suggests that the addition of nutrients of microorganisms should be site specific.

The chemical and microbial profiles of the effluent are invaluable indicators of the performance of SSF systems. Monitoring the prokaryotic community in the influent and effluent water alongside with key biological and chemical parameters (e.g. DOC, AOC, ATP, MBC7, and CBP14) provides a comprehensive view of drinking water quality produced as was also advised previously (van der Kooij et al., 2017a). By examining these parameters not only at the SSF stage but throughout upstream processes as well, drinking water utilities can adjust their SSF operations to the influent water quality and to meet specific water quality objectives. Gathering and analyzing this data will also help understanding how the biological and chemical composition of the effluent water correlates with the SSF operational parameters.

5. Conclusions

- The prokaryotic communities in the SCM, influent, and effluent of SSFs develop distinctly, and are shaped by adaptation to the specific SCM environment rather than simply reflecting the influent composition.
- The prokaryotic community in SSFs is influenced by the interplay of source water type, pretreatment and influent biological stability. Full-scale studies can not determine the dominant factor due to the different variables within and among the DWTPs. Pilot and lab-scale

studies varying one factor at a time are recommended to identify the most dominant factor(s).

- DWTPs using dune infiltration produce more biologically stable drinking water, with SSFs acting as a polishing step, while SSFs treating reservoir-stored water actively modify prokaryotic communities in the SSFs and the microbial water quality of the effluent. Consequently, results from studies on SSFs from a single DWTP should not simply be generalized to other plants due to varying local conditions.
- UV-treatment applied before SSFs reduces influent prokaryotic community richness and evenness, also extending its effect to the effluent prokaryotic community composition. This highlights the impact of pre-SSF disinfection on the microbial water quality.
- qPCR analyses show that SSFs effectively reduce the bacterial load in effluent water across all DWTPs, confirming their effectiveness as a filtration technology.

CRediT authorship contribution statement

Valentina Attiani: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Hauke Smidt:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Paul W.J.J. van der Wielen:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Hauke Smidt reports financial support was provided by Nederlandse Organisatie voor Wetenschappelijk Onderzoek Utrecht. Hauke Smidt reports financial support was provided by Dunea. Hauke Smidt reports financial support was provided by Vitens. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was funded by the Netherlands Organization for Scientific Research (NWO) as part of the partnership programme 'Dunea-Vitens: Sand Filtration' (grant 17831). The authors acknowledge Brent Pieterse and Clemy van der Steen-de Kok from Dunea, Fenna Philipse from Waternet, and Jantinus Bruins from WLN for their support during our visits to the drinking water plants, for providing samples, water data, and for the manuscript approval.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2025.123328.

Data availability

Data will be made available on request.

References

Abkar, L., Taylor, A., Stoddart, A., Gagnon, G., 2023. Microbiome and hydraulic performance changes of drinking water biofilters during disruptive events-media replacement, lake diatom bloom, and chlorination. Environ. Sci.: Water Res. Technol. 9 (3), 723–735.

Ahlmann-Eltze, C. and Patil, I. 2021 ggsignif: r package for displaying significance brackets for 'ggplot2'.

Apprill, A., McNally, S., Parsons, R., Weber, L., 2015. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. Aquat. Microb. Ecol. 75 (2), 129–137.

Bai, X., Dinkla, I.J.T., Muyzer, G., 2023. Shedding light on the total and active core microbiomes in slow sand filters for drinking water production. Water Res. 243, 120404.

Barnett, 2021. microViz: an R package for microbiome data visualization and statistics. J. Open Source Softw. 6 (63), 4.

Boon, N., Pycke, B.F.G., Marzorati, M., Hammes, F., 2011. Nutrient gradients in a granular activated carbon biofilter drives bacterial community organization and dynamics. Water Res. 45 (19), 6355–6361.

Campos, L.C., Su, M.F.J., Graham, N.J.D., Smith, S.R., 2002. Biomass development in slow sand filters. Water Res. 36 (18), 4543–4551.

Chen, L., Zhai, Y., van der Mark, E., Liu, G., van der Meer, W., Medema, G., 2021. Microbial community assembly and metabolic function in top layers of slow sand filters for drinking water production. J. Clean. Prod., 126342

Daims, H., Lücker, S., Wagner, M., 2016. A new perspective on microbes formerly known as nitrite-oxidizing bacteria. Trends Microbiol. 24 (9), 699–712.

de Souza, F.H., Roecker, P.B., Silveira, D.D., Sens, M.L., Campos, L.C., 2021. Influence of slow sand filter cleaning process type on filter media biomass: backwashing versus scraping. Water Res. 189.

Di, H.J., Cameron, K.C., Shen, J.-P., Winefield, C.S., O'Callaghan, M., Bowatte, S., He, J.-Z., 2009. Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. Nat. Geosci. 2 (9), 621–624.

Franzmann, P., Skerman, V., 1984. Gemmata obscuriglobus, a new genus and species of the budding bacteria. Antonie Van Leeuwenhoek 50, 261–268.

Gounot, A.-M., 1994. Microbial oxidation and reduction of manganese: consequences in groundwater and applications. FEMS Microbiol. Rev. 14 (4), 339–349.

Haig, S.J., Collins, G., Davies, R.L., Dorea, C.C., Quince, C., 2011. Biological aspects of slow sand filtration: past, present and future. Water Sci. Technol.: Water Supp. 11 (4), 468–472.

Haig, S.J., Quince, C., Davies, R.L., Dorea, C.C., Collins, G., 2015a. The relationship between microbial community evenness and function in slow sand filters. mBio 6 (5), e00729, 00715.

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

Hammes, F., Berger, C., Köster, O., Egli, T., 2010. Assessing biological stability of drinking water without disinfectant residuals in a full-scale water supply system. J. Water Supp.: Res. Technol.—AQUA 59 (1), 31–40.

He, H., Zhen, Y., Mi, T., Fu, L., Yu, Z., 2018. Ammonia-oxidizing archaea and bacteria differentially contribute to ammonia oxidation in sediments from adjacent waters of Rushan Bay. China Front. Microbiol. 9, 116.

Hijnen, W.A.M., Schurer, R., Bahlman, J.A., Ketelaars, H.A.M., Italiaander, R., van der Wal, A., van der Wielen, P.W.J.J., 2018. Slowly biodegradable organic compounds impact the biostability of non-chlorinated drinking water produced from surface water. Water Res. 129, 240–251.

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

Ivanova, A., Kulichevskaya, I., Dedysh, S., 2021. Gemmata palustris sp. nov., a novel planctomycete from a Fen in Northwestern Russia. Microbiology (N. Y) 90, 598–606.

Kaboré, O.D., Godreuil, S., Drancourt, M., 2019. Improved culture of fastidious Gemmata spp. Bacteria using marine sponge skeletons. Sci. Rep. 9 (1), 11707.

Kassambara, A., 2023. ggpubr:"ggplot2" based publication ready plots. R Package Version 0.4. 0. Available online: https://CRAN.R-project.org/package=ggpubr. accessed 12 August 2020.

Knezev, A., 2015. Microbial Activity in Granular Activated Carbon Filters in Drinking Water Treatment. Wageningen University and Research.

Kowalchuk, G.A., Stephen, J.R., 2001. Ammonia-oxidizing bacteria: a model for molecular microbial ecology. Ann. Rev. Microbiol. 55 (1), 485–529.

Kulichevskaya, I., Pankratov, T., Dedysh, S., 2006. Detection of representatives of the planctomycetes in Sphagnum peat bogs by molecular and cultivation approaches. Microbiology (N. Y) 75, 329–335.

Kulichevskaya, I.S., Naumoff, D.G., Miroshnikov, K.K., Ivanova, A.A., Philippov, D.A., Hakobyan, A., Rijpstra, W.I.C., Damsté, J.S.S., Liesack, W., Dedysh, S.N., 2020. Limnoglobus roseus gen. nov., sp. nov., a novel freshwater planctomycete with a giant genome from the family Gemmataceae. Int. J. Syst. Evol. Microbiol. 70 (2), 1240–1249.

Lahti, L., Shetty, S., 2018. Introduction to the microbiome R package. Preprint at https://microbiome.github.io/tutorials.

Larsen, E.I., Sly, L.I., McEwan, A.G., 1999. Manganese (II) adsorption and oxidation by whole cells and a membrane fraction of pedomicrobium sp. ACM 3067. Arch. Microbiol. 171, 257–264.

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

Learbuch, K., Smidt, H., Van der Wielen, P., 2022. Water and biofilm in drinking water distribution systems in the Netherlands. Sci. Total Environ. 831, 154940.

LeChevallier, M.W., Au, K.-K., 2004. Water Treatment and Pathogen Control. Iwa Publishing.

Ley, R.E., Harris, J.K., Wilcox, J., Spear, J.R., Miller, S.R., Bebout, B.M., Maresca, J.A., Bryant, D.A., Sogin, M.L., Pace, N.R., 2006. Unexpected diversity and complexity of the Guerrero Negro hypersaline microbial mat. Appl. Environ. Microbiol. 72 (5), 3685–3695.

Ma, B., LaPara, T.M., Hozalski, R.M., 2020. Microbiome of drinking water biofilters is influenced by environmental factors and engineering decisions but has little influence on the microbiome of the filtrate. Environ. Sci. Technol. 54 (18), 11526–11535.

Maiyo, J.K., Dasika, S., Jafvert, C.T., 2023. Slow sand filters for the 21st century: a review. Int. J. Environ. Res. Public Health 20 (2), 1019.
Martinez Arbizu, P. 2017. pairwiseAdonis: pairwise multilevel comparison using Adonis_. R package version 0.4.1.
McLaren, M. 2023. speedyseq: faster implementations of phyloseq functions.
McMurdie, P.J., Holmes, S., 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8 (4), e61217.
Neuwirth, E., 2022. RColorBrewer: ColorBrewer Palettes. R package Version 1.1.3.
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 (Supplement C), 278–285.
Parada, A.E., Needham, D.M., Fuhrman, J.A., 2016. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. Environ. Microbiol. 18 (5), 1403–1414.
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.
Poncheewin, W., Hermes, G.D.A., van Dam, J.C.J., Koehorst, J.J., Smidt, H., Schaap, P.J., 2020. NG-Tax 2.0: a semantic framework for high-throughput amplicon analysis. Front. Genet. 10, 1366.
Prosser, J.I., Head, I.M., Stein, L.Y., 2014. The prokaryotes: Alphaproteobacteria and Betaproteobacteria. Springer Berlin/Heidelberg, pp. 901–918.
Prosser, J.I., Nicol, G.W., 2012. Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. Trends Microbiol. 20 (11), 523–531.
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41 (Database issue), D590–D596.
Ramiro-Garcia, J., Hermes, G.D.A., Giatsis, C., Sipkema, D., Zoetendal, E.G., Schaap, P.J., Smidt, H., 2016. NG-Tax, a highly accurate and validated pipeline for analysis of 16S rRNA amplicons from complex biomes. F1000Res. 5, 1791.
Ranjan, P., Prem, M., 2018. Schmutzdecke-a filtration layer of slow sand filter. Int. J. Curr. Microbiol. Appl. Sci. 7 (07), 637–645.
Rinke, C., Schwientek, P., Sczyrba, A., Ivanova, N.N., Anderson, I.J., Cheng, J.-F., Darling, A., Malfatti, S., Swan, B.K., Gies, E.A., Dodsworth, J.A., Hedlund, B.P., Tsiamis, G., Sievert, S.M., Liu, W.-T., Eisen, J.A., Hallam, S.J., Kyrpides, N.C., Stepanauskas, R., Rubin, E.M., Hugenholtz, P., Woyke, T., 2013. Insights into the phylogeny and coding potential of microbial dark matter. Nature 499 (7459), 431–437.
Rosario-Ortiz, F., Rose, J., Speight, V., Gunten, U.v., Schnoor, J., 2016. How do you like your tap water? Science (1979) 351 (6276), 912–914.
Seeger, C., Butler, M.K., Yee, B., Mahajan, M., Fuerst, J.A., Andersson, S.G., 2017. Tuwongella immobilis gen. nov., sp. nov., a novel non-motile bacterium within the phylum Planctomycetes. Int. J. Syst. Evol. Microbiol. 67 (12), 4923–4929.
Shetty, S.L.L., 2024. _microbiomeutilities: microbiomeutilities: Utilities for Microbiome Analytics_. R package Version 1.00.17.
Sly, L., Arunpairojana, V., Hodgkinson, M., 1988a. Pedomicrobium manganicum from drinking-water distribution systems with manganese-related “dirty water” problems. Syst. Appl. Microbiol. 11 (1), 75–84.
Sly, L., Hodgkinson, M., Arunpairojana, V., 1988b. Effect of water velocity on the early development of manganese-depositing biofilm in a drinking-water distribution system. FEMS Microbiol. Ecol. 4 (3–4), 175–186.
Team, R.C., 2020. RA language and environment for statistical computing, R Foundation for Statistical. Computing.
Toyofuku, M., Inaba, T., Kiyokawa, T., Obana, N., Yawata, Y., Nomura, N., 2016. Environmental factors that shape biofilm formation. Biosci. Biotechnol. Biochem. 80 (1), 7–12.
Trikannad, S.A., Attiani, V., van der Wielen, P.W.J.J., Smidt, H., van der Hoek, J.P., van Halem, D., 2024. Recovery of microbial biomass and purification performance after scraping of full-scale slow sand filters. J. Water. Process. Eng. 60, 105101.
Trudinger, P., Walter, M., Ralph, B., Marshall, K., 1980. The Role of Surface Attachment in Manganese Oxidation By Freshwater Hyphomicrobia. Springer, pp. 333–337.
van der Kooij, D., 1992. Assimilable organic carbon as an indicator of bacterial regrowth. J.-Am. Water Works Assoc. 84 (2), 57–65.
Van der Kooij, D., 2003. Biodegradable Compounds and Biofilm Formation in Water Treatment and distribution. Water Resources and Water Supply in the 21st Century. Hokkaido University Press, Sapporo.
van der Kooij, D., Bakker, G.L., Italiaander, R., Veenendaal, H.R., Wullings, B.A., 2017a. Biofilm composition and threshold concentration for growth of Legionella pneumophila on surfaces exposed to flowing warm tap water without disinfectant. Appl. Environ. Microbiol. 83 (5).
Van der Kooij, D. and Veenendaal, H.R. (2014b) Microbial Growth in Drinking Water Supplies. Problems, Causes, Controls and Research Needs. Van der Kooij, D. and Van der Wielen, P.W.J.J. (eds), pp. 291–337, IWA Publishing, London, UK.
van der Kooij, D., Veenendaal, H.R., van der Mark, E.J., Dignum, M., 2017b. Assessment of the microbial growth potential of slow sand filtrate with the biomass production potential test in comparison with the assimilable organic carbon method. Water Res. 125, 270–279.
van der Wielen, P.W., Brouwer-Hanzens, A., Italiaander, R., Hijnen, W.A., 2023. Initiating guidance values for novel biological stability parameters in drinking water to control regrowth in the distribution system. Sci. Total Environ. 871, 161930.
Vandermaesen, J., Lievens, B., Springael, D., 2017. Isolation and identification of culturable bacteria, capable of heterotrophic growth, from rapid sand filters of drinking water treatment plants. Res. Microbiol. 168 (6), 594–607.
Weber-Shirk, M.L., Dick, R.I., 1997. Physical—Chemical mechanisms in slow sand filters. J. Am. Water. Works Assoc. 89 (1), 87–100.
Wickham, H., 2016. Ggplot2: Elegant graphics For Data Analysis. Springer, New York.
Wickham, H., François, R., Henry, L., Müller, K., Vaughan, D., 2023. dplyr: A Grammar of Data Manipulation. R package Version 1.1.4.
Wickham, H.V., D., Girlich, M., 2024. tidyr: Tidy Messy Data. R package Version 1.3.1. https://CRAN.R-project.org/package=tidyr.
Zhang, X.C., J. 2024. MicrobiomeStat: statistical methods for microbiome compositional data. R Package Version 1.2. https://cran.r-project.org/web/packages/MicrobiomeStat/index.html.
Zhou, H., He, K., Chen, J., Zhang, X., 2022. LinDA: linear models for differential abundance analysis of microbiome compositional data. Genome Biol. 23 (1), 95.