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Comparing the bacterial growth potential of ultra-low nutrient drinking water assessed by growth tests based on flow cytometric intact cell count versus adenosine triphosphate

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ABSTRACT

The bacterial growth potential (BGP) of drinking water is widely assessed either by flow cytometric intact cell count (BGP_{ICC}) or adenosine triphosphate (BGP_{ATP}) based methods. Combining BGP_{ICC} and BGP_{ATP} measurements has been previously applied for various types of drinking water having high to low growth potential. However, this has not been applied for water with ultra-low nutrient content, such as remineralised RO permeate. To conduct a sound comparison, conventionally treated drinking water was included in this study, which was also used as an inoculum source. BGP_{ICC}, BGP_{ATP}, intact cell-yield (Y_{ICC}), and ATP-yield (Y_{ATP}) were determined for conventionally treated drinking water (Tap-water) and remineralised RO permeate (RO-water). In addition, both BGP_{ICC} and BGP_{ATP} methods were used to identify the growth-limiting nutrient in each water type. The results showed that the BGP_{ICC} ratio between Tap-water/RO-water was ~7.5, whereas the BGP_{ATP} ratio was only ~4.5. Moreover, the Y_{ICC} ratio between Tap-water/RO-water was ~2 ($9.8 \pm 0.6 \times 10^6$ vs. $4.6 \pm 0.8 \times 10^6$ cells/μg-C), whereas the Y_{ATP} ratio was ~1 (0.39 ± 0.12 vs. 0.42 ± 0.06 ng ATP/μg-C), resulting in a consistently higher ATP per cell in RO-water than that of Tap-water. Both BGP_{ICC} and BGP_{ATP} methods revealed that carbon was the growth-limiting nutrient in the two types of water. However, with the addition of extra carbon, phosphate limitation was detected only with the BGP_{ICC} method, whereas BGP_{ATP} was not affected, suggesting that a combination of carbon and phosphate is essential for biomass synthesis, whereas carbon is probably utilised for cellular activities other than cell synthesis when phosphate is limited. It was estimated that the intact cell-yield growing on phosphate would be $0.70 \pm 0.05 \times 10^9$ cells/μg PO_4-P.

1. Introduction

Delivering safe and biologically stable potable water is the aim of drinking water utilities worldwide. Biologically stable drinking water is especially important for non-chlorinated drinking water distribution systems (Van der Kooij and Veenendaal, 2014) to limit bacterial growth that might take place during transport and distribution in bulk water and as biofilm (Chen et al., 2020; Liu et al., 2020; Sousi et al., 2020a). Traditionally, biological stability is assessed by the available nutrients to support bacterial growth in water, namely biodegradable dissolved organic carbon (BDOC) (Servais et al., 1987; Huck, 1990) and assimilable organic carbon (AOC) (Van der Kooij et al., 1982; Hammes and Egli, 2005). In addition to nutrient measurement, biological stability has been recently assessed by direct measurement of the bacterial growth potential (BGP) of water, which is defined as the level of bacterial growth that can occur in water samples under predefined conditions in the laboratory (Prest et al., 2016a).

The BGP can be measured based on cell count by flow cytometry (FCM) or adenosine triphosphate (ATP) by luminometer under various environmental conditions (Prest et al., 2016b; Nescerecka et al., 2018).

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FCM is a rapid bacterial quantification tool based on fluorescence staining of total and intact cells, while ATP is the energy currency of all living cells measured based on bioluminescence analysis, which is an indicator of viability (Abushaban et al., 2019). BGP assessment by FCM or ATP overcomes the limitations of the traditional biological stability assessment methods that are based on plate counting, in terms of rapidity, accuracy, and laborious demands (Hammes et al., 2010; Prest et al., 2016b; Van Nevel et al., 2017). However, there are no guidelines based on FCM or ATP as these parameters are still used for research purposes. Moreover, FCM and ATP measurements are beneficial for the complete detection of bacterial cells in water, allowing for using a natural bacterial inoculum for BGP tests to ensure the consumption of a wider range of organic compounds present in the water (Hammes and Egli, 2005).

Several studies have used both FCM and ATP in field-testing for monitoring water quality in treatment plants and distribution systems (Vital et al., 2012; Farhat et al., 2018), where the relationship between instant FCM and ATP measurements has been investigated. By measuring BGP of different water types, Farhat et al. (2018) found that ATP and FCM results did not show the same trend due to the nature of each method, where ATP measures variable energy carrier compounds within cells, while FCM measures the numerical growth of bacteria. Based on these observations, the authors suggested combining both methods for more insights into bacterial growth potential. However, the reason behind the different BGP trends with FCM and ATP and the interpretation of this difference still need further investigation for conventional drinking water and more especially for ultra-low nutrient drinking water such as remineralised reverse osmosis (RO) permeate. The latter water type is the focus of this study where a very low BGP has been reported for drinking water produced by RO-based treatment (Sousi et al., 2018).

Therefore, the objective of this study was to combine the outcome of BGP obtained by FCM intact cell count (BGP_{ICC}) with that by ATP (BGP_{ATP}). Two water types were used to conduct this comparison, namely: conventionally treated drinking water with a relatively high nutrient content versus ultra-low nutrient water produced by RO-based treatment (RO and remineralisation). The comparison included the ratio between the BGP_{ICC} and BGP_{ATP} of these types of water, as well as the ratio between intact cell-yield (Y_{ICC}) and ATP-yield (Y_{ATP}) obtained for each water type. Moreover, nutrient limitation was investigated in each water type with both BGP_{ICC} and BGP_{ATP}.

2. Materials and methods

2.1. Water samples

This study was conducted at the Oasen drinking water treatment plant located in Kamerik, the Netherlands, which supplies 340 m^3/h of drinking water. The detailed description of treatment and water quality is given in Sousi et al. (2020a). In short, the plant currently treats anaerobic groundwater by conventional means comprising dry sand filtration (rapid sand filters fed with spray aerated water), pellet softening, rapid sand filtration, activated carbon filtration, and UV disinfection. The finished conventionally treated drinking water (Tap-water) is stored in the clean water reservoir, from which samples were collected during this study. A pilot-scale advanced treatment scheme (7 m^3/h) is installed at the same location to treat anaerobic groundwater directly by reverse osmosis (RO) with a total recovery of 75%. Thereafter, RO permeate is post-treated with ion exchange, remineralisation by calcite contactors, magnesium doing, and tower aeration. Additional details about the conventional and RO-based water treatment schemes can be found in the supplementary information (Table S1). The blank was prepared by correcting the mineral content of water collected directly after RO filtration (i.e., RO permeate) at the laboratory using ultrapure chemical stock solutions, where the final concentrations were as follows: $NaHCO_3$ (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 RO-water. The water quality analysis of Tap-water and RO-water, including LC-OCD analysis and AOC determined according to Van der Kooij et al. (1982), is shown in the supplementary information (Table S2). The cell count in RO permeate (2×10^3 total cells/mL and 10^3 intact cells/mL) was lower than that reported in other studies (Dixon et al., 2012; Thayanukul et al., 2013; Buysschaert et al., 2018; Fujioka et al., 2018), which might be attributed to the anaerobic operation of RO in this study that resulted in limited bacterial growth on RO membranes, or different methods used for cell counting.

2.2. Bacterial growth potential (BGP) test

The bacterial growth potential (BGP) of water was measured according to Sousi et al. (2018). In short, samples of Tap-water and RO-water were collected in AOC-free glassware that is treated at 550 °C for 6 h. Thereafter, samples were pre-treated at the laboratory by pasteurisation (70 °C for 30 min) to inactivate indigenous bacteria before inoculating with ~104 ICC/mL (ICC: intact cell count) of a natural bacteria consortium originating from Tap-water, where using RO permeate bacteria as an inoculum is not recommended due to their limited ability to consume complex organic carbon (Sousi et al., 2020b). Pre-treated samples were distributed between three individual AOC-free vials (i.e., triplicate measurements per sample), incubated in the dark at 30 °C, and lastly measured for intact cell count (ICC) or ATP over a growth period of 20 days. BGP was expressed as the maximum intact cell count or ATP concentration obtained during the incubation period. BGP based on intact cell count by FCM is denoted as BGPICC whereas BGP based on ATP is denoted as BGPATP. Moreover, a broth of trace elements was used for growth limitation experiments, where two stock solutions were prepared (pH ~7): stock solution A containing 5 mg/L CoCl2.6H2O and 10 mg/L H3BO3; and stock solution B containing 500 mg/L MnSO4.7H2O, 10 mg/L ZnSO4.7H2O, and 300 mg/L FeSO4.7H2O. The stock solutions were kept in the dark at room temperature. Aliquots of 4 and 3.7 mL/L from stock solutions A and B, respectively, were added in water samples, resulting in final concentrations of 5 μg/L Co, 6.5 μg/L B, 359 μg/L Mn, 8.5 μg/L Zn, 215 μg/L Fe, and 345 μg/L S. Moreover, adding phosphate and nitrogen was accompanied with the addition of 29.2 μg/L K, as elaborated in the following sections.

2.3. Intact cell count (ICC)

ICC was measured using flow cytometry (BD Accuri C6® FCM, BD Biosciences, Belgium) coupled with DNA staining (a mix of SYBR® Green I and propidium iodide stains) as previously described by Prest et al. (2016a). The detection limit is 10^3 ICC/mL. Moreover, data obtained by FCM, namely the ratio of high and low nucleic acid bacteria (HNA and LNA) as well as forward and sideward scatter signals (FSC and SSC), were analysed for both water types as described by Wang et al. (2009).

2.4. Adenosine triphosphate (ATP)

Intracellular ATP from microorganisms was measured according to the filtration-based method described by Abushaban et al. (2019) using a Water-Glo testing kit (lysis reagent and detection reagent) and a GloMax®-20/20 Luminometer (Promega Corp., USA). The detection limit is 0.1 ng ATP/L. An ATP calibration line was prepared for each water type (Tap-water and RO-water) to convert the measured emitted light (relative light units, RLU) into intracellular ATP concentration, as shown in the supplementary information (Fig. S1).

2.5. Experimental approach

Experiments were conducted with two types of water: conventionally treated water (Tap-water) and ultra-low nutrient drinking water prepared by remineralising RO permeate (RO-water). Samples of Tap-water and RO-water were pasteurised and inoculated with bacteria originating from Tap-water as described in the previous section. Thereafter, six different nutrient combinations (carbon, phosphate, and nitrogen) were added to these samples prior to BGP measurement, as shown in Fig. 1, where zone A represents the actual BGP of Tap-water or RO-water without the addition of nutrients (results presented in Fig. 2); zone B represents BGP measurement with the addition of C:N:P up to a ratio of 100:10:1 according to bacterial elemental composition (Hammes and Egli, 2005) (results presented in Fig. 5); and zone C represents BGP measurement with the addition of extra carbon considering a C:N:P ratio of 100-300:10:1 (results presented in Fig. 6). Nutrients were added from the following stock solutions: 0.219 g/L KH_2PO_4 (for phosphate addition), 3.607 g/L KNO_3 (for nitrogen addition), and 1,000 ± 50 mg/L Ac-C (for carbon-acetate addition). Bacterial yield (Y) is calculated from the slope of the linear increase in ICC or ATP with the standard carbon addition.

Based on the BGP results, the following calculations were made:

Ratio BGP_{ICC} = (BGP_{ICC} of Tap-water) / (BGP_{ICC} of RO-water)

Ratio BGP_{ATP} = (BGP_{ATP} of Tap-water) / (BGP_{ATP} of RO-water)

Ratio Y_{ICC} = (Y_{ICC} of Tap-water) / (Y_{ICC} of RO-water)

Ratio Y_{ATP} = (Y_{ATP} of Tap-water) / (Y_{ATP} of RO-water)

In addition to the previous calculations, the growth-limiting nutrient was determined using BGP_{ICC} and BGP_{ATP} as described in Table S3. Fig. 1 represents the experimental approach followed in this study.

2.6. 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). In addition, a simple linear correlation between two quantitative variables was applied. Calculations for statistical analysis were conducted using the Microsoft Excel software (version 2013) considering 95% confidence interval (alpha of 0.05).

3. Results

The ratio of BGP_{ICC} and BGP_{ATP} between Tap-water and RO-water

The BGP_{ICC} of Tap-water and RO-water without the addition of any nutrient were $436 \pm 20 \times 10^3$ and $58 \pm 3 \times 10^3$ ICC/mL, respectively, as shown in Fig. 2A, which resulted in a BGP_{ICC} ratio of about 7.5 between the two water types. The corresponding BGP_{ATP} under the same conditions were 20.10 ± 1.40 and 4.32 ± 1.10 ng ATP/L, respectively, as shown in Fig. 2B, the ratio of which was about 4.5. The results of BGP_{ICC} and BGP_{ATP} clearly demonstrated that the ratio of BGP between Tap-water and RO-water was significantly influenced by the parameter used (i.e., ICC by FCM vs. ATP). Moreover, based on the aforementioned observations, the ATP per cell for Tap-water (4.6×10^{-17} g ATP/cell) was considerably lower than that of RO-water (7.4×10^{-17} g ATP/cell).

3.1. The intact cell-yield (Y_{ICC}) and ATP-yield (Y_{ATP}) based on the growth curves of Tap-water vs. RO-water

The BGP_{ICC} and BGP_{ATP} were determined for each carbon concentration (0-300 µg/L Ac-C) based on the 14 days' growth curves of ICC (Fig. 3) and ATP (Fig. 4). The ATP concentration after pasteurisation on the first test day was <0.1 ng/L in both water types. However, the corresponding ICC in RO-treated water was $<10^3$ ICC/mL, whereas Tap-water contained around 100×10^3 ICC/mL of pasteurisation-resistant cells, which could not grow during the growth test period (Sousi et al., 2020b).

For RO-water, it was observed that the growth peak was quicker obtained with ATP than ICC (3 days vs. 4–7 days) for all carbon concentrations. Similar phenomenon was observed for Tap-water when >5 µg/L Ac-C was added, where a second ATP peak was also observed after 7–10 days. However, for Tap-water with low carbon concentrations (0–5 µg/L Ac-C), both ATP and ICC peaks were obtained after 7–10 days. In addition, Figs. 3 and 4 clearly demonstrate that ATP concentration significantly dropped after reaching the peak, especially with the addition of high carbon concentrations, irrespective of the type of water. On the contrary, ICC was maintained around the peak value until day 14 when considering low carbon concentrations (0–100 µg/L Ac-C), whereas a drop in ICC was observed after day 4–6 at carbon concentrations of 200 and 300 µg/L Ac-C. The analysis of FCM data showed that 80% of the cells growing in Tap-water and RO-water were HNA bacteria.

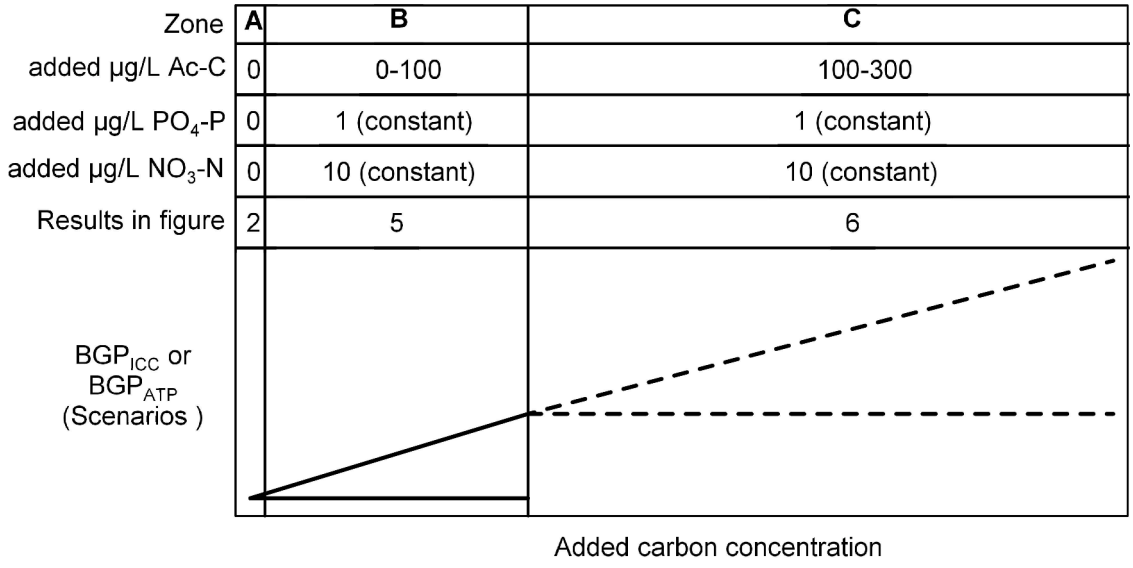

Fig. 1. The experimental approach and test zones (A, B, and C) showing the possible scenarios for the BGP of conventionally treated water (Tap-water) and remineralised RO permeate (RO-water) with the addition of nutrients. Zone A: BGP without the addition of nutrients (results given in Fig. 2); zone B: BGP with the addition of C:N:P up to a ratio of 100:10:1 (results given in Fig. 5); and zone C: BGP with the addition of extra carbon considering a C:N:P ratio of 100–300:10:1 (results given in Fig. 6).

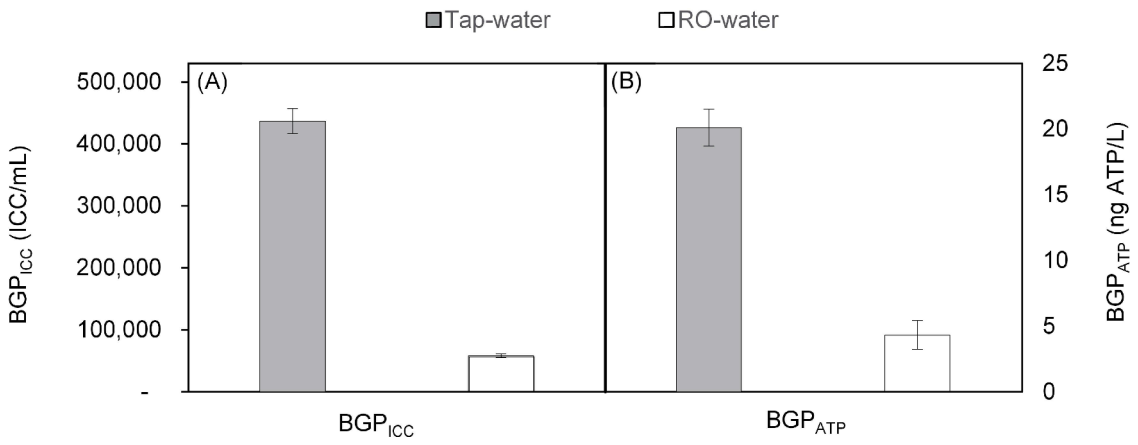

Fig. 2. The reduction in BGP_{ICC} (7.5 times, A) and BGP_{ATP} (4.5 times, B) obtained by applying RO treatment (RO-water) as compared with conventional water treatment (Tap-water) (zone A in Fig. 1).

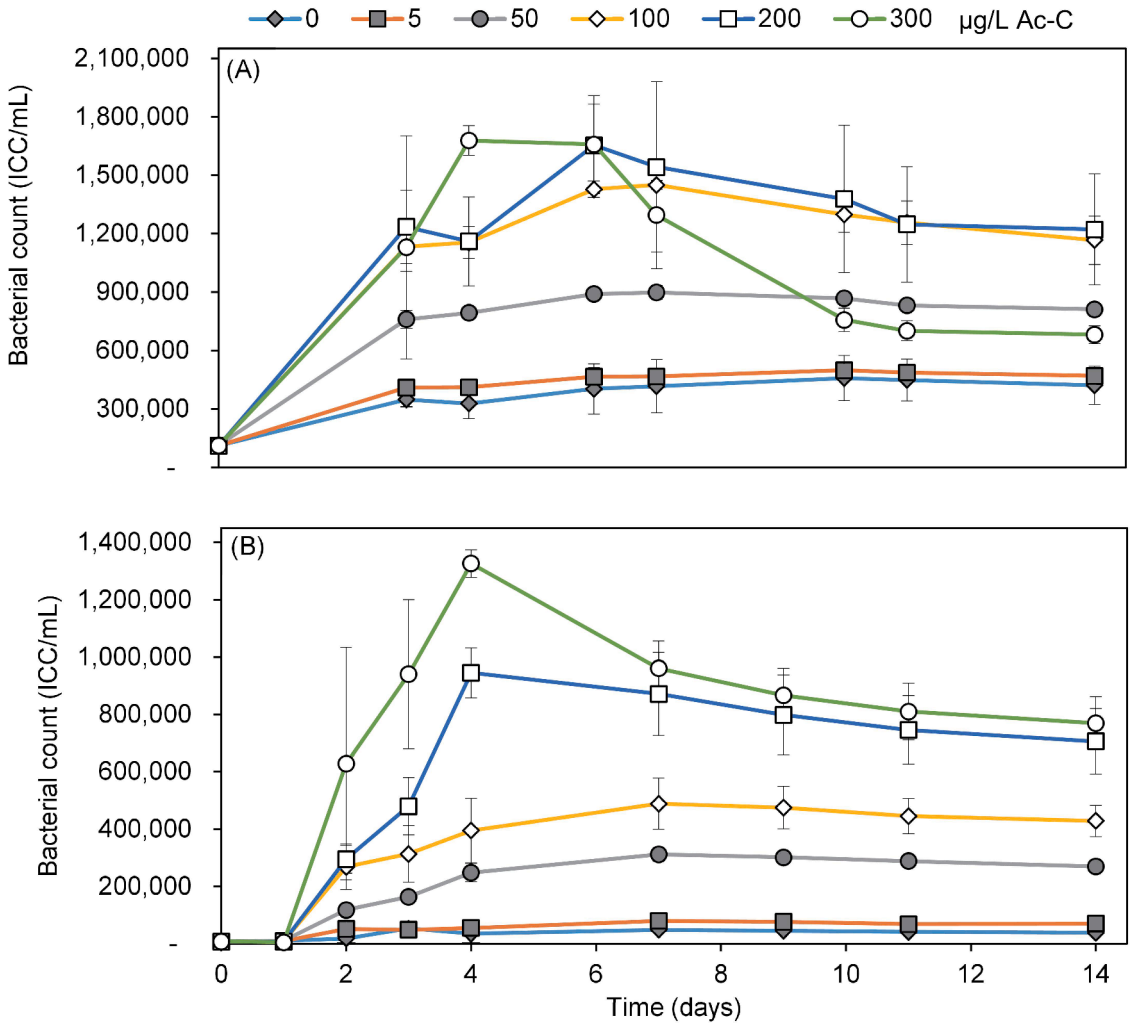

Fig. 3. The growth (30 °C; 14 days) of bacteria originating from conventionally treated water in their own water (A) and RO-treated water (B) obtained by FCM after inoculating with ~10^4 ICC/mL. Each curve represents the growth on a certain carbon concentration (0–300 µg/L Ac-C). Error bars represent the standard deviation of triplicate samples.

However, both FSC and SSC signals were higher for RO-water than Tap-water at all carbon concentrations, indicating larger bacterial cells in RO-water, i.e., an average of 2200 vs. 880 for FSC and 2100 vs. 1200 for SSC, respectively (Table S4).

Y_{ICC} and Y_{ATP} for both water types were determined based on the slope of the linear increase in BGP_{ICC} and BGP_{ATP} with the standard addition of carbon up to 100 µg/L Ac-C (Fig. 5), where carbon was not limiting the growth due to the addition of phosphate (1 µg/L PO_4-P) and

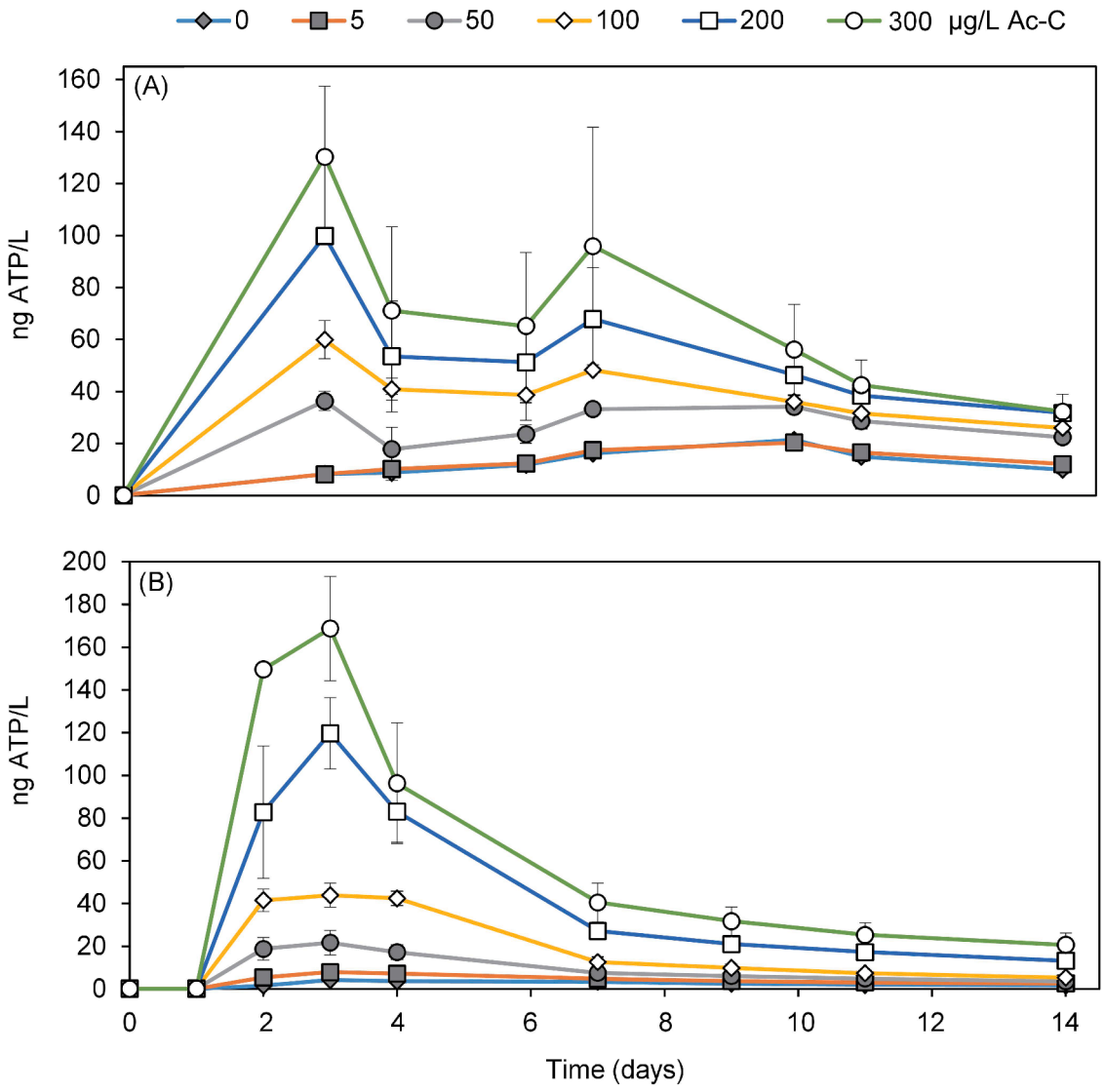

Fig. 4. The growth (30 °C; 14 days) of bacteria originating from conventionally treated water in their own water (A) and RO-treated water (B) obtained by ATP. Each curve represents the growth on a certain carbon concentration (0–300 µg/L Ac-C). Error bars represent the standard deviation of triplicate samples.

nitrogen (10 µg/L NO_3-N) according to the ratio of C:N:P = 100:10:1. The Y_{ICC} for RO-water was 4.6 ± 0.1 × 10^6 cells/µg-C, which was not influenced by the addition of trace elements (K, Co, H_3BO_3, Mn, Zn, Fe, and S, Fig. S2), indicating that these trace elements were not the limiting factor for bacterial growth even in the ultrapure RO-treated water. For Tap-water, the Y_{ICC} was 9.8 ± 0.6 × 10^6 cells/µg-C, which was ~2 times higher than that in the RO-water (Fig. 5A). Conversely, the Y_{ATP} for both water types was similar (ratio of ~1), which was 0.39 ± 0.12 and 0.42 ± 0.06 ng ATP/µg-C for Tap-water and RO-water, respectively (Fig. 5B).

Based on the ICC and ATP results, the maximum ATP per cell was calculated for each carbon concentration (Fig. S3). The ATP per cell values for RO-water were consistently 2–4 times higher than these for Tap-water, where the spike of carbon in RO-water resulted in an increase of 50% in ATP per cell, even at 5 µg/L Ac-C. While for Tap-water, the ATP per cell values were stable around the same level for the whole carbon range of 0–300 µg/L Ac-C.

3.2. Growth-limiting nutrient identified by BGP_{ICC} and BGP_{ATP} for Tap-water vs. RO-water

As shown in Fig. S4, both BGP_{ICC} and BGP_{ATP} of Tap-water without the addition of nutrients were comparable to these of Tap-water with 1 µg/L PO_4-P and 10 µg/L NO_3-N added. Fig. S5 shows similar observations for RO-water. Therefore, both BGP_{ICC} and BGP_{ATP} revealed that carbon was the growth-limiting nutrient in Tap-water as well as RO-water.

When carbon was added, clear differences were observed (Fig. 6). For Tap-water, a linear increase in BGP_{ICC} was observed until the addition of 100 µg/L Ac-C when the ratio of added C:N:P was 100:10:1 (Fig. 6A, left). Afterwards, a plateaued curve was established, where the difference in BGP_{ICC} was insignificant for 100, 200, or 300 µg/L Ac-C addition (P <0.05) (Fig. 6A, right). The BGP_{ICC} of Tap-water increased from 454 ± 65 × 10^3 ICC/mL with no carbon addition to the level of 1500 × 10^3 ICC/mL with the addition of 100, 200, or 300 µg/L Ac-C, indicating that there might be other elements (e.g., phosphate) limiting cell multiplication. On the contrary, such a trend was not observed for BGP_{ATP}. With the addition of carbon from 0 to 300 µg/L Ac-C, the BGP_{ATP} of Tap-water showed a linear increase from 21.41 ± 1.62 ng ATP/L to 130.32 ± 27.14 ng ATP/L (Fig. 6B). The different trends observed for BGP_{ICC} and BGP_{ATP} indicate that though the cell number reached a stable level due to the limitation on cell multiplication, the cellular activity kept increasing. To obtain a holistic view, the actual C:N:P ratio was calculated by including the internally available nutrients in Tap-water and RO-water (Table S5). The original AOC in Tap-water

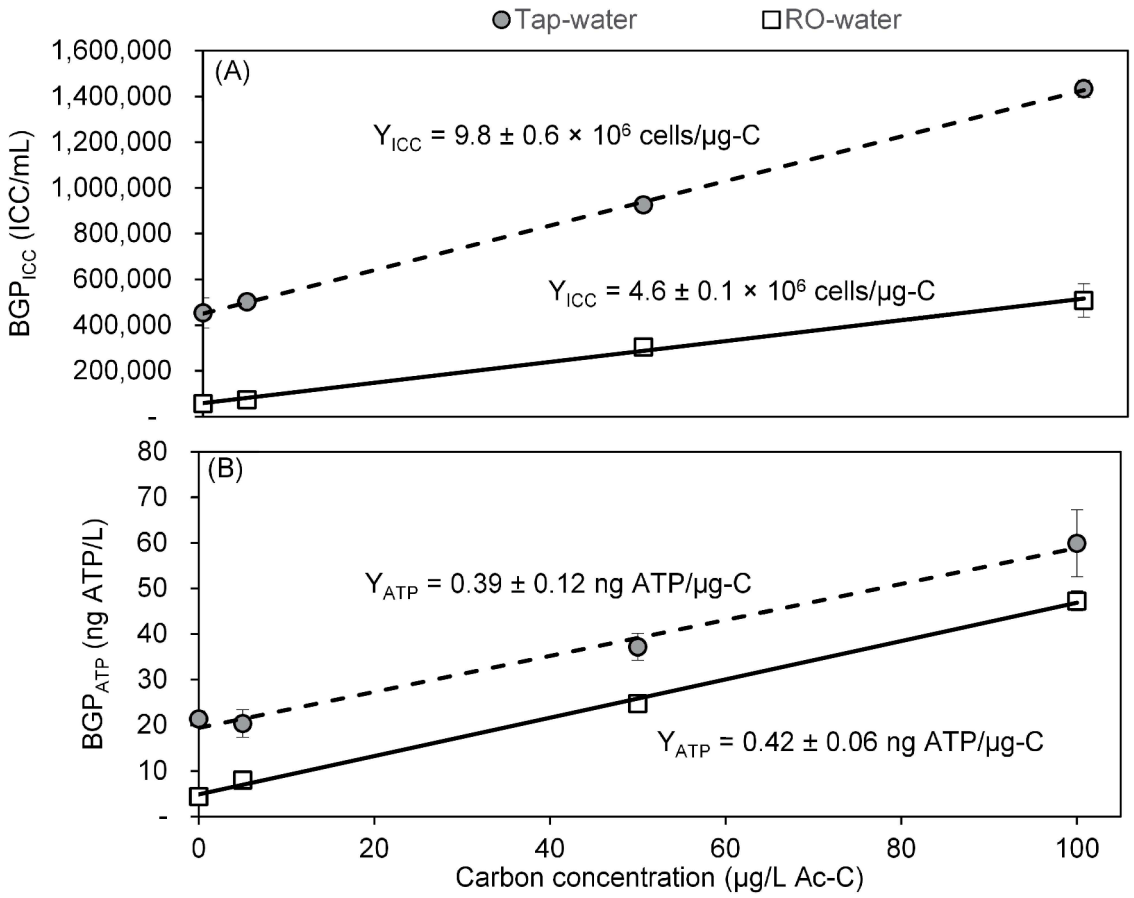

Fig. 5. The intact cell-yield (Y_{ICC}, cells/µg-C) and ATP-yield (Y_{ATP}, ng ATP/µg-C) obtained for conventionally treated water (Tap-water) and laboratory-remineralised RO permeate (RO-water) at a carbon range of 0 (no carbon added) to 100 µg/L Ac-C. Phosphate (1 µg/L PO_4-P) and nitrogen (10 µg/L NO_3-N) were added to all samples (zone B in Fig. 1).

was ~45 µg/L Ac-C (~10 times higher than AOC P17 and NOX) considering the Y_{ICC} obtained in Fig. 5A, meaning that the actual C:N:P ratio was 145:2910:~2 with the addition of 100 µg/L Ac-C.

On the other hand, for RO-water, both BGP_{ICC} and BGP_{ATP} increased linearly along the whole range of carbon addition from 0 to 300 µg/L Ac-C (Fig. 6A and B), indicating that there was no growth or activity limitation observed for RO-water. Remarkably, the BGP_{ICC} for RO-water was approaching but did not reach the cell number limitation observed for Tap-water (1,327 ± 48 × 10^3 vs. 1,500 × 10^3 ICC/mL).

4. Discussion

Intact cell count (ICC) and ATP measurements were used to evaluate the bacterial growth potential of conventionally treated water (Tap-water) and ultra-low nutrient water prepared by remineralising RO permeate (RO-water). Combining the results obtained by intact cell count and ATP allowed for integral understanding of the bacterial growth characteristics of each water type. In addition, trace elements, *N, P,* and different concentrations of acetate carbon were added for performing a matrix of BGP tests, which allowed for the investigation of growth limiting factors and the comparison between ICC and ATP for measuring bacterial growth dynamics.

4.1. Reduction in BGP_{ICC} and BGP_{ATP} achieved by RO-based treatment

In the present study, both BGP_{ICC} and BGP_{ATP} showed that RO-based treatment (RO-water) resulted in a significantly lower BGP than that of conventional treatment (Tap-water) (Fig. 2), which is in line with previous findings on RO performance for controlling bacterial growth (Park and Hu, 2010; Thayanukul et al., 2013; Sousi et al., 2020a). This could be explained by the high AOC rejection efficiency of RO membrane (Escobar et al., 2000; Hong et al., 2005). In addition, although the same bacterial consortium was inoculated in both water types, the difference in water matrix might have also contributed to the reduction in BGP achieved by RO treatment, such as the difference in inorganic and/or trace elements. For example, the conductivity in RO-water was 3 times lower than that of Tap-water (20.9 mS/m vs. 63.2 mS/m, Table S2). The BGP tests performed on RO-water with addition of Ca, Mg, HCO_3^-, and other essential trace elements (i.e. K, Co, Zn, Fe, Mn, S, and hydrogen borate) confirmed that none of these elements was the growth limiting factor in the ultra-low nutrient RO permeate. Based on the previous observations, it is hypothesised that bacterial in RO-treated water might be limited not only by organic compounds but also by certain inorganic constituents that were present in Tap-water at extremely low concentrations, which were not included in this study.

However, the results revealed that the magnitude of BGP reduction was dependent on the parameter used, where BGP_{ICC} showed a higher reduction ratio (7.5 times) compared with BGP_{ATP} (4.5 times). The different degree of bacterial growth measured by ICC and ATP was previously reported, and attributed to the discrepancy of the two measurements and the possibility of missing the ATP peak because of the rapid increase and collapse in ATP values when readily available carbon such as acetate is added (Farhat et al., 2018). Such discrepancies were observed in the present study, where the measurement of bacterial growth with the addition of acetate carbon revealed that the ATP peak was obtained after 3 days while the ICC reached the peak within 4–7 days (Figs. 3 and 4). Moreover, Farhat et al. (2018) argued that both

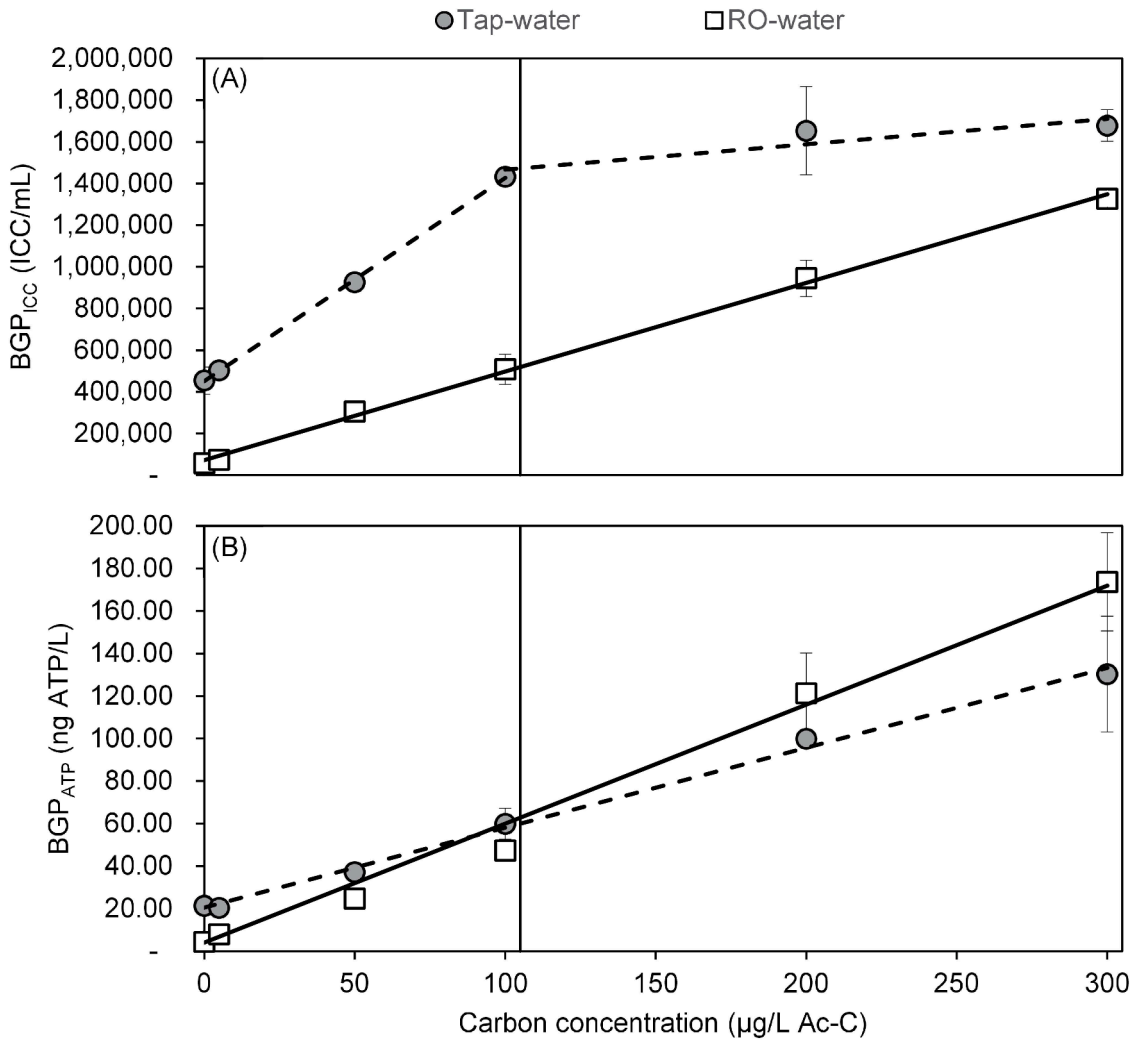

Fig. 6. The differences observed in intact cell-yield (Y_{ICC}, A) and ATP-yield (Y_{ATP}, B) between conventionally treated water (Tap-water) and RO-treated water when phosphate was theoretically limiting bacterial growth: 100–300 µg/L Ac-C, 1 µg/L PO_4-P, and 10 µg/L NO_3-N (zone C in Fig. 1).

methods are accurate and reliable, while the definition of growth in terms of cell multiplication or active biomass formation dictates which method evaluates the growth better, and suggested that the combination of BGP_{ICC} and BGP_{ATP}, together with the parameter ATP per cell offers more insights into growth potential.

Although the same bacteria originating from conventionally treated water were used as an inoculum in the present study, the ATP per cell of RO-water was consistently higher (2–4 times) than that of Tap-water, indicating that there might be different bacteria thrived in the two water types. The higher ATP per cell in RO-water was in line with the FCM data (FSC and SSC signals), which were higher in RO-water compared with Tap-water, affirming the larger cell size in RO-water (Wang et al., 2009; Sousi et al., 2020a). It is well known that the level of ATP production is influenced by environmental conditions (Hespell and Bryant, 1979; Boström and Törnblom, 1990), where ATP production would be affected when cells are subjected to conditions that are different from their original environment, which could explain the higher ATP per cell in RO-water compared with Tap-water. Moreover, the standard carbon addition tests showed that the Tap-water bacterial consortium inoculated in RO-water converted carbon into ATP for bio-activities (e.g., synthesis of reserve materials, mobility (Hammes et al., 2010; Mempin et al., 2013)) as efficient as in their own water, where a comparable Y_{ATP} was observed in both water types. However, two ATP peaks were observed in Tap-water, corresponding to the added readily available organic carbon (first peak) and the natural organic compounds present in water (second peak) (Van der Kooij et al., 2017). The second peak was not observed in RO-water because of its ultra-low nutrient content, specifically complex organic compounds as measured by LC-OCD (Table S2). The concentration of ATP quickly dropped after the first peak in both water types, which could be attributed to the rapid response of ATP production to environmental changes i.e., the addition and consumption of acetate in this case. The drop in intracellular ATP concentrations could imply increasing extracellular ATP during growth (Mempin et al., 2013; Ihssen et al., 2021), or an actual decrease in intracellular ATP due to physiological reasons (i.e., less active cells). The quick drop in intracellular ATP was not accompanied with a significant decrease in cell numbers, indicating that cells were maintaining themselves with low levels of energy and bioactivities.

Bacterial growth in term of intact cell count, and thus intact cell-yield, was significantly affected by the change in the surrounding environment (Tap-water vs. RO-water), where the rate of cell synthesis in RO-water was hindered. According to this comparison, it is suggested that bacteria inoculated in an unfavourable environment (RO-water) tend to covert available nutrients into energy source (ATP) for surviving rather than synthesising new cells. Although the same inoculum was used, there might be different bacteria thrived in Tap-water and RO-water, especially considering the significantly different ATP per cell between the two water types. For future research, it is recommended to investigate the dynamics of bacterial communities and identify which members become dominant during bacterial growth in each water type,

which will be valuable for understanding bacterial growth from community composition and structure level. This is especially important considering that each water type contained different bacterial communities (Sousi et al., 2020a).

4.2. The role of phosphate in bacterial growth and the maximum intact cell-yield per μg PO_4-P

In the present study, both BGP_{ATP} and BGP_{ICC} clearly showed that carbon was the growth limiting nutrient in Tap-water and RO-water. However, different trends of bacterial growth were observed when >100 μg/L Ac-C was added, which could be attributed to the phosphate limitation established in Tap-water. This is because nitrogen limitation could be clearly excluded as a result of the data presented in Table S2, i.e., excess NO_3-N at 2910 and 260 μg/L in Tap-water and RO-water, respectively. Additionally, no growth-limitation effect was observed for a wide range of trace elements (K, Ca, Mg, Mn, Co, Zn, Fe, H_3BO_3, and S, Fig. S2), where even in RO-water, there were sufficient trace elements for cell multiplication until the addition of 300 μg/L Ac-C (Fig. 6A). Therefore, trace elements limitation was excluded. As such, the actual C:P ratio when changing from carbon-limited (Fig. 1, zone B) to carbon-excess (Fig. 1, zone C) conditions in Tap-water was 145:2, which is between the Redfield ratio of 100:1 (Hammes and Egli, 2005) and the 50:3 ratio for exponentially growing cells under carbon-limited conditions (Egli, 2009). This complies with the consensus of variable microbial cell composition, which is highly dependent on the cultivation conditions (Herbert, 1961).

The difference regarding the BGP_{ICC} and BGP_{ATP} trends of Tap-water with phosphate limitation suggested that phosphate is an essential element necessary for biomass synthesis (Jansson, 1988; Miettinen et al., 1997), but phosphate limitation did not influence the formation of bacterial ATP, because carbon was the main nutrient needed by bacteria to produce ATP required for bio-activities regardless whether new biomass was synthesised or not (Giorgio and Cole, 1998). In other words, this finding indicates that BGP_{ATP} cannot be used to detect phosphate limitation in water or to measure growth potential in term of cell synthesis when phosphate is the growth limiting nutrient.

Interestingly, the maximum number of cells obtained in Tap-water at 100 μg/L Ac-C addition and in RO-water at 300 μg/L Ac-C addition was in the same range (1.3–1.5 × 10^6 ICC/mL). Therefore, it is reasonable to hypothesise that this is the maximum intact cell count which could be synthesised out of the total available phosphate (i.e., phosphate already present in water as well as the added 1 μg/L PO_4-P). Assuming that the already present phosphate in water was around the method detection limit of 1 μg/L PO_4-P (reported values were 0.8–0.9 μg/L PO_4-P for both water types), intact cell-yield growing on phosphate could be estimated in the range of 0.70 ± 0.05 × 10^9 cells/μg PO_4-P, indicating that significant bacterial growth could be promoted at extremely low phosphate concentrations (Miettinen et al., 1997).

To the best of our knowledge, this is the very first study discussing the potential intact cell-yield quantified by FCM on phosphate in drinking water, where there was no data available for comparison. The reported yield based on the standard plate count method was about 3.73 × 10^5 CFU/μg PO_4-P (Lehtola et al., 1999), which is much lower than that obtained based on FCM. This difference might be caused by the fact that only Pseudomonas fluorescens P17 was used and less than 1% of bacteria in water could be plate cultivated (Hammes and Egli, 2005; Van Nevel et al., 2017). It is worthwhile to mention that a different cell-yield based on phosphate can be obtained for other types of water, which was observed in this study for cell-yield based on carbon (i.e., different cell-yield between Tap-water and RO-treated water).

4.3. Practical insights

BGP test: BGP_{ICC} and BGP_{ATP} are increasingly applied for the assessment of biological stability of drinking water. In general, it is agreed that both methods are rapid, accurate, and reproducible, where BGP_{ICC} measures cell synthesis and BGP_{ATP} measures cellular activity (Vital et al., 2012; Prest et al., 2016a; Van der Kooij et al., 2017; Farhat et al., 2018; Sousi et al., 2020a). As reported in this study and elsewhere, the combination of BGP_{ICC} and BGP_{ATP}, in addition to the traditional methods for cell measurement such the determination of cell density and bio-volume, can provide more insights into the bacterial growth, e.g., in-depth understanding of cell growth stages and the role of growth limiting nutrients. In addition, the present study has also demonstrated BGP_{ATP} could not reveal the BGP of phosphate-limited samples. Therefore, for the choice of BGP method, BGP_{ICC} would be suitable for all cases, while BGP_{ATP} should be applied only on the carbon-limitation cases. Since most of drinking water is carbon-limited (Van der Kooij et al., 1982; Huck, 1990; Schurer et al., 2019; Sousi et al., 2020a), both methods are suitable to be used for bacterial growth potential assessment.

Microbially available phosphate (MAP). As discussed in this study and elsewhere, it is clear that even a very low concentration of phosphate (<1 μg/L PO_4-P) can promote extensive microbial growth (e.g. >10^6 ICC/mL) (Lehtola et al., 1999; Nescerecka et al., 2018). However, the traditional method for phosphate measurement with the current detection limit of 1 μg/L PO_4-P can hardly be helpful for bacterial growth evaluation. Using Pseudomonas fluorescens P17 and plate count, Lehtola et al. (1999) was able to develop a sensitive bioassay for determining MAP in water, with a detection limit of 0.08 μg/L PO_4-P. The present study demonstrated that by using natural bacterial consortium as an inoculum and applying FCM for intact cell quantification, the MAP bioassay could be significantly improved regarding the representativity and sensitivity (i.e., lower limit of detection). Such a bioassay will be especially valuable for the ultra-low nutrient drinking water, where phosphate concentration is below the current detection limit, but the bacterial growth in water might be phosphate-limited. Another advantage of such a bioassay is that it can be used to measure all types of phosphate that are of importance for bacterial growth in drinking water.

5. Conclusions

The assessment of bacterial growth potential (BGP) using both flow cytometric intact cell count (ICC) and adenosine triphosphate (ATP) is especially useful as additional complementary information can be obtained from the combined tests (BGP_{ICC} and BGP_{ATP}). Comparing conventionally treated drinking water (Tap-water) and ultra-low nutrient water prepared by remineralising RO permeate (RO-water) using both methods revealed that:

- Although the same bacterial inoculum (originating from Tap-water) was used, the BGPICC ratio between Tap-water/RO-water was about 7.5, whereas the BGPATP ratio was about 4.5.
- Regarding the yield of bacteria growing on acetate, a comparable ATP-yield was obtained for Tap-water and RO-water (0.39 ± 0.12 vs. 0.42 ± 0.06 ng ATP/μg-C, respectively), whereas the intact cell-yield was significantly different (9.8 ± 0.6 × 106 vs. 4.6 ± 0.8 × 106 cells/μg-C).
- A consistently higher ATP per cell was observed for RO-water compared with Tap-water, which could be attributed to the fact that the inoculum used was adapted to a significantly higher salinity and a broader range of trace elements than these present in remineralised RO permeate. This indicates that bacterial growth in ultra-low nutrient water could be limited not only by organic compounds, but also inorganic constituents.
- Carbon was identified as the growth-limiting nutrient in the two types of water studied by both BGPICC and BGPATP. With the addition of extra carbon, phosphate limitation was detected with BGPICC but not BGPATP, suggesting that a combination of carbon and phosphate is essential for the synthesis of new cells, whereas

carbon is probably used as an energy source for other bacterial activities measured by ATP when phosphate is limited.

- The intact cell-yield growing on phosphate was estimated at 0.70 ± 0.05 × 109 cells/μg PO4-P, indicating that significant bacterial growth could be promoted when extremely low concentrations of phosphate are available.

Declaration of Competing Interest

None.

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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.2021.117506.

References

Abushaban, A., Salinas-Rodriguez, S.G., Mangal, M.N., Mondal, S., Goueli, S.A., Knezev, A., Vrouwenvelder, J.S., Schippers, J.C., Kennedy, M.D., 2019. ATP measurement in seawater reverse osmosis systems: eliminating seawater matrix effects using a filtration-based method. Desalination 453, 1–9. https://doi.org/10.1016/j.desal.2018.11.020.

Boström, B., Törnblom, E., 1990. Bacterial production, heat production and ATP-turnover in shallow marine sediments. Thermochim. Acta 172, 147–156. https://doi.org/10.1016/0040-6031(90)80568-J.

Buysschaert, B., Vermijs, L., Naka, A., Boon, N., De Gusseme, B., 2018. Online flow cytometric monitoring of microbial water quality in a full-scale water treatment plant. npj Clean Water 1, 16. https://doi.org/10.1038/s41545-018-0017-7.

Chen, L., Ling, F., Bakker, G., Liu, W.T, Medema, G.J., van der Meer, Walter, Liu, G., 2020. Assessing the transition effects in a drinking water distribution system caused by changing supply water quality: an indirect approach by characterizing suspended solids. Water Research 168, 115159.

Dixon, M.B., Qiu, T., Blaikie, M., Pelekani, C., 2012. The application of the bacterial regrowth potential method and fow cytometry for biofouling detection at the Penneshaw desalination plant in South Australia. Desalination 284, 245–252. https://doi.org/10.1016/j.desal.2011.09.006.

Egli, T., Schaechter, M., 2009. Nutrition, microbial. Encyclopedia of Microbiology, (3rd ed.). Academic Press, Oxford, pp. 308–324.

Escobar, I.C., Hong, S., Randall, A.A., 2000. Removal of assimilable organic carbon and biodegradable dissolved organic carbon by reverse osmosis and nanofiltration membranes. J. Membr. Sci. 175, 1–17. https://doi.org/10.1016/S0376-7388(00)00398-7.

Farhat, N., Hammes, F., Prest, E., Vrouwenvelder, J., 2018. A uniform bacterial growth potential assay for different water types. Water Res. 142, 227–235. https://doi.org/10.1016/j.watres.2018.06.010.

Fujioka, T., Hoang, A.T., Aizawa, H., Ashiba, H., Fujimaki, M., Leddy, M., 2018. Real-time online monitoring for assessing removal of bacteria by reverse osmosis. Environ. Sci. Technol. Lett. 5, 389–393. https://doi.org/10.1021/acs.estlett.8b00200.

Giorgio, P.A.D., Cole, J.J., 1998. Bacterial growth efficiency in natural aquatic systems. Annu. Rev. Ecol. Syst. 29, 503–541. https://doi.org/10.1146/annurev.ecolsys.29.1.503.

Hammes, F., Goldschmidt, F., Vital, M., Wang, Y., Egli, T., 2010. Measurement and interpretation of microbial adenosine tri-phosphate (ATP) in aquatic environments. Water Res. 44, 3915–3923. https://doi.org/10.1016/j.watres.2010.04.015.

Hammes, F.A., Egli, T., 2005. New method for assimilable organic carbon determination using flow-cytometric enumeration and a natural microbial consortium as inoculum. Environ. Sci. Technol. 39, 3289–3294. https://doi.org/10.1021/es048277c.

Herbert, D., 1961. The chemical composition of micro-organisms as a function of their environment. Symp. Soc. Gen. Microbiol. 11, 391–416.

Hespell, R.B., Bryant, M.P., 1979. Efficiency of rumen microbial growth: influence of some theoretical and experimental factors on YATP. J. Anim. Sci. 49, 1640–1659. https://doi.org/10.2527/jas1979.4961640x.

Hong, S., Escobar, I.C., Hershey-Pyle, J., Hobbs, C., Cho, J., 2005. Biostability characterization in a full-scale hybrid NF/RO treatment system. J. Am. Water Work. Assoc. 97, 101–110.

Huck, P.M., 1990. Measurement of biodegradable organic matter and bacterial growth potential in drinking water. J. Am. Water Work. Assoc. 82, 78–86. https://doi.org/10.1002/j.1551-8833.1990.tb06995.x.

Ihssen, J., Jovanovic, N., Sirec, T., Spitz, U., 2021. Real-time monitoring of extracellular ATP in bacterial cultures using thermostable luciferase. PLoS One 16, e0244200. https://doi.org/10.1371/journal.pone.0244200.

Jansson, M., 1988. Phosphate uptake and utilization by bacteria and algae. Hydrobiologia. 170, 177–189. https://doi.org/10.1007/978-94-009-3109-1_11.

Lehtola, M.J., Miettinen, I.T., Vartiainen, T., Martikainen, P.J., 1999. A new sensitive bioassay for determination of microbially available phosphorus in water. Appl. Environ. Microbiol. 65, 2032–2034. https://doi.org/10.1128/AEM.65.5.2032-2034.1999.

Liu, G., Zhang, Y., Liu, X., Hammes, F., Liu, W.T., Medema, G.J., Wessels, P., van der Meer, W., 2020. 360- Degree Distribution of Biofilm Quantity and Community in an Operational Unchlorinated Drinking Water Distribution Pipe. Environmental Science and Technology 54(9), 5619–5628.

Mempin, R., Tran, H., Chen, C., Gong, H., Kim Ho, K., Lu, S., 2013. Release of extracellular ATP by bacteria during growth. BMC Microbiol. 13 (301) https://doi.org/10.1186/1471-2180-13-301.

Miettinen, I.T., Vartiainen, T., Martikainen, P.J., 1997. Phosphorus and bacterial growth in drinking water. Appl. Environ. Microbiol. 63, 3242–3245. https://doi.org/10.1128/AEM.63.8.3242-3245.1997.

Nescerecka, A., Juhna, T., Hammes, F., 2018. Identifying the underlying causes of biological instability in a full-scale drinking water supply system. Water Res. 135, 11–21. https://doi.org/10.1016/j.watres.2018.02.006.

Park, S.K., Hu, J.Y., 2010. Assessment of the extent of bacterial growth in reverse osmosis system for improving drinking water quality. J. Environ. Sci. Health Part A 45, 968–977. https://doi.org/10.1080/10934521003772386.

Prest, E.I., Hammes, F., Kötzsch, S., Van Loosdrecht, M.C.M., Vrouwenvelder, J.S., 2016a. A systematic approach for the assessment of bacterial growth-controlling factors linked to biological stability of drinking water in distribution systems. Water Sci. Technol. Water Supply 16, 865–880. https://doi.org/10.2166/ws.2016.001.

Prest, E.I., Hammes, F., Van Loosdrecht, M.C.M., Vrouwenvelder, J.S., 2016b. Biological stability of drinking water: controlling factors, methods, and challenges. Front. Microbiol. 7 (45) https://doi.org/10.3389/fmicb.2016.00045.

Schurer, R., Schippers, J.C., Kennedy, M.D., Cornelissen, E.R., Salinas-Rodriguez, S.G., Hijnen, W.A.M., Van der Wal, A., 2019. Enhancing biological stability of disinfectant-free drinking water by reducing high molecular weight organic compounds with ultrafiltration posttreatment. Water Res. 164, 114927 https://doi.org/10.1016/j.watres.2019.114927.

Servais, P., Billen, G., Hascoët, M.C., 1987. Determination of the biodegradable fraction of dissolved organic matter in waters. Water Res. 21, 445–450. https://doi.org/10.1016/0043-1354(87)90192-8.

Sousi, M., Liu, G., Salinas-Rodriguez, S.G., Chen, L., Dusseldorp, J., Wessels, P., Schippers, J.C., Kennedy, M.D., Van der Meer, W., 2020a. Multi-parametric assessment of biological stability of drinking water produced from groundwater: Reverse osmosis vs. conventional treatment. Water Res. 186, 116317 https://doi.org/10.1016/j.watres.2020.116317.

Sousi, M., Liu, G., Salinas-Rodriguez, S.G., Knezev, A., Blankert, B., Schippers, J.C., Van der Meer, W., Kennedy, M.D., 2018. Further developing the bacterial growth potential method for ultra-pure drinking water produced by remineralization of reverse osmosis permeate. Water Res. 145, 687–696. https://doi.org/10.1016/j.watres.2018.09.002.

Sousi, M., Salinas-Rodriguez, S.G., Liu, G., Schippers, J.C., Kennedy, M.D., Van der Meer, W., 2020b. Measuring bacterial growth potential of ultra-low nutrient drinking water produced by reverse osmosis: effect of sample pre-treatment and bacterial inoculum. Front. Microbiol. 11 (791) https://doi.org/10.3389/fmicb.2020.00791.

Thayanukul, P., Kurisu, F., Kasuga, I., Furumai, H., 2013. Evaluation of microbial regrowth potential by assimilable organic carbon in various reclaimed water and distribution systems. Water Res. 47, 225–232. https://doi.org/10.1016/j.watres.2012.09.051.

Van der Kooij, D., Veenendaal, H.R., van der Kooij, D., van der Wielen, P.W.J.J., 2014. Regrowth problems and biostability assessment in the Netherlands. Microbial Growth in Drinking-Water Supplies: Problems, Causes, Control and Research Needs. IWA Publishing, London, pp. 291–337.

Van der Kooij, D., Veenendaal, H.R., Van der Mark, E.J., Dignum, M., 2017. Assessment of the microbial growth potential of slow sand filtrate with the biomass production potential test in comparison with the assimilable organic carbon method. Water Res. 125, 270–279. https://doi.org/10.1016/j.watres.2017.06.086.

Van der Kooij, D., Visser, A., Hijnen, W.A.M., 1982. Determining the concentration of easily assimilable organic carbon in drinking water. J. Am. Water Work. Assoc. 74, 540–545. https://doi.org/10.1002/j.1551-8833.1982.tb05000.x.

Van Nevel, S., Koetzsch, S., Proctor, C.R., Besmer, M.D., Prest, E.I., Vrouwenvelder, J.S., Knezev, A., Boon, N., Hammes, F., 2017. Flow cytometric bacterial cell counts challenge conventional heterotrophic plate counts for routine microbiological drinking water monitoring. Water Res. 113, 191–206. https://doi.org/10.1016/j.watres.2017.01.065.

Vital, M., Dignum, M., Magic-Knezev, A., Ross, P., Rietveld, L., Hammes, F., 2012. Flow cytometry and adenosine tri-phosphate analysis: alternative possibilities to evaluate major bacteriological changes in drinking water treatment and distribution systems. Water Res. 46, 4665–4676. https://doi.org/10.1016/j.watres.2012.06.010.

Wang, Y., Hammes, F., Boon, N., Chami, M., Egli, T., 2009. Isolation and characterization of low nucleic acid (LNA)-content bacteria. ISME J. 3, 889–902. https://doi.org/10.1038/ismej.2009.46.