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Further developing the bacterial growth potential method for ultra-pure drinking water produced by remineralization of reverse osmosis permeate

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ABSTRACT

Ensuring the biological stability of drinking water is essential for modern drinking water supply. To understand and manage the biological stability, it is critical that the bacterial growth in drinking water can be measured. Nowadays, advance treatment technologies, such as reverse osmosis (RO), are increasingly applied in drinking water purification where the produced water is characterized by low levels of nutrients and cell counts. The challenge is, therefore, how to measure the low bacterial growth potential (BGP) of such ultra-pure water using the available methods which were originally developed for conventionally treated drinking water. In this study, we proposed a protocol to assess BGP of ultra-pure drinking water produced by RO and post-treatment (including remineralization). Natural bacterial consortium from conventional drinking water was added to all water samples during this study to ensure the presence of a wide range of bacterial strains. The method development included developing an ultra-pure blank with high reproducibility to lower the detection limit of the BGP method ($50 \pm 20 \times 10^3$ intact cells/mL) compared with conventional blanks such as bottled spring water, deep groundwater treated by aeration and slow sand filtrate of surface water supply. The ultra-low blank consists of RO permeate after adjusting its pH and essential mineral content under controlled laboratory conditions to ensure carbon limitation. Regarding the test protocol, inoculum concentrations of $>10 \times 10^3$ intact cells/mL may have a significant contribution to the measured low levels of BGP. Pasteurization of water samples before measuring BGP is necessary to ensure reliable bacterial growth curves. The optimized method was used to assess BGP of ultra-pure drinking water produced by RO membranes and post-treatment (including remineralization), where the BGP has decreased more than 6-fold to a level of $90 \pm 20 \times 10^3$ intact cells/mL compared with conventionally treated water ($630 \pm 70 \times 10^3$ intact cells/mL).

1. Introduction

Bacterial (re)growth in drinking water supply systems may lead to problems, such as health threats by (opportunistic) pathogens, aesthetic deterioration of water taste and odour, bio-corrosion of pipes and fittings, and nitrification processes (Volk and LeChevallier, 1999; Berry et al., 2006). Currently, this unwanted bacterial growth is being managed either by maintaining disinfectant residuals which may react with organic compounds in drinking water and form hazardous disinfection by-products (DBPs) (Havelaar et al., 2000; Sadiq and Rodriguez, 2004; Gopal et al., 2007), or through producing biologically stable drinking

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water that contains low concentrations of readily available nutrients for bacterial growth (van der Kooij, 2000; Hammes et al., 2010; Liu et al., 2013b), e.g., assimilable organic carbon (AOC) below 10 μg ac-C eq/L (van der Kooij, 1992).

To understand the bacterial (re)growth during distribution and to develop efficient management strategies, it is essential to be able to measure and quantify this (re)growth. Until now, several batch tests have been developed to assess the potential of drinking water to support bacterial growth using different techniques to measure the increase in cell abundance. Those techniques include: turbidity (Withers and Drikas, 1998; Page et al., 2002), colony count (van der Kooij et al., 1982; Joret et al., 1991; van der Kooij, 1992; Sack et al., 2010, 2011), biomass volume (Servais et al., 1987), cell number (Hammes and Egli, 2005; Dixon et al., 2012; Park et al., 2016; Prest et al., 2016) and cell activity based on adenosine tri-phosphate (ATP) (van der Kooij and Veenendaal, 2001, 2014; van der Kooij et al., 2017). Employing flow cytometry (FCM) for cell count was evaluated and applied in BGP tests (Dixon et al., 2012; Prest et al., 2016) because of its advantages of being simple, rapid, reproducible and informative (Prest et al., 2013; van Nevel et al., 2017).

Regardless of the test type and the parameter to be measured, each conducted test to assess the potential of drinking water to support bacterial growth requires using a blank, ideally nutrient-free water, which defines the detection limit of the method to obtain reliable and reproducible results. Bottled spring water (Evian, France) has been widely investigated and used as a blank for BGP measurements due to its low nutrient content (Hammes and Egli, 2005; Vital et al., 2007; Bucheli-Witschel et al., 2012; Elhadidy et al., 2016; Prest et al., 2016; Farhat et al., 2018). In addition, two water types collected at water treatment plants in the Netherlands, namely deep groundwater treated by aeration and slow sand filtrate of surface water supply, have been used as blanks because of their low nutrient content (Bereschenko and Hornstra, 2014; Guo et al., 2014; Mikkers and Magic-Knezev, 2014; van der Kooij et al., 2014). Table 1 presents the characteristics of each blank.

Although the lowest reported BGP of those blanks is 100×10^3 cells/mL, they are still adequate and reliable blanks for drinking water produced by conventional treatment processes with BGP reaching levels of $300–700 \times 10^3$ cells/mL (Prest et al., 2016; Nescerecka et al., 2018). However, this becomes problematic if the BGP of treated drinking water decreases below 100×10^3 cells/mL as the measurements will no longer be reliable. Drinking water produced by reverse osmosis (RO) is an example of such water with considerably low BGP (Park and Hu, 2010; Dixon et al., 2012) due to the high efficiency of RO to retain bacterial cells (Madaeni, 1999; van der Bruggen and Vandecasteele, 2003; Park and Hu, 2010) and the AOC content (Escobar et al., 2000; Hong et al., 2005; Thayanukul et al., 2013). Being unable to measure the low levels of BGP with high reliability might limit our understanding of the bacteriological water quality of such ultra-pure water and factors affecting it. For instance, post-treatment of RO permeate including remineralization and aeration may have a significant impact on its original quality, which cannot be affirmed with conventional blanks. Therefore, it is highly necessary to lower the detection limit of the BGP method, by introducing an ultra-pure blank with a much lower BGP. This is especially true when looking at the increasing number of advanced treatment processes in water utilities, including RO treatment plants (Villacorte et al., 2015), in response to more stringent water quality regulations, advances in water treatment technologies, and increasing awareness of water quality deterioration during distribution (Liu et al., 2017). Moreover, there are other variations in the test conditions of current BGP methods, all of which may influence the measurement and detection limit of the method, such as (i) inactivation procedures of indigenous bacteria (e.g. pasteurization, filtration or no pre-treatment) and (ii) introduction of various inoculum types (if applicable). For instance, pasteurization is needed to inactivate natural bacteria before inoculating with pure bacterial strains for AOC measurements (van der Kooij et al., 1982; van der Kooij, 1992; Sack et al., 2010, 2011), whereas filtration is mainly practiced when total inactivation of natural bacteria is unnecessary, which is especially true when using a natural bacterial consortium for inoculation (Hammes and Egli, 2005; Farhat et al., 2018).

The main objective of the present study is to develop a protocol to assess the BGP of ultra-pure drinking water. The research focused on: (i) lowering the detection limit of the method by using RO permeate, after adjusting its chemical water quality at the laboratory, as a blank, (ii) assessing the need for pre-treatment (i.e., pasteurization) of water samples prior to measuring BGP, and (iii) evaluating the contribution of inoculation to the BGP measurements. Finally, the developed protocol was applied to evaluate the change in BGP of remineralized RO permeate from a pilot treatment system operated at a drinking water pumping station in the Netherlands.

2. Materials and methods

2.1. Conventional and RO-based water treatment

This study was conducted at one of the water treatment plants of Oasen Drinkwater (the Netherlands). Currently, the anaerobic groundwater is treated by conventional water treatment processes at a capacity of 340 m^3/h. The treatment comprises aeration, rapid sand filtration, softening, granular activated carbon (GAC) filtration, and UV disinfection. At the same location, a pilot-scale RO-based water treatment unit (7 m^3/h) is operated in parallel, which consists of anaerobic RO filtration (Hydranautics ESPA2-LD-4040) with 75% total recovery, followed by ion exchange (1.4 m^3 of LEWATIT S 2568 synthetic resin) for further removal of ammonium, remineralization using traditional calcite contactors for the addition of calcium (40 mg/L Ca^{2+}) and hydrogen carbonate (122 mg/L HCO_3^-), magnesium dosing (4 mg/L Mg^{2+}), and lastly tower aeration (packed with 1.24 m^3 of 38-8 plastic Raflux-Rings) for stripping out methane gas (CH_4) present naturally in the groundwater and excess carbon dioxide (CO_2) dosed before the calcite contactors for efficient dissolution of calcite grains. The term post-treatment used

Table 1
Characteristics of the conventional blanks used for biological stability assessment tests.

Water	pH (−)	DOC (mg/L)	AOC (μg ac-C eq/L)	BPP_{14} (d.ng ATP/L)	BGP ($\times 10^3$ cells/mL)
Bottled spring water (Evian, France)	~7.90	<0.2	10–52[a]	n.a.[c]	133 ± 18 (net growth)
Deep groundwater treated by aeration	~7.95	0.17–0.4	~1[b]	5–10	100 - 350 (max. growth)
Slow sand filtrate of surface water supply	~8.45	1.7–2.3	3–5[b]	30–40	250 - 600 (max. growth)

DOC: dissolved organic carbon; AOC: assimilable organic carbon; BPP: biomass production potential; BGP: bacterial growth potential.
[a] AOC (natural bacterial consortium) (Hammes and Egli, 2005).
[b] AOC (P17/NOX) (van der Kooij et al., 1982).
[c] n.a., not available.

during this study refers to all the treatment units after RO filtration, including ion exchange, remineralization and aeration. The final pH of the product water of both treatment schemes is 7.8 ± 0.2.

2.2. Water samples

Water samples, including conventionally treated groundwater (CTW), groundwater collected directly after RO filtration (RO permeate) and post-treated RO permeate at the site, i.e., RO permeate after passing ion exchange, remineralization and aeration (site-Remin), were collected at the water treatment location in the period between October 2016 and April 2017. In addition, four AOC-low waters were tested as blanks for the BGP measurements, including conventional blanks, i.e., bottled Evian water (BEW), deep groundwater (DGW) treated by aeration and slow sand filtrate (SSF) of surface water supply, and the ultra-pure blank proposed in this study, i.e., RO permeate after making the necessary chemical adjustments of pH and mineral content at the laboratory (as described in the following sections and referred to as lab-Remin). The total number of water samples collected during the study period was 69 including the site samples and blanks. A list of the 6 water types of the current study and their characteristics is given in the supplementary information (Table S1).

2.3. Experimental approach

The experimental approach is shown in Fig. 1. Firstly, the ultra-pure blank (lab-Remin) was compared with conventional blanks (BEW, DGW and SSF), specifically in terms of BGP. Secondly, the factors affecting BGP of the lab-Remin blank were studied, including the undesired addition of nutrients during chemical adjustments of the lab-Remin blank which was assessed by varying the concentrations of $NaHCO_3$, namely: 61, 122 (selected for this study), 183 and 244 mg/L HCO_3^-. The pH remained constant at 7.8 ± 0.2 to guarantee that any differences can be attributed to chemical impurities. A similar approach was followed to study the undesired addition of nutrients during inoculating the lab-Remin blank with a natural bacterial community from fresh CTW. This effect was assessed for several inoculum concentrations, namely: 0.5 (lowest), 1, 2, 5, 10 (selected for this study), 20 and 50 (highest, to ensure obtaining a significant impact) × 10^3 intact cells/mL, as compared to the non-inoculated lab-Remin blank. The theoretical increase in BGP due to inoculation ($\Delta BGP_{consortium}$) can be calculated according to Equation (1).

$$\Delta BGP_{consortium} = \left(V_1/V_2\right) \times BGP_{CTW} \quad (1)$$

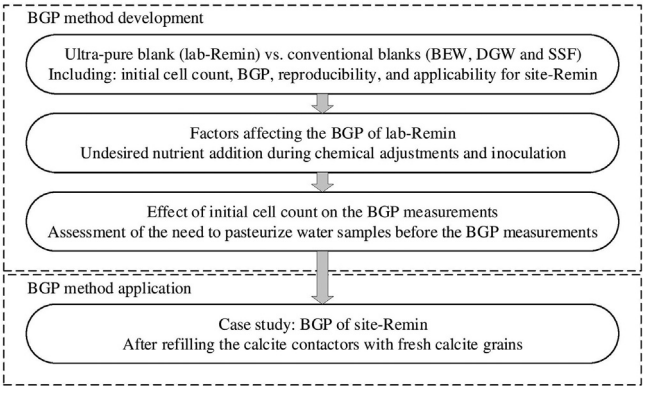

Fig. 1. The experimental approach to develop and apply the bacterial growth potential (BGP) method for ultra-pure drinking water after RO and post-treatment.

In this expression, V_1, V_2 and BGP_{CTW} represent volume of CTW added, volume of the lab-Remin blank, and the average measured BGP of CTW, respectively.

Thirdly, the effect of initial cell count on bacterial growth was studied by analyzing all water samples with and without pasteurization and observing the resulting bacterial growth curves. Lastly, the developed method was applied to assess the BGP of ultra-pure drinking water produced by RO membranes and post-treatment (site-Remin) collected at different times (i.e., 0, 1, 2, 3, 24, 48, 72, 96, 264 and 336 h) after refilling the calcite contactors with fresh calcite grains. The purpose of the latter test was to confirm the applicability of the developed method and ultra-pure blank to reliably assess the BGP of such drinking water.

2.4. Bacterial growth potential (BGP) method

2.4.1. AOC-free materials

Glassware used for sampling (Duran® graduated clear glass bottles with screw plastic cap) and incubation (clear glass vials with screw plastic cap) of water samples was made AOC-free as described in previous studies (Weinrich et al., 2009; Prest et al., 2016). In short, the glassware was washed with a cleaning solution (Alconox® detergent, 10 g/L in ultrapure water), rinsed three times with ultrapure water (Milli-Q® water, Merck Millipore), air-dried overnight, and heat treated in a muffle oven at 550 °C for 6 h. The plastic lids were cleaned by soaking in heated (60 °C) sodium persulfate solution ($Na_2S_2O_8$, 100 g/L) for 1 h, then rinsing three times with ultrapure water and air drying. Moreover, all preparations and procedures for the BGP test were conducted in a clean laboratory environment to avoid air-borne contamination.

2.4.2. Preparation of stock solutions

To create a lab-Remin blank, four different inorganic stock solutions were prepared in AOC-free bottles using ultrapure water (i.e., Milli-Q water) with final concentrations of 67.2 g/L $NaHCO_3$ (for pH adjustment and buffer addition), 294 g/L $CaCl_2 \cdot 2H_2O$ and 67 g/L $MgCl_2 \cdot 6H_2O$ (for calcium and magnesium addition), 0.219 g/L KH_2PO_4 (for phosphate addition), and 3.607 g/L KNO_3 (for nitrogen addition). The prepared stock solutions were kept in the fridge at 4 °C and were used for multiple experiments. Reagent grade chemicals (>99% purity) were used throughout this study (J.T.Baker® Reagents Salts, ACS Grade, the USA).

2.4.3. Preparation of blanks

The ultra-pure blank, lab-Remin, was prepared by adjusting the pH and mineral content of RO permeate by the addition of 2.5 μL/mL of $NaHCO_3$ and 0.5 μL/mL of $CaCl_2$ and $MgCl_2$ stock solutions to achieve identical concentrations of the corresponding elements in site-Remin (final pH of 7.8 ± 0.2, buffer of 122 mg/L HCO_3^-, final concentrations of 40 mg/L Ca^{2+} and 4 mg/L Mg^{2+}). Moreover, phosphorus (5 μg P/L) and nitrogen (50 μg N/L) were added to the blanks (BEW, DGW, SSF and lab-Remin) to guarantee growth limitation by organic carbon up to 500 μg C/L (C:N:P = 100:10:1, (Hammes and Egli, 2005)). P and N were added by dosing 0.1 μL/mL of both KH_2PO_4 and KNO_3 stock solutions. The blanks are made carbon-limited to ensure detecting any potential carbon contaminations during the handling of samples, which comes in different forms such as: (i) carbon attached to the glassware and caps, (ii) volatile carbon present in the laboratory environment, and (iii) carbon contamination present in reagent grade chemicals used in the laboratory, in addition to the original carbon content of the blanks.

2.4.4. Samples pre-treatment and inoculation

To evaluate the influence of initial cell count on the BGP measurements, all water types (CTW, site-Remin, BEW, DGW, SSF,

and lab-Remin) were analyzed both with and without pasteurization at 70 °C for 30 min followed by cooling the samples down to room temperature in an ice bath before any further handling. A natural bacterial inoculum from CTW collected during each sampling campaign was added to all water samples whether pasteurized or not to ensure the presence of a broad bacterial community. The final concentration of inoculum in samples was ~10×10^3 intact cells/mL as suggested elsewhere (Hammes and Egli, 2005; Farhat et al., 2018). The added volume of CTW for inoculation was determined by its initial cell count (measured by FCM), and it was around 18 ± 2 μL/mL (~1.75% v/v) throughout the study period. No further amendments have been performed on the practical samples (CTW and site-Remin) to measure the actual BGP of these waters, whereas P and N have been added to the blanks (see section 2.4.3). Routinely, addition of the different chemicals and inoculum was done using pipettes with sterilized plastic tips which were rinsed 10 times with ultrapure water before using to avoid AOC leaching into the water samples.

2.4.5. Test handling procedures

After performing the aforementioned adjustments, each water sample was transferred into three AOC-free glass vials by direct pouring of 20 ± 2 mL per vial (volume was measured using a reference vial). The glass vials containing the samples were incubated at 30 °C in the dark under static conditions. Aliquots were poured from the incubated vials into 1.5 mL Eppendorf tubes to perform FCM analysis on day 0 (initial count), 1, 3, 6, 8, 10, 13, 16 and 20. BGP was expressed as the maximum count obtained during the 20-day incubation period.

2.4.6. Flow cytometry (FCM) measurements

Flow cytometry (FCM) coupled with fluorescence staining has been selected for this study to quantify cell counts in water samples. The FCM (BD Accuri C6® FCM, Belgium) is fitted with a 50 mW laser with emission wavelength of 488 nm, green fluorescence intensity detector (FL1 channel, 533 ± 30 nm), red fluorescence intensity detector (FL3 channel, > 670 nm), and sideward and forward scattered light intensity collectors. The staining protocol described by Prest et al. (2013) was applied during this study where two staining solutions were prepared: *(i)* SYBR® Green I (SG) 1:100 diluted in filtered (IC Millex – LG, 0.2 μm, Millipore) DMSO for total cell count, and *(ii)* a mix of SYBR® Green I and propidium iodide (SGPI) with a PI working concentration of 0.3 mM and 1:100 diluted SG in filtered DMSO for intact cell count. The staining protocol includes preheating of 500 μL of sample to 35 ± 2 °C for 5 min, staining either with SG or SGPI (10 μL/mL) depending on the desired measurement, incubating in the dark at 35 ± 2 °C for 10 min, and lastly measuring with FCM at 35 μL/min flow rate and 50 μL of analyzed sample volume (identical settings to volumetric calibration of FCM) with setting a threshold on FL1 channel of green fluorescence to 700. Data acquisition was performed using BD Accuri CFlow® software where a digital gate was set on FL1/FL3 density plot to distinguish the stained bacterial cells from inorganic particles of water samples and instrument noise. The FCM detection limit is 2×10^3 cells/mL. Samples were diluted when bacterial counts exceeded 200×10^3 cells/mL.

2.5. Statistical analysis

Different statistical tools were applied using Microsoft Excel, including: (i) *Q-Q* plot, *Chi-Square* test and *Kolmogorov-Smirnov* test to check the normality of data, and (ii) student's *t*-test and *ANOVA* test to determine the significance of differences when comparing 2 or more than 2 samples, respectively. Simple linear regression analysis was conducted for quantitative correlation between two variables. A significance level (Alpha) of 0.05 was considered.

3. Results

3.1. Laboratory remineralized RO permeate (lab-Remin) as an ultrapure blank for the bacterial growth potential (BGP) measurements

As shown in Fig. 2, the BGP of conventional blanks expressed as absolute maximum growth obtained during a 20-day period of incubation was in the range of 80–220, 100–210, and $375–500 \times 10^3$ intact cells/mL for bottled Evian water (BEW), deep groundwater (DGW) treated by aeration and slow sand filtrate (SSF) of surface water supply, respectively. Regarding site water samples, conventionally treated groundwater (CTW) has an average BGP of $630 \pm 70 \times 10^3$ intact cells/mL, whereas the BGP of RO-treated groundwater after post-treatment (site-Remin) was reduced considerably (more than 6-fold) to $90 \pm 20 \times 10^3$ intact cells/mL, which can no longer be affirmed by the BGP detection limit using conventional blanks. Noteworthy, laboratory remineralized RO permeate (lab-Remin) has a BGP of $50 \pm 20 \times 10^3$ intact cells/mL, which is significantly lower than that of conventional blanks ($P < 0.05$, *ANOVA* and Tukey-Kramer *post-hoc* tests), and site-Remin ($P < 0.05$, student's *t*-test).

Furthermore, the lab-Remin blank showed better reproducibility and the standard deviation (~20×10^3 intact cells/mL) was at least 2-fold lower compared with conventional blanks (>50×10^3 intact cells/mL). This observation can also be confirmed by the constant initial cell count of the lab-Remin blank (~2×10^3 intact cells/mL, the detection limit of FCM) measured on different sampling dates, whereas up to 40% variation was observed in the initial (total and intact) cell count of conventional blanks (comparable averages of ~$60–80 \times 10^3$ intact cells/mL for BEW, DGW and SSF, Fig. S1).

3.2. Influence of chemicals addition and inoculation on the BGP of lab-Remin blank

The chemical stocks used to adjust pH and mineral content of RO permeate contain impurities which can be biodegradable and lead to increase the BGP of lab-Remin blank. However, the results showed that the added volumes of $NaHCO_3$ stock solution to obtain final concentrations of 61, 122, 183 and 244 mg/L HCO_3^- (final pH of 7.8 ± 0.2 in all cases) led to insignificantly different ($P > 0.05$, *ANOVA* test) BGP of lab-Remin blank (Fig. S2).

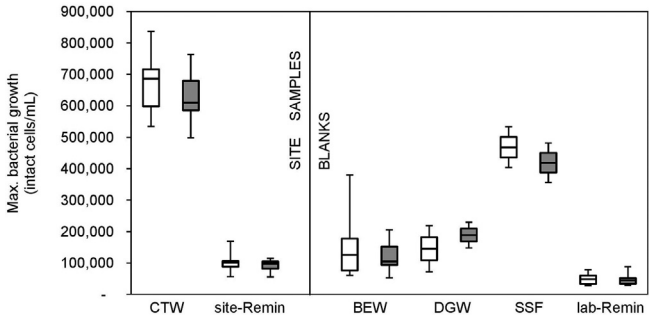

Fig. 2. BGP of non-pasteurized (□) and pasteurized (■) water samples: CTW (conventionally treated groundwater, n = 16), site-Remin (post-treated RO permeate at the site, n = 13), BEW (bottled Evian water, n = 7), DGW (deep groundwater treated by aeration, n = 2), SSF (slow sand filtrate of surface water supply, n = 2), and lab-Remin (lab-remineralized RO permeate, n = 19). Each sample was inoculated with CTW. BGP is expressed as absolute maximum growth of intact bacterial cells obtained during the incubation period (20 days at 30 °C). Error bars represent the variation of "n" number of BGP tests.

Introduction of biodegradable compounds can also occur when inoculating the lab-Remin blank with natural bacteria of fresh CTW, which may lead to additional BGP. Theoretically, this additional BGP depends on inoculum volume and can be calculated (Equation (1)) as shown in Table 2 ($\Delta BGP_{consortium}$) for several inoculum concentrations (namely: 0.5, 1, 2, 5, 10, 20 and 50×10^3 intact cells/mL corresponding to inoculum volume).

The final inoculum concentration selected for this study is 10×10^3 intact cells/mL as suggested elsewhere (Hammes and Egli, 2005; Farhat et al., 2018) to ensure the presence of sufficient cells to initiate growth. The theoretical increase in BGP due to the selected inoculum concentration, comparing with non-inoculated lab-Remin blank, was in the range of $11–14 \times 10^3$ intact cells/mL (Table 2), which was also observed experimentally with a high significance level ($P < 0.05$, student's t-test) by conducting a large number of tests (n = 18 tests in triplicate, Fig. 3b). However, this increase was experimentally insignificant ($P > 0.05$, student's *t*-test) with a small number of tests (n = 1 test in triplicate, Fig. 3a). The large number of BGP tests needed is attributed to the higher standard deviation of BGP measurements (~20×10^3 intact cells/mL) comparing with the target theoretical increase in BGP. For instance, the measured increase in BGP due to inoculating with 50×10^3 intact cells/mL (~80×10^3 intact cells/mL) was according to the theoretical calculations ($58–70 \times 10^3$ intact cells/mL > standard deviation of BGP measurements), as shown in Table 2, even with a small number of tests (n = 1 test in triplicate, Fig. 3a).

Although the increase in BGP due to inoculation can be theoretically less by decreasing the inoculum concentration to 5×10^3 intact cells/mL or even lower (Table 2), still a larger number of BGP tests (n > 150 tests in triplicate, based on student's *t*-test calculations) will be needed to detect this increase experimentally, which is beyond the scope of this study. As a result, the BGP of lab-Remin blank without and with inoculation with the selected concentration (10×10^3 intact cells/mL) was 35 ± 10 and $50 \pm 20 \times 10^3$ intact cells/mL, respectively. Therefore, contribution of inoculum to the measured BGP of lab-Remin blank is about 20–30%.

3.3. Effect of initial cell count on the BGP: is there a need for pasteurization?

The typical shape of bacterial growth curves was not observed when the initial cell count is higher than the maximum bacterial growth that can be maintained by nutrients in water. This effect was occasionally observed for non-pasteurized site-Remin samples (Fig. 4a), where the initial cell count of non-pasteurized samples ($C_{0,\ non\text{-}pasteurized} = 170 \times 10^3$ intact cells/mL) was substantially higher than the maximum bacterial growth that could be maintained by the nutrients available for bacteria ($C_{max} = 110 \times 10^3$ intact cells/mL), and thus, bacterial counts decreased immediately after incubation. This was observed when fresh calcite grains were used in the remineralization step and the attached bacteria may have washed-out. Unlike the non-pasteurized samples, pasteurized site-Remin samples resulted in the typical bacterial growth curves where growth parameters could be calculated (Table 3). An additional example of this case is given in Fig. S3. In contrast to the previous case, non-pasteurized as well as pasteurized site-Remin samples resulted in the typical bacterial growth curves when the calcite filter was in operation for several weeks (Fig. 4b), and the bacterial growth parameters could be calculated for both water samples (Table 3). In this case, the maximum growth level for both non-pasteurized and pasteurized samples was identical (C_{max} = ~98×10^3 intact cells/mL) regardless of the difference in the initial cell count.

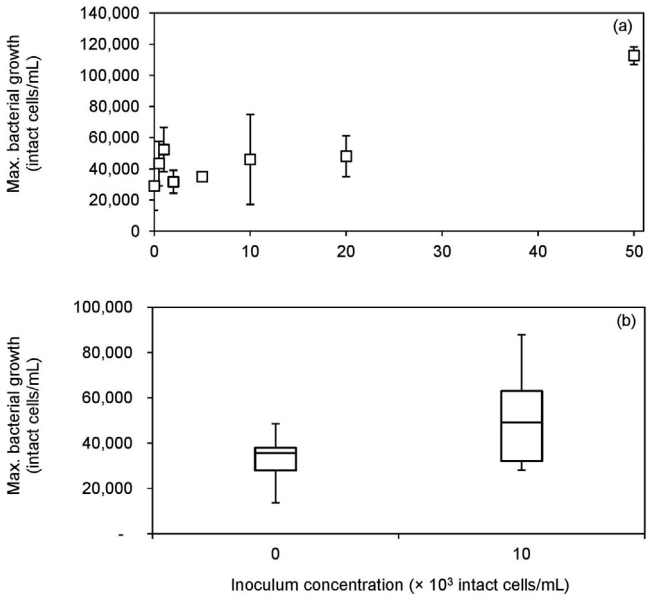

Fig. 3. BGP of the lab-Remin blank (laboratory remineralized RO permeate) with (a) different concentrations of CTW inoculum (n = 1 sample in triplicate), and (b) 10×10^3 intact cells/mL concentration of CTW inoculum (n = 18 samples in triplicate) compared with non-inoculated blank. BGP is expressed as absolute maximum growth of intact bacterial cells during the incubation period (20 days at 30 °C). Error bars represent the variation of "n" number of measurements.

3.4. Case study: monitoring BGP of drinking water produced by RO and post-treatment (site-Remin)

Fig. 5 shows the BGP results of pasteurized and inoculated (with 10×10^3 intact cells/mL of the original water and CTW) site-Remin samples at different time intervals after refilling the calcite contactors with fresh calcite grains. BGP decreased dramatically from ~470×10^3 intact cells/mL for the sample collected immediately

Table 2
The theoretical ($\Delta BGP_{consortium}$) and experimental increase in the BGP of lab-Remin blank due to inoculation.

Inoculum concentration ($\times 10^3$ intact cells/mL)	$\Delta BGP_{consortium}$ ($\times 10^3$ intact cells/mL)	Experimental increase in BGP[a] ($\times 10^3$ intact cells/mL)	*P*-value[b]
0.5	~0.5	14	0.1530
1	~1	23	0.0643
2	2–3	3	0.4017
5	6–7	6	0.3222
10	11–14	17	0.2108
20	22–27	19	0.0914
50	58–70	80	0.0005

[a] Increase in BGP comparing with non-inoculated lab-Remin blank (calculated based on Fig. 3a).
[b] Significant experimental increase if *P*-value < 0.05.

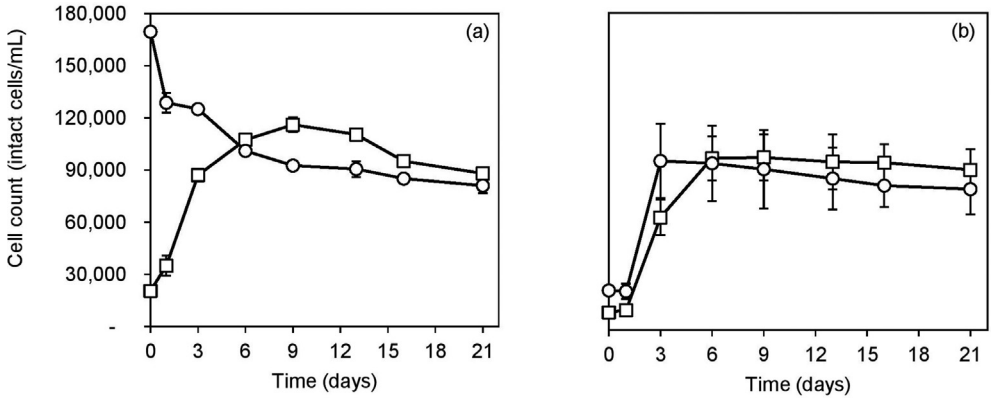

Fig. 4. Examples of bacterial growth curves in pasteurized (—□—) and non-pasteurized (—○—) site-Remin samples analyzed on two different dates: (a) with fresh calcite grains ($C_{0,non\text{-}pasteurized} > C_{max}$) and (b) when the calcite contactors have been in operation for several weeks ($C_{0,non\text{-}pasteurized} < C_{max}$). Each sample was inoculated with CTW. Error bars represent the standard deviation of triplicate measurements.

Table 3
Bacterial growth parameters of non-pasteurized and pasteurized site-Remin samples under different operating conditions.

Case[a]	Cell count ($\times 10^3$ intact cells/mL)			Specific growth rate, μ (/day)
	Initial	Maximum	Net growth[b]	
Case a: non-pasteurized	170	170	0	n.a.[c]
Case a: pasteurized	20	115	95	0.49
Case b: non-pasteurized	22	96	74	0.74
Case b: pasteurized	10	98	88	0.85

[a] Case a refers to Fig. 4a and case b refers to Fig. 4b.
[b] Net growth = maximum cell count – initial cell count.
[c] n.a., not applicable.

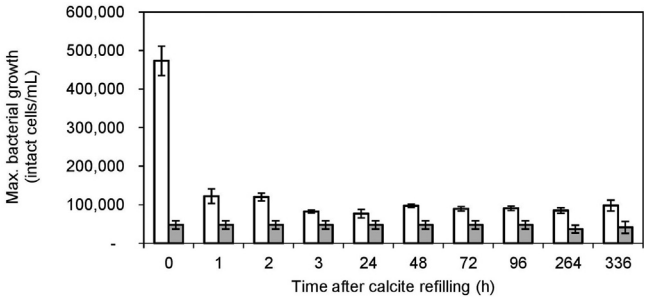

Fig. 5. BGP of pasteurized and inoculated lab-Remin blanks (laboratory remineralized RO permeate, ■) and site-Remin (post-treated RO permeate at the site, □) collected at different times after refilling the calcite contactors with fresh calcite grains (n = 3, each). Each sample was inoculated with CTW. BGP is expressed as absolute maximum growth of intact bacterial cells during the incubation period (20 days at 30 °C). Error bars represent the standard deviation of triplicate measurements.

after starting the operation with the fresh calcite grains to a stable level of 80–110 $\times 10^3$ intact cells/mL after 3 h of operation. A similar trend was observed for non-pasteurized and inoculated (with 10×10^3 intact cells/mL of CTW) site-Remin samples (Fig. S4).

Similar trends were observed for the dissolved organic carbon (DOC) and initial cell count of non-pasteurized samples. The DOC concentrations and initial cell counts decreased from 3.1 to <0.3 mg/L and from 280 to 20×10^3 intact cells/mL over time, respectively (Fig. S5).

For this monitoring test, lab-Remin blanks were measured on each sampling day and the average resulting BGP was ~50×10^3 intact cells/mL, which was significantly ($P < 0.05$, student's *t*-test) lower than the BGP of site-Remin samples after reaching stability (80–110 $\times 10^3$ intact cells/mL).

4. Discussion

4.1. Composition of the ultra-pure lab-Remin blank and factors influencing the BGP measurements at low levels of nutrients

It is proposed to use a lab-Remin blank (which is RO permeate after remineralization in the laboratory to adjust pH to 7.8 ± 0.2 and mineral content) as an ultra-pure blank for BGP measurements of very low nutrient drinking water produced by desalination technologies. The adjustments of the lab-Remin blank and the addition of phosphorus and nitrogen ensure that bacterial growth is limited by carbon. The pH adjustment of the lab-Remin blank is necessary since low pH may hinder the ability of bacteria to effectively hydrolyse nutrients (Russell and Wilson, 1996), and it requires complex adaptive strategies to maintain a neutral intracellular pH (Dilworth and Glenn, 1999). This was confirmed by the observation of no significant growth with the original pH (5.5) of RO permeate in the present study (data not shown). These results might explain the very low bacterial yield observed in the study of Elhadidy et al. (2016) for synthetic water comprising Milli-Q water, nitrogen, phosphorus and carbon (acetate) without pH adjustments. Besides, mineral addition is essential because the low ionic strength of RO permeate may cause osmotic shock in aquatic microorganisms (Kaplan et al., 1993). The BGP of lab-Remin might also be limited by the absence of trace elements required for bacterial growth, such as Fe and Mn (Durand and Kawashima, 1980; Takashima et al., 1990). In this regard, it is assumed that the minimum required concentrations of such elements are added to lab-Remin when inoculating with CTW which contains sufficient amounts of those elements (Table S1). This was confirmed by the observation of no additional growth in the inoculated lab-Remin blank when a trace elements broth (Fe, Zn, B and Co up to 200 μg/L) was added (Fig. S6).

Additionally, Church et al. (2000) also found that similar Fe concentration to that available here in the lab-Remin blank after inoculation (0.15 μg/L Fe) is not growth-limiting. All the aforementioned adjustments, together with the addition of phosphorus and nitrogen, guarantee that the BGP of lab-Remin ($50 \pm 20 \times 10^3$ intact cells/mL) is limited by the organic carbon content rather than the inorganic constituents. Therefore, any potential carbon contamination that may significantly influence the low levels of BGP can be detected.

The addition of chemicals to adjust water quality of RO permeate is a source of contamination, where even using reagent grade chemical stock solutions (>99% purity) may cause unwanted addition of nutrients that could increase the BGP of lab-Remin blank. It is ideal to use AOC-free chemical stock solutions for necessary water quality adjustments. However, they are commercially not available (Kaplan et al., 1993), because chemical manufacturing for laboratory and commercial purposes involves the usage of additives and impurities (Patnaik, 2003). Nonetheless, the results revealed that there was no significant effect of $NaHCO_3$ chemical impurities (>99.5% purity) on the BGP of lab-Remin blank. Therefore, other chemical stocks (i.e., reagent grade $CaCl_2$ and $MgCl_2$, >99% purity) are assumed to have insignificant effects as well, as their final concentration (~145 mg/L $CaCl_2 \cdot 2H_2O$ and 35 mg/L $MgCl_2 \cdot 6H_2O$) in the lab-Remin blank was lower than that of $NaHCO_3$ (~170 mg/L $NaHCO_3$).

Although RO permeate should be totally bacteria-free due to the smaller pore size of RO membranes (<1 nm) comparing with bacterial cells (typically > 200 nm), it still contains bacteria that are able to grow and reproduce. This is a common observation in several RO studies (Park and Hu, 2010; Dixon et al., 2012; Fujioka et al., 2018). The practical aspects, such as leakage in membrane systems (e.g., O-rings of interconnectors) (Liu et al., 2013a; Pype et al., 2016) or bacterial growth in the permeate side, are possible reasons for this observation. Despite the bacterial content of RO permeate, fresh bacterial inoculum of CTW was always added to the lab-Remin blank to ensure the presence of a broad bacterial community in addition to its original cell load. As a result of using fresh CTW for inoculation, unwanted increase in nutrient content of the lab-Remin blank could occur. The consistent and simple approach in this study of inoculating with fresh CTW ensures the viability and activity of cells, even though protocols to prepare AOC-free natural bacterial inoculum are suggested (Hammes and Egli, 2005). The additional BGP of lab-Remin blank caused by increase in nutrients due to inoculation could be quantified ($\Delta BGP_{consortium}$, Table 2) as the characteristics of the inoculum are known. Therefore, the measured BGP of lab-Remin blank can be corrected where the actual BGP can be expressed as $BGP_{actual} = BGP_{measured} - \Delta BGP_{consortium}$. This correction can be applied for all inoculated blanks and samples but it will be more critical in the case of ultra-low-nutrient water such as the lab-Remin blank, where $\Delta BGP_{consortium}$ can reach 30% of the measured BGP when inoculating with 10×10^3 intact cells/mL of CTW, and the site-Remin samples, where this percentage decreases to 10–15%. However, this additional BGP is insignificant for CTW samples (less than 2%). In this study, no correction was made because all the blanks and samples were inoculated with the same concentration of CTW bacteria (10×10^3 intact cells/mL), and hence, the absolute differences will remain unchanged with and without correction.

Interestingly, the results (Table 2) showed that the increase in BGP of the lab-Remin blank after inoculation is solely attributed to nutrient content of CTW inoculum. This suggests that the indigenous bacteria in RO permeate are capable of utilizing all the nutrients available in the lab-Remin blank, and thus, the BGP of the non-inoculated lab-Remin blank reflects the total nutrient content of the RO permeate. However, we argue here to inoculate the lab-Remin blank in all cases (for any future study) before measuring BGP to ensure no growth limitation occurs because of the limited diversity of the bacterial strains in RO permeate.

4.2. Comparing the ultra-pure lab-Remin blank with conventional blanks

In the present study, the detection limit of the BGP method was further decreased to $50 \pm 20 \times 10^3$ intact cells/mL after using the ultra-pure lab-Remin blank comparing with conventional blanks, namely: bottled Evian water (BEW), deep groundwater (DGW) treated by aeration, and slow sand filtrate (SSF) of surface water supply, which have BGP ranging from $100–600 \times 10^3$ intact cells/mL, as reported elsewhere (Vital et al., 2007; Bucheli-Witschel et al., 2012; Bereschenko and Hornstra, 2014; Guo et al., 2014; Mikkers and Magic-Knezev, 2014; van der Kooij et al., 2014; Prest et al., 2016) and confirmed here. The lower BGP of the lab-Remin blank comparing with conventional blanks was found whether pasteurization was applied before measuring BGP or not (Fig. 2). This observation can be explained by the lower organic nutrient content (DOC) of the lab-Remin blank (<0.3 mg/L, detection limit) comparing with the conventional blanks (~0.5 mg/L). Furthermore, the measured BGP of the lab-Remin blank with flow cytometry can be expressed as AOC concentration using the reported yield factor of 1×10^7 cells/μg ac-C eq (Hammes and Egli, 2005; Prest et al., 2016). The estimated AOC concentration of the lab-Remin blank is 5 ± 2 μg ac-C eq/L and is lower than the reported AOC content of BEW (10–52 μg ac-C eq/L (Vital et al., 2007; Bucheli-Witschel et al., 2012)) as measured using a natural bacterial consortium according to Hammes and Egli (2005). However, the estimated AOC concentration of the lab-Remin blank is slightly higher than that of DGW and SSF (1–4 μg ac-C eq/L (Bereschenko and Hornstra, 2014; van der Kooij et al., 2014)) as measured using pure bacterial strains (P17 and NOX) according to van der Kooij et al. (1982). The latter appears to contradict the BGP results obtained in this study where the lab-Remin blank was found to support much lower bacterial growth than DGW and SSF. The reason is that the AOC measuring protocol has a pronounced effect on the obtained concentrations, as previously highlighted (Ross et al., 2013). The critical methodological aspects are the pre-treatment of water samples and the inoculum type where the pure bacterial stains (P17 and NOX) are known to lead to lower AOC content compared to the natural bacterial communities (Hammes and Egli, 2005). However, the estimated AOC content of BEW, DGW and SSF, using the measured BGP in the present study and the yield factor of 1×10^7 cells/μg ac-C eq (Hammes and Egli, 2005; Prest et al., 2016), is in the range of 10–60 μg ac-C eq/L and is significantly higher than that of the lab-Remin blank.

Another advantage of the lab-Remin blank compared to conventional blanks is the high reproducibility. The results show that the lab-Remin blank is more reproducible (standard deviation of $\sim 20 \times 10^3$ intact cells/mL) compared to BEW, DGW and SSF (standard deviation of $>50 \times 10^3$ intact cells/mL). The reproducibility of the lab-Remin blank can be attributed to high nutrient removal efficiency and constant performance of RO treatment regardless of feed water quality and environmental factors (Escobar et al., 2000; Hong et al., 2005; Park and Hu, 2010; Thayanukul et al., 2013). For conventional blanks, they are prone to water quality changes influenced by operating conditions, for instance, switching extraction wells in the case of groundwater and fluctuating treatment efficiency. This explanation can be confirmed by the lower variation in the measured initial cell count of RO permeate comparing with that of conventional blanks (up to 40%). The higher reproducibility of the lab-Remin blank implies that any contamination during BGP measurements could be detected more easily

comparing with conventional blanks.

Although the BGP of lab-Remin blank might be overestimated due to the addition of phosphorus and nitrogen, it is still lower than the actual BGP of ultra-pure drinking water produced by RO and post-treatment including remineralization (site-Remin) measured without any amendments. Using a lab-Remin blank, the BGP of the site-Remin samples could be reliably measured and was considerably lower (more than 6-fold) than that of conventionally treated water from the same groundwater source. The difference in BGP observed between the remineralized RO permeate under controlled conditions (lab-Remin blank) and at the site (site-Remin samples) in this study indicate that leaching of nutrients could occur during post-treatment of RO permeate at the site (e.g., leaching of AOC and microbially available phosphorus (Lehtola et al., 2002) from the calcite grains). This leaching was mainly observed when the calcite contactors were refilled with fresh calcite grains to remineralize RO permeate as a higher DOC content was measured in the instant effluent. Preventing water quality deterioration of RO permeate during post-treatment processes is a challenging from a practical standpoint, where RO permeate comes in contact with different materials (e.g., chemicals, fittings, pipes) which might leach nutrients.

Despite the increase in the BGP of RO permeate after the post-treatment processes, the BGP of site-Remin samples is still significantly lower (more than 6-fold) than that of CTW. The considerable reduction in BGP reflects the effectiveness of RO treatment in retaining bacterial cells and AOC content (Madaeni, 1999; Escobar et al., 2000; van der Bruggen and Vandecasteele, 2003; Hong et al., 2005; Park and Hu, 2010; Thayanukul et al., 2013; Belila et al., 2016) from the water to be treated, and thus limiting bacterial growth. AOC concentration in the site-Remin samples is estimated at 9 ± 2 μg ac-C eq/L using yield factors reported by Hammes and Egli (2005) and Prest et al. (2016), and is in line with the findings of Park and Hu (2010). Although higher AOC concentrations have been reported for RO permeate (32–60 μg ac-C eq/L) (Hong et al., 2005; Meckes et al., 2007; Thayanukul et al., 2013), it comprised the lowest AOC concentrations among all the tested waters in those studies. The variation in AOC content of RO permeate can be largely attributed to the different methods applied to measure it (Ross et al., 2013) as discussed above. Moreover, different design of RO systems in the aforementioned studies (including post-treatment) may also affect the final AOC concentration.

4.3. Is there a need for pasteurization of water samples as part of the BGP method?

Pasteurization is applied in several biological stability assessment methods to guarantee complete inactivation of indigenous bacteria before inoculating with pure bacterial strains (van der Kooij et al., 1982; Joret et al., 1991; van der Kooij, 1992; Sack et al., 2010, 2011). Some researchers argued that there is no need for pasteurization to measure the extent of indigenous bacterial growth (Prest et al., 2016) because of its potential effects on modifying the nutrients composition (e.g., proteins denaturing) which may increase the uncertainty of the measured values (Ross et al., 2013). However, in the present study, pasteurization of water samples prior to BGP measurement was found to be necessary, even when considering indigenous bacteria for inoculation, to avoid the possible influence of abnormally elevated initial cell count on the typical shape of bacterial growth curves, and hence, BGP outcome (Table 3). This was occasionally observed for non-pasteurized site-Remin samples when using fresh calcite grains for remineralization where bacteria attached to the grains were washed-out (Fig. 4a). Similar fluctuations in cell concentrations in the effluent of biological filters have been previously observed and linked to the operating conditions (Servais et al., 1994; Velten et al., 2011).

However, under normal operating conditions (Fig. 4b), pasteurized and non-pasteurized samples after the calcite contactors reached comparable maximum levels regardless of their initial cell counts as shown in Table 3. This indicates that expressing the BGP results as the absolute maximum growth, rather than the net bacterial growth, is a better reflection of the actual total nutrient content of these water samples. For instance, considering net growth implies that part of the nutrients that are utilized by the cells initially present in the sample is totally neglected. This becomes even more critical and misleading when comparing waters with different initial levels of bacterial cells, which is the case of this study (i.e., CTW and site-Remin samples). Based on the aforementioned discussion, the comparable maximum growth levels in water samples without and with pasteurization, under normal operating conditions, suggests that any modification to the nutrient content due to pasteurization is limited. However, further research is recommended to investigate in depth the potential effect of pasteurization on BGP measurement. Another advantage of pasteurization, besides the fact that it controls the initial cell count in all samples quantitatively, is that it ensures that the bacterial species used for the test are the species that naturally grow in water. The case of an abnormal initial cell count (Fig. 4a) implies that the bacterial species in the non-pasteurized sample are mostly attached bacteria onto calcite grains which may differ from the bulk water bacteria (Martiny et al., 2005; Bonadonna et al., 2009), and consequently have different yields. As a result, the attached bacteria may dominate the growth characteristics of the water samples, rather than the bulk water bacteria which are the target of the test. This is especially critical when bulk water bacteria vary significantly from bacteria that may be released from a filter bed.

Alternatively, pre-treatment of water samples with 0.22 μm pore size filtration is another option to obtain a clear distinction between the initial and final cell count during the growth of natural bacterial consortia (i.e., net growth and specific growth rate) (Percherancier et al., 1996; Hammes and Egli, 2005). However, pre-treatment by pasteurization is preferred over 0.22 μm filtration in this study because of the ultra-low nutrient concentration and high sensitivity of RO permeate samples (i.e., lab-Remin and site-Remin) as even minor leaching of nutrients from the flushed filters can have significant influences on BGP measurement (as similarly observed for the influence of inoculation, i.e., $\Delta BGP_{consortium}$ Table 2).

4.4. Practical implications of measuring low levels of BGP

A new ultra-pure blank for the BGP method based on a laboratory remineralized RO permeate is proposed and applied in this study to assess the BGP of water produced by a pilot-scale RO system. There is no doubt that measuring the changes in microbiological water quality is essential for understanding and managing biological stability of drinking water. Thus, using an ultra-pure blank (including the lower detection limit and better reproducibility) makes it possible to push forward the monitoring and understanding of bacterial growth and microbiological quality of drinking water. This is especially true for drinking water with ultra-low levels of nutrients and cell counts produced by RO systems. Moreover, the lower detection limit of the BGP method can capture the potential of bacterial water quality deterioration after RO treatment.

However, it should also be clarified that the measurable increase in cell number does not necessarily mean that drinking water is biological unstable, or that there are problems associated with

drinking water bio-safety. For instance, despite the release of cells and nutrients from the calcite contactors leading to measureable levels of bacterial growth, the finished water has 6-fold lower growth potential compared with conventionally treated water. The lower detection limit of the BGP methods has improved the sensitivity so that small water quality changes can be measured and acted upon.

Other researchers have suggested that a combination of cell number and bacterial community assessment can offer more insight into both the process of bacterial (re)growth and whether there is improvement or deterioration regarding health related biosafety issues (Prest et al., 2014; Liu et al., 2018). Besides the biological stability (including both bacterial count and community), it is also interesting to look into how applying advanced water purification technologies can help to control the growth of target (opportunistic) pathogens. For instance, the relationship between BGP of total cells and BGP of *Legionella* spp., *Aeromonas* spp. or other pathogens (Vital et al., 2010), and to what extent drinking water with ultra-low levels of nutrients will be able to limit their growth in case of pipe cracks and bacterial intrusion into drinking water, especially when considering that organic carbon is not the growth-limiting factor for a group of the opportunistic pathogens, e.g., *Legionella pneumophila* (Williams et al., 2015).

5. Conclusions

The conclusions that can be drawn from this study are:

- The ultra-pure blank proposed in this study, laboratory remineralized RO permeate, has much lower BGP ($50 \pm 20 \times 10^3$ intact cells/mL) and higher reproducibility than conventional blanks ($100–600 \times 10^3$ intact cells/mL), which led to lowering the detection limit of the BGP method.
- Depending on the concentration of the natural bacterial consortium used as inoculum ($>10 \times 10^3$ intact cells/mL), it may have a significant influence on the measured BGP.
- Pasteurization of water samples prior to measuring BGP is necessary to lower the initial number and ensure similar communities of cells in all samples. Further research is needed to assess the potential effect of pasteurization on denaturing the organic nutrient content of water.
- Expressing BGP results as the absolute maximum growth, rather than the net growth, is a better reflection of the actual nutrient content of the water. This becomes even more critical when comparing the BGP of water with different backgrounds (e.g., initial number of cells).
- The BGP of finished drinking water has been reduced more than 6-fold, after applying RO and post-treatment comparing with conventional treatment of groundwater, from $630 \pm 70 \times 10^3$ to $90 \pm 20 \times 10^3$ intact cells/mL.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2018.09.002.

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