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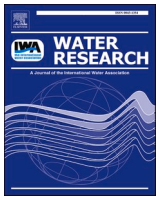

Investigating spatial and temporal dynamics in microbial community composition of multiple full-scale slow sand filters in drinking water treatment

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ABSTRACT

Slow sand filters (SSFs) are essential for producing high-quality and sustainable drinking water, relying on chemical, physical, and microbial processes to remove nutrients, organic matter, and pathogens. Despite numerous studies on the physical and chemical mechanisms in SSFs, the microbial processes and dynamics remain poorly understood. This study bridges this knowledge gap by investigating the spatial and temporal dynamics of prokaryotic communities within SSFs, by analysing different depths and the top layer, the Schmutzdecke (SCM), over time in full-scale SSFs from different drinking water treatment plants in The Netherlands. Utilising 16S ribosomal RNA gene-targeted amplicon sequencing and quantitative PCR, we observed a horizontally uniform prokaryotic community at each depth at all analysed SSFs, suggesting effective influent water and nutrient distribution, regardless of filter size or influent inlet design. Vertically, however, the prokaryotic composition varied significantly, with the SCM showing higher biomass and diversity compared to the deeper layers. This study identified a core prokaryotic community, including the families *Nitrospiraceae, Pirellulaceae, Nitrosomonadaceae, Gemmataceae*, and *Vicinamibacteriaceae*, consistent across various depths and SSFs, and in the SCMs of different ages. Their presence suggests a central role in supporting key biological processes in SSFs such as organic matter degradation and nitrification. Additionally, the relative abundance of archaea increased with sand depth in all SSFs, suggesting their adaptation to lower-nutrient conditions found in deeper layers. Analysis of the SCM over time showed that after scraping, the prokaryotic community gradually adapted, with minimal biomass increase during the first 3.6 years, eventually evolving into a mature, diverse, and even prokaryotic community. Our findings highlight the presence of spatially distinct microbial communities at various depths of SSFs, suggesting the removal of specific compounds in distinct sand layers. Moreover, the persistence of a core prokaryotic community across different SSFs, SCM maturation stages, and even after disturbances like scraping, demonstrates that the biology in SSFs is resilient and likely ensures reliable SSF performance. It also implies possibilities for earlier SSF operational restart after cleaning than is conventionally done, but with continuous monitoring of water quality parameters to ensure microbial safety. These findings lay the groundwork for future research to focus on these microorganisms and their functional potential.

1. Introduction

Access to clean drinking water is essential for public health. Slow sand filtration is a widely used, cost-effective and sustainable technology for producing high-quality drinking water, often without the addition of chemical disinfectants. This treatment effectively removes organic compounds and biomass without the need for chlorination. Slow sand filters (SSFs) function through a combination of biological, physical, and chemical mechanisms that occur throughout the sand bed (Graham and Collins, 2014; Haig et al., 2011; Huisman and Wood, 1974; Maiyo et al., 2023). While the physical and chemical processes in SSFs have been well characterized, the biological component, particularly the

Abbreviations: SCM, Schmutzdecke; DWTP, Drinking water treatment plant; SSF/s, Slow sand filter/s; BDOC, Biodegradable dissolved organic carbon; AOC, Easily assimilable organic carbon; AOA/AOB, Ammonia-oxidizing archaea/bacteria.

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microbial processes, remains relatively underexplored. Modern molecular techniques that are available nowadays allow for a more detailed investigation of microbial communities and their responses to operational conditions, offering new insights into how these communities support SSF performance (Attiani et al., 2025; Bai et al., 2023, 2024; Haig, 2014).

Traditionally, the Schmutzdecke (SCM), a biologically active layer that develops at the sand surface, has been considered the primary driver of SSF performance (Hijnen et al., 2004; Huisman and Wood, 1974). However, recent studies employing microbial molecular techniques have revealed a greater microbial diversity within the SCM than previously documented (Attiani et al., 2025; Bai et al., 2023, 2024; Haig, 2014). These microbial communities are shaped by factors such as influent water characteristics, treatment processes, and sand type, and they support a range of metabolic functions involved in complex organic matter degradation and nutrient cycling (Chen et al., 2021; de Souza et al., 2021; S. Oh et al., 2018). Microbial diversity is increasingly recognized as critical to SSF performance. The presence of diverse and even microbial communities in SSFs has been associated with enhanced resilience and metabolic versatility, improving treatment performance under variable environmental conditions (Haig et al., 2015; Lautenschlager et al., 2014; Tardy et al., 2014; Yachi and Loreau, 1999).

In addition, the role of deeper sand layers in SSF performance is increasingly acknowledged. Studies on pilot- and full-scale SSFs showed that microbial community structure was strongly influenced by depth, with biomass decreasing as filter depth increases (Campos et al., 2002; Chen et al., 2021; de Souza et al., 2021; Haig et al., 2014; Huisman and Wood, 1974). However, some key microbial taxa persist throughout the sand bed and may contribute significantly to water treatment, especially in pathogens and nutrient removal (Trikannad et al., 2023, 2024).

Despite these advancements, knowledge gaps remain in understanding how microbial communities are spatially and temporally organized across full-scale SSFs operated under real-world conditions, and which are the focus of our study. Specifically, we addressed the following research questions: 1) How do SSF design features such as surface area and influent inlet configurations, influence horizontal microbial community distribution? 2) Does vertical stratification result in reproducible patterns of microbial community composition across different drinking water treatment plants (DWTPs)? 3) Is there a core microbial community consistently present across SSFs and sand depths, despite observed variations in overall microbial community composition? 4) How does the SCM microbial community develop over time after scraping, from early recolonization to maturity?

To answer these questions, we applied 16S ribosomal RNA (rRNA) gene amplicon sequencing and quantitative PCR (qPCR) to sand and water samples from full-scale SSFs from different DWTPs in The Netherlands. Our aim was to provide insights into the microbial ecology underlying SSF function and to suggest future optimization of design, operation, and maintenance of SSF systems used for drinking water production.

2. Materials and methods

2.1. Slow sand filters characteristics

Sand and water samples were collected from nine full-scale SSFs across four DWTPs in The Netherlands. Specifically, SSFs 4D and 5B were located at DWTP Monster, SSFs 3, 5, 6, 7A and 9A were located at DWTP Scheveningen, SSF 7.2 was located at DWTP Katwijk and SSF 2 was located at DWTP De Punt. All SSFs were operated indoors as the last step in the drinking water treatment train. Consequently, the effluent of the SSFs is directly distributed as drinking water without a disinfectant residual to consumers. The selected SSFs differed in their age, location, water source, surface area, the point at which the influent enters the SSF, pretreatments and operational parameters (Table 1, **Table S1, Table S2 and Fig. S1**).

2.2. Water and sand sampling

The water influent and effluent from the SSFs were collected in duplicates of 1 L by using sterile plastic bottles (Identipack, Netherlands) before and, when possible, after the scraping procedure that removed the top layer (SCM) of the SSF (**Table S2**). The water samples were transported to the laboratory in Styrofoam boxes containing icepacks within 24 h from sampling and once arrived in the laboratory they were immediately filtered over a 0.2 μm filter (Isopore TM PC membrane, 47 mm hydrophilic, Merck, Millipore) to collect the microorganisms. Depending on the operational conditions of each SSF, sand samples were obtained in a different manner. For the SSFs where the SCM (0–2 cm) was scraped (Monster_4D, Monster_5B, Katwijk_7.2, Scheveningen_5 and De Punt_2), sand samples were collected across the filter surface from three points A/B/C and over depths (**Fig. S1**), 2–3 h before filter scraping using a sterile stainless-steel peat sampler (Royal Eijkelkamp, Netherlands) (**Fig. S2**). In these cases, the drinking water production was paused, and the water level of the supernatant water was lowered until the top layer of the sand bed was exposed. In addition, the water samples from the influent and effluent of these SSFs (except Katwijk_7.2) were collected up to six days before scraping. These samples were used to investigate spatial variability of the prokaryotic community within the SSFs. The sand cores were subsequently divided into three sections representing different depths of the sand bed (0–2 cm, 10–15 cm, and 20–25 cm) (**Table S2**) and placed in 50 ml sterile Falcon tubes.

To explore the temporal changes in prokaryotic communities, we analysed SCMs at different maturation stages from five SSFs at the DWTP Scheveningen (SSFs 3, 5, 6, 7A and 9A). These SSFs have comparable environmental and operational parameters such as water source, treatment steps, cleaning procedure, water influent temperature and filtration rate (**Table S1** and **S2**). However, they differed in time elapsed since the last SCM scraping. The SCM of SSF Scheveningen_5, representing the youngest stage, was sampled at 8, 15, 28 and 52 days after scraping. SSFs Scheveningen_3, 7A and 9A, representing the middle

Table 1
Slow sand filter characteristics and samples collected. *Filter age at the moment of sampling since the last scraping.

DWTP	De Punt	Katwijk	Monster		Scheveningen						
SSF	2	7.2	5B	4D	5		7A	3	9A	6	
Filter age (years)*	4.8 years	1.2 years	1.5 years	1.5 years	5.6 years	8/15/28/52 days	1.6 years	2.7 years	3.6 years	4.5/4.6/4.7 years	5.3 years
Water influent type											
Filtration rate (m/h) average	0.17	0.29	0.35	0.35	0.20	0.20	0.20	0.22	0.21	0.27	0.21
Surface area (m^2)	1205	600	375		2362		1295	2598	1575	2362	
Water influent temperature (C°)	9.9	10.6	15.8	14.9	12	11.8	11.2			11.8	15.2
Sand sampling points	A/B	A/B/C				C					
Sand depths (cm)	0–2, 10–15, 20–25 cm	0–2, 10–15, 20–25 cm				0–2 cm					

stage, were sampled 1.6, 2.7 and 3.6 years after scraping, respectively. Finally, the SSFs Scheveningen_6 and 5, representing the oldest SCM, were sampled 4.5, 4.65, 4.7, and 5.3 years after scraping (Scheveningen_6), and 5.6 years after scraping (Scheveningen_5). As these SSFs were operational during sampling, it was not possible to pause drinking water production and lower the supernatant water. Therefore, only the SCM was collected from sampling point C (**Fig. S1**) without lowering the supernatant level, using a 50 ml sterile Falcon tube attached to a sterile stick. Additionally, sand samples from 10 to 15 cm and 20–25 cm depths of SSF 5 were included for a more comprehensive analysis, as previously described. All water and sand samples were transported in Styrofoam boxes equipped with icepacks and stored at −20 °C within 24 h until DNA extraction.

2.3. Molecular analysis and data processing

DNA isolation, qPCR, 16S rRNA gene-amplicon library preparation, sequencing, and following data processing were conducted as outlined in Attiani et al. (2025). In short, qPCR was used to determine total bacterial 16S rRNA gene copy numbers, as a measure for bacterial abundance, in the sand samples. The V4 region of the 16S rRNA gene was amplified and subsequently sequenced on an Illumina Novaseq 6000 platform at Novogene (Cambridge, United Kingdom). Amplicon sequence variants (ASVs) were identified from the sequence data on a per sample basis. The obtained 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 PRJEB84957.

The structure and diversity of the microbial community within and across SSFs were evaluated using microbial ecology diversity indices. Alpha diversity, which measures diversity within individual samples, was assessed using the Shannon index (which describes both the richness and evenness of taxa in a sample), Chao1 (which only describes richness), and Pielou's evenness (which only describes evenness), were used to assess microbial diversity within individual sand layers (Thukral, 2017). Richness refers to the number of different taxa (e.g., ASVs) present in a sample, while evenness describes how evenly the individual taxa (e.g., ASVs) are distributed among all taxa. These metrics were visualized using boxplots. Beta diversity, which captures differences between microbial communities across samples, was assessed using Principal Coordinates Analysis (PCoA) based on weighted UniFrac distances, which account for both the relative abundance and phylogenetic relatedness of ASVs (Lozupone et al., 2007). In these plots, each point represents a microbial community (i.e., a sample), and the distance between points reflects differences in community composition and phylogeny, with longer distances between points representing larger differences in community composition and phylogeny. Beta diversity was used to compare microbial communities across different depths, sampling points, and SSFs.

2.4. Statistical analyses

The statistical analyses were performed in R with packages *vegan* and *stats* (4.3.3) (R Core Team 2020) and for a more in-depth description of statistical methods employed in microbial ecology, the reader is referred to Paliy & Shankar (2016). 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 assess whether community composition significantly differs between predefined groups (beta diversity) with the number of permutations set at 999. To confirm that the PERMANOVA results were not influenced by differences in group dispersions, we performed a homogeneity of multivariate dispersions test. This analysis assesses whether the variance around group centroids in multivariate space is comparable across groups, ensuring that observed differences reflect true compositional variation rather than dispersion effects.

Before conducting statistical tests on the qPCR and alpha diversity data, we assessed variance and normality of the data within each group using the Levene's and Shapiro-Wilk's tests, respectively. If normality and homogeneity of variance assumptions were not met, the Kruskal-Wallis test, followed by Dunn's post hoc test, was used to compare the groups. If the data met the assumptions of normality and homogeneity of variance, one-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) post hoc test was implemented. When the data met the normality assumption but failed the homogeneity of variance test, Welch's ANOVA followed by the Games-Howell post hoc test was utilised.

Differential abundance analyses 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 (∼depth+(1|samplingpointsand)) and ∼young_middle_old+(1|sandagedaysyears) were employed for the spatial and temporal variability analysis respectively. Analysis parameters of both models included a prevalence filter of 0.1, a mean abundance filter of 0.015, and p values adjusted using the Benjamini-Hochberg method with an alpha of 0.05.

The core microbiota was defined as the prokaryotic taxa, identified at the family level, that were consistently found across all samples of a defined subset with a minimum of 0.5 % relative abundance. The analysis of the core microbiota was performed in R using the packages *microbiome*, and *ggplot2* was used for visualization.

3. Results

3.1. Horizontal variability

The qPCR analysis revealed that, for none of the depths of any of the SSFs analysed, the total bacterial 16S rRNA gene copy numbers of the sand were significantly different ($p > 0.05$) between the horizontally dispersed sampling points A, B and C, which were located at increasing distances from the influent inlet (Fig. 1**A, Table S3**). In addition, the beta diversity analysis showed that the prokaryotic community composition of the sand at sampling points A, B and C for each depth of all SSFs was not significantly different from each other (PERMANOVA; $p > 0.05$) (Fig. 1**B, Table S4**). Further analysis of homogeneity of multivariate dispersions tests supported these results, indicating no significant variance in dispersion across the horizontally dispersed sampling locations (A/B/C), nor in the pairwise comparisons between any pairs of these sampling points at any depth ($p > 0.05$).

3.2. Vertical variability

3.2.1. Biomass and prokaryotic community structure

The SCM layer had significantly higher 16S rRNA gene copy numbers than the deeper layers ($p < 0.05$), indicating that most prokaryotic biomass resided in the top layer of the SSFs (Fig. 2**A** and **Table S6**). Furthermore, the 16S rRNA gene sequence analysis showed that the alpha diversity (**Fig. S3** and **Table S5**), measured using the Shannon index (which accounts for both evenness and richness), was significantly higher in the SCM compared to the deeper sand layers ($p < 0.05$), except at SSF Katwijk_7.2. When assessing only richness (Chao1 index), the number of ASVs did not differ significantly across all depths at SSFs DePunt_2, Monster_4D, and Monster_5B ($p > 0.05$). However, at Katwijk_7.2, the SCM exhibited significantly lower richness than the deepest layer, while the opposite was observed at Scheveningen_5. In addition, the Pielou's index, as a measure for evenness, indicated that the SCM layer had significantly higher evenness than the deeper layers across all SSFs ($p < 0.05$), although the increase in evenness at Katwijk_7.2 was not statistically significant. Consequently, SSFs at DePunt_2, Monster_4D, and Monster_5B showed a similar number of ASVs

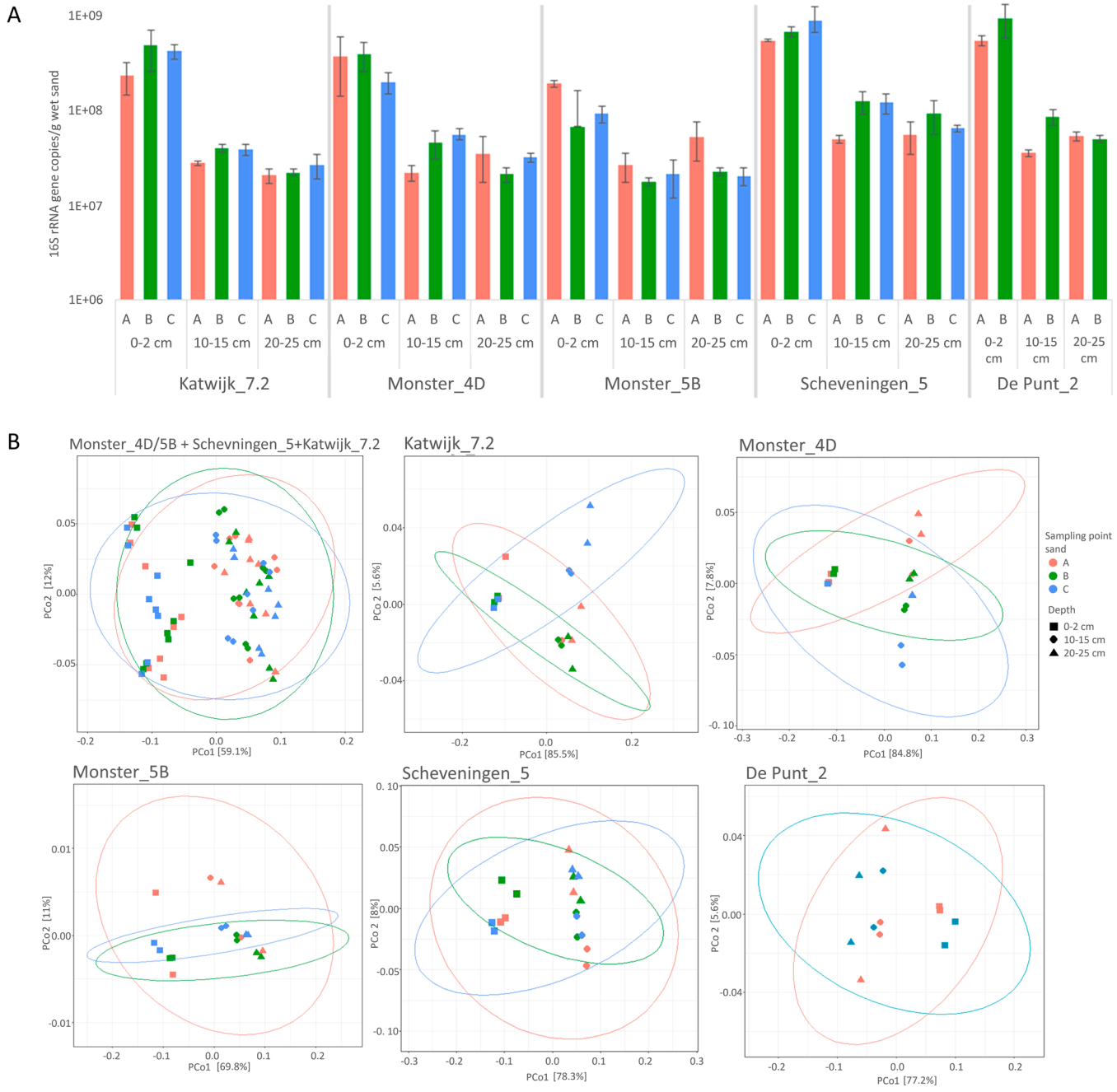

Fig. 1. Bacterial 16S rRNA gene copies and Principle Coordinates Analysis (PCoA) of prokaryotic community composition of sand samples that were taken at different horizontally dispersed locations (A, B and C) and vertically dispersed locations (0–2 cm, 10–15 cm, 20–25 cm) in SSFs from drinking water treatment plants Katwijk, Monster, Scheveningen and De Punt. **A)** Total bacterial 16S rRNA gene copies from each sampling point from the different SSFs. **B)** PCoA plot of prokaryotic communities based on pairwise weighted UniFrac distances.

across depths, but the ASVs were more evenly represented in the top 2 cm compared to the deeper layers. In contrast, at Scheveningen, the SCM displayed both greater richness and evenness than the deepest layers.

The beta diversity analysis for each SSF separately demonstrated that in all analysed SSFs the SCM layer had a unique composition that clustered separately from the two deeper layers, which were more similar to each other. PERMANOVA confirmed the significance of depth in shaping the prokaryotic community in SSFs ($p < 0.05$) (Fig. 2**B** and **Table S7**). A comparative analysis of all sand samples revealed two primary patterns of clustering. First, the samples clustered in two groups: one group consisting of sand from the SSF in De Punt, and the other group comprising sand from the SSFs in Monster, Scheveningen, and Katwijk. Second, within these two groups, the sand samples differentiated further based on depth, with the SCM layers distinctly separated from the 10–15 and 20–25 cm layers, which closely grouped together (Fig. 3). To more effectively visualize changes in the abundance of predominant taxa across different sand depths and in water, the relative abundances of the top 20 taxa from all SSFs are presented in **Fig. S5**.

3.2.2. *Differential abundance analysis*

To determine the taxa that significantly differed across depths in SSFs, we conducted differential abundance analyses for each SSF using the linear regression framework for differential abundance analysis (LinDA) (**Fig. S4 A-O**). The results showed that several ASVs were found to be relatively more abundant in the SCM than in deeper layers of the different SSFs. These included one ASV from the genus *Schlesneria*, one

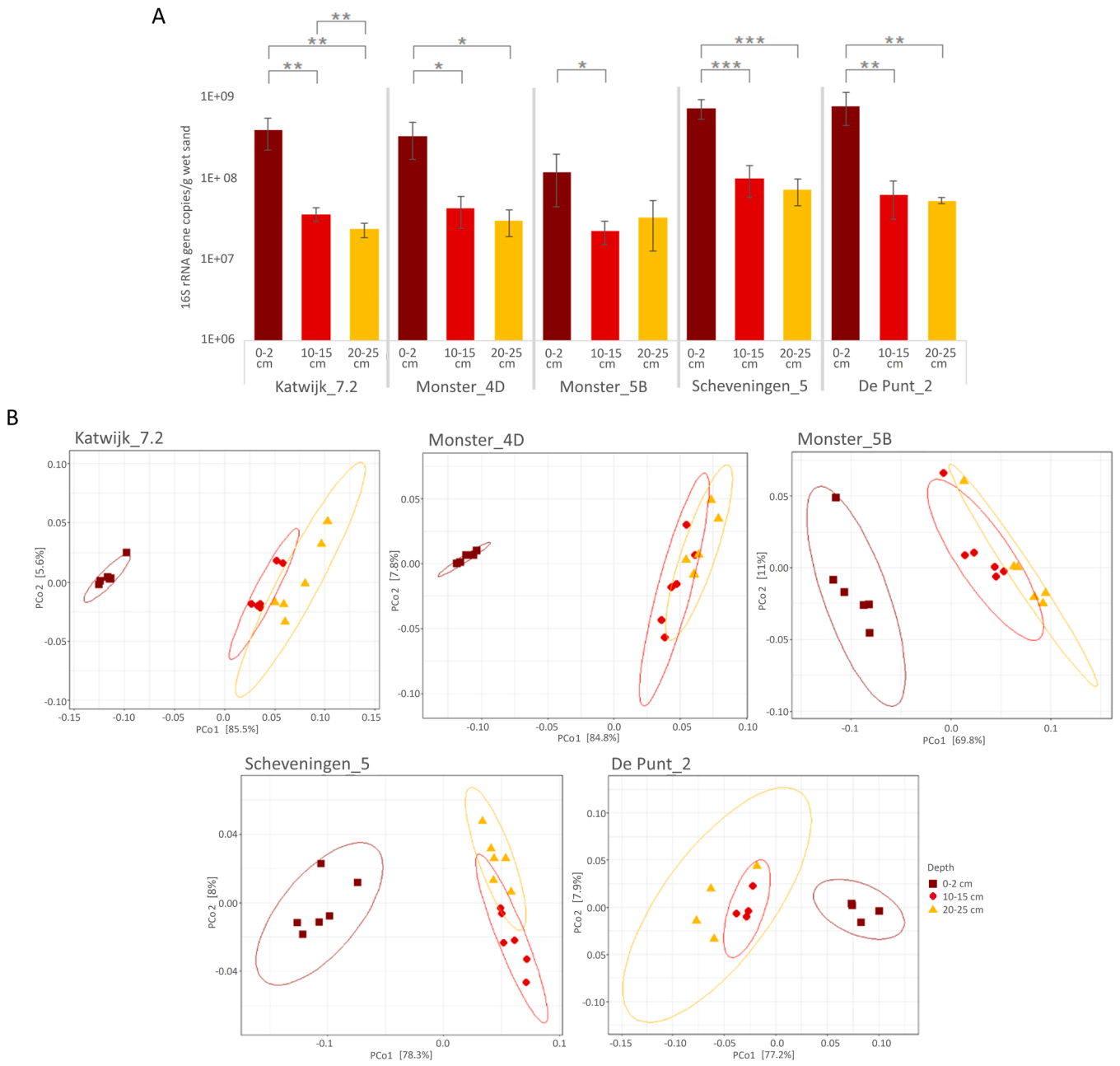

Fig. 2. Bacterial 16S rRNA gene copies and Principle Coordinates Analysis (PCoA) of prokaryotic community composition of sand samples that were taken at different depths (0–2 cm, 10–15 cm, 20–25 cm) of the sand bed from drinking water treatment plants Katwijk, Monster, Scheveningen and De Punt. **A)** Total bacterial 16S rRNA gene copies from each depth from the different SSFs. Statistical significance symbols * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. **B)** PCoA plot of prokaryotic communities based on pairwise weighted UniFrac distances.

from the NS9 marine group, and one from the *Blastocatellaceae* family at Katwijk_7.2, one ASV from the A4b family at Monster_4D, one ASV belonging to the A4b family and one to the *Blastocatellaceae* family at Monster_5B, one ASV from the PLTA13 order at Scheveningen_5, and one ASVs from the bacterialp25 class and another from the *Vicinamibacteriales* order at De Punt_2. Additionally, one or two ASVs belonging to the *Nitrospira* genus was relatively more abundant in the SCM compared to the deeper layer at all SSFs.

The results of the differential abundance analysis also showed a progressive significant increase in relative abundance of ASVs belonging to the archaeal family *Nitrosopumilaceae* within the deepest layers (10–15 and 20–25 cm) compared to the SCM of the sand bed across all SSFs. At De Punt the enriched ASV could be assigned to the genus *Nitrosarchaeum*. SSF Scheveningen_5 displayed a unique pattern, with the *Nitrosopumilaceae* being the third most relatively abundant ASV in the deepest layers compared to the SCM, following ASVs from the *Pedosphaeraceae* and *Entotheonellaceae* families. In contrast, *Nitrosopumilaceae* was the most relatively abundant ASV in the deepest layers compared to the SCM across all other SSFs. The SSFs Scheveningen_5, Monster_4D and Katwijk_7.2 shared a higher relative abundance of ASVs within the order PLTA13 in the deepest layers compared to the SCM layer.

A comparison of the 10–15 cm with the 20–25 cm depth in SSFs revealed fewer or no ASVs with different relative abundances between those two layers than between the SCM and either of the deeper layers. This finding confirms that the prokaryotic communities in the two deeper layers were more comparable to each other than to the community in the SCM layer.

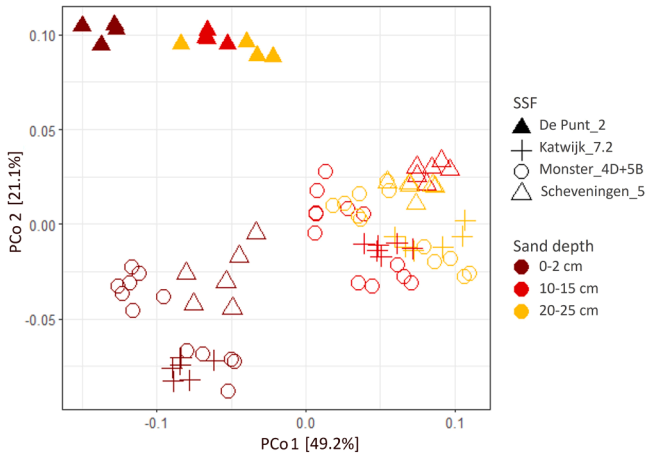

Fig. 3. Principle Coordinates Analysis (PCoA) plot of prokaryotic communities based on pairwise weighted UniFrac distances for sand samples from different depths from the drinking water treatment plants Katwijk, Monster, Scheveningen and De Punt.

For most SSFs, the relative abundance of archaea in sand increased with depth, and they were more prevalent in water effluent than influent, with the highest relative abundance observed in the effluent (**Fig. S6**). Archaeal sequences retrieved from the water samples belonged predominantly to the *Woesearchaeales* order, *Thermoplasmatota* phylum, CG1–02–32–21 family, *Nitrosarchaeum* genus (within the *Nitrosopumilaceae* family), and *Nanoarcheota* phylum. In contrast, the sand bed displayed a more selective preference for certain archaeal taxa. Next to the presence of the *Nitrosopumilaceae* and the genus *Nitrosarchaeum* within this family, the *Woesearchaeales* order and *Bathyarchaeis* class were observed in some sand samples as well. In SSF Katwijk_7.2, the *Woesearchaeales* order showed an increasing trend with depth, whereas in SSF Monster_5B, the *Bathyarchaeis* class was notably enriched in some samples of the top layer. archaea were not detected in the top 2 cm of SSF Monster_4D and in the water influent from DePunt_2.

3.2.3. Core microbiota

ASVs belonging to the families *Nitrospiraceae, Gemmataceae, Pirellulaceae, Nitrosomonadaceae, Vicinamibacteriaceae, Blastocatellaceae*, and the order *Vicinamibacteriales* constituted the shared core microbiota present in sand samples from all depths at all SSFs at relative abundance of at least 0.5 %. In addition, there were also unique core taxa present at each SSF or at groups of SSFs undergoing the same treatment process. For example, the order PLTA13, TRA3–20 family and RCP2–54 phylum were predominant core taxa over all depths in the SSFs from Monster, Katwijk, and Scheveningen, which all treat dune-infiltrated surface water in the same manner, but they were not a core taxon over all depths in SSF De Punt_2. De Punt_2 distinctive core microbiota over all depths included the bacteria25 class, the order *Rokubacteriales*, and families such as *Pyrinomonadaceae, Chthoniobacteraceae, Dadabacterales, Xanthobacteraceae*, and *Methyloligallaceae* (Fig. 4**A-F**).

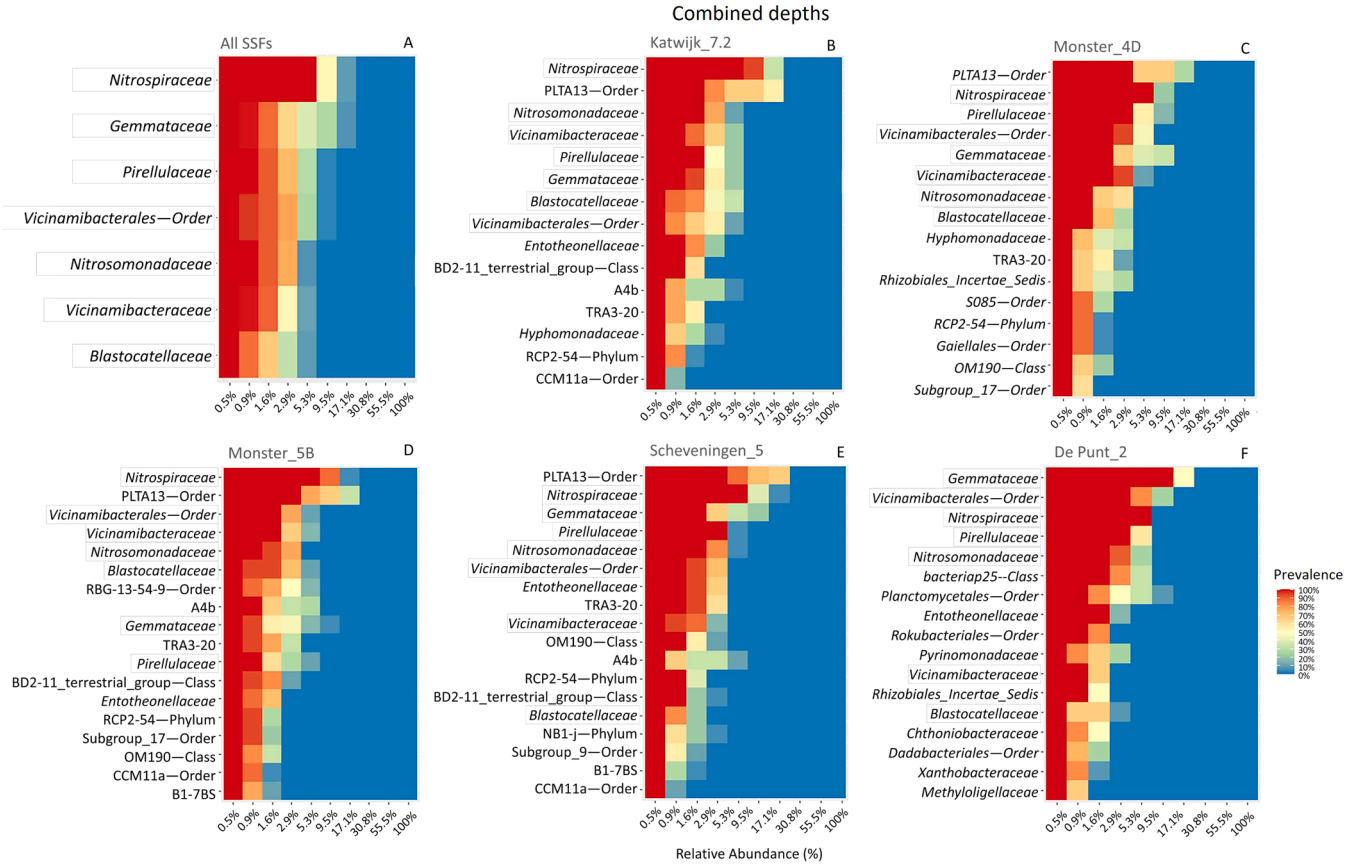

Fig. 4. Relative abundance of core microbiota taxa in sand samples shared by all three depths that were sampled from SSFs at drinking water treatment plants Katwijk, Monster, Scheveningen and De Punt. **A)** all SSFs, **B)** samples from SSF Katwijk_7.2, **C)** SSF Monster_4D, **D)** SSF Monster_5B, **E)** SSF Scheveningen_5, and **F)** SSF DePunt_2. The core microbiota was defined as the taxa present in all samples of a specific dataset with at least 0.5 % relative abundance. The colour scale indicates the prevalence at a given relative abundance (x-axis) of a specific taxon across all samples of the subset, (i.e. a prevalence of 100 % means that a taxon is present at a specific relative abundance in all samples of the dataset). The taxa highlighted with a grey rectangle represent the core taxa that are consistently found in all analysed slow sand filters.

3.3. Temporal variability in the SCM

3.3.1. Biomass and prokaryotic community structure

The qPCR results showed that the 16S rRNA gene copy numbers in the SCM from SSFs that aged 4.5 to 5.6 years after scraping were significantly higher than those in the SCM from SSFs that aged 8 to 52 days or 1.6 to 3.6 years after scraping ($p < 0.05$) (Fig. 5**A** and **Table S8**). Furthermore, the 16S rRNA gene copy numbers in the middle-aged SSFs

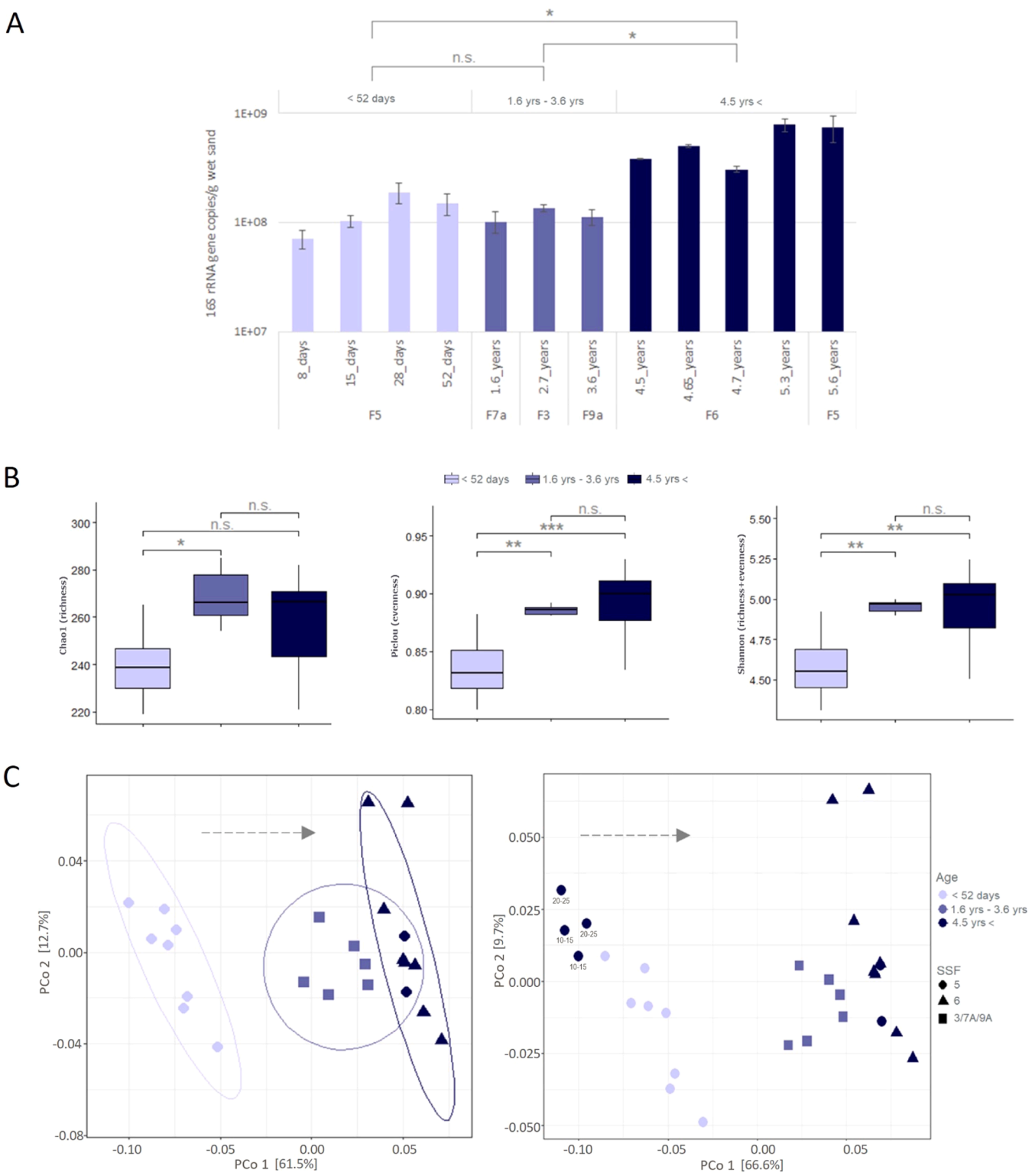

Fig. 5. Bacterial 16S rRNA gene copies and prokaryotic community composition in SCM samples of SSF from three different age classes (8–52 days, 1.6–3.6 years and 4.5–5.6 years after scraping). **A)** Total bacterial 16S rRNA gene copies; statistical significance symbols * $p \leq 0.05$, n.s. $p > 0.05$. **B)** Alpha diversity of prokaryotic communities using Chao1, Pielou's and Shannon index; statistical significance symbols * $p \leq 0.05$, n.s. $p > 0.05$. **C)** PCoA plot of the beta diversity of prokaryotic communities based on pairwise weighted UniFrac distances; the left plot shows only the SCM and the right plot shows the SCM samples and the sand from 10–15 and 20–25 cm of SSF filter 5.

(1.6 to 3.6 years after scraping) were not significantly different from those in the SCM of young-aged SSFs (8 to 52 days after scraping) ($p > 0.05$).

Alpha diversity analyses (Fig. 5**B** and **Table S9**) showed that the Shannon index was significantly lower in the SCM of young-aged SSFs compared to the SCM of middle- and old-aged SSFs ($p < 0.05$). The same result was obtained for evenness, whereas the microbial community richness parameter Chao1 was only significantly different between the SCM of young- and middle-aged SSFs ($p < 0.05$). The Shannon index, richness and evenness parameters were not significantly different between SCM of middle- and old-aged SSFs ($p > 0.05$).

The beta diversity analysis demonstrated that there was a significant distinction between the prokaryotic community composition in the SCM of young-, middle- and old-aged SSFs (PERMANOVA; $p < 0.05$) with a trend from young-aged to middle-aged to old-aged SSFs (Fig. 5**C** and **Table S10**).

3.3.2. Differential abundance analysis

The most pronounced result from the differential abundance analysis was a significant higher relative abundance of one ASV from the *Nitrospira* genus and one from the A4b family as the SCM aged from 8–52 days to 1.6–3.6 years after scraping and from 1.6–3.6 years to 4.5–5.6 years after scraping ($p < 0.05$) (**Fig. S7A-C**). One ASV belonging to the order PLTA13 was significantly more enriched in the young-aged SCM compared to the middle-aged SCM, while in turn one ASV of the PLTA13 order was more enriched in the SCM of middle-aged SSFs compared to old-aged SSFs ($p < 0.05$).

3.3.3. Core microbiota

Our analysis identified core taxa that were consistently present at 0.5 % relative abundance or more in the SCM of SSFs during the whole aging period (**Fig. S8**). The most abundant taxa in the core microbiota were the *Nitrospiraceae* family and the PLTA13 order, appearing in all samples with a relative abundance of 2.9 % or more. Additional abundant taxa in the core microbiota included the *Gemmataceae, Pirellulaceae, Nitrosomonadaceae*, and *Vicinimibacteraceae* families, along with the *Vicinamibacterales* order, each present in all samples with at least 1.8 % relative abundance. Core taxa observed at relatively low abundance (0.5 to 1.7 %) comprised the A4b, *Hyphomicrobiaceae*, TRA3–20, *Blastocatellaceae, Hyphomonadaceae*, and *Enthoenellaceae* families, the Subgroup_17 order, the BD2–11 terrestrial group and OM190 classes, and the RCP2–54 and *Latescibacterota* phyla.

4. Discussion

4.1. Spatial distribution of prokaryotic communities in SSFs

4.1.1. Horizontal uniformity

In this study we observed that the 16S rRNA gene copy numbers, as indicator of biomass, and the prokaryotic community composition in the sand of SSFs from different DWTPs in The Netherlands were comparable across various horizontal sampling points at different distances from the influent water inlet. This demonstrates a uniform distribution of prokaryotic communities across the horizontal space of an SSF. Remarkably, this uniformity persisted regardless of SSF surface size. Even in SSFs with extensive surface areas, such as Scheveningen_5 and DePunt_2, there were no significant differences in prokaryotic community composition attributable to the horizontally dispersed sampling points. This uniform distribution was also observed in SSF systems with varying influent inlet designs. This indicates uniform infiltration across SSFs, irrespective of size or inlet configuration. Additionally, these outcomes indicate that the prokaryotic community in the SSF sand is both robust and stable despite possible variations in the chemical and microbial profiles of the water influent. Contrastingly, studies on full-scale SSFs in the UK demonstrated that the distance from the influent inlet significantly impacted the microbial community structure in the sand; particularly samples closest to the inlet had different community structures than those further from the inlet (Haig et al., 2015). This apparent discrepancy with our study is not entirely surprising as SSFs in the UK were sampled after chlorine had been added to the influent water for decommissioning, and sand nearest to the inlet was also closest to the point where chlorinated influent water was introduced. Furthermore, the SSFs in that study differed from those in our study as SSFs in the UK were operated outdoors and exposed to more variable environmental conditions including seasonal temperature fluctuations, algae growth due to direct sunlight exposure and contamination from wildlife (e.g. bird faeces).

In contrast, positioning SSFs indoors as the final step in the filtration process, a common practice in The Netherlands, likely contributes to maintain more stable environmental conditions and a more consistent prokaryotic community. This stability promotes microbial evenness and enhances overall performance across the horizontal space of SSFs. As an even microbial community has been shown to be positively associated with SSF performance (Haig et al., 2015), this operational approach not only protects the microbial community in the sand bed from external fluctuations and wildlife contamination but may also improve the overall functionality of the system.

4.1.2. Vertical variability

While the prokaryotic community composition in the sand was horizontally homogeneous across all sand layers, we observed that this composition was significantly impacted by the sampling depth in all SSFs, thus showing vertical variability. The bacterial biomass content was higher in the SCM layer than in the deeper layers, as also previously demonstrated (Campos et al., 2002; Chen et al., 2021; de Souza et al., 2021). Moreover, the biomass concentrations in the deeper layers, specifically those at 10–15 cm and 20–25 cm, were more similar to each other than to the SCM, suggesting that below the more metabolic active SCM layer, the metabolic activity is lower and remains more stable over the filter bed.

Apart from SSF Katwijk_7.2, the SCM layers of the SSFs exhibited significantly greater diversity (measured by the Shannon index) compared to the deeper sand layers as previously observed in similar SSFs (Chen et al., 2021). Because the richness parameter (Chao1 index) was not significantly different, but the evenness parameter (Pielou's index) was, we conclude that the higher Shannon diversity observed in the SCM compared to the deeper layers is attributed to a more even distribution of the ASVs in terms of their relative abundance. We hypothesize that this greater evenness is likely driven by a broader nutrient composition in the SCM layer compared to the deeper layers. In the SCM layer, a more diverse spectrum of biodegradable dissolved organic carbon (BDOC) might be available, supporting a more diverse microbial community. Conversely, in the deeper layers, where the BDOC composition might be less diverse due to BDOC consumption in the SCM layer, more specialised BDOC degraders may dominate, leading to lower evenness. A greater diversity, including a more even microbial distribution is generally associated with higher metabolic diversity and ecological stability (Tardy et al., 2014; Yachi and Loreau, 1999), which is crucial for effective water treatment in SSFs (Haig et al., 2015).

Based on the beta diversity analyses, the sand samples clustered in two groups: one consisting of sand from the filter DePunt_2, and the other including sand from the filters at Monster, Scheveningen, and Katwijk. This observed clustering can most probably be attributed to the different source water used and differences in pretreatment before SSF as was shown previously for these treatment plants (Attiani et al., 2025). Within these two groups, however, the sand samples differentiated based on depth, with the SCM layers distinctly separated from the 10–15 and 20–25 cm layers, which closely grouped together. These findings show that source water and pretreatment processes used before SSF predominantly influence SSF microbial communities, followed by filter depth. However, within a treatment plant filter depth plays a pivotal role in shaping the microbial community composition by a clear

distinction between the top and deeper layers in all SSFs analysed.

Previous studies on rapid sand filters (RSFs) have shown that microbial community composition varies significantly with filter depth (Abkar et al., 2023; Ma et al., 2020), likely due to differences in nutrient availability, redox conditions, and hydraulic retention time. Our results indicate that SSFs also exhibit distinct microbial stratification, supporting the hypothesis that depth plays a critical role in shaping microbial dynamics. Unlike RSFs, SSFs operate at slower flow rates (de Moel et al., 2006), promoting longer microbial retention times and biofilm development, which may further enhance these depth-dependent variations. These findings underscore the importance of considering microbial stratification when evaluating SSF performance and resilience. Another important difference is that RSFs are one of the first treatment steps, whereas SSFs are often the last step (de Moel et al., 2006) and, as a result, RSFs are fed with other BDOC concentration and composition than SSFs, which is likely to also impact the microbial ecology in these filters. Consequently, care should be taken to directly compare the microbial ecology in RSFs and SSFs.

A recent study on the same SSFs (Attiani et al., 2025) revealed that the relative abundance of certain taxa varied depending on the sample type (SCM and water influent or effluent). Extending these observations to deeper sand layers, our study confirms that *Gemmataceae, Nitrospiraceae*, and *Nitrosomonadaceae* are not only more abundant in the SCM than in the water effluent, but also remain prevalent in deeper layers (**Fig. S5**). This new finding suggests that these three taxa thrive by forming biofilms and remaining attached to the sand. In contrast, *Comamonadaceae*, which were shown to be abundant in the water influent and SCM but not in the water effluent, showed a clear decline with increasing sand depth. This supports the hypothesis that they originate from the influent, are removed in the SCM and do not persist throughout the filter bed, thereby not being preferentially transported to the effluent. *Vicinamibacteriaceae* and *Vicinamibacterales* were found to be more abundant in the water effluent than in the influent (Attiani et al., 2025). Our data not only confirm this observation but also reveal an increase in their abundance with depth, indicating that they thrive within the sand bed and are likely released into the effluent. This positions the sand bed as a probable source of these taxa. Thus, taxa are preferentially filtered out or preferentially grow in the different sand layers, which in turn influences the composition of the effluent water.

4.1.3. Differentially abundant taxa at different sand depths and SSFs

ASVs belonging to *Nitrospira* were relatively more abundant in the SCM layer of all SSFs compared to the deeper layers. This pattern likely reflects its role in nitrification (Daims et al., 2015; Koch and Lücker et al., 2019), as in the SCM environment *Nitrospira* spp. can benefit from a continuous supply of nutrients, including ammonia, from the influent water. Additionally, ammonia could be released from the turnover of microbial biomass, which is generally more active and abundant in the SCM layer. The SCM layer when compared to the deepest layers of SSFs at Monster, Scheveningen and Katwijk, exhibited a higher relative abundance of shared ASVs belonging to *Nitrospira* (common across all SSF), A4b (Monster 4D and 5B), and *Blastocatellaceae* (Katwijk_7.2 and Monster_4D). Remarkably, the SCM layer in Katwijk showed a significantly higher relative abundance of ASVs belonging to *Schlesneria* and NS9 marine group family, which were not observed in the other SSFs. SSFs Monster, Scheveningen and Katwijk use the same source water and pretreatment. The only difference is that Monster and Scheveningen both implement softening and powdered activated carbon (PAC) dosage followed by aeration prior to the SSF, whereas Katwijk employs PAC just before the SSF. As there are no other differences between these three plants, the difference in PAC dosage and aeration most likely resulted in the observed difference in more relatively abundant ASVs in the SCM between Katwijk and Scheveningen/Monster. At De Punt as well the ASVs with higher relative abundance in both the SCM and deeper layers in SSFs were different from those observed in the SSFs of the other three plants (*Nitrosarchaeum* and bacterial class p25). De Punt uses a very different source water (reservoir surface water) and treatment train (including BAC filtration and UV disinfection) than Katwijk, Scheveningen or Monster, which is the likely cause for these differences in the microbiology of the SSFs.

Overall, the differential abundance analysis revealed that while there was considerable variation in ASVs belonging to different taxa between the SCM and deeper layers, the microbial communities at the 10–15 cm and 20–25 cm depths were relatively uniform with a consistent bacterial biomass and similar enriched taxa, indicating a shared ecological environment in the deeper layers. This suggests that the deeper layers maintain consistent microbial communities across different DWTPs, contributing to the stability and efficiency of the SSF system. In contrast, the SCM layer hosts more distinct, site-specific communities influenced by factors such as source water treatment, operational conditions, nutrient availability, and influent water composition. Notable differences exist between SSFs that handle water post-UV disinfection and reservoir storage versus those processing non-disinfected water following dune infiltration. These findings emphasize the need to study multiple SSFs with diverse operational conditions and water sources to better understand microbial community variability and function, as single-plant studies do not provide generalizable insights.

4.1.4. Other factors influencing prokaryotic communities across SSFs

Besides depth, the treatment processes and water source used are also significant factors influencing the prokaryotic community structure in the sand (Fig. 3). The contribution of treatment processes and source water was evidenced by the distinct prokaryotic community composition of the sand at De Punt, which undergoes different treatment processes compared to Monster, Scheveningen, and Katwijk. The latter three do not only share similar treatment processes but also utilise the same water source. As demostrated by Attiani et. al (2025), the combination of different treatment processes and source waters can shape the prokaryotic community composition of the SCM layer in full-scale SSFs, and affects the biological stability of the produced drinking water. Biological stability is crucial for mantaining microbiological water quality and preventing the growth of pathogens in the distribution system. Drinking water produced at a DWTP using dune-infiltrated surface water, without the application of ozonation or UV disinfection before SSF, showed lower AOC concentrations and, consequently, a reduced potential for microbial regrowth. In contrast, water produced at a DWTP treating reservoir-stored surface water, combined with either ozonation or UV disinfection SSF, exhibited higher AOC concentrations and a greater potential for microbial regrowth in the distribuition system (Attiani et al., 2025; van der Kooij et al., 2017). The findings of our current study suggest that treatment processes applied and water source used are not only important factors to shape the prokaryotic community of the SCM layer, but also for the deeper layers.

It has been observed that seasonal variation can impact both temperature, microbial activity and BDOC concentration and composition in surface water that is treated to drinking water (Hijnen et al., 2018; Schurer et al., 2022; van der Kooij et al., 2015). Seasonal variation can, thus, be a potential confounding factor influencing microbial community composition. However, our sampling was conducted at a specific time of year for each SSF, which limits our ability to directly assess seasonal effects, particularly for DWTP De Punt. That said, samples from Scheveningen, Katwijk, and Monster were collected across different seasons, yet their microbial communities still showed greater similarity to each other than to that of De Punt. Specifically, De Punt and Katwijk were sampled in April, Scheveningen in February, and Monster in November. This suggests that seasonality is not a primary driver for the observed community differences between these SSFs. One possible explanation is that the influent water treated by these SSFs, which is dune-infiltrated, experiences only slight temperature and BDOC fluctuations throughout the year (± 5 °C), contributing to a more stable environment within the SSFs. However, we cannot draw definitive conclusions about SSFs treating reservoir-stored influent, such as De

Punt, because we only had a single sampling point in time from that location.

4.1.5. Core microbial community across SSFs

Although each SSF had its specific microbial community composition profile and some variations in relative abundance of microbial groups across sand layers, a core sand prokaryotic community was consistently identified across all layers. Specifically, the families *Nitrospiraceae, Pirellulaceae, Nitrosomonadaceae, Gemmataceae*, and *Vicinamibacteriaceae* were present in all sand samples from every depth of all SSFs analysed, each at a relative abundance of at least 0.5 %. Their stable presence under different environmental conditions within the SSFs suggests their essential role in the microbial ecosystem and the microbial processes in SSFs. The *Nitrospiraceae* and *Nitrosomonadaceae* families are both known for their involvement in nitrification. Their contribution to the core prokaryotic community in SSFs underscores the importance of nitrification in SSFs to obtain high drinking water quality. Effective ammonia removal during treatment helps preventing the breakthrough of harmful ammonia and/or nitrite to drinking water (Bouwer and Crowe, 1988; Trikannad et al., 2024; Wilczak et al., 1996). The *Gemmataceae* family is widely distributed across various environments, including freshwater ecosystems like lakes and rivers and terrestrial habitats (Brummer et al., 2004; Ivanova et al., 2021; Pollet et al., 2011; Wang et al., 2002). Known representatives of the *Gemmataceae* are strictly aerobic, chemoorganotrophic bacteria capable of degrading biopolymers such as cellulose, chitin, xylan, and pectin that are found in biofilm structures like those formed on sand beds (Kulichevskaya et al., 2020).The *Vicinamibacteriaceae* family includes aerobic, chemoorganotrophic bacteria typically found in soils that form strong aggregates and likely utilise amino acids, peptides, and fatty acids, as well as various sugars and complex proteinaceous substrates (Dedysh et al., 2022; Huber et al., 2016; Serrana and Watanabe, 2022; Vieira et al., 2017). Members of the *Pirellulaceae* family are primarily chemoorganotrophic and aerobic, although some species are facultatively anaerobic. They are predominantly found in aquatic environments but also inhabit peatlands, soils, and activated sludge (Bondoso et al., 2017; Chouari et al., 2003; Kulichevskaya et al., 2022; Rensink et al., 2020). *Pirellulaceae* can utilise a variety of sugars, polysaccharides, and other carbon sources (Chen et al., 2021; Kulichevskaya et al., 2022; Kumar et al., 2021; Zhang et al., 2019). Additionally, *Pirellulaceae* have been identified as abundant taxa in SSFs (Bai et al., 2024; Chen et al., 2021). The metabolic versatility of bacterial species within the *Gemmataceae, Vicinamibacteriaceae* and *Pirellulaceae* in degrading BDOC suggests that the core microbiota in SSFs plays a pivotal role in removing a variety of BDOC compounds from the water. Still, due to the limited number of cultivated representatives, identification to the genus level remains challenging, underscoring the need for further cultivation efforts of these apparent BDOC degrading bacteria to better understand their exact role in BDOC removal in SSFs. In all SSFs treating dune-infiltrated water, the order PLTA13 is predominant in the sand layers along with the core microbiota. However, the specific roles of PLTA13 members remain unclear due to their status as uncultivated organisms and their classification at a high taxonomic order level.

The presence of core microbial groups across all sand layers and SSFs analysed, along with their correspondence with findings from previous studies, shows the presence of a robust prokaryotic community in SSFs that enhances the ecological stability and resilience of these sand filter ecosystems in treatment. This resilience is crucial for sustaining continuous and effective filter operation, especially in response to operational disturbances such as scraping or changes in environmental conditions like temperature and nutrients. Our results together with those of Trikannad et al. (2024) showed that irrespective of operational and environmental conditions SSFs continuously removed (biodegradable) dissolved organic carbon and ammonia in both the SCM and the deeper layers. This finding highlights that also the deeper sand layers play a crucial role in contributing to SSF resilience and performance even after cleaning procedures that remove the SCM layer to prevent clogging. A study by Bai et al. (2023), on an SSF from another treatment plant in The Netherlands, also observed that the families *Nitrospiraceae, Pirellulaceae, Gemmataceae, Nitrosomonadaceae*, and *Vicinamibacteriaceae* belonged to the core microbiota. These authors confirmed via both 16S rRNA gene and 16S rRNA amplicon sequencing methods that these core taxa likely also represent the active members of the SSF community. Studies on SSFs from other countries also found a core microbiota in SSFs, including the phyla *Pseudomonadota, Nitrospirota*, and *Acidobacteriota,* that we also identified in SSFs in The Netherlands (de Souza et al., 2021; Lautenschlager et al., 2014; Oh et al., 2018). These findings demonstrate that SSFs seem to harbour a core microbiota across treatment plants in The Netherlands and abroad.

4.2. Temporal distribution of prokaryotic communities

We observed that during the initial 3.6 years after the SCM layer was scraped from the SSFs, the prokaryotic community primarily changed in composition rather than increasing the biomass. Immediately after scraping, the community composition, richness and evenness of the newly developing SCM layer resembled that of the deeper layers, specifically the 10–15 and 20–25 cm layers of a mature filter. This was expected since the upper 10 cm of the sand is removed during scraping, exposing the 10 cm depth layer as the new top layer at the SSF surface. The prokaryotic community of this newly exposed layer then gradually changed over 3.6 years to again resemble the community of a mature SCM.

Other studies also demonstrated that after scraping the prokaryotic community of the SCM layer it is capable of recovering, stabilizing and subsequently evolving towards a more diverse and even composition in time that reflects mature SCM layers (de Souza et al., 2021; Haig et al., 2015). In those studies, it was observed that scraping the SCM layer from SSFs primarily reduced the community evenness, with a more pronounced effect on the upper than the lower layers, but not so much on the community composition or richness.

Although the community composition and diversity in the SCM layer increased during the first 3.6 years after scraping, the results from the differential abundance analysis showed that only a few ASVs belonging to different taxa were differentially abundant between the different stages of SCM maturation. This means that most prokaryotic taxa were already present in the newly developing SCM after scraping, but that the relative abundance of these taxa changed during the maturation period. It also showed that the temporal variation did not result in as many differences in the community composition of the sand as the spatial variation with depth did.

4.3. The ecological niche of archaea in SSFs

Studies that analysed the microbial communities in SSFs often only focused on the top layer of SSFs, where they reported archaeal abundances below 1 %, suggesting that archaea do not play an important role in SSFs (Bai et al., 2023; Oh et al., 2018). We also observed low relative abundance of archaea compared to bacteria in the SCM layer ($<$ 0–1.9 % vs 95–99.5 %). However, the relative abundance of archaea increased up to 10 % with depth in the sand bed, and up to 14 % in the effluent water. These findings indicate that archaea in the deeper layers might play a role in the performance of SSFs that has been overlooked up till now. Studies on freshwater lake sediments also showed that the relative abundance of archaea increases with sediment depth (Lloyd et al., 2013; Wurzbacher et al., 2017). This pattern could be attributed to some members of the archaea being adapted to environments characterised by nutrient depletion where they can maintain viability with an extremely low metabolic rate (Hoehler and Jørgensen, 2013; Schippers et al., 2010). This allows them to survive in nutrient-poor, oligotrophic settings which are likely to occur in lower sediment (or SSF) layers, because most nutrients might have been consumed in the top layer (Aslan, 2008).

The *Nitrosopumilaceae* was the archaeal taxon with the highest relative abundance in the sand filters. In some samples, ASVs have been identified to *Nitrosarchaeum*, a genus within this family. Known representatives of the *Nitrosopumilaceae* family are involved in ammonium oxidation. The increased relative abundance of *Nitrosopumilaceae* over the sand bed indicates that they might be more adapted to lower ammonia concentrations as the ammonia-oxidizing bacteria (AOB) observed in the SCM layer might have reduced the ammonia concentration in the water that reaches the deeper layers (**Fig. S6C**). This aspect is further confirmed by previous findings that AOB and ammonia-oxidizing archaea (AOA) are adapted to higher and lower ammonia concentrations, respectively (Baolan et al., 2012; Wright and Lehtovirta-Morley, 2023). The predominance of *Nitrosarchaeum* at De Punt_2 could be associated with pre-treatment processes, as members of this genus were reported to be enriched in biofilms in UV-disinfected drinking water systems, compared to those in non-disinfected systems (Inkinen et al., 2021). AOA have been identified before in drinking water distribution systems and groundwater treatment facilities, where they can play a crucial role in ammonia removal during biological filtration processes (Kasuga et al., 2010; van der Wielen et al., 2009).

The relative abundance of archaea in the influent or effluent water in the analysed SSF was generally similar between treatment plants and showed an increase in relative abundance in the effluent over the influent. The water samples exhibited a more diverse archaeal community than the sand. The lower diversity of archaea in the sand suggests that only some members present in the water can adhere and grow in the biofilms around the sand grains, whereas others travel with the water through the sand filters and ending up in the effluent. Additionally, both the water and sand samples from De Punt_2 exhibited lower archaeal diversity compared to other SSFs, which may be caused by using UV disinfection of the SSF influent water. Although it has been mentioned before that the genus *Nitrosarchaeum* might be more UV-resistant (Inkinen et al., 2021), others reported that UV disinfection of drinking water can reduce both the abundance and diversity of the total archaeal community in drinking water distribution systems when compared to systems that were not treated with UV disinfection (Bautista-de los Santos et al., 2016; Inkinen et al., 2021).

Up till now, archaea in drinking water systems have largely been neglected. Consequently, the exact roles and significance of the members of this domain we observed in drinking water treatment are still mainly unknown, and their impact may vary depending on factors such as the treatment process used, the characteristics of the source water, and operational conditions. In addition, the current sequencing approaches may not capture the full diversity of Archaea, as they typically have compact genomes and a lower ribosomal copy number compared to bacteria (Koonin and Wolf, 2008; Pan et al., 2023). Further research will be essential to better understand the exact role archaea play in biological filtration systems in drinking water treatment and their dynamics in relation to physical/chemical and operational conditions.

4.4. Practical implications and future recommendations

By revealing spatial and temporal patterns in microbial communities across full-scale systems, this study offers valuable insights for optimizing SSF design, operation, and maintenance. Our results demonstrated that prokaryotic communities in the SCM and deeper sand layers are uniformly distributed across the horizontal surface of SSFs, regardless of surface area, depth, or inlet design. This consistency suggests that even larger SSFs or those with varying inlet configurations can achieve homogenous microbial development. This insight supports that the size and inlet design of SSFs can be applied flexibly and optimally adapted to the treatment plant without compromising on biological performance of the SSF.

In contrast to the horizontal uniformity observed across SSFs, clear vertical stratification was evident, with the SCM layer exhibiting greater microbial diversity and evenness than the deeper sand layers. This suggests a functional zoning, where the SCM supports more active and diverse metabolic processes, while the deeper layers might contribute to long-term stability and resilience and can take over the function of the SCM after scraping. Preserving this stratified microbial structure is, therefore, essential, as disruption through practices such as backwashing or aggressive sand bed cleaning may negatively affect filter performance and recovery. Consequently, before implementing new cleaning procedures, water utilities should carefully evaluate the potential impact of new cleaning procedures on the microbial community structure and the quality of the water produced. Maintenance strategies that protect this ecological layering can help sustain the functional integrity and efficiency of SSFs over time.

Following SCM scraping, we observed that the microbial biomass increased only marginally over a 3.6-year recovery period, while microbial diversity and evenness gradually improved. This indicates that the establishment of a stable, functionally diverse community, rather than biomass accumulation, is a more accurate marker of SSF recovery, maturity and performance. Moreover, given that a stable core microbial community persists throughout this period, SSFs might be returned to operation sooner than the conventional two-month waiting period post-scraping, especially under consistent operational and environmental conditions. However, this should be done with caution: we strongly recommend continued monitoring of key water quality parameters such as AOC, ammonia, and faecal indicator or pathogen breakthrough to ensure safety and performance.

While 16S rRNA gene sequencing reveals microbial community composition in SSFs, it offers limited direct functional insight. Inferring metabolic roles solely from taxonomy is unreliable, as significant functional diversity often exists within the same order, family, or genus. Therefore, elucidating the functional potential of the SSF microbial community, including the specific roles of bacteria and archaea, requires future research to prioritize direct functional assessment. We recommend an integrated strategy, combining multi-omics approaches (metagenomics, metatranscriptomics, proteomics) with advanced cultivation techniques. This direction will likely yield a more comprehensive understanding of the metabolic processes and functional roles of microbial taxa within SSFs.

5. Conclusions

- The minimal influence of horizontal sampling location on prokaryotic communities, regardless of depth, dimension, or inlet design, indicates a horizontally uniform environment throughout the filter bed and potentially uniform filtration performance. This uniformity can allow design flexibility.
- Depth is a significant factor shaping the prokaryotic community in SSFs, thus different prokaryotes presumably perform distinct metabolic functions, or similar functions with distinct kinetics, such as the increased presence of archaeal ammonium oxidizers in deeper layers.
- A core prokaryotic community, including *Nitrospiraceae, Pirellulaceae, Nitrosomonadaceae, Gemmataceae*, and *Vicinamibacteriaceae*, is consistently present across all sand samples and depths. We conclude from this finding that these sand filter ecosystems have a robust prokaryotic community that enhances their ecological stability and resilience and suggests an essential role of the microbial ecology in SSF function. Moreover, these families could serve as biomarkers for filter performance and stability, with routine monitoring providing early warning and performance insights. Validating this approach requires studying dysfunctional SSFs. Further research into their roles could optimize SSF performance and troubleshooting.
- Beside the core microbiota, each SSF/plant has also specific communities, indicating that the conditions result in both uniform and site-specific community members, which makes it difficult to draw general conclusions when only one treatment plant is investigated, pressing the need that full-scale studies should include multiple DWTPs.

- After SCM scraping, SSFs over 3.6 years gradually develop a greater diversity and evenness prokaryotic community with minimal biomass increase, suggesting that stable core community, rather than biomass growth, is crucial for performance. This stability suggests that SSFs could potentially return to production earlier than the typical two-month waiting period, provided key water quality parameters, such as AOC and pathogen breakthrough, are monitored to ensure safety.

CRediT authorship contribution statement

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

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Supplementary materials

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Data availability

Data will be made available on request.

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