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Multi-parametric assessment of biological stability of drinking water produced from groundwater: Reverse osmosis vs. conventional treatment

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ABSTRACT

Although water produced by reverse osmosis (RO) filtration has low bacterial growth potential (BGP), post-treatment of RO permeate, which is necessary prior to distribution and human consumption, needs to be examined because of the potential re-introduction of nutrients/contaminants. In this study, drinking water produced from anaerobic groundwater by RO and post-treatment (ion exchange, calcite contactors, and aeration) was compared with that produced by conventional treatment comprising (dry) sand filtration, pellet softening, rapid sand filtration, activated carbon filtration, and UV disinfection. The multi-parametric assessment of biological stability included bacterial quantification, nutrient concentration and composition as well as bacterial community composition and diversity. Results showed that RO permeate remineralised in the laboratory has an extremely low BGP ($50 \pm 12 \times 10^3$ ICC/mL), which increased to $130 \pm 10 \times 10^3$ ICC/mL after site post-treatment. Despite the negative impact of post-treatment, the BGP of the finished RO-treated water was >75% lower than that of conventionally treated water. Organic carbon limited bacterial growth in both RO-treated and conventionally treated waters. The increased BGP in RO-treated water was caused by the re-introduction of nutrients during post-treatment. Similarly, OTUs introduced during post-treatment, assigned to the phyla of Proteobacteria and Bacteroidetes (75–85%), were not present in the source groundwater. Conversely, conventionally treated water shared some OTUs with the source groundwater. It is clear that RO-based treatment achieved an extremely low BGP, which can be further improved by optimising post-treatment, such as using high purity calcite. The multi-parametric approach adopted in this study can offer insights into growth characteristics including limiting nutrients (why) and dominating genera growing (who), which is essential to manage microbiological water quality in water treatment and distribution systems.

1. Introduction

Water utilities are aiming at producing biologically safe and stable drinking water, i.e., water that does not promote excessive bacterial growth during distribution. To achieve that, source water undergoes different treatment processes to remove bacteria and growth-promoting nutrients. The multi-barrier treatment approach is especially needed in the Netherlands, where chlo-

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rine is neither used for primary disinfection nor for maintaining disinfectant residual in the distribution network. Groundwater accounts for about two-thirds of total drinking water in the Netherlands (van der Kooij and Veenendaal, 2014; Vewin, 2017), and typically contains high concentrations of methane and inorganic compounds (Prest et al., 2016b). Groundwater is conventionally treated by aeration, rapid sand filtration, activated carbon filtration, and sometimes UV disinfection before pumping the water into distribution systems (de Vet et al., 2010; van der Kooij et al., 2017). Though drinking water of high quality is produced and supplied in the Netherlands, the assimilable organic carbon (AOC) may occasionally be higher than the biological stability guideline of 10 µg-C/L (van der Wielen and van der Kooij, 2010). To ensure the best microbiological water quality (AOC ~1 µg-C/L), Oasen drinking water utility is constructing a treatment plant based on reverse osmosis (RO) filtration, which can efficiently reduce cell count, AOC, and bacterial growth potential (BGP) (Escobar et al., 2000; Park and Hu, 2010; Dixon et al., 2012; Thayanukul et al., 2013). One of the clear advantages of applying RO in drinking water treatment is that it can achieve a significant reduction in biofilm formation compared to conventional treatment, which controls the growth of *L. pneumophila* to a large extent (Learbuch et al., 2019). To comply with drinking water regulations, post-treatment of RO permeate is necessary for remineralisation (e.g., calcium and magnesium), maintaining chemical stability, and improving taste (Vingerhoeds et al., 2016). However, the post-treatment steps are likely to re-contaminate RO permeate by introducing organic and inorganic components, such as the observed increase in BGP caused by the re-introduction of growth-promoting nutrients in the permeate (Sousi et al., 2018). Until now, the potential negative effects of post-treatment on biological stability and its control strategies are still poorly documented. Several methodologies and protocols have been developed for the assessment of drinking water biological stability, including measuring the nutrients as AOC and biodegradable organic carbon (BDOC), and enumerating bacteria as BGP and biofilm formation potential (BFP). Recently, bacterial community profiling has been introduced as a complementary tool, which elevates the assessment of biological stability to include both quantity and community aspects. Lautenschlager et al. (2013) and Prest et al. (2014) applied a multi-parametric approach that combined flow cytometric cell counting and 16S rRNA sequencing, which examined different aspects of microbiological water quality and captured the changes that cannot be reflected by single parameter studies. As the type and composition of nutrients are important for both bacterial growth and shaping the bacterial community (Elhadidy et al., 2016; Nescerecka et al., 2018), including a detailed analysis of carbon, phosphate, and nitrogen fractions in the multi-parametric approach is necessary to understand the driving force for bacterial growth. Such an integral multi-parametric approach will be especially powerful for comparing treatment plant performance, and/or diagnosing problems related to bacterial growth in drinking water.

The objective of this study was to conduct a multi-parametric comparison of biological stability of drinking water produced from anaerobic groundwater by RO-based treatment (RO filtration and post-treatment) and conventional treatment. The integral comparison includes the changes in bacterial quantification and growth potential, nutrient concentration and composition, and the bacterial community composition and diversity across steps in the different treatment lines, based on which the treatment performance was evaluated and recommendations were given on optimising the quality of RO-treated drinking water.

2. Materials and methods

2.1. Water samples

The Kamerik drinking water treatment plant (Oasen Drinking Water Company, Gouda, Netherlands) currently produces 340 m^3/h of drinking water from anaerobic groundwater (AGW) by conventional water treatment processes, which are given in Fig. 1A in the following order: spray aeration on the surface of rapid sand filters (so-called dry sand filtration, DSF), tower aeration, pellet softening (SOF), carry-over submerged rapid sand filtration (RSF), granular activated carbon filtration (ACF), and UV disinfection (UVD) before storing the conventionally treated water (CTW) in the clean water reservoir. Installed in parallel for research purposes, a pilot-scale advanced treatment line with a capacity of 7 m^3/h treats the same source water with the following processes (Fig. 1B): anaerobic RO filtration (RO) with a total recovery of 75%, followed by post-treatment comprising anaerobic ion exchange (IEX) to remove residual ammonium, remineralisation using anaerobic calcite contactors (CC) to correct the calcium and bicarbonate concentrations to the required level (40 mg/L Ca^{2+}, 122 mg/L HCO_3^-), magnesium dosing ($MgCl_2$, 4 mg/L Mg^{2+}), and tower aeration for the introduction of oxygen and the removal of methane and excess carbon dioxide. The finished drinking water after RO filtration and all post-treatment processes is denoted as site-Remin and has a final pH of 7.8 ± 0.2. The full treatment details are given in Table S1 (supplementary information). Water samples were collected after each treatment step in both the conventional and RO-based treatment lines. The main sampling campaign was conducted in March 2019 where all the analyses mentioned in the following section were performed. An additional campaign was conducted in May 2019 where only intact cell count (ICC) and ATP analyses were performed.

2.2. The multi-parametric comparison components

2.2.1. Intact cell count (ICC) and adenosine triphosphate (ATP)

Intact cell count (ICC) was measured using flow cytometry (BD Accuri C6® FCM, BD Biosciences, Belgium) coupled with DNA staining as previously described by Prest et al. (2016a). In short, each sample (500 µL per replicate) was stained with 5 µL of a mix of SYBR® Green I and propidium iodide stains and heated for 10 min before FCM measurement using similar settings and gating. The FCM detection limit is 10^3 ICC/mL, which was determined using ultrapure water (Milli-Q® water, Merck Millipore). Moreover, the size of bacteria was classified between large and small using high and low nucleic acid (HNA and LNA) characteristics obtained by the FCM (Wang et al., 2009).

Microbial intra-cellular ATP was measured according to the filtration-based method described by Abushaban et al. (2019) using the Water-Glo testing kit (lysis reagent and detection reagent) and GloMax®-20/20 Luminometer (Promega Corp., USA). The detection limit is 0.1 ng ATP/L.

2.2.2. Bacterial growth potential (BGP)

BGP of water was measured according to Sousi et al. (2018). In short, samples were taken in AOC-free glassware, pre-treated by pasteurisation (70 °C for 30 min) to remove indigenous bacteria, inoculated with a natural bacterial consortium (~10^4 ICC/mL) originating from CTW freshly collected at each sampling campaign, distributed into three individual AOC-free vials (i.e., triplicate measurements per sample), incubated in the dark at 30 °C, and lastly measured for ICC over a growth period of 3 weeks, commonly on

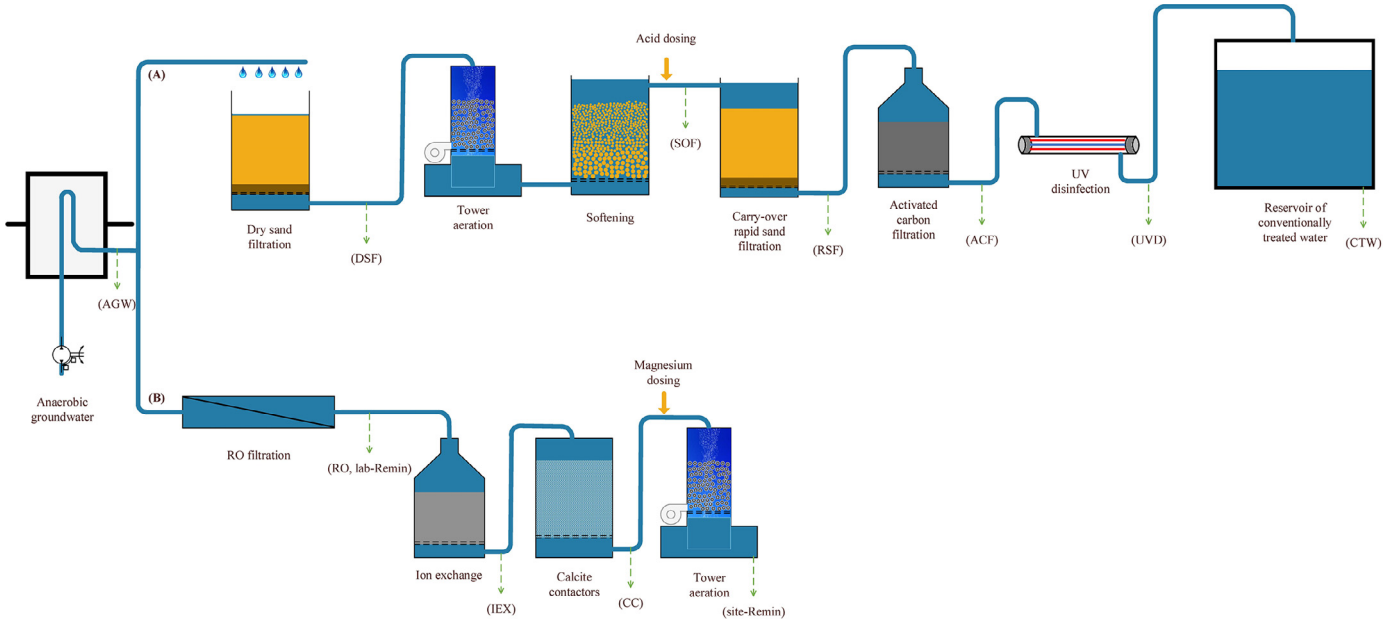

Fig. 1. Full-scale conventional (A) and pilot-scale RO-based (B) water treatment lines at the drinking water treatment plant. Sampling locations (dashed arrows) and codes (between brackets) are indicated.

day 0, 1, 3, 6, 8, 10, 13, 16, and 20. The results were expressed as the maximum cell count obtained during the incubation period (BGP_{max}). The blank was prepared by adjusting the mineral content of RO permeate at the laboratory using chemical stock solutions with high purity: $NaHCO_3$ (pH of 7.8 ± 0.2, 122 mg/L HCO_3^-), $CaCl_2$ (40 mg/L Ca^{2+}), and $MgCl_2$ (4 mg/L Mg^{2+}). The blank (laboratory-remineralised RO permeate) is denoted as lab-Remin, and was spiked with KH_2PO_4 (5 μg/L PO_4-P) and KNO_3 (50 μg-N/L) to ensure that carbon was the growth-limiting nutrient, unless otherwise mentioned. Ion exchange effluent samples were also remineralised with the same concentration of $NaHCO_3$, $CaCl_2$, and $MgCl_2$ to ensure that bacterial growth is not limited by minerals. It is worthwhile to mention that the BGP of anaerobic groundwater (AGW) might be underestimated because of the very high nutrient content (e.g., phosphate and humic substances), which might have formed complexes (e.g., with iron) when oxygen was introduced during the test, and thus, limiting the bioavailability of those nutrients.

2.2.3. Bacterial growth-promoting nutrients

Each water sample was analysed for organic carbon (C) and phosphate (PO_4-P) concentrations. Liquid chromatography – organic carbon detection (LC-OCD) analysis was performed at Het Waterlaboratorium (Haarlem, Netherlands) to measure the concentration of carbon fractions as described by Huber et al. (2011). The carbon fractions were distinguished based on their molecular weight (MW), and they are (from largest to smallest): biopolymers (proteins and polysaccharides), humic substances, building blocks, low molecular weight acids, and neutrals. The reporting limit of LC-OCD analysis is 100 μg-C/L for biopolymers and 200 μg-C/L for the other fractions.

Phosphate was measured at the Rijkswaterstaat laboratory (Lelystad, Netherlands) using the ascorbic acid method. The method implied the reaction of ammonium molybdate and antimony sodium tartrate with orthophosphate (PO_4^{3-}) in an acidic medium to form a complex that was reduced to a blue-coloured compound by ascorbic acid. The blue colour intensity was measured within 30 min by spectrophotometry (880 nm), and then converted to a phosphorus concentration using a calibration line. The detection limit was 0.3 µg/L PO_4-P.

Table 1
BGP test matrix to identify the bacterial growth-limiting nutrient in water samples.

Test #	C ($C_2H_3NaO_2$)	P (KH_2PO_4)	N (KNO_3)	TE*	Investigation
1	–	–	–	–	Actual BGP
2	–	+	+	+	C-limited BGP
3	+	–	+	+	P-limited BGP
4	+	+	–	+	N-limited BGP
5	+	+	+	–	TE-limited BGP
6	+	+	+	+	Positive control

* Trace elements including Co, B, S, Mn, Zn, and Fe.

To identify bacterial growth-limiting nutrients, BGP of water samples was measured with the addition of different combinations of nutrients as previously described by Prest et al. (2016a), and shown in Table 1. The used nutrient stocks were carbon (1.07 g/L $C_2H_3NaO_2$), phosphate (0.219 g/L KH_2PO_4), nitrogen (3.61 g/L KNO_3), and a broth of trace elements (5 mg/L $CoCl_2.6H_2O$, 10 mg/L H_3BO_3, 10 mg/L $CaSO_4.5H_2O$, 500 mg/L $MnSO_4.7H_2O$, 10 mg/L $ZnSO_4.7H_2O$, 300 mg/L $FeSO_4.7H_2O$). Nutrients were added according to the ratio of C:N:P =100:10:1 (Hammes and Egli, 2005). The blank (lab-Remin) and samples of the finished drinking water produced by the RO-based and conventional treatment lines (site-Remin and CTW, respectively) were tested.

2.2.4. DNA extraction, sequencing, and data processing

Genomic DNA extraction was performed as previously described by Liu et al. (2018). In short, water samples (500 mL, duplicate) were filtered using 0.22 µm cellulose ester membranes (GPWP04700, Millipore, Ireland), and afterwards pre-treated using FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH, USA) as demonstrated in the instruction manual. Thereafter, 16S rRNA gene of the extracted genomic DNA was amplified, targeting the V3–V4 regions (a primer set of 341F: 5'-CCTACGGGNGGCWGCAG-3' and 785R: 5'-GACTACHVGGGTATCTAATCC-3'). Illumina MiSeq platform (300 bp paired reads) was used for sequencing at BaseClear (Leiden, Netherlands), where the Illumina sequencing adaptors were appended to the 5' end. The sequencing data is available in the NCBI database (BioProject ID: PRJNA631515) and information about the sample origin is given in Table S2.

The sequences generated from the Illumina Miseq analysis of the 16S rRNA gene amplicons were processed (i.e., filtered, clustered, and taxonomically assigned and aligned) using the Quantitative Insights Into Microbial Ecology (QIIME2, v2018.6) pipeline with the default settings. Raw sequences were first processed using DADA2, including quality filtering, denoising, paired-end sequence merging, and chimera filtering. DADA2 generated unique amplicon sequence variants that were equivalent to 100% similarity operational taxonomic units (OTUs) in the conventional practice. In this publication, the term OTU is used for the purpose of simplicity. Taxonomy was assigned using q2-feature-classifier, customized for the primer set used in this study with Silva SSU database release 132. Multiple sequence alignment and phylogenetic tree construction were performed using the QIIME2 plugin q2-phylogeny. Alpha and beta diversity analyses were performed using the QIIME2 plugin q2-diversity. Weighted and unweighted UniFrac distance matrices were constructed from the phylogenetic tree (Liu et al., 2020). OTUs with relative abundance in the samples of >0.5% were considered dominant. The absolute abundance of selected OTUs was calculated by multiplying their relative abundance by total cell count (TCC) obtained using FCM as proposed by Props et al. (2017).

2.3. Statistical analysis

The significance level of observed differences between samples was examined using Student's *t*-test and one-way analysis of variance (ANOVA) test after affirming the data normality (Q-Q plots, Chi-squared tests, and Kolmogorov-Smirnov tests). Canonical correspondence analysis (CCA) was carried out using XLSTAT (version 2019.4.2.63762) to investigate the influence of nutrients (LC-OCD fractions and phosphate) on microbiological parameters and community composition.

Principal coordinates analysis (PCoA) was conducted to visualise (dis)similarities of DNA sequences based on weighted UniFrac distance matrix, where the significance of (dis)similarities has been tested using permutational multivariate analysis of variance (PERMANOVA).

3. Results

3.1. Intact cell count (ICC) and ATP concentration across treatment lines

The trends in intact cell count (ICC) and ATP concentration across the conventional and RO-based treatment lines from two sampling campaigns are shown in Fig. 2 and Fig. S1, where similar trends were observed except for concentrations in the source anaerobic groundwater (AGW). ICC and ATP concentration in the source anaerobic groundwater (AGW) were 215 ± 40 × 10^3 ICC/mL and ~0.5 ng ATP/L (Fig. 2), which increased by a factor 3 and 28, respectively, after dry sand filtration (DSF) and softening (SOF) in the conventional treatment line where oxygen was introduced in the water. Thereafter, ICC decreased along the following conventional treatment units reaching 390 ± 11 × 10^3 ICC/mL in the finished conventionally treated drinking water (CTW). This decrease in ICC was accompanied with a considerable decrease (~70%) in ATP to the range of 4–5 ng ATP/L after RSF and until the end of the conventional treatment (CTW).

In the RO-based treatment line, the ICC after RO filtration and ion exchange were lower than the FCM detection limit (<10^3 ICC/mL, >99.6% removal), which increased to 30 ± 5 × 10^3 ICC/mL after calcite contactors (CC) and further to 90 ± 1 × 10^3 ICC/mL after tower aeration (site-Remin). Similarly, ATP dropped below the detection limit after RO filtration (<0.1 ng ATP/L) and remained at this level after ion exchange (IEX). Thereafter, it increased after post-treatment with CC to 2 ng ATP/L and further to 7.5 ng ATP/L in the finished RO-treated water after tower aeration (site-Remin).

The percentage of high nucleic acid bacteria (HNA bacteria) within ICC decreased across the conventional treatment from 60% after DSF and SOF to 40–45% after RSF and until CTW. However, this percentage increased across the RO-based treatment and reached up to 85% in CC and site-Remin.

A good correlation was found between HNA bacteria and ATP (Fig. S2, R^2 = 0.77, RO-based treatment; R^2 = 0.65, conventional treatment). Remarkably, ICC in the finished RO-treated water was >75% lower than that of conventionally treated water, whereas the contrary was observed for ATP, which resulted in a significantly higher (P < 0.05) ATP per cell value for RO-treated water than conventionally treated water (average of two sampling campaigns; 9.07 × 10^{-17} vs. 1.71 × 10^{-17} g ATP/cell, Table S3), indicating different community composition between the two treatment lines.

3.2. BGP and nutrient composition of conventionally treated and RO-treated water

The profiling of the two treatment lines showed considerably different degrees of BGP and nutrient removal. The conventional treatment line reduced the BGP by ~60% (from 1,250 ± 100 × 10^3 in AGW to 450–550 × 10^3 ICC/mL across the different treatment steps), where the BGP of conventionally treated water (CTW) was 515 ± 5 × 10^3 ICC/mL (Fig. 3A). Meanwhile, DOC decreased from 7.2 mg/L in AGW to 6.0 mg/L in CTW (Table 2). Notably, the humic substances, which accounted for >70% of DOC in AGW, showed the highest removal in the conventional treatment line (from 5.2 mg/L to 4.3 mg/L). Phosphate was also considerably reduced, mainly during DSF (>98%, from 553 µg/L PO_4-P in AGW to 7 µg/L PO_4-P in DSF), reaching down to 1 µg/L PO_4-P in CTW (Table 2). Similarly, ammonium was also reduced below 0.02 mg/L NH_4-N (limit of detection) by the conventional treatment (Table 2). The results showed that nitrification was the main mechanism for ammonium removal, where ammonium (NH_4^+) in AGW (2.90 ± 0.10 mg/L NH_4-N) was completely converted into nitrate (NO_3^-) in CTW (2.77 ± 0.40 mg/L NO_3-N). Methane, which was present at 2,000–4,000 µg-CH_4/L in AGW, was reduced to 10–20 µg-CH_4/L in CTW.

The RO-based treatment showed a substantial BGP reduction (>96%) from ~1,250 ± 100 × 10^3 ICC/mL in AGW to ~50 ± 12 × 10^3 ICC/mL in lab-Remin (i.e., RO permeate after remineralisation at the laboratory, Fig. 3B). However, the BGP increased by 160% after remineralisation using calcite contactors (CC) and tower aeration (site-Remin), reaching 130 ± 10 × 10^3 ICC/mL. The LC-OCD analysis revealed that all organic matter fractions were considerably retained by RO filtration to levels below the reporting limit (Table 2). Despite the increase in BGP after post-treatment, there was no detectable increase in any DOC fraction by the LC-OCD. For phosphate, a sharp decrease from 553 µg/L PO_4-P to 1 µg/L PO_4-P was observed after RO filtration, followed by an increase across the post-treatment to 7 µg/L PO_4-P (Table 2). In contrast to conventional treatment, nitrification was insignificant within the RO-based treatment line, where ammonium in AGW was mostly retained by the RO membrane (0.17 ± 0.02 mg/L NH_4-N in RO permeate), and was further removed by absorption in ion exchange resins (<0.02 mg/L NH_4-N). This resulted in a low concentration of nitrate in RO-treated water (0.23 ± 0.05 mg/L NH_4-N) (Table 2). Methane in RO-treated water was at similar concentrations as in CTW (Table 2).

The investigation of the growth-limiting nutrient (Fig. 4) revealed that the growth in the examined water types was limited by organic carbon. For all samples, the difference between the actual BGP (i.e., without nutrient addition to the sample) and the C-limited BGP (i.e., samples spiked with all nutrients except for carbon) was insignificant (P > 0.05). Contrarily, the BGP of sam-

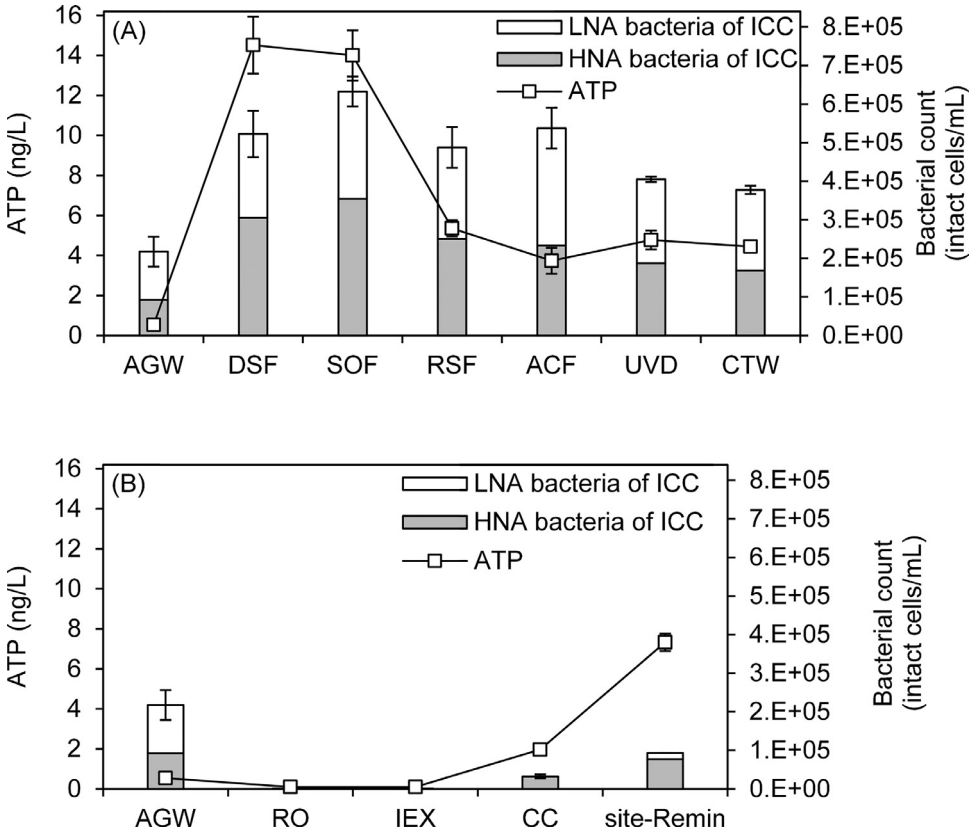

Fig. 2. ATP concentration, HNA bacterial count, LNA bacterial count, and intact cell count (HNA + LNA) of water samples after each treatment step in the conventional (A) and RO-based (B) treatment lines. Error bars represent the standard deviation of triplicate measurements.

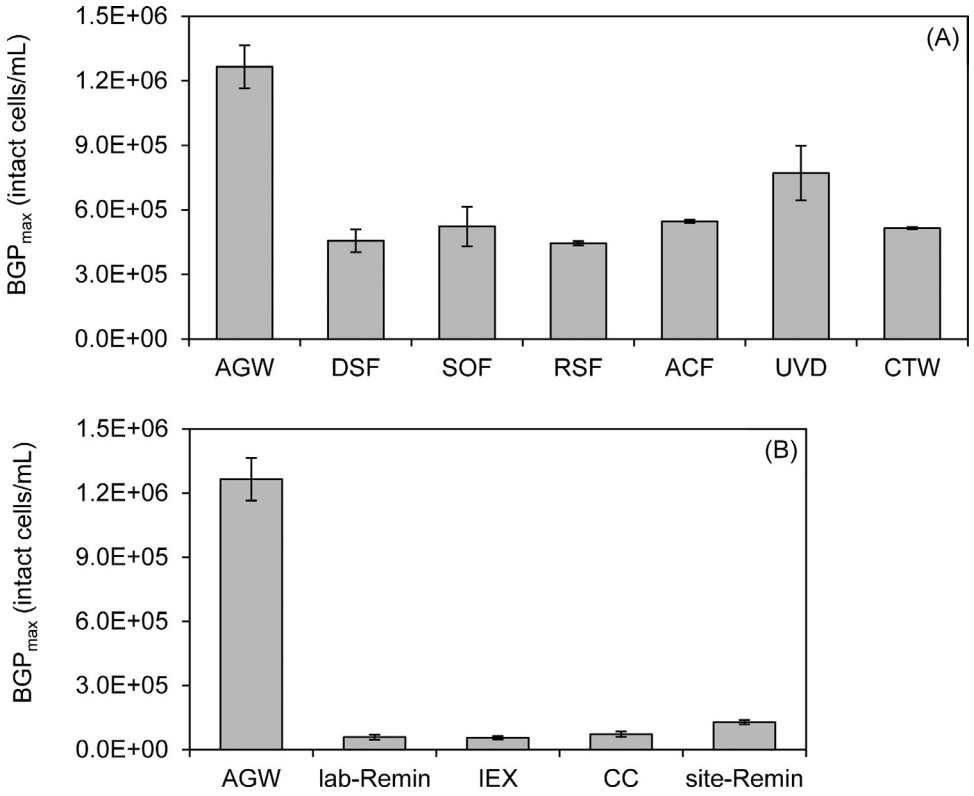

Fig. 3. Bacterial growth potential (BGP) at each step of the conventional (A) and RO-based (B) treatment lines. BGPs of RO permeate and ion exchange effluent were measured after remineralisation at the laboratory (i.e., lab-Remin and IEX respectively). Error bars represent the standard deviation of triplicate measurements.

Table 2
The concentration of carbon (LC-OCD fractions), phosphate, nitrogen (ammonium, nitrite, and nitrate), and methane at each step of the conventional and RO-based treatment lines.

Treatment	Sample type	Carbon (µg-C/L)[a]						Nitrogen (mg-N/L)[b]			Methane (µg-CH_4/L) [c]	Phosphate (µg/L PO_4-P)[d]
		DOC	Bio-polymers	Humic Substances	Building Blocks	Neutrals	Acids	Ammonium (NH_4^+)	Nitrite (NO_2^-)	Nitrate (NO_3^-)		
Source water	AGW	7,242	3	5,202	1,110	869	0	2.90 ± 0.10	<0.003	<0.23	2,000–4,000	553 ± 17
Conventional line	DSF	7,237	16	5,170	1,095	809	0	0.82 ± 0.15	0.022 ± 0.004	n.a.	n.a.	6.8 ± 0.6
	RSF	6,636	8	4,610	1,151	801	0	<0.02	<0.003	n.a.	n.a.	1.1 ± 0.1
	ACF	6,105	6	4,486	1,027	652	0	n.a.	n.a.	n.a.	n.a.	0.7 ± 0.1
	CTW	5,987	10	4,323	994	636	0	<0.02	<0.003	2.77 ± 0.40	10–20	1.1 ± 0.1
RO-based line	RO	36	4	0	2	12	3	0.17 ± 0.02	n.a.	n.a.	n.a.	0.9 ± 0.1
	IEX	32	0	0	1	66	2	<0.02	n.a.	n.a.	n.a.	0.9 ± 0.1
	CC	20	3	0	5	7	2	<0.02	n.a.	n.a.	n.a.	7.0 ± 0.5
	site-Remin	27	13	0	6	22	3	<0.02	<0.003	0.23 ± 0.05	<5–14	7.3 ± 0.1

n.a., not measuredGraphical.
[a] The reporting limit is 100 µg-C/L for biopolymers and 200 µg-C/L for the other LC-OCD fractions.
[b] Limit of detection: 0.02 mg-N/L for ammonium, 0.003 mg-N/L for nitrite, and 0.23 mg-N/L for nitrate.
[c] Limit of detection: 5 µg-CH_4/L.
[d] Limit of detection is 0.3 µg/L PO_4-P. The concentrations after SOF and UVD are 3.8 ± 0.1 and 1.1+0.1, respectively.

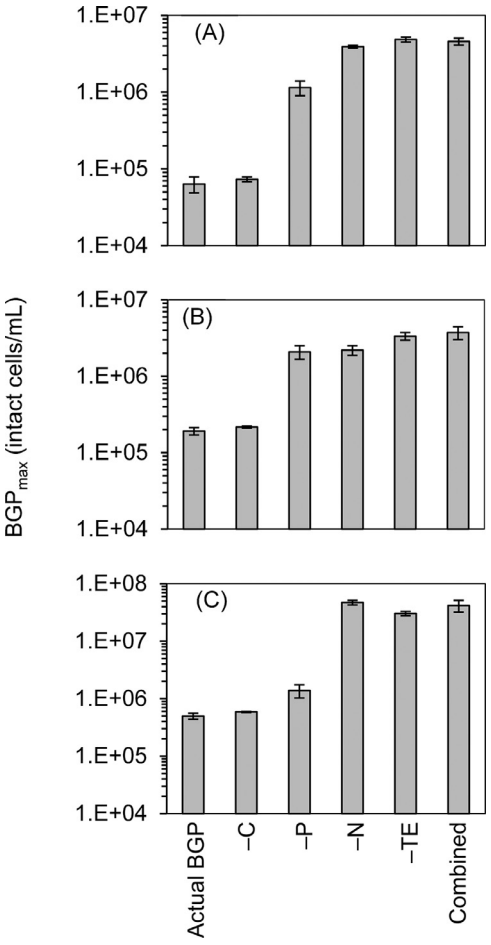

Fig. 4. BGP of lab-Remin (the blank, A), site-Remin (B), and CTW (C) with the addition of different nutrients as given in Table 1 (Lower nutrient concentrations were added in the case of lab-Remin and site-Remin). Acutal BGP: no nutrients added, –C: no carbon added, –P: no phosphate added, –N: no nitrogen added, –TE: no trace elements added, and Combined limitation: all nutrients added. Error bars represent the standard deviation of triplicate measurements.

ples with limited phosphate, nitrogen, and trace elements (Fe, Mn, Zn, Co, and B) was significantly ($P < 0.05$) higher than the actual BGP of the corresponding sample. Interestingly, the P-limited BGP of site-Remin was 50% higher than that of CTW and lab-Remin.
Bacterial community

In total, 29526 sequences were generated for 22 samples, which were assigned to 295 bacterial genera. The rarefaction curve became plateaued after 1750 and 650 sequences were retrieved for conventional and RO-based treatment lines, respectively, indicating that sufficient sample coverage was obtained (Fig. S3). Library size per sample is given in Table S7, which showed that few sequences were obtained for RO-treated water due to the too low DNA content in the sample. The average sequences per sample for the entire dataset was 1400, which was low compared to other recent studies.

Beta diversity. The similarity in the communities obtained for the duplicate samples reflected high reproducibility and reliability (Fig. 5, PCoA plot) of the present study. Moreover, the plot shows that the treatment shaped the composition of bacterial communities, where the observed differences between the three clusters were significant as affirmed using PERMANOVA ($P < 0.05$, Table S4). The bacterial community of source water (AGW) were clearly distinguished from that of treated water, especially for the RO-based treatment. For the conventional treatment, the bacterial communities shifted across the steps, where water after DSF, SOF,

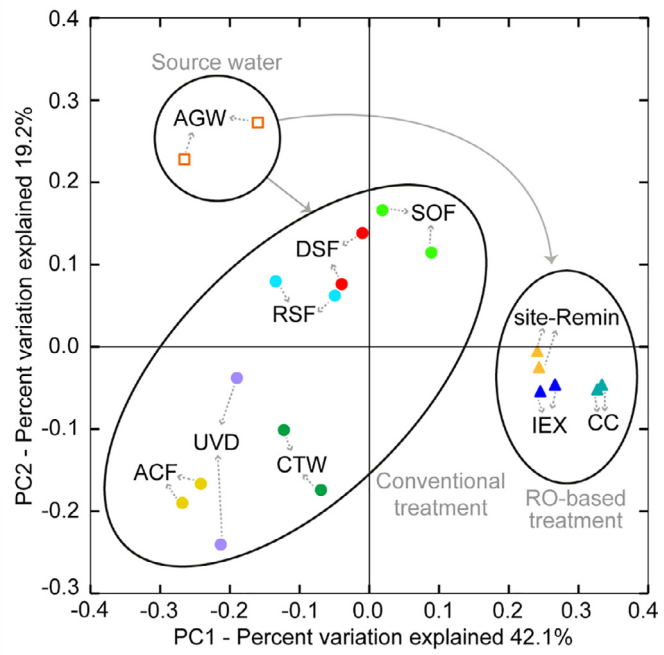

Fig. 5. PCoA plot of weighted UniFrac distances for samples collected from source water (AGW), conventional treatment line (DSF, SOF, RSF, ACF, UVD, and CTW), and RO-based treatment line (IEX, CC, and site-Remin). DNA sequences of RO permeate were too few.

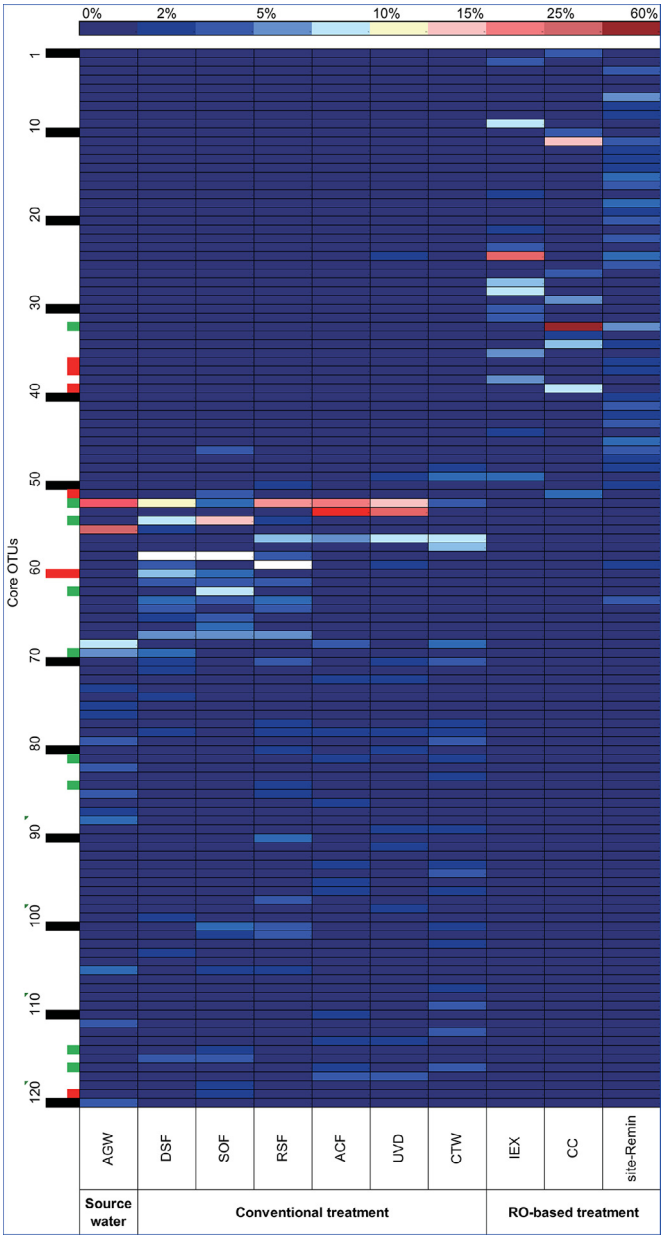

Fig. 6. Heatmap showing the dominant OTUs (genus level) and their relative abundance (average of duplicate) in water samples. DNA sequences of RO permeate were too few. The full taxonomy information of the dominant OTUs is given in Table S5. Black marks on the *y*-axis represent the OTU number given, red marks represent methanotrophs, and green marks represent nitrifiers. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

and RSF contained similar communities, but different from those detected after ACF, UVD, and CTW. For the RO-based treatment, the communities of post-treated RO permeate were closely clustered, indicating a high similarity among the samples after IEX, CC, and site-Remin.

Alpha diversity. Looking into the dominant OTUs (genus level, relative abundance >0.5%) among all water samples, there were 197 OTUs assigned to 120 genera as shown in the heatmap (Fig. 6, Table S5 for taxonomy information). The Venn diagrams (Fig. S4) show that no dominant OTUs were shared between source (anaerobic) groundwater (AGW; 15 dominant OTUs) and water after any steps in the RO-based treatment line (IEX, CC, and site-Remin; 14, 10, and 33 dominant OTUs, respectively). Conversely, there were three dominant OTUs shared between AGW and CTW (23 dominant OTUs), which were assigned to the class of Deltaproteobacteria, Parcubacteria, and Omnitrophicaeota, whereas the only OTU shared between CTW and site-Remin was assigned to the family of Oligoflexaceae.

Community composition. The column plots at phylum, class, and order levels are presented in Fig. S5. At phylum level, Proteobacteria was significantly present in all samples irrespective of the treatment, where the relative abundance ranged from 10% in source water (AGW) to 20–50% after conventional treatment and 55–80% in water after RO-based treatment. Patescibacteria was more abundant in conventionally treated water (20–60%) than RO-treated water (<5%). Omnitrophicaeota and Nitrospirae were dominant in source water only (AGW, ~30%), where their relative abundance decreased to 1–19% after conventional treatment, and below 0.5% after RO-based treatment.

At genus level, OTUs belonging to families characterised as methanotrophs (methane-oxidising bacteria) were detected in both treatment lines after steps where oxygen was introduced (Fig. 6): 4 OTUs for conventional treatment exclusively after DSF and SOF, 4 OTUs for RO-based treatment exclusively after CC and site-Remin, among which 1 OTU was shared by all (Table S6). Interestingly, all 8 OTUs were assigned to type I methanotrophs that belong to Methylomonaceae, including Methylovulum spp. (1%), Methylobacter spp. (6%), Methyloglobulus spp. (2%), Crenothrix spp. (2–3%), and other uncultured genera (2–5%). OTUs belonging to the families which characterised as nitrifiers were specifically found in the water after the first steps of conventional treatment (i.e., DSF, SOF, and RSF, Fig. 6) with different relative abundances. The OTUs were assigned to ammonium-oxidising bacteria (AOB, e.g., Nitrosomonas 6%) and nitrite-oxidising bacteria (NOB, e.g., Candidatus Nitrotoga 1% and Nitrospira 2–10%). Conversely, none of them were found in RO-treated water except for a poorly documented OTU, namely, GOUTA6 belonging to AOB of Nitrosomonadaceae (Table S6). The absolute abundance of methanotrophs and nitrifiers after each treatment step is shown in Fig. S6. The methanotrophs

ranged from 3–70 × 103 ICC/mL, and the nitrifiers ranged from 6–165 × 103 ICC/mL, both of which were more abundant in both treatment lines after oxygen was introduced.

There were several genera present in RO-treated water known for iron, manganese, and sulfur oxidation such as Sulfuricurvum (12%), Ferritrophicum (5%), Leptothrix (5%), Geobacter (3%), and Geothrix (2%). Similarly, OTUs belonging to families of characterised iron-oxidisers (e.g., the Family Gallionellaceae), were found in the conventional treatment line.

3.3. Correlation between nutrients and microbiological parameters

The influence of nutrients (LC-OCD fractions and phosphate) on microbiological parameters (ICC, ATP, and BGP) and community composition (OTUs with relative abundance >0.5%) was investigated using CCA. The results revealed that bacterial growth either within the treatment units (ICC and ATP) or at the laboratory (BGP) was strongly correlated to the concentration of DOC fractions, more specifically building blocks, humic substances, and neutrals, rather than phosphate (Fig. S7A). The growth was not influenced by biopolymers and acids because their concentrations were below the reporting limit for all water samples. Regarding community composition (Fig. S7B), about 50% of the dominant OTUs found in samples were strongly correlated to the concentration of building blocks, humic substances, and neutrals (group 1), whereas 23% were fairly well correlated to phosphate (group 2). However, no clear trend was observed with respect to which OTUs could grow on each nutrient fraction.

4. Discussion

The multi-parametric comparison approach applied in this study enabled better understanding of the microbiological water quality at different stages of the treatment. This approach included measuring several microbiological parameters and studying the relationship between them. The parameters included are the dynamics of bacterial load measured as numbers (cell count), bacterial activity (ATP concentration), and composition (16S rRNA gene sequencing) across treatment, as well as the growth potential of these bacteria in water (BGP) and the driving factors for this growth (nutrients). This multi-parametric approach was used to compare conventionally and RO-treated drinking water as discussed below.

4.1. Characterisations of bacterial cells: ICC, ATP and ATP per cell

As quantified by cell count and ATP concentration, bacteria were better retained by the RO membrane (>99.6% removal), where the values in RO permeate were below the detection limit (<10^3 ICC/mL; <0.1 ng ATP/L). This complies with the fact that RO membrane is an effective barrier for microorganisms (Madaeni, 1999; Pype et al., 2016). However, this superior RO permeate quality could be influenced by the post-treatment processes (Sousi et al., 2018), which was also observed in the present study as increased ICC and ATP after the pilot-scale remineralisation (calcite contactors) and tower aeration units. The deterioration of biological water quality could be attributed to practical factors, such as bacteria sloughing off the surface biofilm of calcite grains and/or packing material filling the aeration tower.

Interestingly, it is noticed that the increase in ATP was particularly correlated to the increased percentage of HNA bacteria within ICC for both RO-treated water (R^2 = 0.77) and conventional treatment (R^2 = 0.65), which agrees with previous findings (Siebel et al., 2008; Liu et al., 2013) and can be attributed to the high activity and large cell size of HNA bacteria (Lebaron et al., 2001; Proctor et al., 2018). The changes in HNA bacteria and ATP across the treatment lines can be explained by the nature of treatment applied. For instance, the highest removal of HNA bacteria by the conventional treatment line (~30%) occurred within the rapid sand filters, where physical retention of larger bacterial cells is more efficient (Vital et al., 2012; Fujioka et al., 2019) and significant ATP reduction could be obtained (Liu et al., 2013).

Regarding the calculated ATP per cell, the obtained values (ranging from 0.70–11.32 × 10^{-17} g ATP/cell) were in line with previous findings of Hammes et al. (2010) for different aquatic environments (average; 1.75 × 10^{-10} nmol/cell equivalent to 8.89 × 10^{-17} g ATP/cell), but lower than reported values for bacteria growing under starvation condition (up to 30 × 10^{-17} g ATP/cell) (Webster et al., 1985). The latter value was obtained for specific bacterial species and might not be applicable to others, and these values were calculated based on heterotrophic plate count, which would significantly underestimate the cell count and result in higher ATP per cell values (van Nevel et al., 2017). Comparing the two treatment lines in the present study, the ATP per cell of the post-treated RO permeate was >5 times higher than that of conventionally treated water (9.07 × 10^{-17} vs. 1.71 × 10^{-17} g ATP/cell). This could be partially explained by the percentage of large and active cells (i.e., HNA bacteria) in each water type (>85% vs. ~40–45%). Nonetheless, the low ATP per cell for the conventionally treated water was in line with previously reported values for water after granular activated carbon filtration (Magic-Knezev and van der Kooij, 2004). Moreover, the composition of bacterial communities present in water could influence ATP per cell values (Eydal and Pedersen, 2007). As confirmed by the bacterial community analysis, members of the Patescibacteria, which (remarkably) pass 0.2 µm and even 0.1 µm filters (Herrmann et al., 2019), were more abundant in conventionally treated water (20–60%) than in post treated RO permeate (<5%). On the other hand, members of Proteobacteria and Bacteroidetes, that are generally known for large cell size based on FCM findings (Proctor et al., 2018), accounted for 75–85% of bacterial communities in RO-treated water.

4.2. BGP and the factors driving bacterial growth (nutrients)

The present study also confirmed the effectiveness of RO filtration in controlling the BGP of water because of its high efficiency of nutrient removal (>97% carbon, >99.5% phosphate), which complies with previous studies (Jacobson et al., 2009; Park and Hu, 2010; Thayanukul et al., 2013). Though the post RO treatment caused an increase in BGP, the BGP of RO-treated water was still lower than that of conventionally treated water by a factor of 4. As reported in a recent study with the same water, ~10 times lower biofilm formation potential (BFP) was found for RO-based treatment compared to that of conventionally treated water (Learbuch et al., 2019), indicating even higher efficiency in controlling biofilm formation by RO filtration and possibly different growth dynamics between planktonic bacteria and biofilm.

The increased BGP after post-treatment can be attributed to the introduction of growth-promoting nutrients during those processes, as confirmed by phosphate measurement which increased more than 7 times (from <1 to 7 µg/L PO_4-P) after calcite contactors. As demonstrated by the nutrient limitation tests, the P-limited BGP of site post-treated RO permeate was higher than that of lab post-treated RO permeate and conventionally treated water (2,089 × 10^3 vs. 1,144 × 10^3 vs. 1,383 × 10^3 ICC/mL), confirming again the higher phosphate concentration in site post-treated RO permeate (compared to lab-Remin and CTW) introduced by calcite contactors. Though the introduction of carbon was not detectable using LC-OCD analysis since the concentrations both before and after the post-treatment were below the reporting limit, it was confirmed by the nutrient limitation tests, where carbon was the

growth-limiting nutrient before and after the post-treatment regardless of the BGP value. The carbon limitation has been commonly found in many types of water (Huck, 1990; Hu et al., 1999; van der Kooij, 2000; Liu et al., 2015; Prest et al., 2016a), as observed also for water types tested in this study even when the phosphate concentration was very low (<1 µg/L PO_4-P). Moreover, the carbon limitation in all samples was confirmed with the canonical correspondence analysis (CCA) without identifying which carbon fraction was the most relevant for growth, where a previous study showed that these bacteria could grow on carbon with different molecular characteristics ranging from readily available to more complex organic carbon (Sousi et al., 2020). Similarly, the link between the presence of different OTUs and carbon fractions needs to be further explored.

4.3. Shifts in bacterial communities

The present study showed that both the diversity and composition of bacterial communities were largely influenced by water treatment processes, which complies with previous studies on different drinking water treatment processes (Ivone et al., 2013; Liao et al., 2015; Li et al., 2017; Liu et al., 2018). Since RO filtration could effectively remove bacteria present in source water, the developed bacterial community in post-treated RO permeate was completely different from the source. This can be used as a control for the observations in the conventional treatment scheme. For example, RO filtration completely removed the dominant phyla (i.e., Nitrospirae and Omnitrophicaeota) from source anaerobic groundwater. However, only the strictly anaerobic members of the Nitrospirae, e.g., *Thermodesulfovibrionia* (Garrity et al., 2001), were effectively removed in the very first conventional treatment step (i.e., DSF). Similarly, the relative abundance of the Omnitrophicaeota decreased across the conventional treatment line because most of the species within this phylum were anaerobic, which could not survive in oxygen-rich fresh water (Rivas-Marín and Devos, 2018).

Moreover, it is interesting to observe the shifts in methane-oxidising bacteria (MOB), ammonium-oxidising bacteria (AOB) and nitrite-oxidising bacteria (NOB) across the treatment lines. The high concentration of CH_4 (2–4 mg/L) in source groundwater and the introduction of oxygen during aeration contributed to the presence of MOB in both conventional and RO-based treatment schemes (Nicol et al., 2003; DiSpirito et al., 2016). Only type I methanotrophs were detected (e.g., *Methylovulum* spp., *Methylobacter* spp., and *Methyloglobulus* spp.), which is typical for drinking water (Lipponen et al., 2004). The presence of only type I methanotrophs indicates that the residence time of methane within the aeration towers was short, as those bacteria are known to rapidly oxidise methane in an oxygen-rich environment (Graham et al., 1993; Hanson and Hanson, 1996). Since methane is the sole carbon source for these bacteria, their presence in the DSF and SOF treatment steps indicates that methane was considerably removed in these treatment units, resulting in ~20 µg/L CH_4 in the following conventional treatment steps. The nitrification activity within the conventional treatment line was clearly higher than that in the RO-based line. For example, the AOB (e.g., *Nitrosomonas* spp.) and NOB (e.g., *Candidatus Nitrotoga* spp., *Nitrospira* spp.) (Prosser et al., 2014; Koch et al., 2015; Kitzinger et al., 2018) were detected in conventional treatment units (DFS, SOF, and RSF), but not in the RO-based scheme. The same observation was confirmed by the complete oxidation of ammonium present in source groundwater (2.90 ± 0.10 mg-N/L) to nitrate in the conventionally treated water (2.77 ± 0.40 mg-N/L) (Table 2). Whereas the nitrification activity within the RO-based treatment scheme was minimal, where AOB and NOB were absent because the ammonium was physically retained by RO membrane (to 0.17 mg-N/L), and further absorbed by post ion exchange (<0.02 mg-N/L) without conversion to nitrate. Lastly, the presence of several bacterial genera in RO-treated water that are known to be involved in the oxidation of iron, manganese, and sulfur, e.g., *Sulfuricurvum, Ferritrophicum, Leptothrix, Geobacter*, and *Geothrix* (Hedrich et al., 2011; Schmidt et al., 2014), indicates that these metals were re-introduced during post-treatment. Nonetheless, the presence of such genera in conventionally treated anaerobic groundwater (e.g., the Family Gallionellaceae) has been previously reported (Hallbeck and Pedersen, 2014).

4.4. Practical insights for managing microbiological water quality

Despite the influences of post-treatment on the quality of RO permeate, the finished RO-treated water had much lower cell count and BGP compared with conventionally treated water. To mitigate the negative influences of post-treatment, it is recommended to use high quality calcite grains for remineralisation to prevent the introduction of organic and inorganic nutrients. Furthermore, aeration towers should be maintained and cleaned more frequently to reduce bacterial growth on the packing material. It should be noted that the current study was conducted with groundwater and the sampling period did not cover the whole seasons. The observations might be different for surface water systems subjected to significant seasonal variations, for which a sampling program including different seasons is high recommended. Though the effect of seasonal variations on the analysed parameters was beyond the focus of this study, negative influences of post-treatment were previously demonstrated throughout the year (Sousi et al., 2020).

The multi-parametric evaluation discussed above offered an integral understanding of the stability and changes in microbiological water quality between two treatment lines considering the bacterial quantity and activity, nutrient concentration and composition as well as bacterial community composition and diversity. Beyond the simple number of cells and/or bioactivity, the comprehensive dataset obtained in this study allows in-depth assessment of which bacteria are growing and why in each step of the treatment process. Besides regular monitoring, the methodology proposed is especially useful when water quality deteriorates during treatment and remedial actions are required to manage biological water quality. Although multi-parametric monitoring is expensive, it is recommended as a complementary approach when water quality deterioration is detected during regular monitoring.

For the microbiology of drinking water distribution system, attributed to its high efficiency in removing nutrients, the RO-based treatment will be useful in controlling the growth of both planktonic bacteria and biofilm. Regarding the microbes, the comparison between conventional and RO-based treatment demonstrated the possible quantitative and qualitative control of drinking water microbiology by using RO membranes as an absolute barrier for cells. Considering produced water is the seed for the microbes to thrive in water, biofilm, and loose deposits in the downstream drinking water distribution system (Liu et al., 2018), it could be a reasonable vision to manage the microbial ecology of drinking water distribution systems by introducing probiotic microbes, rather than microbial contamination, through post RO treatment to occupy the niches and form a predefined microbial ecology (Wang et al., 2013).

5. Conclusions

The following conclusions can be drawn based on this study:

- RO filtration significantly reduced the BGP of source groundwater to an extremely low level of 50 ± 12 × 103 ICC/mL in lab-remineralised RO permeate, which increased again to 130 ± 10 × 103 ICC/mL when RO permeate underwent site post-treatment.

- Despite this increase, the BGP of post-treated RO permeate was >75% lower than that of conventionally treated water (130 ± 10 × 103 vs. 450–550 × 103 ICC/mL).
- Carbon was the bacterial growth-limiting nutrient for both RO-treated and conventionally treated water. Phosphate did not limit bacterial growth even at very low concentrations (<1 µg/L PO4-P).
- The type of water treatment shaped the bacterial community of the finished treated water. Some genera were shared between source anaerobic groundwater and conventionally treated water, whereas the bacterial genera in post-treated RO permeate were mainly introduced during post-treatment.
- Beyond quantitative assessment, the multi-parametric approach suggested in this study is useful in understanding and managing microbiological water quality in drinking water treatment and distribution systems.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2020.116317.

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