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Microbiological changes along a modular wastewater reuse treatment process with a special focus on bacterial regrowth

Andreas Nocker, Lorenz Schulte-Illingheim, Hubert Müller, Anja Rohn, Barbara Zimmermann, Anil Gaba, Andreas Nahrstedt, Hooman Mohammadi, Yannick Tiemann and Kerstin Krömer

ABSTRACT

Water reuse is becoming an increasing necessity due to depleted water resources or increased water demand. A treatment process on a pilot scale was designed to produce different water qualities for different applications in industry or agriculture. We report here microbiological changes along the modular process using treated municipal wastewater effluent as raw water. Treatment technologies included coagulation, ultrafiltration (UF), reverse osmosis (RO), quartz sand, activated granular activated carbon (GAC) filtration and disinfection. Elimination of traditional hygiene indicator bacteria was already achieved by ultrafiltration as the first barrier. Profound changes by each treatment step also applied to the microbiome. Total and intact cell concentrations as quantified by flow cytometry underwent a strong decline after UF and RO, whereas biological stabilization was achieved through quartz sand filtration and GAC passage. Interestingly assimilable organic carbon (AOC) was still present even after RO at levels that allowed substantial regrowth of bacteria. Overall, UF and RO led only to a 0.43 and 0.78 log decrease in intact cells concentrations in stagnated water after regrowth compared with 6.5 log intact cells/ml in the stagnated raw water. Temperature was shown to be an important parameter determining the microbiome of the regrown population. Regrowth could, however, be efficiently suppressed by monochloramine.

Key words | bacterial regrowth, flow cytometry, process monitoring, water recycling, water reuse

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HIGHLIGHTS

- A versatile water reuse process was established producing different water qualities (MULTI-ReUse).
- Elimination of traditional hygiene indicator bacteria was already achieved by ultrafiltration as the first barrier. This predestined ultrafiltration as a critical control point for high-resolution process monitoring by online flow cytometry that produced data on total and intact cells in high temporal resolution.
- Offline cytometry allowed the assessment of the change in regrowth potential along the treatment process.
- Despite strong reductions in bacterial concentrations by UF and RO, the suppression of bacterial numbers was not sustainable as the regrowth potential (as determined by offline flow

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doi: 10.2166/wrd.2020.012

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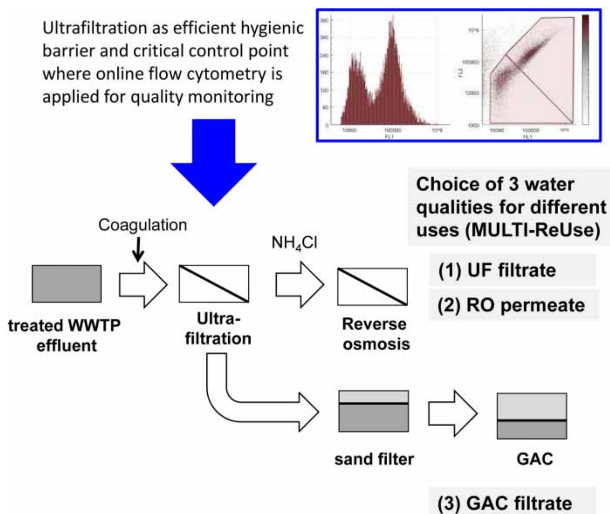
cytometry) was only reduced moderately (on a log scale) despite a strong reduction of DOC (on a linear scale).

- Flow cytometry proved a very sensitive diagnostic tool both in an offline and online format.

GRAPHICAL ABSTRACT



INTRODUCTION

Climate change leads to prolonged dry weather periods and extending semi-arid regions and poses an increasing stress for cities, industries and agriculture in many regions worldwide (Ormerod 2016; Faour-Klingbeil & Todd 2018). Also, in Germany that has previously not been associated with water shortage, local water limitations have become more obvious in the recent dry summers. To avoid excessive stress for groundwater sources, water reuse is increasingly debated especially for industrial uses and irrigation (Drewes & Horstmeyer 2015; Schmid & Bogner 2018; Drewes *et al.* 2019). Given the different types of water uses and the different corresponding water quality requirements, water treatment concepts should ideally be flexible and adjustable to needs (Becker *et al.* 2019). Further challenges consist in the allocation of variable demand-driven water volumes at competitive prices.

To gain experience with how to accomplish different quality requirements in such a scenario, a project named 'MULTI-ReUse' was launched. A coastal region with competing water uses (demand from water-intensive industries versus demand for drinking water) was chosen for a demonstration. As groundwater use would come with the risk of saltwater intrusion due to its direct proximity to the North Sea coast, this region in the northwest of Germany does not have its own drinking water supply. Instead, drinking water is transported over long distances. Whereas relatively low water qualities would be sufficient for most industrial applications and agriculture, high-quality drinking water is used in many cases. Water reuse would, thus, be an immediate alleviation on drinking water demand, groundwater resources elsewhere and energy transport costs. Aims of the project consisted of the (1) optimization and validation of modular treatment trains to achieve different water qualities, (2) improvement of process monitoring strategies, (3) assessment of economic and ecological aspects and socio-cultural acceptance and (4) development of marketable treatment solutions for globally relevant and typical reuse applications.

For this purpose, a German multicenter research consortium developed, demonstrated and evaluated a combination of different water treatment technologies to polish treated

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municipal wastewater treatment plant (WWTP) effluent to achieve different water qualities. The project was performed in collaboration with the local water supplier Oldenburgisch-Ostfriesischer Wasserverband (OOWV) in Lower Saxony. The treatment train of a pilot plant included, among other technologies, coagulation, ultrafiltration (UF), reverse osmosis (RO), quartz sand filtration, granular activated carbon (GAC) filtration and disinfection. Using a modular approach, wastewater was treated to different quality standards for different applications: (1) particle-free water for washing and cooling processes, (2) RO treated water free of ions and micropollutants for industrial processes and special uses and (3) particle-free, biologically stable water with low content of organic micropollutants for agricultural irrigation and groundwater infiltration.

While other members of the consortium worked on other aspects, this study aimed at mapping microbiological changes along the treatment train. Cultivation approaches were supported by cultivation-independent techniques to assess microbiological changes along the treatment process and the effect of different treatment steps. Applied monitoring technologies included flow cytometry (offline and online) to detect changes in total and intact cell concentrations (TCC and ICC) and high-throughput sequencing to capture changes in the microbiome along the treatment process. Both diagnostic methods have been shown to be useful tools to capture microbiological changes along treatment processes (Prest *et al.* 2014; Van Nevel *et al.* 2017; Hull *et al.* 2019). For flow cytometric analysis, the analysis of freshly taken water samples that reflect the microbiological status of the water at the time point of sampling (day 0) was supplemented with the analysis of the same samples after an incubation for 7 days (day 7) at defined temperatures. The day 7 values capture the regrowth potential of the bacteria contained in the corresponding water sample and reflect the planktonic bacterial concentrations that are obtained within the time stagnation time (Gillespie *et al.* 2014). The bacterial levels obtained after the 7 days are supposed to correlate with the assimilable organic carbon (AOC) and nutrient levels contained in the sample (Farhat *et al.* 2018). Indirectly the approach, therefore, also allows conclusions on the efficiency of nutrient removal.

In summary, this study aimed at monitoring changes in the numbers of bacteria along a modular water reuse treatment process and the composition of the bacterial population. The analysis was not limited to the microbiological status of the water directly after sampling, but also tried to cover the changes in that water after stagnation.

METHODS

Water recycling pilot plant

The pilot plant assembled by De.EnCon GmbH and IWW was accommodated in two containers located on the site of a municipal WWTP in the village of Nordenham in Lower Saxony. The region was chosen as it is characterized by competing water demands between drinking water supply and industrial uses. The treated wastewater effluent served as raw water for the pilot plant. The optimization phase over approximately 1 year was followed by a 6 months demonstration phase. Only data from the demonstration phase are shown. The treatment technologies included, among others, coagulation ($FeCl_3$), ultrafiltration (UF, flux of 60 L/(m^2 h) with 4.8 m^3/h per UF module, 90.9% recovery), low-pressure reverse osmosis (RO, flux of 16 L/(m^2 h) with 0.5 m^3/h per RO line, each with 3×4″ modules, 75% recovery), quartz sand filtration and GAC filtration, UV disinfection and optional disinfection with monochloramine to reduce RO membrane biofouling (Figure 1).

Two UF modules and two RO lines, each with three modules and recycling of concentrate, were operated in parallel to test different process conditions during an initial test

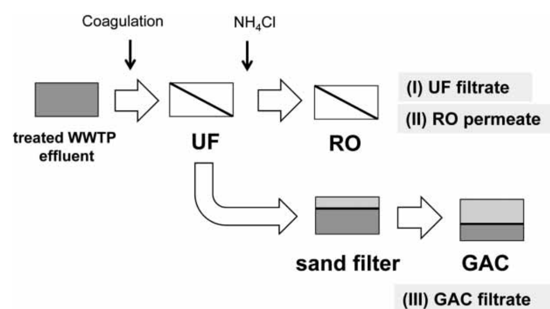

Figure 1 | Simplified schematic presentation of the modular treatment process of the MULTI-ReUse pilot plant for the production of three different water qualities: (1) UF filtrate, (2) RO permeate and (3) GAC filtrate. UF, ultrafiltration; RO, reverse osmosis; GAC, granular activated carbon. Optional UV disinfection to disinfect UF filtrate and RO permeate prior use was not considered in this diagram.

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phase. Operating conditions were more constant during the demonstration phase that forms the basis for the data shown here. Monochloramine was generated to give a final concentration of 1 mg/L and added to the filtrate storage tank of UF 1, whereas UF 2 filtrate was not chemically disinfected. A detailed process and operation description is given elsewhere (Nahrstedt *et al.* 2020).

Preparation of AOC-free glassware

An alkaline solution of potassium permanganate was made by dissolving (A) 30 g of permanganate (cat. nr. 105082, Merck KGaA, Germany) and (B) 100 g of sodium hydroxide pellets (cat. nr. 106498, Merck KGaA, Germany) separately in each 500 ml deionized water. The latter is strongly exergonic and was allowed to cool down. The two solutions were subsequently combined in a 1 L bottle and stored in the dark prior to use. 50 ml borosilicate glass vials (cat. nr. 7612150, Th. Geyer GmbH & Co.KG) used for flow cytometry samples and other borosilicate glassware that should be AOC free were filled approximately one-third of the total volume with this alkaline solution and sealed with appropriate caps. Glassware was subsequently shaken in regular intervals for at least 15 min to ensure contact of the entire surface area with the solution. The permanganate solution was decanted (for recycling purposes) and glassware and caps were rinsed three times with tap water and twice with deionized water. Dried glassware and caps were wrapped in aluminum foil. Glassware was muffled at 280 °C for at least 8 h or overnight, caps were heated at 180 °C during that time.

Sampling procedure

Water samples were taken from designated sampling valves after disinfection with isopropanol (70%) followed by desiccation for at least 2 min (following guidelines by DIN EN ISO 19458) and flushing with 2 L of water. For cultivation, water was sampled using 500 ml polypropylene sampling bottles (cat. nr. 295348, Aqua Laborservice, Wertheim, Germany) containing thiosulfate. For flow cytometry, water was sampled in 50 ml AOC-free borosilicate glass vials containing thiosulfate and sealed with a cap with Teflon sealant. Vials were filled up to a 20 ml mark. The final thiosulfate concentration in the sample was 18 mg/L, an effect of thiosulfate on the regrowth potential was experimentally excluded. For sequencing, water was sampled in 1 L glass bottles (SCHOTT, Jena, Germany). All sampling bottles were sterile. Samples were transported cold (6 ± 2 °C) in styropor containers containing cooling elements and processed within 24 h.

Cultivation

Culture analysis was performed by the laboratory of the Public Health Department of Aurich. The following organisms were quantified: colony counts 22 and 36 °C (both DIN EN ISO 6222:1999-07), *Escherichia coli* and coliforms (both DIN EN ISO 9308-2:2014-06), intestinal enterococci (DIN EN ISO 7899-2:2000-11), *Clostridium perfringens* (DIN EN ISO 14189:2016-11) and *Legionella* spp. (DIN EN ISO 11731:2017-05).

Offline flow cytometry

Fluorescent dyes used in this study were SYBR Green I (10,000× stock, Invitrogen™) and propidium iodide (PI, 1 mg/ml, Invitrogen, Thermo Fischer). Water samples were processed undiluted or 10× diluted with 0.1 μm filtered mineral water (Evian, Evian-les-Bains, France) in case the total signal exceeded approximately 5.000 signals/s. SYBR Green I was diluted to a working stock concentration of 100× using dimethylsulfoxide (DMSO, Sigma-Aldrich) and stored at −20 °C until use. Aliquots (250 μl) of water samples were transferred into 96-well plates (cat. nr. 601808, HJ-Bioanalytik GmbH). To determine total cell concentrations (TCC), 200 μl sample aliquots from this plate were transferred into the wells of a second 96-well plate with pre-aliquoted 2 μl of the 100× SG working stock solution followed by thorough mixing by pipetting up and down several times using a multichannel pipette. To determine ICC, 200 μl sample aliquots from the first plate were transferred into the wells of a second 96-well plate with pre-aliquoted 2.4 μl mixture of a 100× SG I and PI in the ratio of 5:1 again followed by thorough mixing. Final concentrations of fluorescent dyes were 1× SG and 3 μM PI. Staining was performed at 37 °C for 13 min in an incubator. Data were collected using an ACEA NovoCyte® benchtop instrument equipped with a 488 nm laser (OLS OMNI Life Science, Bremen, Germany). A NovoSampler® Pro enabled

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analysis on a 96-well plate basis. Data were analyzed using the instrument-specific NovoExpress software and a gating procedure similar to the one described by Gatza *et al.* (2013).

Online flow cytometry

Online flow cytometry was performed using an Online Bacteria Analyzer (OBA, ONTRONIX AG, Switzerland). A stain solution with a total volume of 100 ml was prepared by mixing 2 ml PI (1 mg/ml), 0.1 ml SYBR Green I (10,000×), 10 ml DMSO and 87.9 ml ultrapure water to give concentrations of 10× SYBR Green I, 30 μM PI and 10% DMSO. This solution was kept in a designated storage compartment in the instrument at 10 °C for a maximum of 4 months. This stain solution was mixed with the water samples (volume of 90 μl) at a ratio of 1:9 to obtain final concentrations of SYBR Green I of 1× and of PI of 3 μM. Incubation of the dye-sample mixture was performed at 37 °C for 13 min. Measurements were typically taken every 2 h. When analyzing samples containing monochloramine, the stain solution was dosed with thiosulfate to a concentration of 180 mg/L to ensure a final thiosulfate concentration in the water sample of 18 mg/L for disinfectant neutralization.

Illumina sequencing

Genomic DNA was extracted from filtered water samples using the Aquadien™ DNA Extraction and Purification Kit (cat. nr. 3578121, Bio-Rad Laboratories GmbH, Feldkirchen, Germany). Illumina sequencing (MiSeq run nano V2, 250 bp paired-end) was performed by Microsynth AG (Balgach, Switzerland) after amplification of 16S rRNA genes using standard universal primers 341F_ill (5′-CCTACGGNGGCWGCAG-3′) and 802R_ill (5′-GAC-TACHVGGGTATCTAATCC-3′; Klindworth *et al.* 2013) amplifying the V3/4 region of the 16S rRNA gene. We received de-multiplexed raw reads from the sequencing company. After removing indices with bbduk (http://jgi.doe.gov/data-and-tools/bbtools/), reads were processed in mothur (version 1.43.0) following the MySeq SOP (Schloss *et al.* 2009; Kozich *et al.* 2013). After trimming, de-noising and chimera removal, sequences were clustered into operational taxonomic units (OTUs) using a 97% similarity cutoff. To compare the microbial diversity across samples, we sub-sampled and rarefied the dataset to the lowest number of sequences per sample, which was 4,072 sequences. This led to a total amount of 2,919 individual OTUs in all samples. Species richness, Simpson and Shannon indices as a measure for microbial Alpha diversity and Bray Curtis as a measure for Beta diversity were calculated in RStudio (version 1.1.463 and R version 3.5.2) using the package Phyloseq (version 1.24.2) (McMurdie & Holmes 2013). Phyloseq in combination with ggplot (version 3.2.1) and Inkscape (version 0.92) (http://www.inkscape.org/) were used for plotting and illustration.

RESULTS AND DISCUSSION

Assessment of the hygienic status

The most important criterion of water is its hygienic safety. Traditional bacterial hygiene indicators and bacterial colony counts as quantified by culture methods were abundant in treated wastewater effluent serving as raw water for the pilot plant (Table 1). Microbiological raw water quality underwent strong variations as indicated by minimum and maximum values. Independent of these variations, UF was shown to be highly efficient at removing all studied hygiene indicators with only one sporadic finding of one intestinal Enterococcus in a single sample out of $n = 17$. This finding suggests that a very good hygienic status was already accomplished after the first barrier. Also, total colony counts dropped substantially although the filtrate compartment was as expected not sterile.

Hygiene indicators were also found absent in the subsequent treatment process. Nevertheless, the water contained an autochthonous bacterial population that underwent profound changes along the treatment train in terms of numbers and composition. Assessment of colony counts was supported by flow cytometry to quantify the entire bacterial waterborne population independent of culturability.

Microbiological changes monitored by offline flow cytometry

Analysis of water (transported cold to the laboratory and analyzed within 24 h after sampling) provided the bacterial concentration reflecting the time point of sampling (day 0).

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Table 1 | Traditional bacterial hygiene indicators and total colony counts in treated WWTP effluent (=raw water) and in UF filtrate

		Raw water		UF filtrate	
		Average	Maximum/Minimum	Average	Maximum/Minimum
E. coli	MPN/100 ml	79,100	198,600/20,600	0	0/0
Enterococci	MPN/100 ml	164,900	2,200,000/600	0	1/0
Clostridium perfringens	MPN/100 ml	64,300	810,000/4.200	0	0/0
Legionella spp.	MPN/100 ml	>100	>100/0	0	0/0
Colony counts 22 °C	CFU/ml	24,600	96,000/5,800	2	10/0
Colony counts 36 °C	CFU/ml	42,600	290,000/6,900	120	700/0

Average numbers are shown for the pilot plant demonstration phase between June 2018 and June 2019 ($n = 17$) with corresponding maximum and minimum values owing to changes in raw water quality.

Whereas bacterial concentrations in the UF feed water were comparable with the raw water (approximately 10^7 suspended cells/ml), UF resulted in a substantial decrease in TCC and ICC by an average of approximately 3.5 log units or 99.97% (Figure 2(a)). Bacterial concentrations tended to increase again within a UF filtrate storage tank and during passage to the RO module. As expected, bacterial concentrations in RO permeate were very low and near or under the detection limit of the method (approximately 100–1,000 cells/ml depending on the cleanliness of the flow cytometer at the time point of sample analysis).

Biological stabilization of UF filtrate via quartz sand filter and GAC passage led to a recovery of bacterial concentrations to approximately 10^6 cells/ml by enrichment of flow-through water with bacteria sessile on these filters. Lowest bacterial concentrations were, thus, contained in water qualities II and I, followed by water quality III. This was in agreement with colony counts that showed the same tendency although on a lower part of the log scale (Figure 2(b)). Comparing bacterial numbers obtained with flow cytometry and culture, between 0.001% (22 °C, quartz sand filter runoff) and 5.9% (36 °C, UF filtrate after storage tank) of intact bacteria were culturable (Figure 2(c)). Overall the percentages of culturable bacteria were thus subject to substantial variations over the treatment train. With the exception of the raw water, more bacteria were culturable at 36 °C than at 22 °C (Figure 2(b) and 2(c)). In the raw water, the proportion of culturable bacteria at the two incubation temperatures was comparable with 0.361% (at 22 °C) and 0.351% (at 36 °C). Based on flow cytometry and culture data, biologically stabilized water after GAC (=water quality III) contained 0.8 log units less bacteria than the raw water based on ICC data, but 3 log units less bacteria based on colony counts. Judgment of treatment efficiency, therefore, greatly depended on the chosen diagnostic method. Given the strong variation of the proportion of culturable bacteria over the treatment process, cultivation-independent quantification by flow cytometry gave a better overview of overall changes in bacterial numbers.

Distinction between membrane integrity breach and regrowth

Especially in the first weeks of the operation of the pilot plant, treatment suffered from technical process interruptions. During that time, bacteria were found in significant numbers in the UF filtrate leading to the question about their origin. Although a retention of *Pseudomonas diminuta* of 9.7 log units was measured for the type of UF membrane used (according to the ASTM standard F838-05, data not shown), a breach could not be excluded. Apart from mere quantification, offline flow cytometry proved useful in this respect. Bacteria in UF filtrate predominantly belonged to the high nucleic acid (HNA) population (data not shown). Low nucleic acid (LNA) bacteria as abundant in UF feed water, on the other hand, were absent in the filtrate. UF filtrates from modules with impaired integrity, on the other hand, had been reported to contain primarily LNA bacteria that have a higher probability to pass through due to their smaller size (Buysschaert *et al.* 2018). In fact, HNA bacteria can already be separated from LNA bacteria by means of a 0.4 μm filter (Proctor *et al.* 2018). The pore size of the UF

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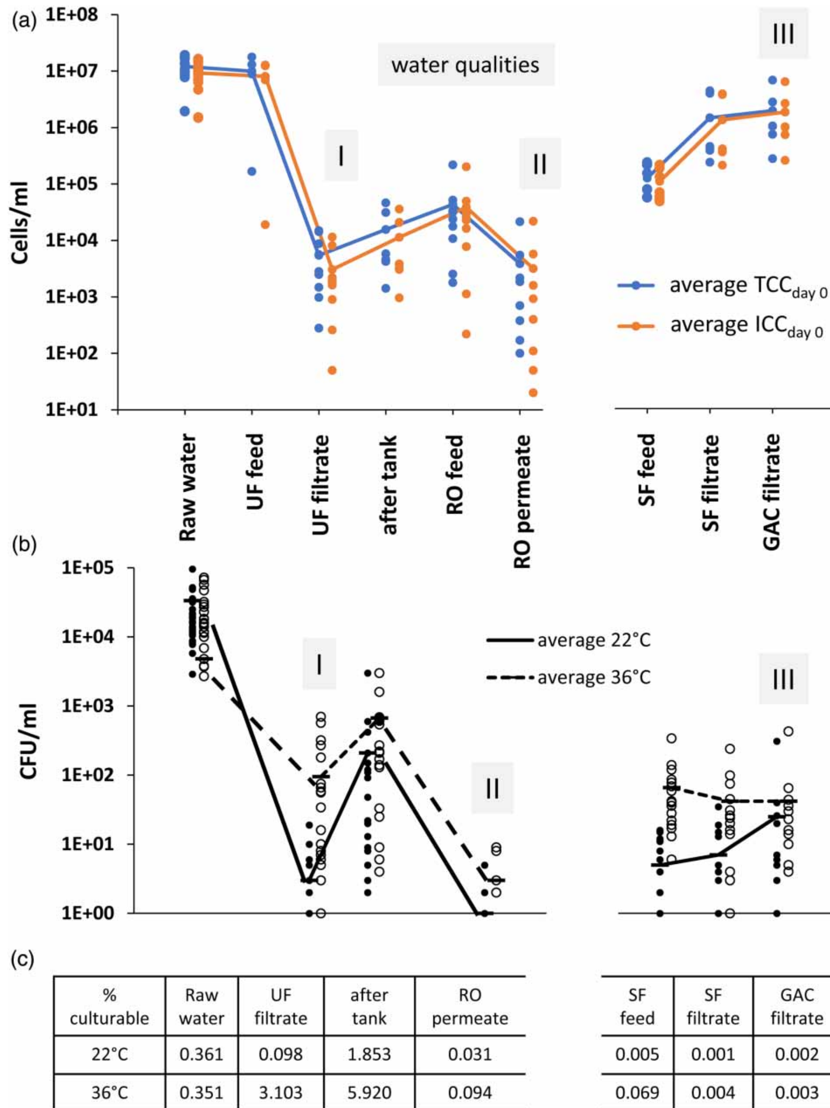

% culturable	Raw water	UF filtrate	after tank	RO permeate	SF feed	SF filtrate	GAC filtrate
22°C	0.361	0.098	1.853	0.031	0.005	0.001	0.002
36°C	0.351	3.103	5.920	0.094	0.069	0.004	0.003

Figure 2 | Changes of (a) total cells, (b) culturable bacteria and (c) proportion of culturable bacteria at different steps of water treatment. (a) Total and intact cell concentrations at the time point of sampling (day 0) as determined by flow cytometry. (b) Colony counts at 22 and 36 °C as determined by cultivation. Samples were transported cold to the laboratory and analyzed within 24 h so that the concentrations reflect the microbiological *status quo* of day 0. Individual dots show individual sampling events, lines combine arithmetic mean values. The line is interrupted as the sand filter is fed with UF filtrate. (c) Average relative proportions of culturable bacteria based on comparison of A and B. TCC, total cell concentrations; ICC, intact cell concentrations; CFU, colony-forming units; UF, ultrafiltration; RO, reverse osmosis; SF, quartz sand filter; GAC, granular activated carbon.

membrane used here was 0.02 μm. The observation that UF filtrates in our study were dominated by HNA bacteria made us exclude the possibility of a breach in membrane integrity and assign their presence to regrowth during water stagnation. This view was supported by the finding that the composition of the bacterial community in the filtrate was profoundly different from the one in the feed water (see section Changes of the microbiome along the treatment process). Bacterial concentrations in our study tended to be elevated when residence time in filtrate compartments was high or when the filtration process was intermittently interrupted leaving time for regrowth. This also applied to the intermittent presence of small numbers of bacteria in the RO permeate, which is in line with the growth potential on the permeate side reported in earlier studies (Park & Hu 2010). As for UF, we hypothesize the bacteria that colonize the surfaces of the filtrate or permeate compartment to be the source of bacteria in the bulk water.

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Changes of the microbiome along the treatment process

Changes in microbial community composition were monitored using Illumina sequencing of 16S rRNA gene amplicons. Great microbial diversity (Shannon index: $H' = 5.96$; Simpson index: $D = 0.99$) was observed for the raw water with an observed richness of 1,068 unique OTUs belonging to 34 different detected bacterial phyla (Figure 3(a)). Diversity ($H' = 2.23$; $D = 0.77$) and observed richness (186 OTUs) were strongly reduced by UF. The resulting bacterial community was very distinct from the raw water and suggested *Pseudomonas* to be among the dominant genera (Supplementary Figure S1). As members of *Pseudomonas* spp. are well known to be sessile and to form biofilms (Tolker-Nielsen *et al.* 2000), the result might reflect enrichment of the filtered and thus bacteria-stripped water by new bacteria attached to the surfaces on

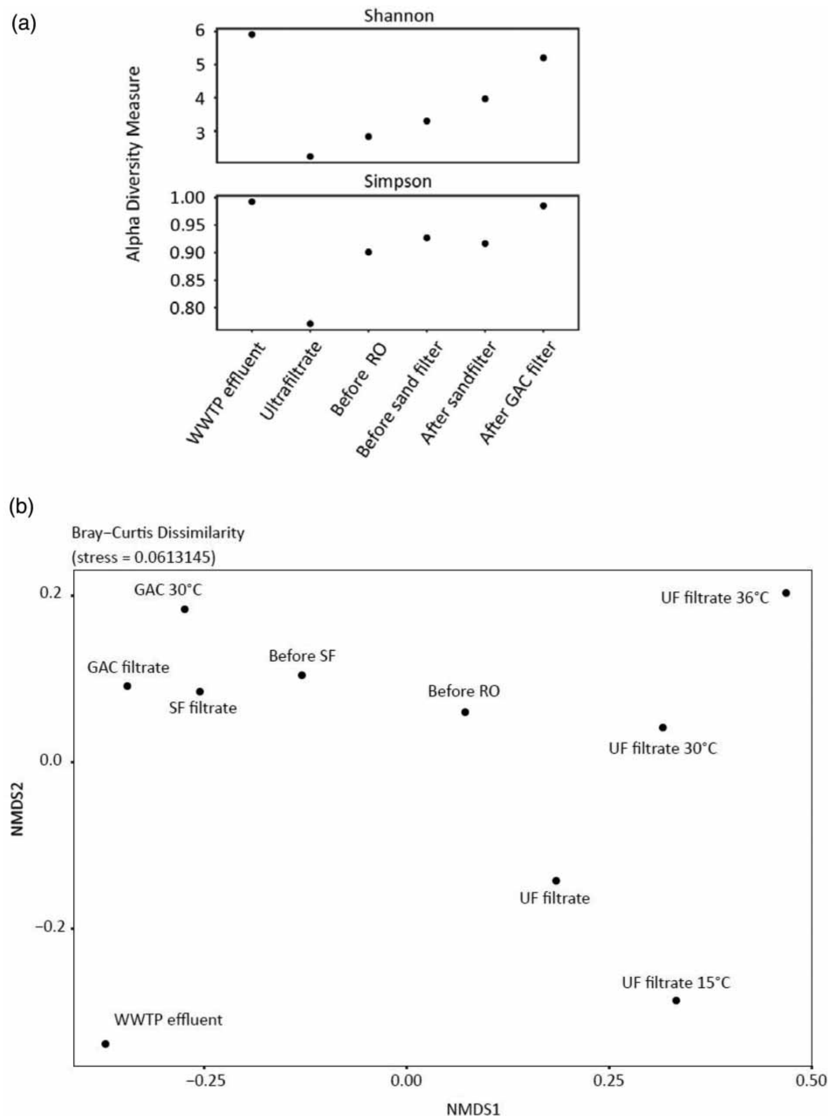

Figure 3 | Relative changes of the bacterial microbiome along the treatment process based on 16S rRNA gene sequencing by Illumina. (a) Change of Shannon and Simpson alpha diversity indices at different points of the treatment train. (b) Bray-Curtis dissimilarity index for water directly after different treatment steps and in part after regrowth. UF filtrate was incubated at 15, 30 or 36 °C for 7 days, GAC filtrate was incubated at 30 °C. No results were obtained for the RO permeate due to the very low numbers of bacteria and insufficient DNA concentrations obtained from this sample. GAC, granular activated carbon; RO, reverse osmosis; SF, quartz sand filtration; UF, ultrafiltration.

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the filtrate side. The bacterial community underwent further change within the UF filtrate tank and on its way to RO, consistent with a moderate increase in cell numbers (Figure 2). In case of the sand filter and GAC passage of UF filtrate, diversity and species richness (407 and 600 OTUs after sand filter and GAC, respectively) increased significantly in agreement with biological stabilization. Overall, the bacterial microbiome was shown to be highly dynamic and to undergo various substantial changes along the treatment train reflected in a Bray-Curtis dissimilarity plot (Figure 3(b); heat map shown in Supplementary Figure S1). The bacterial communities in water qualities I, II and III appeared highly distinct from the one of the raw water.

The bacterial community can undergo further change during stagnation as shown for UF filtrate incubated over 7 days at either 15, 30 or 37 °C. The temperature of incubation strongly influenced the community composition of stagnated UF filtrate (heat map shown in Supplementary Figure S2). Stagnation of GAC filtrate led to less dissimilarity probably because the original sample was closer to biological stability.

It has to be pointed out that sequence analysis was only performed on a single set of samples, so variations over the duration of the demonstration phase cannot be excluded.

Process monitoring by online flow cytometry

Online flow cytometry was used to monitor ICC in high resolution with data points generated every 2 h. ICC in August and September 2019 are shown as an example. In this time period, the effect of monochloramine was tested to suppress biofouling on the RO membrane. For this purpose, monochloramine was added to UF-1 filtrate (prior to the filtrate storage tank) to obtain a final concentration near 1 mg/L, whereas UF-2 filtrate did not receive disinfectant (Figure 4).

Monochloramine was initially dosed nearly continuously over 22 h/day. Dosing time was subsequently gradually reduced with the intention to define the necessary dosing time interval that still achieves a microbiological effect. ICC values were around 2 log units lower for UF-1 filtrate compared to UF-2 demonstrating the effect of monochloramine on the integrity of bacteria contained in the water. Reduction of dosing times to 19, 16 and 13 h/day still achieved a comparable disinfection effect whereas with 10 h/day a risk for rising bacterial concentrations was given. Further reduction of the dosing time to 7 h/day led to an increase in ICC values. As a moderate ICC increase was also seen for UF-2, part of the increase in ICC could also have been caused by changes in the raw water quality with the nutrient content of treated wastewater effluent typically being unknown. Independent of the precise cause, a monochloramine dosing time below 13 h/day was seen unable to suppress such scenarios. Overall, the data resolution of online flow cytometry and the ability to compare two parallel processes proved very useful for process control as it enabled to directly measure the biological consequences of changing process parameters or natural water quality alterations. Like for every flow cytometer, regular

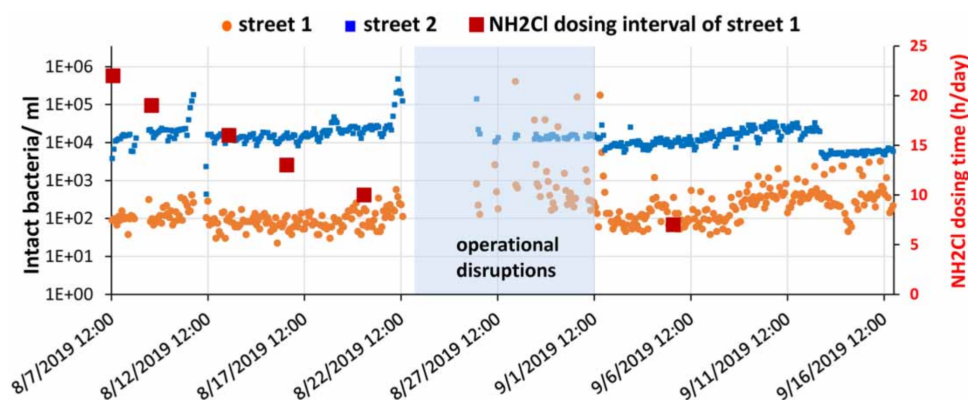

Figure 4 | Online flow cytometric monitoring of intact cell concentration in water after ultrafiltration in two parallel treatment trains. Water from street 1 received monochloramine for indicated dosing times, whereas water from street 2 was not subject to disinfection. Dosing time in street 1 commenced with 22 h/day and was gradually reduced in steps by 3–7 h/day. The shaded time period indicates operational disruptions.

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optimized clean intervals were necessary to prevent overestimation of bacteria due to internal fouling events and to ensure correctness of data. The greatly improved process control does, of course, not eliminate the need for traditional hygienic testing as flow cytometry results as applied here do not give information about the species composition of the water.

Regrowth potential after different treatment steps

Flow cytometric day 0 values of water samples reflecting the microbiological status at the time point of sampling were supplemented with so-called day 7 values. The latter was generated by re-measurement of samples that were stored for 7 days at 22 °C. The underlying idea was that in the case that AOC and other nutrients are present in the water, the autochthonous bacteria can propagate and recover to a cell concentration that is supported by the given AOC/nutrient level. Day 7 values should, thus, represent the full regrowth potential of the water (Figure 5) and are shown together with average TCC and ICC values measured at day 0 (reproduced from identical average values from Figure 2).

As often observed for raw waters, bacterial $TCC_{day\ 7}$ and $ICC_{day\ 7}$ concentrations in treated WWTP effluent moderately declined over the incubation time of 7 days. This decline owes itself to the nearly complete conversion of LNA into HNA bacteria. As the total carbon pool remains identical, it supports less HNA bacteria (which tend to be bigger in size) than the total number of LNA and HNA bacteria that were present in the original sample. In other words, the pool of small LNA bacteria converts into a smaller number of bigger HNA bacteria. The same effect could be seen in UF feed samples that were nearly identical with raw water.

Strong deviations between day 0 and day 7 cell numbers were observed in the subsequent treatment train. Whereas flow cytometry of day 0 samples showed a strong reduction of bacterial concentrations following UF and RO filtration (Figure 2(a) and reproduced in Figure 5), the decline was much less pronounced in stagnated day 7 samples (Figure 5). Regrowth was not surprising as the downstream filter compartments were not sterile. Compared to raw water

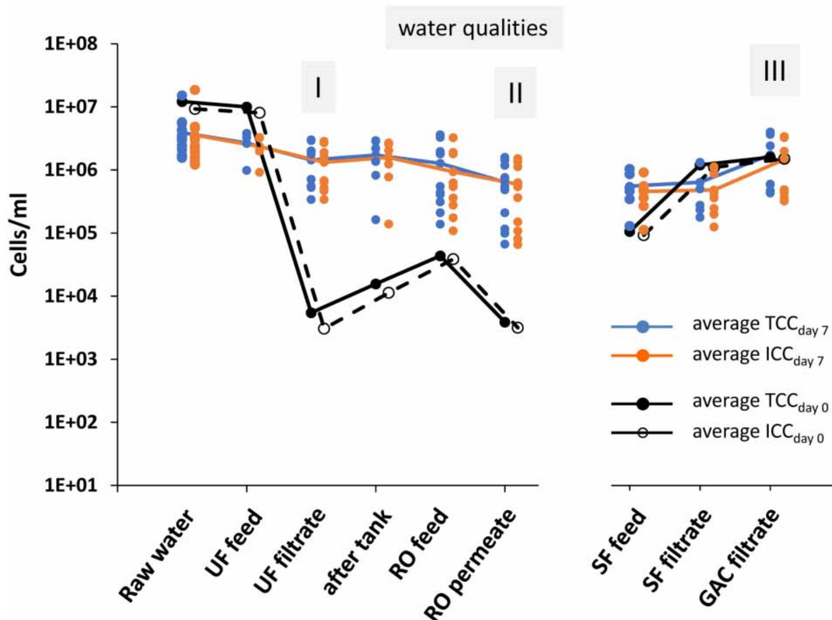

Figure 5 | Change of (a) total and intact cell concentrations after 7 days of incubation at 22 °C over the treatment train. $TCC_{day\ 7}$ and $ICC_{day\ 7}$ values were determined by flow cytometry and represent the maximal total and intact cell concentrations that are supported by the nutrients contained in the corresponding water. Individual dots show individual sampling events, lines combine arithmetic mean values. The line is interrupted as the sand filter is fed with UF filtrate. Average $TCC_{day\ 0}$ and $ICC_{day\ 0}$ values (identical with Figure 2) are shown for easy comparison of day 7 and day 0 values. TCC, total cell concentrations; ICC, intact cell concentrations; UF, ultrafiltration; RO, reverse osmosis; SF, quartz sand filter; GAC, granular activated carbon.

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(stagnated under the same conditions), intact cells ($ICC_{day\ 7}$) only declined by 0.43 and 0.63 log units in regrown UF filtrate and regrown RO permeate, respectively.

Strong regrowth as seen for UF filtrate and RO permeate can imply the risk of excessive biofilm formation, biofouling, clogging of tubes and nozzles (Prest *et al.* 2016). An in-line candle cartridge pre-filter installed to reduce fouling potential of the subsequent RO membrane needed to be exchanged regularly due to visible biofilm formation and increased filter resistance (data not shown). During the time when monochloramine was dosed, the filter installed in street 2 (that did not receive disinfectant) was much more prone to biofouling and needed to be exchanged more often than the filter in street 1 that was dosed with monochloramine. Interestingly, reducing disinfectant dosing time to 10 h and especially to 7 h shortened the lifetime of the cartridge filter, which was in line with online flow cytometry results.

Comparison of $ICC_{day\ 7}$ values with traditional AOC

As bacterial concentrations are limited by AOC, the data suggest AOC removal or regrowth potential removal in the given ranges. DOC in comparison was on average reduced by approximately 17% (10.7 mg/L) and 96% (<0.5 mg/L) after UF and RO, respectively, compared to the average DOC concentration in raw water (12.8 mg/L). To address the comparability of the $ICC_{day\ 7}$ values with traditional AOC assessment (van der Kooij 1992), both parameters were compared for a selected set of identical samples on one particular day. As the two parameters have different scales, their changes relative to the values from raw water (100%) are shown as percentages together with the absolute values (Table 2). Whereas UF led to a reduction of both parameters in samples taken prior to a storage tank, values increase in samples after that tank. As the water after UF was supplemented with chloramine at the time point when samples were taken and as tank surfaces with biofilm can enrich the water with nutrients, an increase in regrowth potential (after neutralization of monochloramine) along tank passage was conceivable. In RO permeate, traditional AOC was negligible with 0.08 μg ac-C eq/L, whereas $ICC_{day\ 7}$ values were still 6% of those in stagnated raw water. Both parameters increased after GAC passage.

Despite the very low AOC value in RO permeate, the sample contained sufficient nutrients to support regrowth to a concentration of 1.1×10^5 intact bacteria/ml corresponding to approximately 6% relative to the $ICC_{day\ 7}$ value of raw water on that day. This example illustrates that traditional AOC assessment has a detection limit. Whereas the parameter is useful at measurable concentrations, AOC values near zero on a linear scale can still translate to a substantial number of bacteria on a log scale. Even under extremely oligotrophic conditions bacteria can reach concentrations in the range of 10^5–10^6 cells/ml (Egli 2010). It has to be acknowledged in this context that the traditional AOC assay is optimized for potable water. To improve the assay's suitability for reuse applications, the two bacteria *Pseudomonas fluorescens* P17 and *Spirillum* sp. NOX forming the basis of the drinking

Table 2 | Comparison of ICC values obtained after 7 days of incubation at 22 °C ($ICC_{day\ 7}$) and AOC values obtained with the traditional method according to van der Kooij (1992)

	$ICC_{day\ 7}$		AOC (van der Kooij)		DOC*
	Intact bacteria/ml	Value relative to raw water (%)	μg ac-C eq/L	Value relative to raw water (%)	mg/L
Raw water	1.9×10^6		358		12.8
UF filtrate prior tank	1.5×10^6	79	174	49	10.7
UF filtrate after tank	2.7×10^6	142	213	60	n.d.
RO permeate	1.1×10^5	6	0	0	<0.5
GAC filtrate	5.0×10^5	26	180	50	8.7

*The given DOC values represent average values over the entire demonstration phase and give an estimate over the change along the treatment process. They cannot be directly compared to the indicated $ICC_{day\ 7}$ values or AOC that were determined on a specific day for a direct comparison of those two parameters.

Changes of absolute values over the treatment process are shown for one specific sampling day together with relative changes in comparison with the raw water on that day. Average DOC values during the entire demonstration phase are shown as a reference.

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water assay have been supplemented by Zhao *et al.* (2013) with three additional strains together with other modifications such as increased inoculums densities and higher incubation temperatures (25 °C). Although this modified AOC assay has not been applied in our study, the required long starvation times and the fact that even strains with the ability to biodegrade a relatively wide range of organic compounds still have a limited metabolic spectrum (that might not be universally adequate for every reclaimed water at every treatment step) make us consider the presented ICC_{day7} approach as a good alternative. Ideally, the size of the bacteria might be considered in the future as the carbon footprint depends on the size. The $ICC_{day\ 7}$ values represent the maximal bacterial concentrations supported by the given AOC. The metabolic spectrum of the complex autochthonous bacterial population can be assumed to be well adapted to the specific water type. Representation of the regrowth potential as an $ICC_{day\ 7}$ value on a log scale ensures high sensitivity in a range that is not covered by the traditional AOC assay due to its relatively high detection limit. The example shows that a traditional AOC value near 0 μg ac-C eq/L could theoretically translate into any ICC over 5 log units. In other words, a log scale showing actual bacterial concentrations provides more sensitivity in the low nutrient range than a linear scale showing traditional AOC.

Nutrient sources and regrowth control

Whereas the presence of AOC required to support regrowth was not surprising for UF filtrate as nutrients are not retained by UF, the finding of regrowth potential in RO permeate with the $ICC_{day\ 7}$ value in the range between 10^5 and 10^6 cells/ml raised the question about the origin of the nutrients. Average DOC levels <0.5 mg/L in RO permeate were comparable with RO-treated drinking water (Buysschaert *et al.* 2018). Passage of cationic and neutral hydrophilic organic molecules of small size had been reported especially under low flux conditions (Albergamo *et al.* 2019). Another possible source of nutrients might be migration or leaching of assimilable nutrients from polymeric pipe materials into permeate water (Wen *et al.* 2015). To control possible regrowth, the addition of a disinfectant and maintaining an appropriate residual concentration has been proposed (Farhat *et al.* 2018). In our study, regrowth of the autochthonous bacterial flora in UF filtrate could be suppressed for at least 5 days and for ≥14 days by addition of 1 or 2 mg/L free chlorine, respectively (data not shown). If UF filtrate was further processed by RO, monochloramine was preferred due to its lesser reactivity. When dosed at approximately 1 mg/L into UF filtrate, monochloramine in RO feed was around 0.1 mg/L which did not incur measurable damage to the RO membrane. The membrane was found to be partially permeable for monochloramine with remaining residual being able to suppress regrowth for 7 days.

Overall, the necessity for regrowth control with a residual has to be decided on a case-to-case level as bacterial concentrations around 10^6 cells/ml are not uncommon also in stagnated potable water in house plumbing systems (data not shown). Typically, no problems are associated with such systems. Where regrowth control does, however, become relevant is if hygienically relevant bacteria can multiply.

Hygienic challenges

Concerns related to pathogen growth in reclaimed water have been expressed in earlier studies (Jjemba *et al.* 2010; Weinrich *et al.* 2010). Reclaimed water from treated effluent was reported to be susceptible to regrowth of opportunistic pathogens like *Aeromonas*, *Legionella*, *Mycobacterium* and *Pseudomonas* (Jjemba *et al.* 2010) adding to a relatively high biofilm formation potential and problems from smell and color (Oesterholt *et al.* 2007). The major culprit for these problems is seen in high concentrations of biodegradable organic carbon as the source of regrowth (Weinrich *et al.* 2010), but also water after extensive treatment was susceptible. Learbuch *et al.* (2019) demonstrated growth potential of *Legionella pneumophila* (concomitant with biofilm growth) in remineralized RO permeate as a result of PE or PVC-P materials used in the permeate compartment. Also in our study, growth of *L. pneumophila* artificially introduced in low numbers into aliquoted UF filtrate and RO permeate was obtained, although not on a reproducible basis. Uncontrolled day-to-day variations in the nutrient content and composition released from the WWTP, presence and absence of biofilm particles or amoeba and other factors might have played a role. The possible regrowth

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potential of pathogens in different water qualities will need further investigation but warrants the addition of disinfectant with a residual for safety reasons.

Another way to minimize the risk for pathogen regrowth might be obtained by biological stabilization. Least regrowth was obtained after biological stabilization of water (Figure 5) as water passage through the sand and GAC filter resulted in bacterial enrichment with a highly diverse population. It remains to be addressed whether the small regrowth potential applies also to pathogens. GAC filters with an established microflora were shown to have a 'protective' effect in regard to filter colonization by bacterial pathogens such as *Salmonella typhimurium*, *Yersinia enterocolitica* and enterotoxigenic *E. coli*, whereas sterile GAC filters became rapidly colonized by these pathogens (Camper *et al.* 1985). In other words, competition with the indigenous microbial community can be antagonistic to the growth of pathogens in the water. For drinking water treatment, biological stabilization has been proposed to shape the bacterial community composition, which is subsequently only moderately modified during water distribution (Pinto *et al.* 2012; Prest *et al.* 2016). This is in line with our finding that stagnation of GAC filtrate resulted in less dissimilarity than stagnation of UF filtrate (Figure 3(b)).

CONCLUSIONS

Microbiological monitoring of the given treatment process demonstrated an efficient removal of hygienically relevant bacteria with ultrafiltration being an efficient microbiological barrier. The latter predestined UF as a critical control point for high-resolution process monitoring by online flow cytometry. The bacterial microbiome was exchanged multiple times with the most profound changes induced by membrane filtration. Despite profound changes in bacterial concentrations along with treatment, the reduction was not sustainable as the regrowth potential (as determined by offline flow cytometry) was only reduced moderately despite a strong reduction of DOC. Potential growth of pathogens is seen as the greatest hygienic risk and might warrant a disinfectant residual if water is at risk to stagnate for extended times.

ACKNOWLEDGEMENTS

Kathrin Wiede and Dietmar Pütz from IWW are gratefully acknowledged for sample preparation and technical analysis as well as for help in data analysis. We extend our thanks to Mrs Katharine Mende from De.EnCon GmbH for engineering assistance, a number of staff members and students from OOWV who were involved in operating the pilot plant and other members of the consortium for critical discussions. We are further very grateful to the German Ministry of Education and Research (BMBF) that financed this study as part of the MULTI-ReUse (Modular Water Treatment and Monitoring for Wastewater Reuse, 2016–2019) project under grant no. 02WAV1403A ('Future-oriented Technologies and Concepts to Increase Water Availability by Water Reuse and Desalination').

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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First received 11 March 2020; accepted in revised form 20 June 2020. Available online 31 August 2020

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