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Bacterial community dynamics during reclaimed water storage

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ABSTRACT

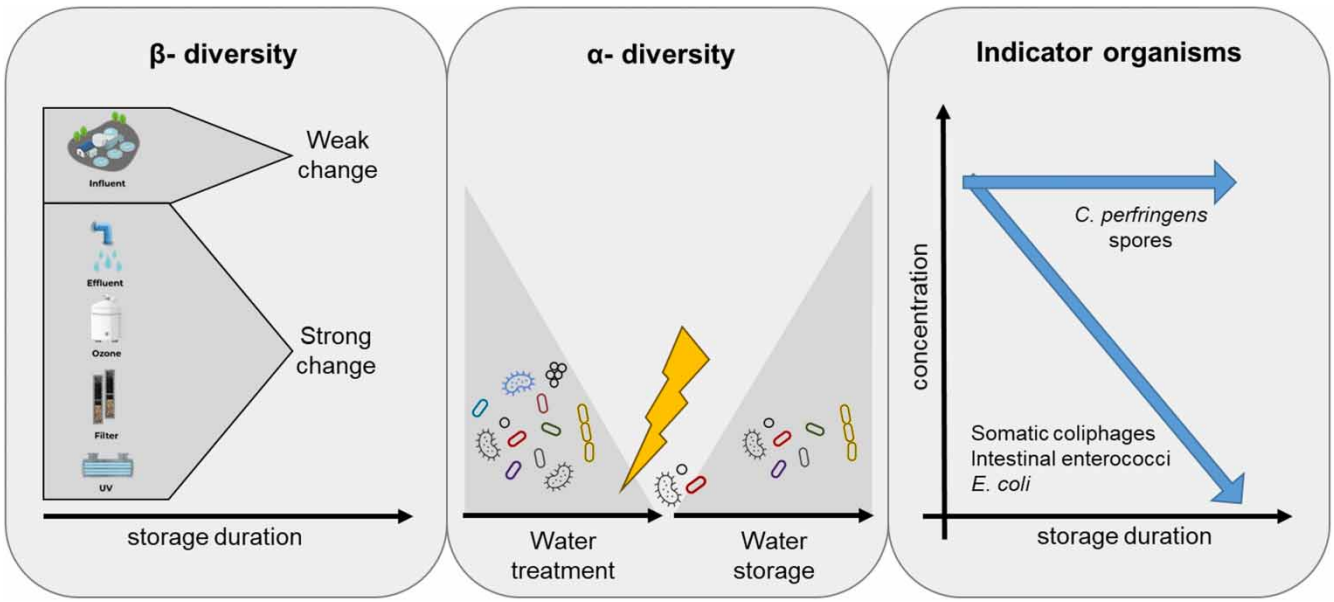
Climate change and industrialization necessitate a reassessment of water management strategies, particularly in agriculture, where reclaimed water supply often fails to meet irrigation needs. Storage can bridge supply gaps but raises concerns about water quality deterioration due to microbial changes and pathogen regrowth. This study examined microbial dynamics and regrowth during reclaimed water storage from a municipal wastewater treatment plant in Germany. The treatment train included ozonation, filtration and UV disinfection, and samples were analyzed using traditional culture methods for indicator organisms (e.g., *Escherichia coli*, *Clostridium perfringens* spores, and somatic coliphages) and 16S rRNA gene amplicon sequencing. Samples were collected throughout the treatment train and stored at 22 °C in the dark for up to 15 days. Results showed effective microbial reduction by treatment, with storage alone achieving similar declines in many cases. While treatment reduced bacterial diversity, storage gradually restored it, forming distinct microbial profiles from the original water quality. Bacterial communities converged during storage, suggesting a succession-like stabilization process. The findings highlight the dynamic nature of reclaimed water microbiomes and the importance of stimulating stable microbial communities to preserve water quality during storage. Advanced treatment should remove contaminants while supporting microbiomes that protect public health and the environment.

Key words: bacterial regrowth, biodiversity, biostability, storage, wastewater, water reuse

HIGHLIGHTS

- Reclaimed water storage alone reduces indicator organisms, sometimes as effectively as wastewater treatment.
- Sequencing reveals broader microbial shifts overlooked by culture methods.
- Microbiome diversity declines after wastewater treatment but gradually restores during storage.
- Reclaimed water microbiomes converge over time, suggesting ecological succession and biostabilization.

GRAPHICAL ABSTRACT



1. INTRODUCTION

The rapidly progressing climate change and industrialization urge the world to fundamentally reassess water supply processes. Implementing water reuse practices as a circular economy strategy can contribute to the long-term sustainability of water resources (Koseoglu-Imer *et al.* 2023). While the reuse of treated municipal wastewater (reclaimed water) specifically for irrigation purposes has long been practiced in many (semi)arid countries, there remains significant potential to extend water reuse efforts in response to the progressing water scarcity in historically water-abundant countries (Giakoumis *et al.* 2020).

In the context of water reuse, wastewater treatment plants (WWTPs) must ensure that the quality of treated wastewater does not adversely affect the environment and consumers upon its discharge. Specifically in agricultural irrigation, it is essential to minimize the risk of transmitting harmful chemicals and microorganisms to humans and animals through crop contamination (Ribeirinho-Soares *et al.* 2022) or through the irrigation procedure itself (e.g., Legionella inhalation in case of spray irrigation). As such, WWTPs serve as important infrastructures responsible for safeguarding and preserving the ecological balance and environmental health. Hence, ensuring the efficient and reliable operation of WWTPs is crucial to upholding water quality standards and meeting environmental protection objectives (Zhang *et al.* 2019b).

A water reuse regulation (EU 2020/741) became norm in the European Union in 2023 and defines requirements for the safe reuse of treated wastewater for agricultural irrigation. It does state minimal requirements for water quality, such as the concentration of, for example, *Escherichia coli* permitted depending on the reclaimed water quality class to be achieved, and also states performance targets concerning the indicator microorganisms *E. coli*, total coliphages (or F-specific coliphages or somatic coliphages) and *Clostridium* spp. spores (or spore-forming sulfate-reducing bacteria) for treatment plant validation monitoring in case of class A-type water (EU Regulation 2020/741).

The temporal availability of reclaimed water for irrigation may not always align with agricultural water demand. Storing reclaimed water helps buffer against fluctuations in both available WWTP effluents and irrigation needs, ensuring a demand-oriented water supply for agriculture. Furthermore, it provides logistical flexibility in water distribution for irrigation, optimizing water management practices on agricultural land. Therefore, ensuring the hygienic safety of reclaimed water remains critical, even during storage for varying periods, ranging from a few hours to several days or even weeks, depending on irrigation requirements (Ribeirinho-Soares *et al.* 2022). However, while the regulation EU 2020/741 addresses reuse water storage in a risk management plan context, there are no microbial target values or alternative opportunistic pathogens specifically enclosed.

In wastewater management and reuse, a key concern is the possible deterioration of water quality due to storage, particularly through changes in microbial communities and pathogen regrowth over time. Such concerns might originate from

drinking water research, where microbial regrowth potential is a widely studied topic and changes in microbial composition due to storage/stagnation and distribution can lead to issues like biofouling, microbially induced corrosion, odor problems, and possible health risks. In reclaimed water, the occurrence of high loads of bacteria and nutrients might be the breeding ground of the trophic chain that consequently results not only in the occurrence of protozoa and invertebrates such as crustaceans (e.g., Asellidae, specifically *Asselus aquaticus*), worms (e.g., annelida), or snails (e.g., mollusca) in distribution systems, but also for bacterial regrowth that might eventually contaminate produce and the environment (Prest *et al.* 2016; German Federal Institute for Risk Assessment 2022). Specifically, bacterial regrowth might depend on the reclaimed water's nutrient composition, which in turn can be influenced by the particular treatment process employed. From a macroecological perspective, disinfection during wastewater treatment can be understood as a disruptive event to the microbial community, with each type of treatment sometimes selectively targeting certain species and potentially reducing microbial diversity (Moreira *et al.* 2021). Distinct bacterial communities in the water are likely to respond differently to disinfection methods and exhibit diverse recovery mechanisms and patterns (Becerra-Castro *et al.* 2016). Prior research has shown that cells capable of surviving a particular disinfection treatment within a treatment plant are capable of regrowth in stored treated water, often reaching levels similar to or above those observed before treatment (Ribeirinho-Soares *et al.* 2022). Once the surviving microbial community is stored, over time, it can gradually rearrange and re-equilibrate through ecological succession (Fierer *et al.* 2010), eventually reaching a new equilibrium that, in theory, should exhibit maximal biostability, characterized by high biodiversity and evenness, and a certain degree of resilience against the intrusion of microorganisms, including pathogens. Succession is a foundational concept in community ecology, widely investigated through both theoretical frameworks and empirical studies, particularly within plant communities, to unravel its underlying mechanisms and dynamics (Connell & Slatyer 1977; Finegan 1984). During the transition toward a new equilibrium, shifts in microbial abundance, viability, and community composition can indicate biological instability, as noted by Prest *et al.* (2016) for drinking water. Stable microbial communities are characterized by rich phylogenetic and functional diversity, engaging in complex interactions, including competition, predation, parasitism, mutualism, and commensalism. These dynamic relationships contribute to the community's resilience, enabling rapid recovery from disturbances, environmental changes, or pathogenic invasions (Do *et al.* 2019; Ribeirinho-Soares *et al.* 2022). Achieving such a stable community could ensure that reclaimed water maintains its microbiological quality throughout transportation and storage, up to its point of use. Becerra-Castro *et al.* (2016) have already claimed that the most effective advanced water treatment technologies would ideally possess the ability to selectively eliminate both chemical and microbial contaminants while preserving a receiving ecosystem's biostability (Becerra-Castro *et al.* 2016; Do *et al.* 2019).

However, these nuanced microbial responses to water treatment and reclaimed water storage cannot be adequately captured using classical cultural approaches, as demanded by the EU regulation (2020/741) (EU 2020/741). While culturing methods offer a direct and effective way to characterize indicator microorganisms, these only represent the smallest fraction of the wastewater microbiome (Stankiewicz *et al.* 2024). The vast majority of bacteria in natural environments prove challenging to culture in the laboratory. Molecular methods have substantially advanced the comprehension of entire microbial communities. Jjemba *et al.* (2010) concluded from their studies that relying on indicator bacteria for monitoring water quality may not adequately reflect the potential downstream risks, especially in reclaimed water distribution systems where nutrient and temperature profiles favor microbial survival and regrowth (Jjemba 2010). Kulkarni *et al.* (2018) have emphasized the importance of adopting approaches that consider the broader microbial community rather than focusing solely on indicator bacteria. They suggested that future reclaimed water treatment technologies and reuse guidelines should encompass a more comprehensive array of bacterial species present in reclaimed water. Such advancements could aid in refining treatment parameters and allow monitoring of microbial community dynamics over time, leading to reuse practices that enhance public and environmental health protection.

This study aimed to examine microbial community dynamics, including potential regrowth and compositional changes within the treatment train of a pilot plant operated at a municipal WWTP in Brunswick, Germany, under varying storage durations. Specifically, the objective was to determine if prolonged storage adversely affects water quality over time, with an emphasis on hygienic safety and microbial stability. To achieve this, both cultural detection of indicator organisms as well as molecular methods (16S rRNA quantitative polymerase chain reaction (qPCR) and 16S rRNA gene amplicon sequencing) were employed. This approach intended to evaluate whether commonly used indicators sufficiently reflect microbial risks, or whether sequencing methods offer valuable additional insights into microbial community shifts across various treatment stages and storage durations. By linking storage duration to microbial regrowth and community structure, the study

aimed to improve understanding of the ecological processes that influence reclaimed water quality beyond conventional detection methods.

2. MATERIALS AND METHODS

2.1. Pilot plant

The pilot plant shown in Figure 1 was used for the advanced treatment of secondary effluent of a municipal full-scale WWTP with a conventional activated sludge treatment in Brunswick, Germany, which has a treatment capacity of approximately 350,000 population equivalents. The pilot plant used a process combination consisting of ozonation, filtration, and UV disinfection. Ozone dosage was regulated based on the abatement of UVA_{254} (ΔUVA_{254}), which was continuously measured at the influent and effluent of the ozonation system. The UVA_{254} setpoint of 47% corresponded to a specific ozone dose of approximately 0.6 mgO_3/mg DOC and was chosen with the target of micropollutant removal. The dual-media filter (sand/anthracite) was used as ozonation post-treatment and was operated with a constant filtration velocity of 10 m/h. The filter was automatically backwashed once a day with air/filtrate. The UV disinfection (Wedeco Spektron® 2e, 50W low-pressure lamp) was operated at a constant power and flow rate ($\approx$ 1.5 m^3/h). Thus, the achieved UV dose varied between 470 and 700 J/m^2, depending on the UV transmittance.

Various analytical chemical parameters, measured at individual sampling points throughout the investigation period, are provided in the Supplementary material (Table S1).

2.2. Sampling and storage

Sterile, organic carbon-free 3.5-L borosilicate bottles were used to collect and store grab samples. Hence, all glassware was washed, rinsed with deionized water and capped with aluminum foil. Glassware was subsequently heated in a muffle furnace at 500 °C for at least 5 h. Caps were similarly washed, rinsed and sterilized at 180 °C for at least 5 h.

The grab samples were taken on three different days from five sampling points: (1) WWTP influent, (2) WWTP secondary effluent (=secondary effluent), (3) ozonation effluent, (4) filtration effluent, and (5) UV disinfection effluent. The sampling valves at the pilot plant were flushed prior to collection to prevent inclusion of stagnant water and biofilm in the samples. The wastewater treatment plant does not have a storage tank or other storage structures. To assess the potential of the water for storage, storage was simulated *in vitro*. The samples were processed within a window of 4 $\pm$ 1 h after collection (point in time = T_0). Subsequently, samples were incubated at 22 °C in the dark and re-analyzed after three (T_3), seven (T_7), and 15 days (T_{15}). These storage conditions were chosen to simulate temperate ambient conditions under which reclaimed water might be stored in covered reservoirs, tanks or distribution systems, preventing photoinactivation and algal growth.

2.3. Indicator organism culture

The detection method employed for somatic coliphages was according to DIN EN ISO 10705-2. In brief, the samples were inoculated with a host *E. coli* (strain WG5) and pour-plated with semisolid modified Scholten's agar mixed with calcium chloride solution in appropriate dilutions. Plaque-forming units (PFUs) were counted after 18 $\pm$ 2 h incubation at 36 $\pm$ 2 °C. Positive (ΦX174 reference phage) and negative controls were always included. Detection of *Clostridium perfringens*

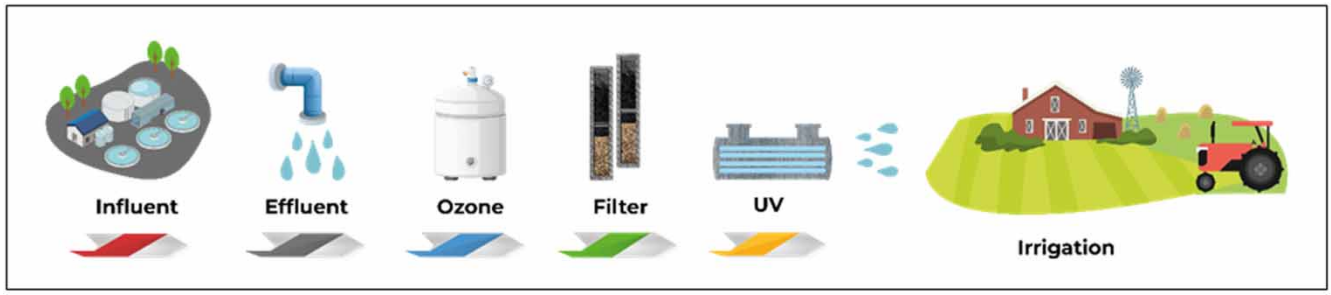


Figure 1 | Treatment train of the conventional WWTP Brunswick (WWTP influent and secondary effluent) and the pilot-scale treatment plant (ozonation, filtration, and UV) with purpose of reclaiming wastewater for agricultural irrigation.

spores was conducted by cultivation based on DIN EN ISO 141189:2016 by pasteurizing the samples at 60 ± 2 °C for 15 min and subsequent membrane filtration or direct plating in appropriate dilutions onto selective tryptose sulfite cycloserine agar supplemented with a *C. perfringens* selective supplement (Merck KGaA), containing 0.4 g/L D-cycloserine and 0.1 g/L 4-methylumbelliferylphosphate disodium salt and incubated at 44 ± 0.5 °C. Characteristic black colonies that fluoresced under UV light were counted after 21 ± 3 h. *E. coli* was cultured according to ISO 9308-3. In brief, samples in appropriate dilutions were deposited into MUG/EC microplates (BioRad Laboratories). Following incubation for 36–72 h at 44 °C, the wells were examined using a UV reader to quantify the number of wells exhibiting fluorescence. Intestinal enterococci were evaluated similarly, following EN ISO 7899-1 and utilizing MUD/SF (BioRad Laboratories) microplates. The detection limit for the microplate assays was <15 MPN/100 mL. In order to achieve a lower detection limit for samples with expected lower concentrations, *E. coli* was also analyzed using method EN ISO 9308-1. One hundred mL samples were filtered onto chromogenic coliform agar and characteristic colonies were counted after incubation at 36 ± 1 °C for 21 ± 3 h. Samples with expected low concentration of enterococci were filtered (100 mL) and filters were deposited onto Slanetz-Bartley agar. After incubation, the filter was transferred onto bile aesculin agar and after 30 min and 44 °C incubation, black imprints were counted on the agar.

2.4. DNA extraction

DNA from all samples was isolated using the NucleoMag DNA Microbiome Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions for processing stool samples with minor adjustments. In case of WWTP influent water, 50 mL of sample were centrifuged and the pellet was mechanically disrupted with ceramic MN Bead Tubes Type A (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. For all effluent samples, 100 mL of water was filtered through a 0.4 μm polycarbonate membrane. The membrane was cut into strips and transferred to the ceramic bead tubes. At the end of the extraction process, the DNA was eluted to a final volume of 100 μL and stored at −80 °C.

2.5. Quantitative polymerase chain reaction

16S rRNA gene qPCR was performed on all samples with the 16S Pan-bacterial control and a spike-in internal positive (inhibition) control DNA (1,000 copies/uL, VetMAX™ Xeno™ Internal Positive Control DNA). The TaqMan assay was performed with the TaqPath™ BactoPure™ Microbial Detection Master Mix (all reagents by ThermoFisher Scientific). The reaction was performed according to the manufacturer's instructions and a thermal cycling protocol including an initial activation step (95 °C, 60 s) followed by 40× denaturation at 95 °C for 1 s and annealing/extension at 60 °C for 20 s. Reactions were run on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories), always in duplicates, including non-template and positive controls.

2.6. Sequencing

16S rRNA libraries were constructed with the Quick-16S NGS Library Prep Kit (Zymo Research Europe GmbH), amplifying the 16S rRNA V3-V4 hypervariable region. Each run included a positive control from the kit itself and a negative control. For qPCR, quality control, and normalization purposes, the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories) was used. After pooling, the library was quantified with a QuBit 4 Fluorometer using the Qubit dsDNA HS Assay Kit (ThermoFisher Scientific) and diluted strictly according to the Illumina protocol for MiSeq sample preparation. Finally, a loading concentration of 10 pM was chosen and a 10% Illumina PhiX spike-in control v3 was added before running it on the Illumina MiSeq platform with the 600-cycle v3 Illumina MiSeq Reagent Kit (Illumina, San Diego, CA, USA).

The bioinformatic analysis included three main parts, starting with preprocessing raw paired-end reads. Subsequently, taxonomies were assigned to the sequences. Finally, the resulting taxa underwent statistical and graphical evaluation. QIIME2 (Bolyen *et al.* 2019) was used for both preprocessing and classification of the data. With the plugin tool DADA2 (Callahan *et al.* 2016) forward and reverse reads were trimmed from the 3' end at position 249, while shorter and low-quality reads were discarded. DADA2 was also used for error correction, as well as merging forward and reverse reads if there was an overlap of at least 12 base pairs and chimera removal.

The processed sequences were clustered into operational taxonomic units (OTUs) of 100% sequence identity and assigned to taxa using a classifier trained on full-length sequences of SILVA (Quast *et al.* 2013). The trained classifier was provided by QIIME2 (Bolyen *et al.* 2019) using scikit-learn 0.24.1 and the plugin tool q2-feature-classifier (Bokulich *et al.* 2018). The main output is a classification of reads at kingdom, phylum, class, order, family and genus levels. Based on the quantified OTUs and

taxa, different diversity indices were calculated using Python and the skbio.diversity library. Prior to diversity analysis, rarefraction was performed to prevent variations due to low sequencing depth.

2.7. Data analysis

The alpha diversity of the bacterial community compositions in the collected samples was analyzed, and the average of three samples from each sampling point was calculated from normalized data for richness, Shannon-Weaver, and Simpson indices. Two-way analysis of variance (ANOVA) was carried out over all samples with sampling time points and sampling sites as independent variables, in combination with a post-hoc Tukey's honestly significant difference (HSD) test. Beta diversity was estimated by normalization of OTU and using the Bray–Curtis Dissimilarity metric. Outputs were visualized by principal coordinate analysis (PCoA) biplot. All plots and statistical analysis were generated using R version 4.3.3 with packages ggplot2 and ape.

3. RESULTS

3.1. Indicator microorganisms

The wastewater treatment plant as well as the pilot-scale system were evaluated for their efficiency to reduce indicator organisms.

(i) Microbial reduction during treatment

Overall, the treatment at the full-scale WWTP showed a reduction in somatic coliphages by almost 2 log, *C. perfringens* spores by 1.5 log, *E. coli* by approximately 2 log and intestinal enterococci by almost 2 log from the WWTP influent to the secondary effluent (Figure 2). The full treatment train was able to reduce somatic coliphages by almost 6 log, *C. perfringens* spores by 4 log, *E. coli* by 7 log and intestinal enterococci by almost 7 log levels from the influent to the effluent of the UV disinfection (Figure 2).

Indicator organisms showed different behavior during storage experiments:

(ii) Storage

Results of the storage experiments showed that somatic coliphages persisted in the full-scale WWTP influent at $<10^6$ PFU/100 mL over the storage period, whereas somatic coliphage concentration decreased significantly and even below the limit of detection after storage in the samples from other sampling points. *C. perfringens* spores persisted in all treatment stages

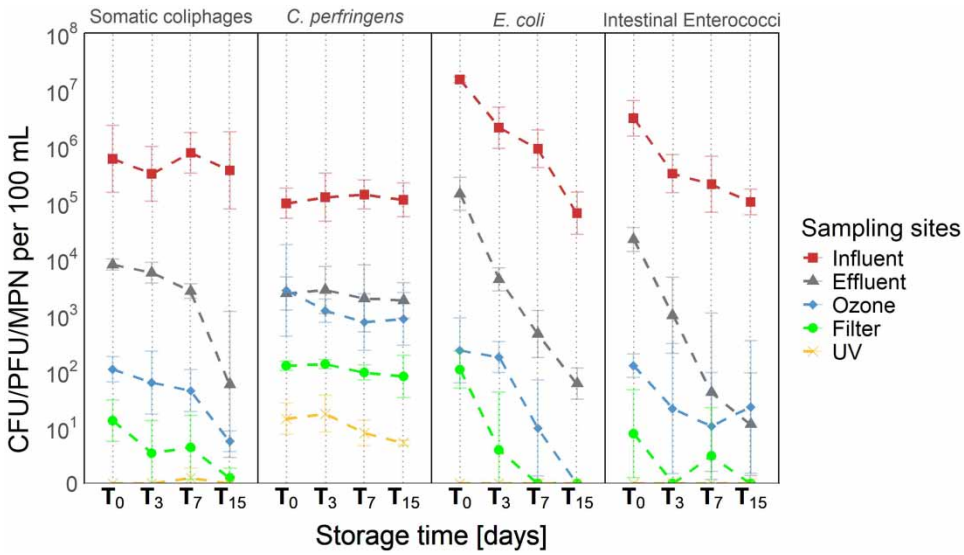


Figure 2 | Arithmetic mean values ($n = 3$) and standard deviations of indicator organism (somatic coliphages, *C. perfringens* spores, *E. coli*, and intestinal Enterococci) culture results per sampling point (WWTP influent, secondary effluent, ozone, filter and UV) in plaques forming units (PFU)/100 mL (somatic coliphages), colony forming units (CFU)/100 mL (*C. perfringens* spores) and most probable number/100 mL (*E. coli* and intestinal enterococci).

throughout the storage period. From all indicator microorganisms, *E. coli* and intestinal enterococci experienced the steepest declines in concentration over the storage period and among all sampling points. In some cases, the decrease in indicator organism concentration over time was greater than the subsequent treatment step itself. For example, storing the WWTP influent for 15 days reduces the *E. coli* concentration to the same level as the WWTP effluent at day 0, and in the case of somatic coliphages, 15 days of storage after ozone treatment could reduce the concentration below the concentration level that would actually be added to the filtration on day 0. Storing the WWTP secondary effluent for 15 days achieved reductions in microbial indicators that were comparable to or exceeded those observed with ozone treatment on day 0.

In summary, the obtained results show that the indicator microorganisms analyzed in this study have either persisted in a certain concentration during storage or tended to decline over the storage period, but that no regrowth of the investigated indicator organisms has taken place at any process stage.

3.2. 16s rRNA qPCR

The results of 16S rRNA concentrations are presented in Figure 3. The highest concentrations were observed in influent samples, with successive reductions along the treatment stages, reaching the lowest levels in the UV effluent (compare T_0 values). The conventional WWTP achieved an average reduction of 2 log in 16S rRNA, while the pilot plant further reduced concentrations by an additional 2 log. During storage, no increase in 16S rRNA concentration was detected, except in the UV effluent, where levels increased by 1.5 log after 3 days of storage, aligning with the average concentrations observed across other sampling sites (excluding WWTP influent), ranging between 10^8 and 10^9 GU/100 mL.

3.3. Sequencing

In total, the 60 sequenced samples generated a total of 3,929,236 input reads. After quality filtering and denoising, all samples passed the minimum quality filter and produced a total of 2,531,729 merged reads. Alpha diversity is depicted by richness (taxa count) as well as Shannon and Simpson indices (Figure 4).

The Shannon index accounts for both richness and evenness (with sensitivity to rare species) and the Simpson index places greater emphasis on evenness over richness (which highlights the dominance of common species rather than rare ones). Beta-diversity analyses are depicted as PCoA plots (Figures 5(a) and 5(b)) with a degree of explained variance of PCoA 1 = 53% and PCoA2 = 18.1% (Figure 5(a)) and PCoA 1 = 42.8% and PCoA 2 = 21.7% (Figure 5(b)).

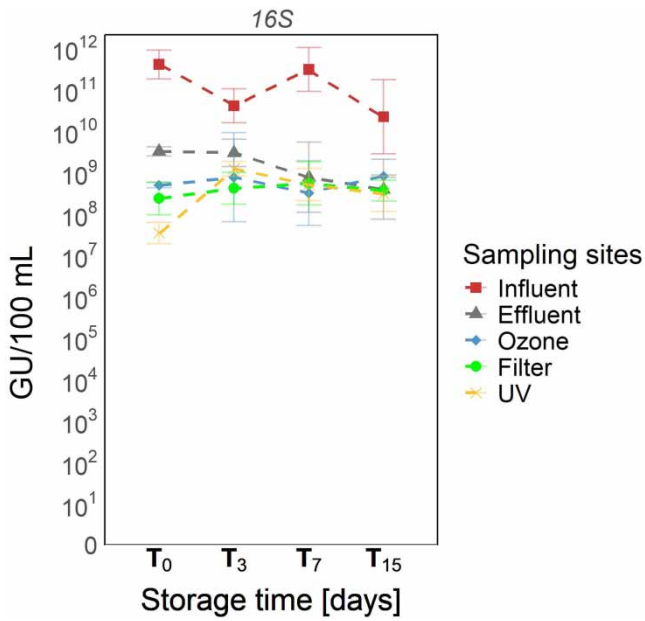


Figure 3 | Arithmetic mean values ($n = 3$) and standard deviations of 16 s rRNA concentrations per sampling point (WWTP influent, secondary effluent, ozone, filter and UV) in genomic units (GU)/100 mL determined with qPCR.

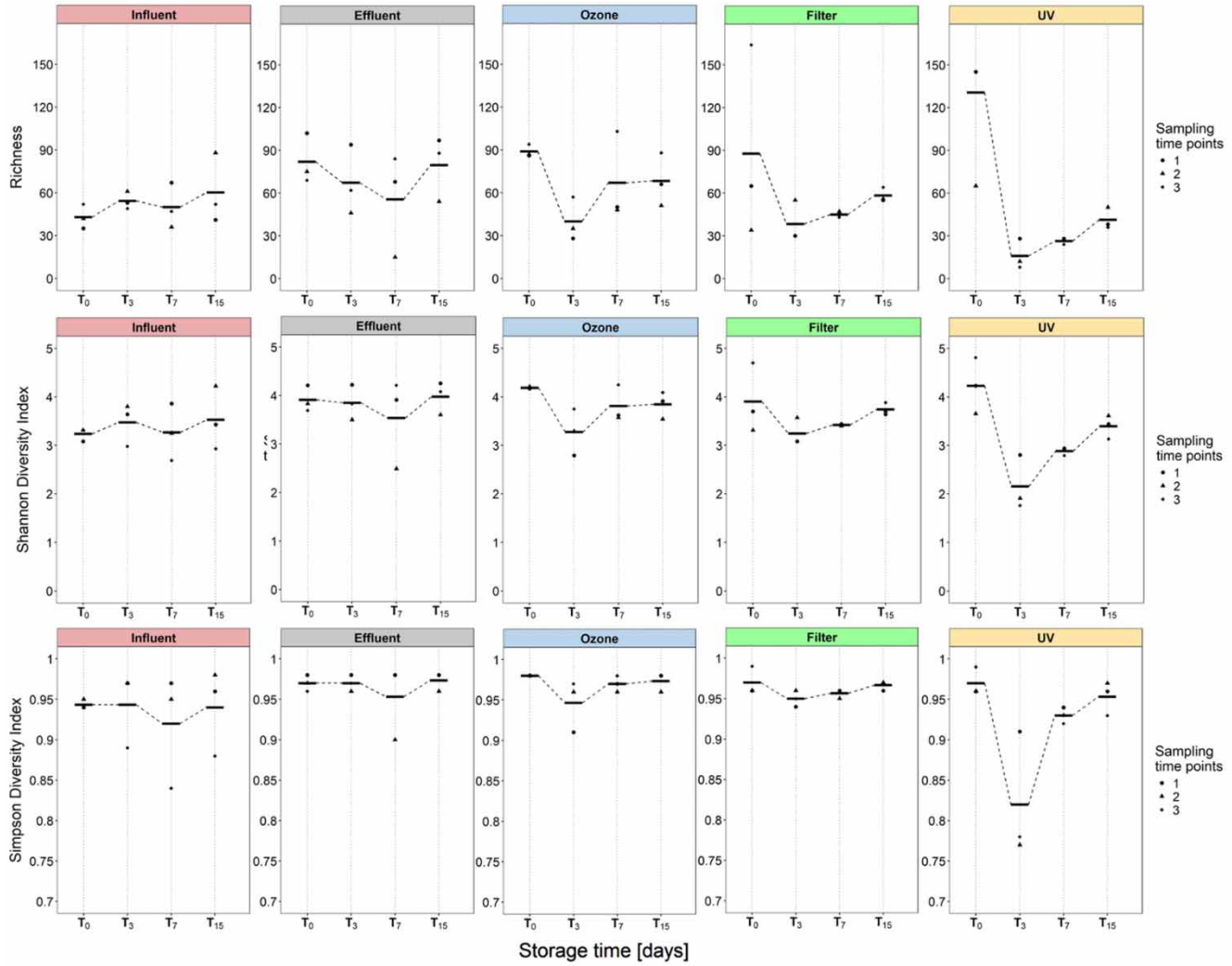


Figure 4 | Alpha diversity indices (upper: richness, middle: Shannon Diversity Index, lower: Simpson Diversity Index) of three independent sampling time points (1 = circle, 2 = triangle, 3 = diamond) along the treatment train and different storage durations (0, 3, 7, and 15 days). Horizontal lines indicate the mean value.

The WWTP influent samples tended to be less rich than the WWTP effluent samples. WWTP influent and effluent samples displayed relatively stable alpha diversity throughout the storage period, although there was notable variance in richness among the three sampling campaigns that increased with time, for example, in the WWTP influent on day 15 and at the WWTP effluent on day 7 (Figure 4). The alpha diversity results suggested a stable microbial composition in the WWTP influent over the storage period, which was further confirmed by rather stable relative abundance of classes throughout the storage duration (Figure 6). Furthermore, beta-diversity analysis (Figure 5(a)) showed no substantial shifts in population composition in the influent over time, as is reflected by the samples' clustering. Overall, the WWTP influent samples differed substantially more from the other sampling points than the other sampling points did from each other. Such differences in richness along the treatment plant were statistically significant (post-hoc Tukey HSD Test, influent vs. UV effluent $p = 0.023$). While Bacilli, Bacteroidia, Campylobacteria, Clostridia, and Gammaproteobacteria predominated in the WWTP influent, the WWTP secondary effluent displayed greater diversity, with dominant classes including Actinobacteria, Alphaproteobacteria, Clostridia, Gammaproteobacteria, Saccharimonadia, and Verrucomicrobiae (Figure 6 and Supplementary material).

In contrast to the samples from the WWTP influent (Figure 5(a)), the WWTP secondary effluent water samples showed a slight shift in community composition on the third day of storage, as indicated by beta-diversity analysis (Figure 5(b)). This

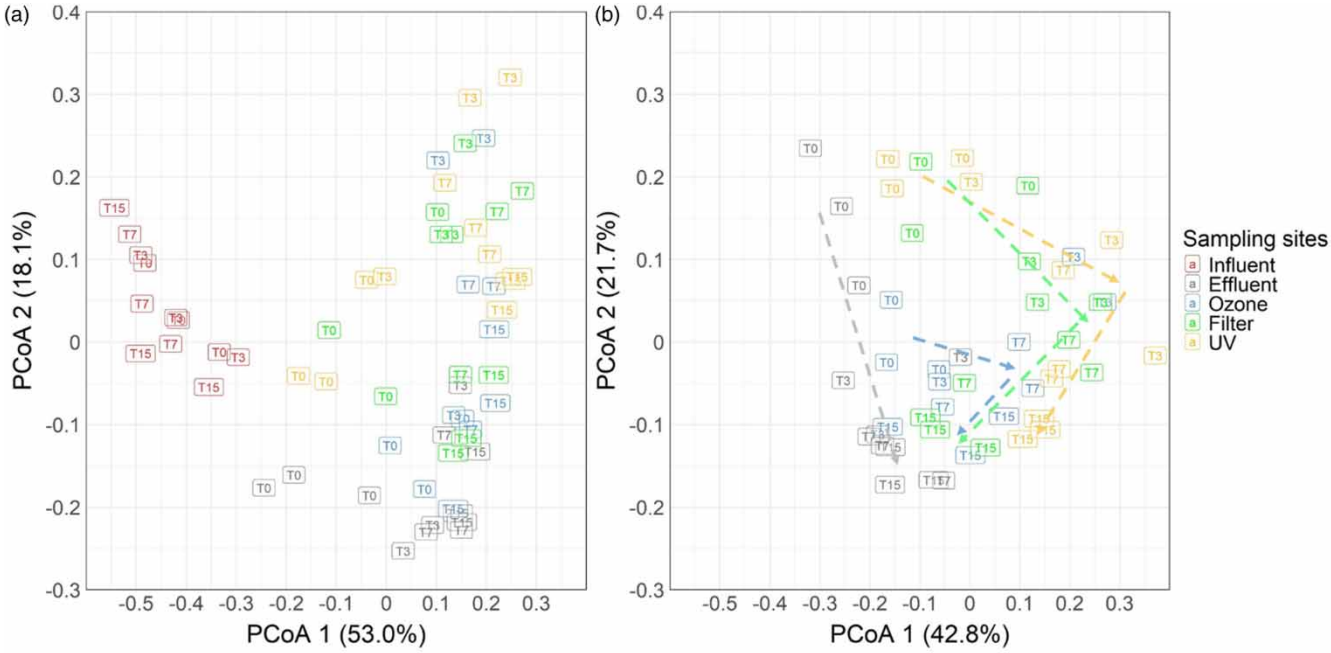


Figure 5 | Principal coordinate analysis biplot based on Bray–Curtis dissimilarities of wastewater samples including the raw wastewater influent (a) and excluding the influent (b) of the conventional WWTP. Strong clustering of the influent has a strong influence on distance matrix generation and PCoA.

shift did not progress further with extended storage, suggesting that a relatively stable bacterial community had already been established after 3 days (Figure 5(b)).

However, the subsequent treatment stages impacted the bacterial community and led to rather substantial changes and dynamics upon storage. The water from the ozonation effluent showed a similar composition to the WWTP secondary effluent, as expected and as exhibited by PCoA (Figure 5(b)). However, the drop in alpha diversity was only visible on day 3 of storage. Alpha diversity augmented again until day 15, with rather minor changes from day 7 onwards (Figure 4). The ozone effluent's bacterial composition on day 3 differed more from the original state than on day 15, suggesting that strong compositional shifts occur rather rapidly after treatment. During storage, Alphaproteobacteria tended to increase in relative abundance compared to T_0. Despite a reduction in their relative abundance shortly after ozonation (T_3 and T_7), Verrucomicrobiae exhibited a resurgence by day 15 of storage (Figure 6 and Supplementary material).

After filtration, alpha diversity over time behaved similarly to the ozonation effluent, showing a drop in diversity between days 0 and 3 that successively increased over the storage period. However, water that passed through the filter was immediately different in bacterial composition from the secondary effluents of the WWTP and the ozonation, respectively (Figure 6). Filter effluent samples on day 3 and 7 were again clearly dominated by Alphaproteobacteria, Clostridia and Gammaproteobacteria. This time, Actinobacteria were present directly after sampling (T_0), diminished in relative abundance on day 3 and 7 but resurged on day 15. Even if those changes were not apparent in species richness, beta-diversity analysis discriminated the difference in bacterial composition on day 0. Interestingly, already on day 3, PCoA revealed a similar composition to samples from the ozonation effluent on days 3, 7, and 15, and variance tended to decrease the longer the water was stored.

The most striking effect was observed after UV treatment. Interestingly the UV effluent's richness was higher compared to other sampling points (Figure 3). The reduction in alpha diversity between day 0 and day 3 was much steeper (post-hoc Tukey HSD Test: $p < 0.001$ for Shannon and Simpson indices) and alpha diversity successively increased until day 15, never reaching original levels. However, the Shannon and Simpson indices on T_0 of the UV effluent were more similar to the ones of the other sampling points, suggesting that the increased richness does not drastically change the overall community structure in terms of evenness. Consequently, the additional taxa introduced in the UV effluent are likely low abundance and do not disrupt the dominance pattern of the main taxa from earlier steps (Figure 6). In the stored UV-treated samples, the dominant classes observed at T_0 shifted by day 3 with an increase in Gammaproteobacteria, which subsequently declined to

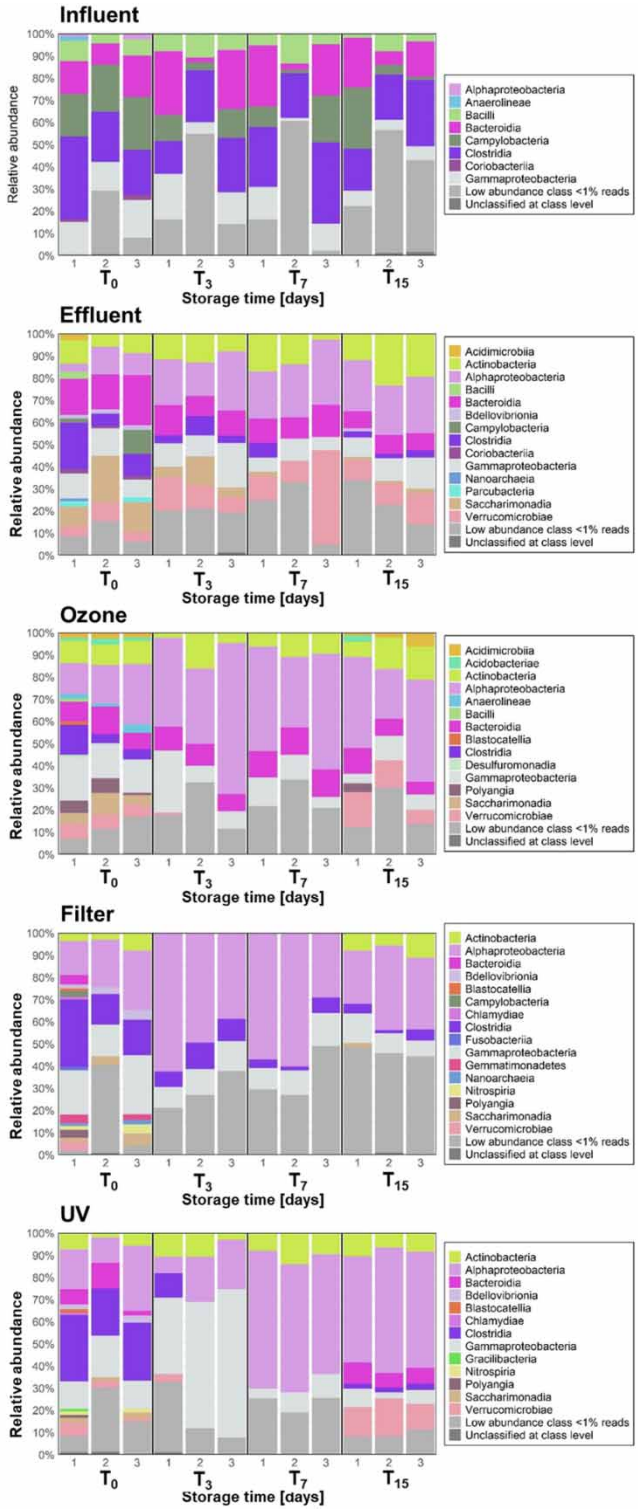


Figure 6 | Relative abundance of classes present in each sampling point on three independent sampling times (1, 2, and 3) and different storage durations (T_0, T_3, T_7, and T_{15}). Low abundance reads (<1%) were summarized as well as reads that were unclassified at the class level.

approximately 10% of the relative abundance by day 7, presumably outcompeted by Alphaproteobacteria, which constituted over 50% of the community. By day 15, other classes, including Bacteroidia, re-emerged, potentially indicating the development of anaerobic conditions during storage (Figure 6). A similar bacterial composition was seen in the filter effluent and

change occurred analogously to patterns of ozone and filter-treated water during the storage period. Again, samples converged on day 15 and were distinct from the original composition at sampling (T_0).

Concluding, all treatment stages altered the bacterial composition in a way that initially decreased alpha diversity, which increased again over time. However, community compositions developed similarly throughout days 3, 7 and 15 of storage, showing that populations converged during storage, irrespective of the applied treatment.

Two-way ANOVA revealed significant differences in Shannon and Simpson indices between storage time ($p < 0.001$ and $p = 0.006$) as well as sampling points ($p = 0.004$ and $p = 0.001$) but for richness only storage time was statistically significant ($p < 0.001$). Post-hoc Tukey HSD test revealed significant changes in all alpha diversity indices from day 0 to day 3, and for days 3–15 (Richness $p = 0.001$ and $p = 0.019$, Shannon $p = 0.001$ and $p = 0.019$, Simpson $p = 0.007$ and $p = 0.024$).

4. DISCUSSION

This study demonstrated that raw wastewater and additionally treated effluent (reclaimed water) did not present any regrowth of indicator organisms during storage. The water thus appears microbiologically stable, or unchanged in quality, when assessed through the limited scope of indicator organism culture methods. However, wastewater is a highly heterogeneous matrix that can contain thousands of different bacterial species per milliliter. Standard culture isolation techniques typically capture only a minuscule portion of this diversity (possibly less than 1%). With the extended analytical range provided by sequencing, we observed significant changes in the microbial composition beyond the insight provided by indicator organism culture.

4.1. Indicator organisms

Indicator organisms concentrations observed in the WWTP influent and effluent samples were generally characteristic of this matrix (Mandilara *et al.* 2006). Somatic coliphages, while abundant in raw wastewater, demonstrated a decline during storage across subsequent treatment steps, with the reduction generally becoming more pronounced after day 7. A recent study by Lee *et al.* (2024) investigated the occurrence and persistence of somatic coliphages in wastewater, also demonstrating their decline during the storage of primary effluent. However, the study also emphasized that somatic coliphages are not a uniform microbial group but taxonomically diverse, noting that the concentration during storage may either increase or decrease, depending on the specific composition of the phage community in the sample. For instance, the family of *Microviridae* tended to persist and increase in relative abundance, while other families, such as *Myoviridae*, showed a tendency to decline (Lee *et al.* 2024), with some phages exhibiting notable resistance to treatment, for example, UV disinfection (Carabias *et al.* 2023).

C. perfringens spores are highly resistant to environmental stresses and serve as conservative indicators for other persistent pathogens such as *Cryptosporidium* spp. and *Giardia lamblia*. Their detection in treated irrigation may indicate potential shortcomings in pathogen removal and represents a potential public health risk when targeting high-quality standards (e.g., class A reclaimed water, EU 2020/741) intended for irrigation of raw-consumed produce. Ingestion of toxigenic Clostridia can cause gastroenteritis, especially in vulnerable populations and settings with inadequate food handling practices. We have seen that even if the treatment steps are able to reduce *C. perfringens* spores throughout the whole treatment train, they persist during storage at constant levels compared to the initial concentration.

Hijnen *et al.* (2009) reported that in river water microcosms and in sand from a slow sand filter, spores of sulphite-reducing Clostridia survived for >10 years with almost unchanged concentrations at 4 and 15 °C storage, and were still able to germinate on culture medium. The tendency to withstand UV disinfection, as seen in our experiments, was also shown in experiments on UV dose responses by Carabias *et al.* (2023). Given the resilience of Clostridia spores to the applied treatment methods, it is evident that the investigated pilot plant would require an additional barrier, such as an enhanced/alternative filtration, to meet the criteria for class A reclaimed water.

We did not observe any regrowth of *E. coli* and intestinal enterococci; instead, their concentration decreased over time. The existing literature on *E. coli* regrowth in reclaimed water presents conflicting findings. Some studies report a significant decline in *E. coli*, total coliform bacteria, *Enterococcus* spp. (Bailey *et al.* 2019) and F+ coliphages (Jeanneau *et al.* 2012), highlighting the stabilizing effect of hydraulic retention time in ponds (Alcalde *et al.* 2003). In contrast, other studies document *E. coli* (Jjemba 2010) and enterococci indicators regrowth, often linking it to environmental factors such as ambient temperature and total dissolved organic carbon (DOC) content (Derry & Attwater 2014). Indicator microorganism culture only shows a small part of the microbial sample composition and also does not account for viable but non-culturable

(VBNC) bacterial states. Numerous bacteria are suspected to be able to transition into a VBNC state during the wastewater treatment process and consequently, as Lin *et al.* (2016a) claim enter reclaimed water systems and serve as 'seeds' for bacterial regrowth.

The observation that storage alone reduced some indicators as effectively as advanced treatment is likely multifactorial, potentially involving die-off due to nutrient depletion (or stoichiometric imbalances), oxygen limitation, and microbial interactions such as predation or competition. Similar to processes in, for example, maturation ponds, these ecological mechanisms may offer cost-effective treatment options in resource-limited settings. However, in Germany, water storage is generally not regarded as a conventional treatment step or standard water management strategy.

Management and monitoring approaches should be tailored to the specific treatment facility and the intended use of the reclaimed water, but more research is needed, particularly with regard to reclaimed water storage. For the pilot plant examined in this study, storage did not adversely impact water quality with respect to indicator organisms, nor did it lead to any observable deterioration.

4.2. Community analysis

Traditional indicator organism culture plays a valuable role in routine monitoring and is widely applied. However, indicators represent only a small fraction of the microbial community and primarily serve as proxies for potential pathogen presence. In contrast, molecular approaches like qPCR and sequencing-based methods can provide a more comprehensive view of microbial dynamics and may improve risk assessment by capturing broader ecological patterns. With genomic sequencing and subsequent diversity analyses we have seen that the water bacterial composition is different for each treatment step. Blair *et al.* (2024) showed recently that treatment train configuration is the most decisive factor explaining observed variation in microbiota, impacting alpha diversity, with more conventional treatment trains yielding lower richness and Shannon diversity compared to more limited treatments. Also, Kulkarni *et al.* (2018) showed that community structure is much more dependent on the applied treatment than on the WWTP influent community structure. Our findings support these statements and show well that not only does the treatment step alter community composition, but also storage times substantially influence the microbiome. In conclusion, the choice of treatment strategies and their combinations could potentially play a critical role in producing biologically stable reclaimed water that is resilient to pathogen intrusion and regrowth.

4.3. Treatment train

4.3.1. WWTP influent and secondary effluent

Indeed, wastewater bacterial communities are complex, with considerable similarities at high taxonomic ranks across various studies in different WWTPs worldwide. Raw wastewater is typically dominated by members of the phyla Proteobacteria, Actinobacteria, and Firmicutes, as well as classes like Bacilli, Clostridia, Bacteroidia, and Alpha-, Beta-, or Gammaproteobacteria (Varela & Manaia 2013; Kulkarni *et al.* 2018; Narciso-da-Rocha *et al.* 2018). This composition was well reflected in the influent of the investigated treatment plant. The WWTP secondary effluent showed higher richness than the influent, which is somewhat unexpected and also stands in contrast to studies by Lin and colleagues (Lin *et al.* 2016b). However, in terms of community composition, the secondary effluent samples align with studies by Do *et al.* (2019), who stated that WWTP effluents show major homologies across the globe and are composed mainly of Proteobacteria, Bacteroides, Actinobacteria, Firmicutes, Tenericutes and Verrucomicrobia. Recently, a core microbiome has been identified for reclaimed water for non-potable reuse that describes organisms that are found in common across microbiomes, for instance, across different reuse water matrices, but do not occur in potable (reuse) water sources. These are Desulfobacterota, Myxococcota, Dependentiae, Chloroflexi, Patescibacteria and Spirochaetota (Blair *et al.* 2024). Those results mostly align well with our findings, apart from Spirochaetota, which were not detected in our samples. Interestingly, according to their findings, Bdellovibrionota, as predatory bacteria, strongly differentiate conventional potable waters from both potable and non-potable reuse waters (Blair *et al.* 2024). Bdellovibrionota were present in the WWTP effluent but seemed to be inactivated by ozone. They reoccurred in the filter effluent and also after UV treatment (Kuroda *et al.* 2023; Zhang *et al.* 2023; Blair *et al.* 2024). Together with parasitic Patescibacteria and predatory Myxococcota, they were found to be associated with a decrease in the available carbon and nitrogen components in wastewater (Kuroda *et al.* 2023). Myxococcoccota were only identified in the ozonation effluent and rarely in the effluents of the filter and UV disinfection, respectively.

4.3.2. Ozone

Ozone is a powerful oxidizing agent that can kill microorganisms, damage nucleic acids, degrade micro-pollutants and oxidize organic compounds, therefore altering the substrate composition of the water (Hammes *et al.* 2010; Prest *et al.* 2016). The oxidation of bacterial cells leads to the leakage of cellular constituents that might be used as nutrients from the surviving or incoming bacterial community for regrowth (Lautenschlager *et al.* 2014; Alexander *et al.* 2016). For instance, both Hammes *et al.* (2010) and Vital *et al.* (2012) observed a 6-fold increase in assimilable organic carbon (AOC) after ozonation during drinking water production, despite no change in DOC concentration, indicating a significant modification in the nutrient composition (Hammes *et al.* 2010; Vital *et al.* 2012). Such disruptive events can allow for surviving microorganisms or new microorganisms to colonize and exploit the altered nutrient pool. Consequently, oxidative processes tend to produce highly unstable water due to increased nutrient availability and the removal or inactivation of existing bacterial cells, which together create a new niche for bacterial growth (Prest *et al.* 2016). Contrary to the expectations, we did not observe any regrowth of indicator organisms after ozonation and also no substantial increase in 16S rRNA concentration in the presumably large new AOC pool. Furthermore, we did not observe any decrease in 16 s rRNA concentration with qPCR during ozonated water storage in contrast to reports by Zhuang *et al.* (2015), but we observed rather stable levels of the gene.

When looking at the bacterial community composition, significant shifts were observed during storage. We observed a similar richness of OTUs on day 0 samples of the ozonation effluent compared to the WWTP secondary effluent but ozonation resulted in an immediate shift in community composition (beta diversity, compare WWTP secondary effluent T_0 and ozonation effluent T_0). Similar observations were reported by Alexander *et al.* (2016), who observed a notable shift in the diversity and relative abundance of several OTUs after ozonation, revealing a reduced diversity in genera after treatment by 50%. However, they operated their system with considerably higher ozone dosage compared to our study (1 g O_3/g DOC vs. 0.6 mg O_3/mg DOC) and applied PMA treatment to only target 16S rRNA of live bacterial cells. Shi *et al.* (2023) undermined these results and found reduced alpha diversity after wastewater ozonation if PMA was applied, as opposed to no difference in richness and diversity if no PMA is applied to ozonated water samples. The absence of PMA treatment in our study could potentially explain the differences observed in our results. We assume that without PMA treatment a turnover in bacterial diversity after ozone treatment is only reflected after some time (here after 3 days of storage) and not immediately after treatment. Presumably, bacterial cell membranes are targeted during ozonation, releasing their DNA contents into the water, which might still contain unfragmented 16S rRNA, allowing such signals to be picked up during the sequencing run. Experiments by Becerra-Castro *et al.* (2016) that used ozone-treated wastewater that was stored over 3 days also exhibited a reduction in OTUs, as seen here, and untreated raw wastewater showed only slight compositional changes, as also seen in our study.

Ozone-induced alterations in microbial communities have been associated with a predominance of Proteobacteria, particularly the opportunistic pathogenic organisms *Pseudomonas aeruginosa* and *Acinetobacter* spp., in ozonated wastewater stored in the dark at room temperature (Ribeirinho-Soares *et al.* 2022). This is analogous to our findings that revealed an increase in Proteobacteria, especially on day 3 of storage. Some phyla were detected in the ozonation effluent (t0) that were not observed in the WWTP secondary effluent (t0), such as Myxococcota, Chloroflexi and Acidobacteriota. This might be explained by the remobilization of biomass that was settled in the ozonation reaction tanks over time, inoculating the ozonation effluent with slightly different phyla.

4.3.3. Filter

Typically, biological filtration steps are applied after primary disinfection processes (e.g., here ozonation) to target both rapidly degradable and more complex organic compounds, for example, low-molecular-weight humic substances (Lautenschlager *et al.* 2014). In theory, this ultimately reduces the downstream regrowth potential of microbial populations (Hammes *et al.* 2006; Lautenschlager *et al.* 2014).

Most literature on the microbial succession and impact of filtration systems comes from drinking water research or water reclamation for potable use, and studies rather focus on slow sand filters. However, compared to drinking water, reclaimed water offers higher bacterial abundance, diversity, and organic matter concentration thus suggesting that it is even more likely to form multispecies biofilms on filters and in reuse systems and also colonize dual-media rapid sand filter (RSF) as the one used in this pilot-scale treatment plant. The filtration effect is not only a physical barrier but also due to the bacterial cells that colonized the filter material and formed biofilms on the particles. This diverse biofilm community can significantly remove organic carbon from the source water (Bar-Zeev *et al.* 2012) since bacteria can degrade various organic components

contained in the water as part of their metabolic activity. As these bacteria routinely detach from filters or are sloughed off as water passes through, they might alter the bacterial community composition in the filtrate (Hammes *et al.* 2010; Pinto *et al.* 2012; Prest *et al.* 2016). The rapid flow rate of water through RSF, up to approximately 300 times higher than in slow sand filters, has led to the implicit assumption that biological effects on water quality in RSF are minimal (Bar-Zeev *et al.* 2012). Our results show that ozonation effluent (t_0) and filter effluent (t_0) have different microbial communities, suggesting a biological impact of the filter. However, our results do not show elevated alpha diversity after filtration; rather they indicate an alteration of the community instead of pure enrichment. Similar effects have been observed in drinking water treatment, where findings suggest that RSF may enrich the destabilized water from the ozone effluent with a modified and more stable microbial community (Prest *et al.* 2016). This community, capable of thriving on a broad nutrient spectrum, is critical in producing biologically stable water. Lautenschlager *et al.* (2014) showed that in drinking water, microbial communities in filter effluents generally reflected the biofilter (rapid sand, granular activated carbon, and slow sand filter) community and therefore concluded that the biofilters considerably shape the effluent community (Lautenschlager *et al.* 2014). The same was shown by Mohr *et al.* (2020), who found that sand filtration for wastewater treatment significantly increased bacterial diversity, supposedly leading to biological stabilization of the reclaimed water. Specifically, filtration through dual-media RSF has been demonstrated to significantly shape bacterial communities, even across seasonal time scales. This process establishes and maintains a stable microbial community that continues influencing water treatment processes downstream of the filtration step (Pinto Xi & Raskin 2012). In drinking water systems, similar to wastewater systems, the microbial community composition is influenced more significantly by the treatment processes applied than by the characteristics of the source water. Consequently, it has been proposed to intentionally manipulate the microbial community within filters to influence the downstream water microbiome. This 'probiotic approach' involves introducing beneficial bacteria into the filtration system, which can outcompete undesirable microorganisms (e.g., pathogens, corrosion-causing bacteria, or odor-producing species), or introducing bacteria that have positive effects (e.g., nutrient cycling) on public health and the environment (Pinto *et al.* 2012). Such attempts were recently made in drinking water, where hydrogen-oxidizing bacteria were introduced to form biofilms on a trickling filter bed, successfully producing biostable drinking water Favere *et al.* (2024). This approach could theoretically be adapted to generate biostable reclaimed water at various stages of treatment or storage, not limited to filter enrichment, thereby offering a potential method for enhancing the stability and quality of water in reuse systems.

4.3.4. Ultraviolet

UV-C light (200–280 nm), as emitted by the utilized UV disinfection in this study, is used to deactivate microorganisms by creating lesions in their DNA, consequently disrupting DNA replication and ultimately causing inactivation. The detection of UV-induced damage in bacteria proves challenging using culture-based methods, as UV exposure can render bacteria non-culturable while they remain viable and potentially infectious (Jungfer *et al.* 2007). The potential for regrowth after UV treatment is linked to bacterial DNA repair mechanisms, including photoreactivation and dark repair. Photoreactivation occurs under near-UV (UV-A) and visible light (310–480 nm), allowing microorganisms to recover activity by repairing DNA lesions. On the other hand, dark repair can occur without light and involves a multi-enzyme process to remove DNA lesions, which could apply to our case since we stored samples in a dark incubator.

Wastewater microbiomes seem to partially exhibit resilience to UV radiation and display a notable potential for regeneration (Süß *et al.* 2009). However, the occurrence of microbiome changes following UV treatment remains a topic of debate in the literature. For instance, Narciso-da-Rocha *et al.* (2018) concluded that UV treatment only leads to minor compositional variations in the bacterial community of wastewater samples directly after UV treatment, characterized by an increase in *Alphaproteobacteria*; this was not the case in our study (Narciso-da-Rocha *et al.* 2018). They also reported that after 3 days of storing the UV-disinfected wastewater, members of *Comamonadaceae* and *Flavobacteriaceae* were most prominent. Contrarily, Becerra-Castro *et al.* (2016) observed that UV treatment reduced sample richness after 3 days of storage, compared to non-treated wastewater, which is analogous to our findings. Furthermore, Kulkarni *et al.* (2018) reported that a treatment plant producing effluent for agricultural irrigation that was irradiated with UV showed considerably lower alpha diversity but, upon storage in an open-air pond, again led to an increase in alpha diversity indices. However, if the increased alpha diversity is due to regrowth or contamination from the environment it is not accounted for experimentally. Nevertheless their findings underscore that reclaimed water storage allows for notable changes in community composition (Kulkarni *et al.* 2018). These observations align with our results, indicating that UV disinfection reduces bacterial diversity and induces major

community shifts during storage. By 15 days, the microbial community composition no longer resembles the state at the time of its discharge.

During storage, the increase in alpha diversity and the reorganization of the bacterial community from beta-diversity analysis over time are thought to indicate an occurring succession, as seen in our storing experiments. Interestingly, succession in our case appeared deterministic, with beta-diversity analysis showing similar community compositions after day 15 of storage, irrespective of the applied disinfection treatment to the effluent of the WWTP. Studies by Zhang *et al.* (2019b) showed that in reclaimed wastewater distribution systems, long-term microbial community succession is the underlying driving factor for biofilm development and reported similar bacterial taxa as we observed: Verrucomicrobia, Proteobacteria, Bacteroidetes, Acidobacteria, and Actinobacteria among others have been reported most dominant in biofilm samples of water reuse treatment systems and were also confirmed by our findings (Zhang *et al.* 2019a).

We propose that future similar studies focus on larger sample sizes and consider differences between aerobic and microaerophilic as well as open-air (light) and closed (dark) conditions during reclaimed water storage, including parallel measurements of nutrients and dissolved oxygen. We did not measure dissolved oxygen contents and nutrient composition during the storage experiments, making it impossible to correlate the occurrence of certain taxa to such data. At times, the community structure consisted of rather anaerobic species, indicating that storage may have caused oxygen depletion. This suggests that the conditions were more representative of closed storage tanks and anaerobic ponds rather than aerobic ones. Additionally, incorporating PMA treatment of samples prior to sequencing could provide valuable insights by distinguishing between live and dead cells, offering a more nuanced understanding of taxa susceptibility to treatment processes beyond what is revealed through traditional indicator organism cultures. Furthermore, including functional profiling in such studies could expand insights into bacterial succession by identifying shifts in metabolic capabilities and network analysis could reveal drivers of succession, such as keystone species or cooperative/competitive interactions during reclaimed water storage. The observed discrepancies between laboratory models and naturally occurring microbes highlight the requirement for real-world evaluation of water treatment and storage systems rather than relying solely on *in vitro* methods using artificially spiked samples.

The presence of higher loads of bacteria in reclaimed water and even regrowth after treatment might not *per se* be an issue, as long as no pathogens in harmful levels for animals, humans, plants and the environment are observed.

5. CONCLUSIONS

This study set out to examine whether prolonged storage of wastewater and reclaimed water (for agricultural reuse) affects water quality, focusing on microbial dynamics through cultural detection of indicator organisms and 16S rRNA gene amplicon sequencing. Findings revealed no regrowth of indicator organisms during 15 days of storage in any of the treatment steps; however, *C. perfringens* spores persisted at constant levels during storage and were not fully eliminated by ozonation, filtration, and UV disinfection. Interestingly, storage alone reduced most indicator organisms, sometimes as effective as treatment. While indicator cultures provided limited insights, sequencing highlighted dynamic microbiome shifts: treatments reduced bacterial diversity, but storage progressively restored it, yielding a unique microbial profile distinct from the initial water quality. During storage, microbial communities tended to converge, irrespective of the applied treatment stages, suggesting biostabilization as a result of ecological succession. This study highlights reclaimed water bacteria as dynamic microbiomes, pointing to the value of promoting biostable and diverse communities to maintain water quality during storage. Advanced treatment systems could benefit from combining effective contaminant removal with strategies that promote beneficial, resilient, biostable microbiomes safeguarding both public health and the environment.

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AUTHOR CONTRIBUTIONS

L.F. contributed to conceptualization, data curation, formal analysis, methodology, investigation, writing – original draft. F.D. contributed to data curation, formal analysis, visualization. J.G. contributed to investigation, writing – reviewing and editing. N.W contributed to formal analysis. M.S. contributed to collecting resources, writing – reviewing and editing. N.Z. contributed to funding acquisition, project administration, writing – review and editing. J.H. collected resources. N.T.M. acquired funds. T.K. contributed to funding acquisition, project administration, supervision, writing – reviewing and editing.

DATA AVAILABILITY STATEMENT

Data cannot be made publicly available; readers should contact the corresponding author for details.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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