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Biological stability shapes habitat-specific microbial community dynamics from treatment to distribution in non-chlorinated drinking water distribution systems

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Maintaining biological stability is essential for high microbiological water quality in drinking water distribution systems (DWDS). Although treatment without residual disinfectant removes biodegradable organic matter to limit microbial regrowth, it remains unclear how improved biological stability influences microbial communities during distribution. We investigated habitat-specific microbial communities in two full-scale DWDS supplied with the same surface water source but treated differently. Conventionally treated drinking water was compared with water produced by managed aquifer recharge and recovery, and the additional effect of ultrafiltration (UF) post-treatment was evaluated. Microbial communities in habitats (bulk drinking water, biofilms and loose deposits) were characterized using 16S rRNA gene amplicon sequencing and integrated with biological stability, physicochemical and cultivation-based measurements. Improved biological stability reduced regrowth potential and *Aeromonas* occurrence while altering microbial communities. Treatment established the initial microbial community, but UF-post treatment and storage reshaped this community, increasing cell numbers and promoting the dominance of *Comamonadaceae* and *Brevundimonas*. During distribution, loose deposits increasingly contributed to bulk drinking water communities, although this effect was reduced after UF. Loose deposits showed stronger seasonal variation than bulk drinking water. These findings show that biological stability shapes microbial communities and identifies loose deposits as an important habitat for understanding and monitoring regrowth.

Drinking water distribution systems (DWDS) need to maintain high microbiological water quality during transportation from water treatment plants to consumers. In systems operated without residual disinfectant (e.g. in the Netherlands, Denmark, and parts of Germany, Switzerland, and Belgium), this depends largely on ensuring high biological stability, i.e. limiting regrowth potential of (micro)organisms, prior to and during transportation and distribution. Biological stability in drinking water produced from surface water is achieved by reducing the concentration of biodegradable organic carbon and other nutrients (such as nitrogen, phosphorous, iron and manganese)[1,2] and is commonly evaluated using parameters such as assimilable organic carbon (AOC), maximum biomass growth in 7 days (MBG_7), the biopolymers (BP) concentration as particulate and/or high molecular weight organic carbon (PHMOC), and the iron accumulation rate (FeAR)[3].

Micro-organisms in DWDS reside in different habitats: suspended in drinking water (DW), attached to or in pipe surface biofilms (BF) and in

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loose deposits (LDs) where they are associated and co-occur with invertebrates[4,5]. The biomass and micro-organisms in the different habitats are part of the DWDS food web and add to the biodegradable organic material. The biomass in BF and LDs could also act as refuge for opportunistic pathogens such as *Legionella pneumophila* and *Pseudomonas aeruginosa* and other unwanted microorganisms or invertebrates that are involved in causing regrowth and/or a decrease of the drinking water stability and microbial water quality[6–9]. This could for instance lead to exceedance of regulatory drinking water standards on regrowth and hygienic indicators, which in the Netherlands are *Aeromonas*, Heterotrophic Plate Count (HPC), and coliforms[10], and are included in legislative routine monitoring of distributed bulk drinking water quality[11,12]. Regrowth of micro-organisms within the DWDS may also cause undesirable water quality conditions, pipe corrosion, consumer complaints on color and odor, and clogged water meters[2,13].

Methods to specifically detect microorganisms related to regrowth have been developed, but these are focused on a minority of the microbial population using cultivation-dependent methods or PCR. These methods therefore hamper evaluation of the role of the microbial population in observed regrowth issues and the ecological interactions of the microbial communities within and between the different habitats during DWDS retention. Microbiome research using 16S rRNA gene amplicon sequencing has brought new understanding to the relationship between the environmental conditions in DWDS, biological stability, and microbial communities. For instance, researchers have demonstrated significant effects of temporal and spatial changes in water quality or pipe diameters on DWDS microbial population diversity and composition[6,14,15]. Bacterial community turnover times were proposed as proxy for temporal and spatial hotspots of biological instability[16].

However, most studies on microbial composition do not link clearly to water treatment strategy, biological stability, and distribution dynamics in the same operation system. This makes it difficult to draw conclusions on the importance of these population dynamics during selective water treatment to manage regrowth processes and biological stability. The knowledge gap arises from the lack of evidence for relationships between biological stability parameters and microbial population dynamics of the different habitats in DWDS. This is primarily due to the difficulty of accessing these habitats within fully operational networks, resulting in most microbial diversity studies in DWDS being limited to bulk water samples[17].

In the current study, we used 16S rRNA gene amplicon sequencing together with physicochemical measurements, biological stability indicators, cultivation-based methods, and source tracking analysis to examine habitat-specific microbial population dynamics in full-scale distribution systems supplied with non-chlorinated drinking water from the same surface water source but treated differently. In one district metered area (DMA), we compared conventionally treated drinking water with drinking water that included managed aquifer recharge and recovery via dune infiltration, and, in a second DMA, we assessed the effect of additional ultrafiltration (UF) post-treatment of the conventionally treated water. The goal of the current study was to answer (1) how treatment strategy affects microbial community composition and diversity in different habitats, (2) how UF treatment, reservoir storage, and distribution reshape these communities over time, and (3) to what extend BF and LDs contribute to the bulk-water microbiome as residence time increases. Answering these questions could help identify how treatment and distribution shape habitat-specific microbial population dynamics under different levels of biological stability. Moreover, it allows interpreting treatment effects in the context of full-scale network dynamics and provides insights into which habitats are most relevant for future monitoring of regrowth and biological stability in DWDS without residual disinfectants.

Results

Water treatment as primary community driver

Water treatment strategy was the dominant determinant of drinking water microbial community composition across the drinking water distribution systems. PCoA showed that all samples clustered mostly according to the treatment plant (DWTP), district metered area (DMA), habitat and whether the water was UF post-treated or not (Fig. 1). Across all samples, water treatment (16%), DMA (12%) and habitat (11%) each explained a significant fraction of the total variance, whereas residence time accounted for only 4% (Table 1). Pairwise PERMANOVA confirmed that the microbial communities differed significantly between all treatment groups and habitats ($p < 0.05$), except for the DWTP-B biofilm, for which only a single sample was available (Table S1).

Treatment-specific microbial community dynamics were further supported by relative and differential abundance analysis of ASVs (Fig. 1). Microbial taxa that are characteristic of the different DWTPs, DMAs, and habitats were determined by their relative abundance (Fig. S2), their differential abundance for each habitat and their contribution to the variance in each habitat for each DWTP and DMA (Fig. S3). Although many ASVs were detected across multiple habitats, several ASVs showed higher relative and differential abundance within specific treatment systems and their habitats and explained most of the variance between treatments and habitats (Fig. 2). These results clearly show how the different DWTPs and DMAs can be distinguished by differential abundances of habitat-specific ASVs. Drinking water from DWTP-A was for instance characterized by relatively high abundances of taxa including *Limnobacter*, *Bdellovibrio* and members of *Caenaercaniphilales*, whereas DWTP-B was enriched in "*Candidatus* Omnitrophus". Likewise, several Planctomycetes-related taxa were

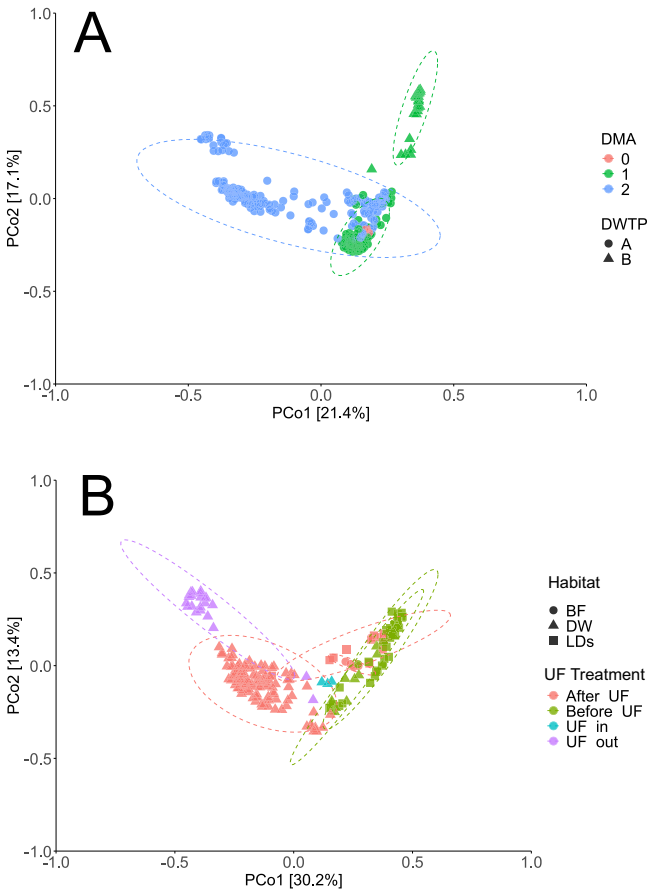

Fig. 1 | Principal coordinates analysis (PCoA) based on a Bray-Curtis dissimilarity matrix showing samples from DMA1 and DMA2 clustering according to DWTP and DMA (**A**; $n = 322$, ASVs=6477) and from only DMA2 clustering according to habitat or UF post-treatment (**B**; $n = 203$, ASVs=5692). BF biofilm, LDs loose deposits, DW drinking water. Ellipses indicate the 95% confidence intervals. Only the first two dimensions that explain most of the variance of the PCoA are shown, and the relative contribution of each axis to the total variance is indicated in percentages.

consistently associated with biofilms and loose deposits. The overlap of ASVs between habitats was probably due to their tight ecological relationships and because some of the collected LD fractions contained flushing water when sampled. Moreover, DW samples may have contained suspended aggregates. Notably, the included DW samples obtained from fire hydrants contained a higher percentage of LD-specific microorganisms (Fig. S1), due to the higher water flow of fire hydrants compared to household taps, as explained in "Methods", section "Drinking Water (DW)".

Table 1 | PERMANOVA partitioning and analysis (adonis test) based on abundance-weighted Bray-Curtis dissimilarity measures of the microbial ASVs obtained from the different DMAs (DMA1 and DMA2, with data from DMA2 before and after UF-post-treatment), habitats, seasons, and residence times (n = 322) with 999 permutations

	Df	Sum of Sqs	*R2*	*F*	*Pr(>F)*
Habitat	2	10.85	0.11	43.40	0.001
DWTP	1	15.42	0.16	123.37	0.001
DMA	2	11.77	0.12	47.09	0.001
Season	3	4.82	0.05	12.85	0.001
UF treatment	3	10.46	0.11	27.91	0.001
Residence_time	12	3.95	0.04	2.63	0.001
Residual	298	37.24	0.39		
Total	321	94.51	1		

The dataset was filtered to a minimum amount of 10,000 reads per sample with a minimum of 0.05% relative abundance per feature and rarified to the minimum amount of reads per sample (25,464 reads).

Biological stability and community changes

Improving biological stability through either MARR treatment (DWTP-B, DMA1-B) or ultrafiltration post-treatment (DWTP-A, DMA2) substantially altered microbial community composition throughout the distribution system. In DMA1-B, the drinking water was swapped from drinking water of DWTP-A to that of DWTP-B, to assess the effect of optimized biological stability on the microbial communities in DMA1 (Fig. 3). Table 2 summarizes the differences in water quality and biological stability of purified drinking water from DWTP-A, after UF treatment (at 'UF-out'), and purified water from DWTP-B for the research period of this work. These parameters showed that water from DWTP-B contained up to tenfold less BPs, up to eight times lower assimilable organic carbon levels (AOC-A3 and AOC-p17/Nox) and up to 43% lower biomass production potential (CBP_{14}) than water from DWTP-A prior to UF post-treatment (Table 2). The BP values for DWTP-B were compliant with the guidance levels for biologically stable drinking water, whereas the values for DWTP-A without UF treatment were not. Dutch guidance values for biologically stable drinking water from surface water are respectively AOC ≤ 10 µg acetate-C/L[18], MBG_7 ≤ 4.5 ng ATP/L, BPs as PHMOC ≤ 47 µg C/L, and FeAR ≤0.34 mg Fe/m^2/day^3. Plate counts of regrowth indicator *Aeromonas* showed lower yearly median numbers (maximum 86 CFU/100 ml for DWTP-B vs 2550 CFU/100 ml for DWTP-A) and a much lower percentage of samples exceeding the technical standard (maximum 4.8% for DMA1-B vs 96.7% for DMA1-A) for distributed water from DWTP-B (DMA1-B) than for DWTP-A (DMA1-A and DMA-2) (Table S2). The higher biological stability of drinking water from DWTP-B was also reflected in the microbial communities. 16S rRNA sequencing results confirmed presence of *Aeromonas* only in distributed water from DWTP-A (DMA1-A and DMA2) (Fig. S4). Moreover, the microbial diversity of DMA1-B showed less

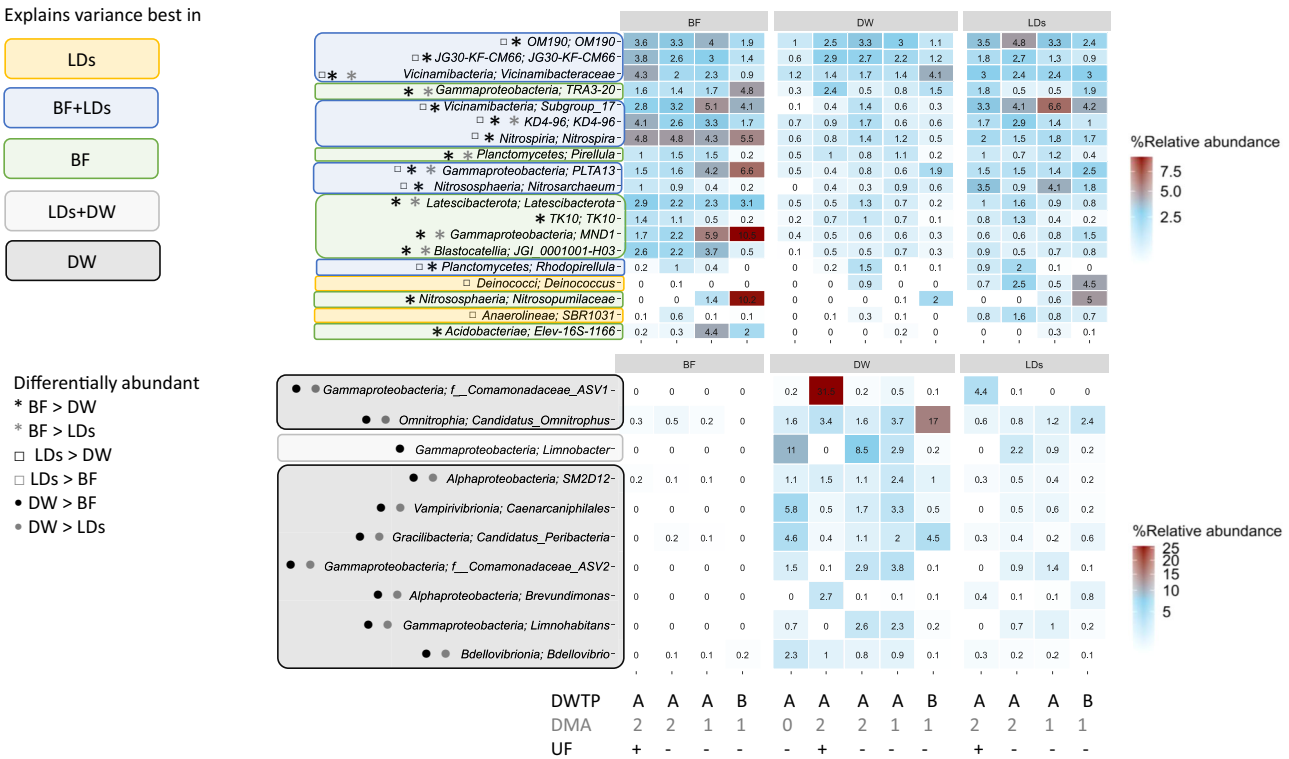

Fig. 2 | Heatmap showing the ASVs that best explained the variance in the different habitats (biofilm; BF, drinking water; DW, loose deposits; LDs) for the different DWTPs (**A** and **B**) and DMAs (1 and 2) and the purified drinking water of DWTP-A (DMA 0). DMA2 is differentiated between the periods before UF (–) and after UF (+). The UF '+' samples include the UF-out samples and the UF '-' samples include UF-in samples. Also shown are the ASVs that were differentially abundant in each habitat, as determined with ANCOM analysis (see legend). n = 329; ASVs=780.

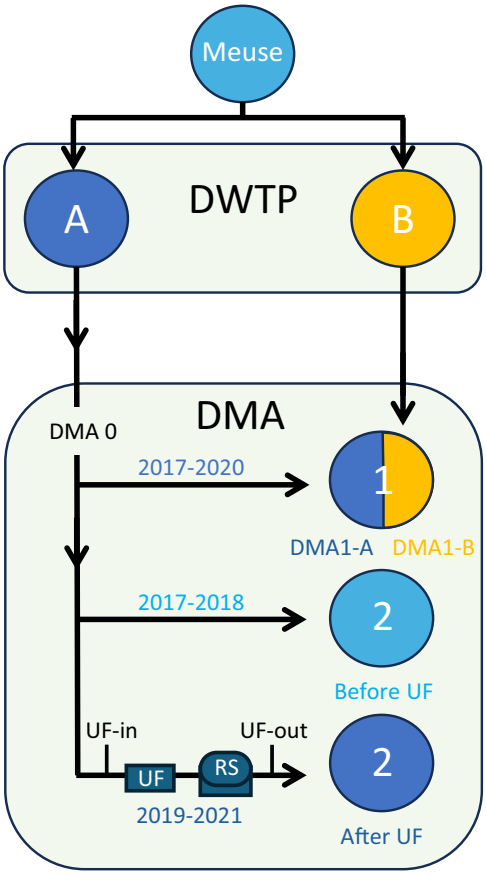

Fig. 3 | Schematic representation of the study design. River Meuse source water was treated in two different drinking water treatment plants (DWTPs); DWTP-A: conventional treatment and DWTP-B: MARR treatment (see materials and methods for full description). After treatment, drinking water was transported to two district metered areas (DMAs): DMA1 and DMA2. Both DMA1 and DMA2 were historically supplied with drinking water from DWTP-A (purified drinking water before distribution here called "DMA0"). During this study of 2017–2020, DMA1 was physically separated into two parts supplied with drinking water from DWTP-A (DMA1-A) and DWTP-B (DMA1-B). DMA2 contained drinking water from DWTP-A that was sampled in the period during conventional treatment before ultrafiltration (Before UF; 2017–2018) and after ultrafiltration post-treatment (After UF; 2019–2021). Samples were also taken from the influent of the UF-post treatment (UF-in) and from the UF permeate after 24 h reservoir storage (RS), here called UF-out. Detailed sample information can be found in the supplementary materials.

Table 2 | Water quality parameters (mean ± standard deviation) for the DW produced by conventional treatment, before the UF posttreatment step (DWTP-A) from 2017 to 2021, the DW after "UF-out" (2019–2021) and the drinking water from DWTP-B (2017–2020)

	DWTP-A (2017–2021)	UF-out (2019–2021)	DWTP-B (2017–2020)
LC-OCD DOC [mg C L^{-1}]	1.7 ± 0.2 (38)	1.7 ± 0.2 (41)	2.8 ± 0.3 (15)
LC-OCD BP [µg C L^{-1}]	76 ± 50 (38)	38 ± 12 (41)	5 ± 4 (15)
AOC-P17/Nox [µg Ac-C L^{-1}]	18 ± 4 (34)	10 ± 2 (19)	10 ± 4 (8)
AOC-A3 [µg BP-C L^{-1}]	8 ± 4 (13)	1 ± 1 (22)	1 ± 2 (10)
CBP_{14} [d ng ATP L^{-1}]	104 ± 33 (43)	86 ± 24 (27)	59 ± 15 (9)
TCC [$\times 10^3$ mL^{-1}]	408 ± 152 (21)	43 ± 67 (76)	286 ± 60 (18)
ICC [$\times 10^3$ mL^{-1}]	31 ± 37 (21)	15 ± 35 (76)	150 ± 54 (18)

The number of measurements (*n*) are given between brackets., LC-OCD DOC = dissolved organic carbon concentration determined by LC-OCD, LC-OCD BP = biopolymer concentration determined by LC-OCD, AOC-A3 and AOC-P17/Nox = assimilable organic carbon, CBP_{14} = biomass production potential determined as cumulative biomass production over 14 days, TCC = total cell count, ICC = intact cell count.

temporal and spatial variability than DMA1-A (Fig. 4) and the total microbial diversity of water from DWTP-B was significantly higher than that of water from DWTP-A in DMA1 and DMA2 (Kruskal-Wallis, $p < 0.05$) (Fig. 4; Table S3). The residence time did not have a significant effect on the microbial communities in DMA1-B, while it was significant for those in DMA1-A and DMA2 that contained water from DWTP-A (Table S4). These spatial and temporal microbial diversity and community differences could be related to the higher biological stability of the water of DWTP-B as compared to water from DWTP-A, and/or to the fact that DWTP-A water was chlorinated just before distribution (without residual disinfectant). Previous findings also showed a strong increase in microbial diversity in pre-treated surface water after MARR which correlated with an increase in biological stability[19]. However, the residence time of water from DWTP-B was much shorter (0–17 h) than the water from DWTP-A (0-93 h) and the sampling frequency of DMA1-A was higher ($n = 82$) than of DMA1-B ($n = 36$). It therefore remains to be determined how the biological stability of DMA1-B would be affected by longer residence times. Overall, it was successful to switch from drinking water with a lower biological stability (DWTP-A) to drinking water with increased biological stability (DWTP-B) to reduce the regrowth conditions in a DMA, even after decades of distributing drinking water from DWTP-A.

A different response was observed following UF post-treatment and reservoir storage in DMA2. DMA2 is an isolated DWDS section supplied exclusively by DWTP-A and was monitored before and after UF post-treatment implementation (Fig. 3). The UF permeate after 24 h of reservoir storage ('UF-out') showed increased biological stability compared to DWTP-A, with BPs lowered by 50%, AOC-A3 by 88%, CBP_{14} by 17%, and total cell counts (TCC) by 90% (Table 2). Correspondingly, *Aeromonas* and heterotrophic plate count exceedances became less frequent after UF implementation (Table S2). Microbial community composition also showed clear clustering based on UF treatment (Fig. 2B). Drinking water samples from DMA2 before and after UF treatment were significantly different, as well as between the UF influent and 'UF-out' (pairwise PERMANOVA; $p < 0.05$, Table S5). The microbial diversity of DW from DMA2 was significantly lower in the period after UF treatment (Kruskal-Wallis $p < 0.05$; Table S3; Fig. 4). UF post-treatment and reservoir storage therefore substantially altered the microbial communities in the DWDS. The decrease in microbial diversity after UF treatment and reservoir storage was caused by the emergence of a hyper-dominant community largely composed of *Comamonadaceae*, *Brevundimonas*, and clade TRA3-20 (Fig. S5). Directly after UF and before storage, the UF permeate showed reduced total and intact cell counts compared to the UF influent (Fig. S6), demonstrating effective cell removal. Yet, total and intact cell numbers rose, with intact cells rising from a median of 590 cells/mL in the UF permeate to 7400 cells/mL in the 24 h reservoir-stored permeate of 'UF-out' (Fig. S6), indicating that the reservoir was not simply preserving the UF-treated community but actively reshaping it. Several mechanisms may explain these observations, including limited microbial growth supported by residual biodegradable substrates and detachment of cells from reservoir biofilms (BFs) or from loose deposits (LDs).

Laboratory contamination was another possibility for this community change as *Comamonadaceae* and *Brevundimonas* were also detected in blank sequencing controls (Fig. S7) and were commonly found in blank samples that could result from ultrapure water contamination or barcode crosstalk[20–22]. However, inspection of blank samples and sequencing runs suggests biological origin since other typical ASVs in blanks were mostly absent from 'UF-out' and other post-UF samples (Figs. S5; S7). Moreover, *Comamonadaceae* abundance varied between sequencing runs and was not

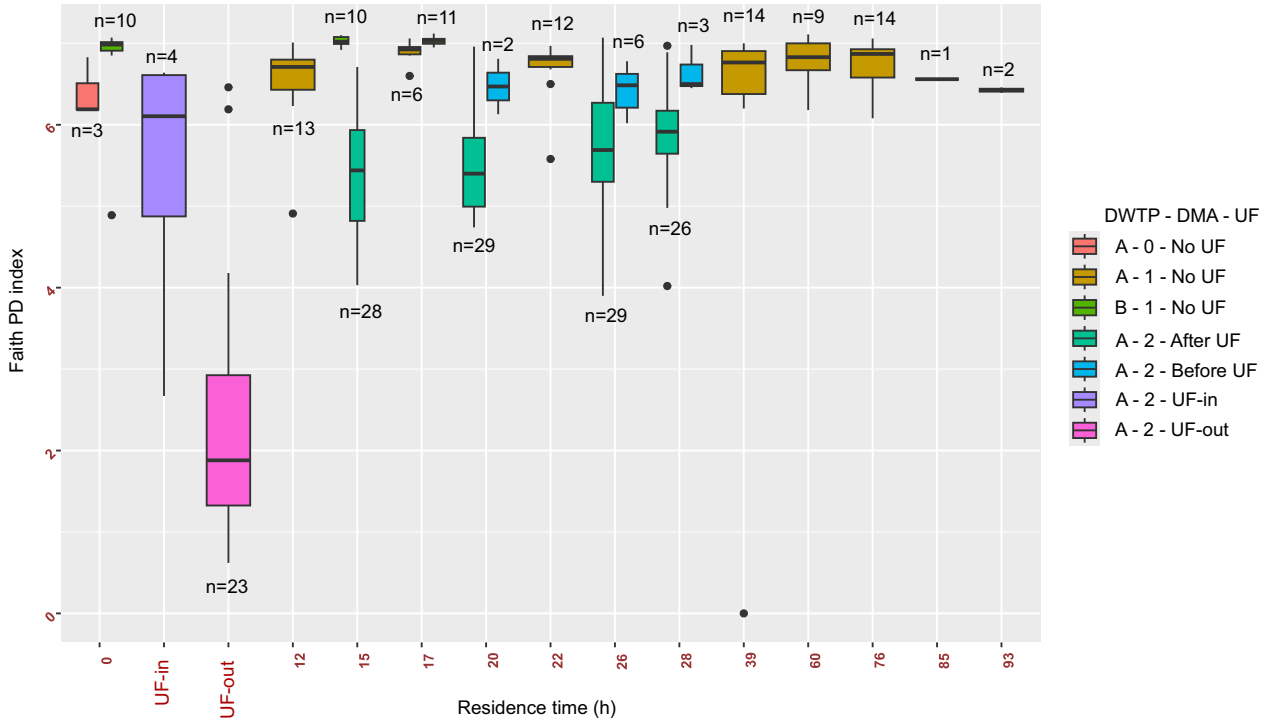

Fig. 4 | Microbial diversity expressed as the Faith's PD index of drinking water samples against residence time (h) in the DMAs. Displayed are DW samples from purified drinking water from source A (DWTP-A, DMA0), from DWTP-A in DMA1 and DMA2 and DWTP-B in DMA1. Samples taken from DMA2 were indicated as "UF-in" (influent of UF), "UF-out" (effluent of UF after reservoir storage), 'Before UF' (period before UF treatment was installed) and 'After UF' (period with UF treated water). The box of the boxplots extends from the first to the third quartile (Q1 to Q3) with a line in the middle that represents the median. The range of values between Q1 and Q3 is the Interquartile range (IQR). The whiskers extending from both ends of the box indicate variability outside Q1 and Q3. The minimum/maximum whisker values are calculated as Q1/Q3 -/ + 1.5 * IQR. Everything outside is represented as an outlier with black dots.

detected in blanks from run 1 but was detected in drinking water samples from the same run (Fig. S7). *Brevundimonas* were consistently found in 'UF-out' across runs, but not in corresponding blanks. These findings rule out contamination or crosstalk as sources of these taxa in "UF-out" but rather point to growth and/or cell release from BF and/or LDs, which coincided with the increases in intact cell numbers during storage. The UF-treated water may have had similar microbial selection pressures as lab-grade ultrapure water, explaining the presence of similar taxa. Others also have identified members of the *Comamonadaceae* family in unchlorinated DWDS[23].

Regrowth after UF treatment has been reported to be possible because dissolved nutrients (especially dissolved low molecular weight organic compounds) can freely pass through the membranes and constitute most of the biodegradable AOC[24]. However, with an assumed growth rate of ~0.30/day and a 24 h storage time, growth alone cannot explain this increase during storage, and biofilm detachment seems additionally important and was previously hypothesized as cause for cell increase after UF[25].

Distribution reshaped the microbiome

The microbial community established during treatment and reservoir storage was subsequently reshaped during residence in the DWDS. Residence time explained only a minor part of the microbial population dynamics across all habitats, DWTPs, and DMAs in space and time (4%; Table 1). However, its influence differed markedly between treatment strategies and explained 15% of the variance of the microbial communities in DMA1-A and 18% in DMA2 before UF treatment. In contrast, this decreased to only 5% following UF treatment in DMA2 (PERMANOVA, adonis tests, Table S4), suggesting that improved biological stability after UF also stabilized the spatial microbial community variations in certain habitats.

To identify which habitats contributed to these changes during residence, microbial source tracking with FEAST was performed[26]. Source tracking analysis focused on DWTP-A water in DMA1-A and DMA2 (with and without UF post-treatment). In DMA1-A, LD-derived microorganisms increased from ~20% at 12 h to ~40-50% after 76 h, whereas DW-derived fractions decreased and BF contributions slightly rose from around 17 h to a stable fraction (Fig. 5A). The unknown fraction remained stable at ~25%. In DMA2 before UF-post-treatment, LD-derived microbes also increased throughout distribution from ~25% at 5 h to ~60-70% at 28 h (Fig. 5B). BF contributions remained consistently minor. The unknown fractions might originate from source water microbes or from early regrowth before sampling. As previous studies found negligible source water contributions to DW bacterial communities[15], the latter explanation seems more likely.

In DMA2 after UF post-treatment at 15 h residence, ~35% of the bulk drinking water microbial community consisted of 'UF-out'-derived microbes while ~25% consisted of LD-derived microbes and remained stable during residence (Fig. 5C). The relatively stable contribution of these LD-derived communities during distribution aligns with residence time explaining only 5% of the variance after UF treatment. These source tracking results are consistent with other observations that the microbial community of stored UF permeate changed again in the DWDS. The drinking water communities in the distribution network after 15 h residence contained a significantly different microbial community than the stored UF permeate ('After UF' vs 'UF out'; pairwise PERMANOVA, Table S5). The stored permeate also showed a significantly lower microbial diversity than the samples during distribution in DMA2 after UF (Kruskal-Wallis $p < 0.05$; Table S3; Fig. 4). Lastly, the number of total cells, and especially intact cells, did not only increase between the UF permeate and the stored permeate in 'UF-out', but also increased with increasing residence time in DMA2 (Fig. S6). Many taxa were removed from drinking water by UF treatment but increased again in relative abundance during distribution, although for most to a lower relative abundance than in the period before UF treatment. These taxa belonged to, for instance, uncultured *Planctomycetes* and *Alphaproteobacteria*, clade OM190, clade JG30-KF-CM66, "*Candidatus* Omnitrophus", *Vicinamibacteriaceae*, clade KD4-96, clade Subgroup 17,

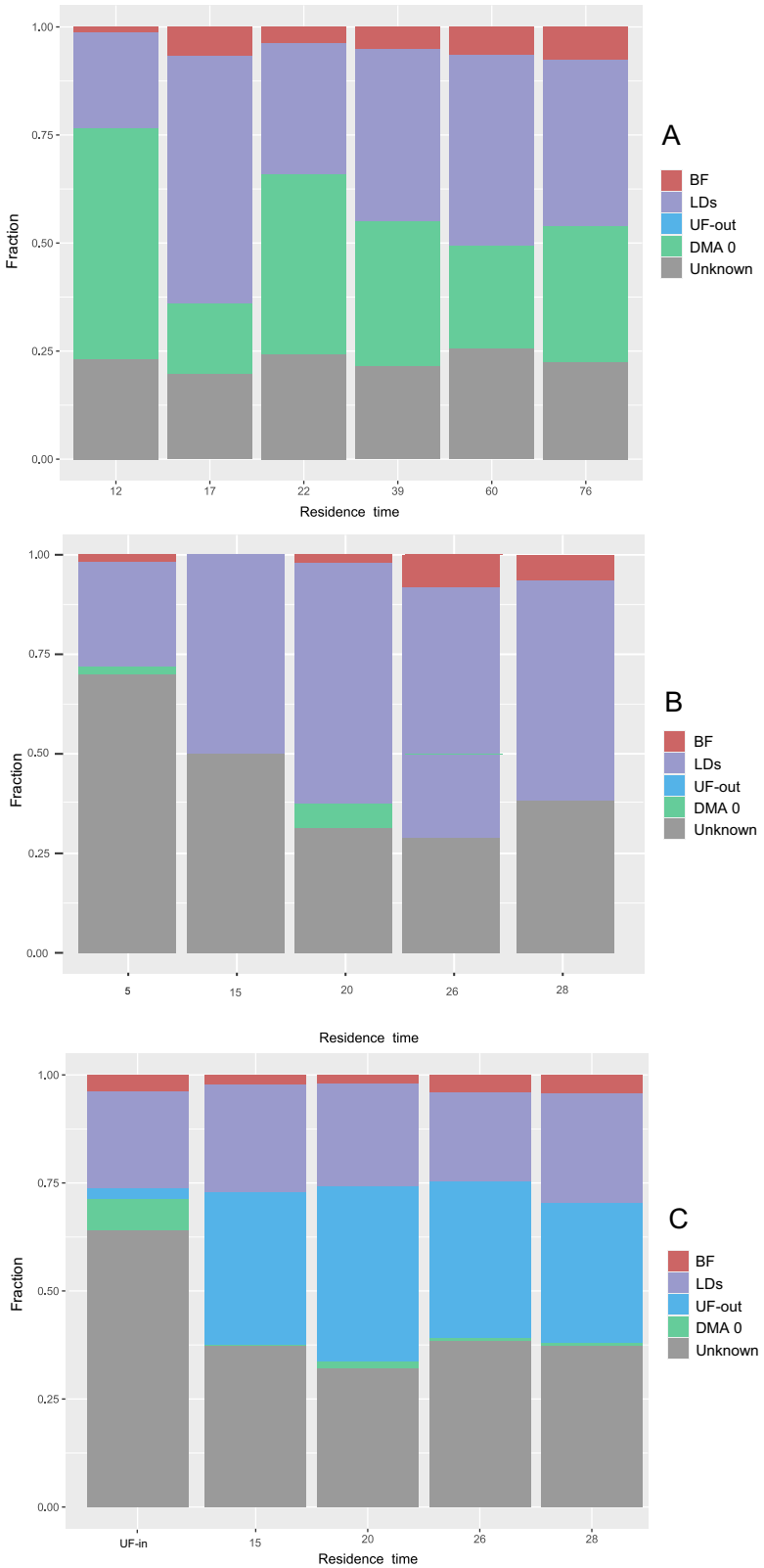

Fig. 5 | FEAST source tracker results showing the fraction of the microbial community of DW during residence in the DMA that derived from the different sources (BF: biofilm, LDs: loose deposits, UF-out: UF effluent, DMA0: purified drinking water before UF). Figure **A**: DWTP-A, DMA1 ($n = 85$); **B**: DWTP-A, DMA2, before UF ($n = 222$); and c: DWTP-A, DMA2, after UF ($n = 222$).

Nitrospira, and uncultured *Bacteroidia* (Fig. S5). Most of these taxa, except for '*Candidatus* Omnitrophus', were specific for BF and/or LDs (Fig. 2), implying that these ASVs indeed derived from the BF and/or LD fractions. This corresponded to our source tracking analysis that showed a smaller but stable LD-derived fraction after UF treatment compared to the period before UF treatment in bulk drinking water. From the taxa that were removed by UF treatment, only the DW-specific *Limnobacter* was not detected at any sampling point during subsequent distribution in DMA2 (Fig. S5). This reshaping of the microbial community composition of the stored UF permeate could have been caused by regrowth or cell release from the BF or LD fractions in the DWDS, as hypothesized previously when UF treatment was applied for drinking water production[25,27,28].

Seasonal variation identified loose deposits as a potential reservoir of biological instability

Seasonal variation affected microbial communities throughout the DWDS but had a substantially stronger influence on loose deposits than on bulk drinking water, further supporting their importance as a habitat contributing to biological instability. Seasonal effects were higher on the microbial communities of loose deposits (16%; Table S6) than bulk drinking water (6%; Table S7). Moreover, *Aeromonas*, which is known to be associated with loose deposits and invertebrate biomass, was primarily detected during spring, summer, and autumn (Fig. S4), consistent with routine cultivation-based monitoring of both *Aeromonas* and HPC (Table S2).

Besides *Aeromonas*, abundance of other microorganisms in DW could therefore also be related to seasonal biological instability and regrowth. Our results showed that *Limnobacter* was also absent in winter and showed a relatively high peak in spring and summer (Fig. S4), as observed in previous research (6: Vavourakis et al., 2020). More ASVs besides *Aeromonas* and *Limnobacter* showed higher relative abundance in spring and/or summer, such as *Limnohabitans*, one ASV of the family *Comamonadaceae*, *Caulobacter*, clade TRA3-20, *Brevundimonas*, clade Subgroup_17 of the family *Vicinamibacteriaceae*, clade Elev-16S-1166, *Nitrospira, Nitrosarchaeum*, and *Deinococcus* (Fig. S4). These ASVs could potentially indicate or contribute to regrowth, especially those that are LD-specific (i.e., Subgroup 17, *Nitrospira*, and *Nitrosarchaeum*).

Discussion

This study investigated how treatment strategies to improve biological stability influence microbial communities in bulk drinking water, biofilms, and loose deposits in full-scale drinking water distribution systems without residual disinfectants. Our results demonstrated that microbial community assembly in non-disinfected DWDS was governed by three successive ecological filters: drinking water treatment, UF-post treatment, reservoir storage, and distribution-system habitats. While primary treatment and UF-post treatment and reservoir storage determined the initial community entering the network, interactions with loose deposits subsequently reshaped this community, particularly under conditions of lower biological stability.

Water treatment had the largest effect on microbial community composition. Source waters and/or water quality resulting from the water treatment processes have been shown before to highly influence the microbial communities in DWDS[29]. Treatment processes therefore establish the microbial community entering the distribution system, while subsequent distribution processes modify this community to a lesser extent. Drinking water produced from surface water by managed aquifer recharge and recovery exhibited higher biological stability, lower *Aeromonas* occurrence, and lower spatial and temporal variation in microbial diversity than drinking water produced from the same surface water but conventionally treated. Ultrafiltration also improved biological stability and significantly altered the microbial communities entering the distribution system.

The genus *Limnobacter* was previously found to be dominant in the same treated surface water as this study from DWTP-A in two DWDS of urban locations (of which one was DMA2) with more regrowth and large invertebrates, while it was absent in a rural DWDS with less regrowth related issues[6]. Furthermore, it was also detected in other DWDS that distributed treated surface water, both non-chlorinated drinking water[30] and chlorinated drinking water[31]. In the latter DWDS, the occurrence of *Limnobacter* was linked to the presence of iron-oxidizing bacteria and the discoloration of water. The genus *Limnobacter* was hypothesized to be an indicator for biological stability when BP concentrations are high[6] and its absence in our results after UF treatment could be explained by the lowered BP concentration.

Treatment alone did not determine the microbial community reaching consumers. After ultrafiltration and 24-h reservoir storage, microbial diversity decreased, intact cell numbers increased, and a limited number of taxa, including *Comamonadaceae* and *Brevundimonas*, became dominant. These results demonstrate that substantial microbial changes can occur after treatment but before entering the distribution system and that the microbial community composition entering the DWDS cannot be assumed to reflect the community immediately leaving the treatment process.

Microbial communities also change during distribution. Source-tracking analysis showed that microorganisms associated with loose deposits increasingly contributed to bulk drinking water with increasing residence time in conventionally treated systems, whereas this effect was much smaller after ultrafiltration. These findings indicate that improving biological stability reduced but did not eliminate microbial community changes during distribution. Our results align with previous studies suggesting that loose deposits significantly affect microbial dynamics due to residence time effects[6], act as microbial reservoirs, and exhibit compositional heterogeneity[15]. Other source tracking studies similarly showed that with residence time, the LD-derived microbial contributions increased, while the DW-derived microbes decreased[15].

Loose deposits showed stronger seasonal variation than bulk drinking water and are associated with microorganisms that indicate periods of increased biological instability, including *Aeromonas*. Seasonal differences affect the invertebrate fraction of LDs and *Aeromonas* growth[32]. *Aeromonas* are predominantly found to be detected in the LDs of the DWDS[7,15,33,34]. Hijnen et al. [7], supported by the data of Ketelaars et al. [32], hypothesized that *Aeromonas* numbers exceed the technical standard in drinking water due to the transmission or release of these bacteria from the biomass of *Asellus aquaticus*, a large invertebrate detected in the LDs that contains high levels of *Aeromonas* bacteria[7,35]. *Aeromonas* numbers are positively correlated with higher concentrations of particulate, slowly biodegradable organic biopolymers (BP) and the biomass production potential in drinking water[35]. *Aeromonas* numbers also coincide with the occasional presence of coliform growth[36]. The stronger seasonal response observed in loose deposits therefore suggests that this habitat acts as an important reservoir of microorganisms contributing to temporal fluctuations in drinking water quality.

Overall, this study shows that improving biological stability influences microbial communities well beyond the treatment plant. Monitoring microbial communities across drinking water, loose deposits and biofilms provide a more complete understanding of biological stability and may support future management of drinking water distribution systems without residual disinfectants. 16S rRNA gene amplicon sequencing offers valuable insights into the microbial ecology of DWDS when combined with physical-chemical, biological stability, and cultivation-based measurements. Spatial and temporal habitat-specific sampling in full-scale operational networks is essential to improve the understanding of the ecology and biological stability in drinking water systems. In our work, microbial communities continued to change after drinking water left the treatment plant and these changes differed between bulk drinking water, biofilms and loose deposits. Future research should focus on identifying the mechanisms underlying these community changes. Non-DNA and PCR biased methods such as metaproteomics combined with stable isotopic probing could prove to be valuable in distinguishing regrowth (the metabolically active microbial communities) from detachment (the transported microbial communities) of the different habitats as a response to biological stability changes. This also could help in quantifying the biomass of each active contributor during DWDS residence, although the protein reference databases are currently not as complete as those of DNA sequencing data. Moreover, besides prokaryotic ecology, eukaryotic community studies are needed to gain full understanding of the ecological food webs in distribution systems, especially in loose deposits where the role of eukaryotes is less understood.

Our source-tracking analyses suggested that loose deposits increasingly influenced the drinking water microbiome during distribution, particularly under conditions of lower biological stability. Controlled laboratory and pilot-scale experiments, combined with full-scale monitoring, are needed to determine the mechanisms governing microbial exchange between bulk

water, biofilms and loose deposits and to establish causal relationships between biological stability parameters and microbial community development.

Lastly, the relationship between loose deposits and current regrowth indicators and biological stability parameters in DWDS would need to be determined to validate the potential role of LD-derived microorganisms in drinking water on biological stability. Ultimately, the determination of other regrowth indicators with molecular DNA techniques could facilitate easier, more sensitive, faster, and therefore earlier detection, than using the current routine monitoring techniques that rely on traditional cultivation methods of less abundant microorganisms such as *Aeromonas* and HPC in bulk drinking water.

Methods

Study design

This study investigated how improvements in biological stability influence habitat-specific microbial communities in full-scale drinking water distribution systems (DWDS). Two different interventions were evaluated. First, drinking water produced by two treatment plants using the same surface water source but different treatment strategies (DWTP-A and DWTP-B) were compared within one DMA (DMA1). Second, ultrafiltration (UF) post-treatment was introduced in a separate DMA (DMA2) to assess how this additional treatment affected biological stability and microbial communities during storage and distribution. Microbial communities were analyzed in three habitats (bulk drinking water, biofilms and loose deposits) and integrated with physicochemical, biological stability and cultivation-based measurements. For a schematic representation of the research area, see Fig. 3.

DWTP-A

A detailed description of DWTP-A can be found in ref. 36 (there named "TP#1"). Briefly, raw river Meuse water is stored for five months in three connected reservoirs after which it is pumped to DWTP-A, treated, chlorinated with ClO_2 (low concentration; 0.05 mg/L) and stored (12 h residence time, no residual disinfectant). After storage, the purified water (here called "DMA0") is transported to both DMA1 and DMA2 of the DWDS by two separate network sections with high-pressure pumping from the production facility DWTP-A.

DWTP-B

DWTP-B uses water from a branch of the river Meuse (Dammed-up Meuse). The average residence time of the Meuse River water in this section is two months. Microsieving, rapid sand filtration, and partial advanced oxidation ($O_3 + H_2O_2 + UV$) are applied before infiltration in the sand dunes with an average residence time of two months (see Timmers et al. [19], for more details). The recovered water is treated with softening (NaOH), powdered activated carbon, aeration, rapid sand filtration, and slow sand filtration, and the purified water is stored in reservoirs until distribution.

DMA1 and DMA2

DMA1 consisted of two parts that were physically separated by valves; one supplied by the usual DWTP-A (DMA1-A), the other supplied with drinking water from DWTP-B (DMA1-B) (Fig. 3). The transport mains from DWTP-A to DMA1 are 40 km in length with 39 h average residence time, ∅1250 mm for the first 26 km, gradually decreasing to ∅800 mm, ∅400 mm and ∅300 mm in the last 7 km. Transport mains from DWTP-B to DMA1 are only 2 km in length with 15 h average residence time (∅500 and ∅250 mm). Before the switch DMA1-B had been supplied with drinking water from DWTP-A for two decades. The biological stability and the microbial community dynamics of the different habitats within DMA1-A and DMA1-B were studied in the same period. The residence time of water from DWTP-B was shorter (0–17 h) than the water from DWTP-A (0–93 h) and the sampling frequency of DMA1-A was higher ($n = 82$) than DMA1-B ($n = 36$).

DMA2 received conventionally treated drinking water from DWTP-A until the end of 2018 ("before UF") and received drinking water post-treated with ultrafiltration (UF) from 2019–2021 ("after UF") (Fig. 3). The UF post-treatment (100 m^3/h capacity) was installed at 4 km distance and 5 h residence time from the high-pressure pump (HPP) of the production facility DWTP-A. The UF influent was sampled regularly ('UF-in'). The UF-permeate was stored in a buffer reservoir (24 h residence time) before sampling ('UF-out') and distribution to DMA2 via transport mains (20 km length, ∅250-300 mm, up to 33 h average residence time). The pore size of the UF membrane was 150 kDa and the filtration flux was 100 L/m^2/h. These specifics were chosen based on the optimum between biopolymer retention and membrane fouling, as determined in a previous pilot study[24].

Sampling methods and processing

Samples were collected from all habitats of the purified water of DWTP-A (here called "DMA0"), DMA1, and DMA2. From DMA0, a total of 3 drinking water samples were collected. From DMA1, a total of 4 biofilms, 12 loose deposits, and 102 drinking water samples were collected. From DMA2, a total of 7 biofilms, 53 loose deposits, and 122 drinking water samples were collected. All samples were transported and stored in the dark at 4 °C and processed within 24 hours after sampling. Detailed information on the samples obtained can be found in the metadata of the supplementary material.

Drinking water (DW)

For DW samples, one liter of drinking water was collected with a standard protocol of sterilization and flushing until the water temperature was constant for 30 s, as described previously[32,37].

From DMA1, a total of 102 DW samples were collected from August 2018 to October 2021 at four sampling cabinets (with continuously flowing sampling ports) within DMA1 and three sampling ports installed along the transportation section (∅ 300–1200 mm) from DWTP-A towards DMA1. Two of the latter sampling ports comprised an RVS lance (∅ 10 mm) installed via one of the air valves present on these large transport pipes and positioned at the heart of the pipe.

The purified DW from DWTP-A (DMA0) was sampled three times from 2017 to 2018 at the high-pressure pumping station (HPP) at a continuously flowing sampling point within the production facility.

From DMA2, a total of 153 DW samples were collected from June 2017 to December 2021, at a continuously flowing sampling point at the high-pressure pumping station (HPP) of the ultrafiltration facility and at four different sampling cabinets with a continuously flowing sampling port within DMA2. The influent of the ultrafiltration installation (UF-in) was sampled four times, from April to August 2019, at a sampling cabinet with a continuously flowing sampling port directly in front of the treatment facility. During the flushing method sampling for loose deposits ("Methods", section "Loose deposits (LDs)"), a drinking water sample was collected from a nearby household tap instead of sample cabinets.

During the COVID-19 pandemic in 2020 and 2021, some DW samples from DMA1 and DMA2 were collected on a tap installed at a hydrant instead of at a household tap. Microbial source tacking analysis using FEAST (fast expectation-maximization for microbial source tracking[26], demonstrated that the DW samples collected on a hydrant contained a larger fraction of microorganisms associated with loose deposits (Fig. S1). Consequently, hydrant DW samples were excluded from source-tracking analyses, and their influence was considered when interpreting microbial community composition.

Loose deposits (LDs)

From DMA1, a total of 12 LD samples were collected at two different locations in August 2020 and August 2021. From DMA2, a total of 59 LD samples were collected at five different locations from 2017 to 2021. The LDs within the distribution networks were sampled once a year using the hydrant flushing method as described previously[31,36]. LDs were collected by water flushing with a flow rate of 1 m/s at a hydrant attached to a 100 mm

(inner diameter) pipe, until a total volume of 1000 L was filtered. This volume was divided in different fractions using a sampling set-up with plankton nets of different mesh sizes (Hydro-bios Kiel, Altenholz, Germany). 90% of the flow (900 L) was filtered over a plankton net with a mesh size of 500 µm to yield the large sediment fraction–this fraction was not used in this study. The remaining 10% of the flow (100 L) was filtered over consecutive plankton nets with a mesh size of 500, 100, and 30 µm to yield samples of the moderate (100–500 µm) and small loose deposits (30–100 µm). The LDs were collected from the plankton nets and resuspended in the drinking water. Additional one-liter samples were collected of the filtered flushing water (< 30 µm, very small loose deposits) and unfiltered flushing water. At the laboratory, the LD suspensions were pretreated with 2 min of high-energy sonication (45%) to release the biomass from the LDs and homogenize the suspensions prior to further analysis.

In a previous study that included the same DWDS[6], differentiation was made between small loose deposits (SLDs; i.e., <30 µm filtered flushing water sample) and total loose deposits (TLDs; i.e., the sediment fractions of 30–100 µm and 100–500 µm). In most other studies, no differentiation is made between these sediment fractions, and the reported LDs usually consist of a flushing sample that contained all loose deposit fractions and flushing water[38–41]. To be consistent with the published literature, the term 'loose deposits (LDs)' is used in this study, which includes all sediment fractions.

Biofilms (BF)

Biofilm (BF) samples were collected by cutting a pipe length of 1 m from the main (110 mm PVC-U). As this is an invasive and labor-intensive sampling method, BF samples were only collected from two locations in DMA1 (total of 4 samples from August 2020 and August 2021) and three locations in DMA2 (total of seven samples from August and September of 2020 and 2021). The pipe segments were stored in a sterile plastic bag and, like all samples, transported in the dark at 4 °C. At the laboratory, four replicate swab samples (10 cm^2) were collected from the bottom, top, left- and right-hand side of the pipe using BBL™ CultureSwab (Becton, Dickinson and Company, NJ, USA). Each swab was sonicated four times (2 min) in 10 mL sterilized water. The replicate samples (4 × 40 mL) were pooled for further analysis, as done previously[6].

Biological stability parameters in drinking water samples

Total and intact cell counts were enumerated for DW samples by flow cytometry according to the protocol and equipment (BD Accuri C6; BD Biosciences, San Jose, CA, USA) described previously[42].

The biopolymer (BP) fraction of the DOC was determined for DW samples with Liquid Chromatography-Organic Carbon Detection (LC-OCD) by DOC-Labor GmbH (Karlsruhe, Germany)[43]. This method is strongly correlated with the particulate and high-molecular organic carbon (PHMOC) method[24,44]. The biological stability of the DW samples was determined using the microbial growth potential methods AOC-P17/NOX easily biodegradable, low-MW compounds[18], (NEN 6271), AOC-A3 slowly biodegradable, high-MW compounds[45] (NEN 6271), and Biomass Production Potential (overall microbial growth potential as cumulative biomass production after 14 days; CBP_{14})[3,46].

Heterotrophic plate counts (HPC22) were determined for DW samples according to NEN-EN-ISO 6222 (CFU/mL, after three days incubation at 22°C)[47] and *Aeromonas* colony counts according to Havelaar et al. [48] and NEN 6263 (CFU/100 mL, after 24 h incubation on ampicillin-dextrin agar at 30 °C of the 0.45 µm pore size membrane)[49].

DNA isolation and 16S rRNA gene amplicon sequencing and analysis

From the collected samples, a maximum of 1 l was filtered through a polycarbonate membrane filter with a 0.2 µm pore size (Track-etch membrane, Sartorius AG, Göttingen, Germany). Filters were stored at −20 °C until DNA extraction and 16S rRNA amplicon sequencing, which was done as described previously, using the primers 515 F and 806R[50] with Illumina overhang adapter sequences, targeting the V4 region of the 16S rRNA gene of both bacteria and archaea[6]. Negative controls or "blanks" (water samples of DNase/RNase free water) were included during filtration and subsequent DNA extraction.

Microbial community analysis was performed on all collected samples as described previously[19]. Briefly, processing of 16S rRNA amplicon sequence data was performed using Qiime2[51]. After demultiplexing using the software of Illumina, paired-end sequences were joined using VSEARCH[52]. Afterward, an initial quality filtering process based on quality scores was applied using the quality filtering approach described previously[53] with default settings. Then, amplicon sequence variants (ASVs) were determined using Deblur[54], where we used a trim length of 250 bp. A phylogenetic tree was generated for phylogenetic diversity analysis, using the MAFFT program for alignment and subsequent FastTree from a masked alignment[55]. Taxonomy was assigned to the generated feature table using the q2-feature-classifier plugin and the readytowear SILVA_132_515F_806R_water-non-saline classifier[56,57] based on the latest SILVA reference database 138.1[58].

Qiime2 output was imported into R studio (version 2024.09.1 Build 394) and R version 4.4.2 (2024-10-31 ucrt). Samples with less than a minimum of 10.000 reads and with less than 0.05% of the total reads were removed prior to data analysis. Further analysis and visualization were done with the R software packages Ampvis2[59], VEGAN[60], and phyloseq[61]. The corresponding metadata and feature tables of the microbial communities are summarized in the supplementary material.

Alpha diversity analysis was done with different diversity parameters, such as Faith's PD index and Shannon diversity, and statistical tests were done using the Kruskal-Wallis H-test. Differences in community composition among samples were quantified using Bray-Curtis dissimilarity calculated from the abundance matrix. Ordination was performed using Principal Coordinates Analysis (PCoA) based on the Bray-Curtis distance matrix with ellipses of 95% confidence intervals that visualize the uncertainty around the centroid (mean) of a group of samples to determine if differences between groups are statistically significant rather than just visual artifacts. Statistical differences in community composition among groups were tested using permutational multivariate analysis of variance (PERMANOVA) on the Bray-Curtis dissimilarity matrix of rarified data (number of rarified reads are mentioned for each result) with 999 permutations and post-hoc Adonis test ($\alpha < 0.05$). Principal Component Analysis (PCA) was used specifically to identify taxa contributing most to the overall variance, as PCA provides explicit species loadings that are not available from distance-based ordinations. The taxa that contribute most to the overall variance of samples are displayed as gray dots to show which ASVs drive community differences[59]. Prior to PCA, species abundance data were Hellinger-transformed to reduce the influence of highly abundant taxa and to meet the assumptions of Euclidean-based ordination. Differential abundance analysis was done using ANCOM with bias corrections, after filtering features (minimum frequency 50 reads in minimum 4 samples) and adding pseudocounts[62,63].

Source tracking analysis was used to estimate the proportional contribution of microbial communities from different habitats (drinking water, biofilms, and loose deposits) to bulk drinking water communities during distribution with fast expectation-maximization for microbial source tracking (FEAST)[26]. This tool quantifies the fraction of each source environment in a target microbial community through a statistical model. We have analyzed the relative contribution of microbial communities from each habitat to the communities in DW with increasing residence time. Given limited biofilm samples from DWTP-B and habitat differences per DWTP and DMA, FEAST analysis focused on DWTP-A water in DMA1-A and DMA2 (with and without UF post-treatment). Microbial community sources for DW in DMA1-A included purified DW from DWTP-A (DMA0) and DMA1-specific BF and LD samples. For DMA2, sources comprised BF and LD samples from DMA2 (pre- and post-UF), purified DW from DWTP-A (DMA0), and DW from 'UF-out'. We assessed the microbial composition of DW in periods before and after UF-post-

treatment using respectively DW samples before UF implementation or after UF implementation as sinks. Hydrant DW samples were excluded due to the higher LD-derived fractions (see Fig. S1 and "Methods", section "Drinking water (DW)").

Data availability

The 16S rRNA amplicon sequence data for this study were deposited in the European Nucleotide Archive (ENA) at the EMBL-EBI under project accession number PRJEB108724. Detailed information on the samples and the metadata can be found in the supplementary material.

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Author contributions

Authors' contributions: CRediTPHAT has performed investigation, formal analysis, data curation, validation, conceptualization, visualization, and writing–original draft and review and editing. WH, LM, JW, and GS have contributed to conceptualization, investigation, resources, supervision, and project administration. MintZ and PHAT performed funding acquisition, and MintZ performed investigation and formal analysis. GE performed methodology, validation, resources. GS, JW, WH, LM, GE and MintZ have all contributed to writing–review & editing.

Competing interests

The authors declare no competing interests.

Additional information

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