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Characterization of microbial regrowth potential shaped by advanced drinking water treatment

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ABSTRACT

Microbial regrowth in premise plumbing is a threat to water safety. Disinfectant residuals are often diminished during water transportation and stagnation, leading to the regrowth of opportunistic pathogens. Although microbial regrowth potential is mostly determined by water treatment, little is known about how each treatment step affects two key factors that contribute to microbial regrowth potential: biodegradable organic matter and microbial abundance. In this study, we operated annular reactors to evaluate the microbial regrowth potential of water shaped after each treatment step in a full-scale drinking water treatment plant with ozonation and biological activated carbon filtration. The assimilable organic carbon and total cell count (TCC) were stable at all treatment steps during the sampling period from July to October 2015. The assimilable organic carbon consumption and TCC net increase in the annular reactors indicated that apparent growth yields (cell number base) of microbial communities were different in each reactor. Regrowth potential evaluated by indigenous microbial community in finished water was reduced to 22% of that in raw water, while 75% of assimilable organic carbon in raw water remained in finished water. It suggested that treatment performance evaluated by indigenous microbial communities was better than that evaluated by assimilable organic carbon.

Key words: advanced drinking water treatment, annular reactor, assimilable organic carbon, microbial regrowth potential

HIGHLIGHTS

- Changes in microbial regrowth potential in water during advanced drinking water treatment were evaluated.
- The apparent growth yields of microbial communities growing in effluent from each treatment step were different.
- Indigenous microbial community in finished water was used to assess the changes in specific regrowth-promoting substrates.

INTRODUCTION

Microbial regrowth in water distribution systems and premise plumbing deteriorates drinking water hygiene and biological safety (Liu *et al.* 2014; Proctor *et al.* 2017; Ling *et al.* 2018; Chan *et al.* 2019). Disinfectant residuals are easily diminished during water distribution through old pipes and intermittent stagnation in premise plumbing (Al-Jasser 2007; Rahmatika *et al.* 2020). The regrowth of opportunistic pathogens, such as *Legionella pneumophila* and *Mycobacterium avium*, significantly impacts human health (van der Wielen & van der Kooij 2013; Gebert *et al.* 2018; Nescerecka *et al.* 2018; Waak *et al.* 2018, 2019; Donohue *et al.* 2019). These opportunistic pathogens in premise plumbing impose a more serious health burden than enteric bacteria for waterborne infectious diseases in the USA (Collier *et al.* 2021). Therefore, the biological stability of drinking water attracts increasing attention.

Microbial regrowth potential is regulated by various factors (Prest *et al.* 2016). The interaction between biodegradable organic matter (BOM) and microorganisms is crucial to understand these phenomena. BOM in drinking water is frequently evaluated by assimilable organic carbon (AOC). The AOC is conventionally determined by the growth of two standard strains: *Pseudomonas fluorescens* P17 and *Aquaspirillum* sp. NOX (van der Kooij *et al.* 1982; van der Kooij & Hijnen 1984). Recently, microbial communities have been also used for AOC

determination instead of the P17 and NOX strains (Hammes & Egli 2005). The AOC level increases and decreases during water treatment. Oxidation treatment, such as ozonation, increases the AOC level compared to that in raw water (Hammes *et al.* 2007; Ramseier *et al.* 2011a; Soonglerdsongpha *et al.* 2011). Subsequent biological treatment, such as biological activated carbon (BAC) filtration and biological sand filtration, effectively reduces AOC (Chien *et al.* 2008; Soonglerdsongpha *et al.* 2011; Basu *et al.* 2016; Kasuga *et al.* 2020). However, these treatments have opposite impacts on microbial abundance. While ozonation is very effective to inactivate and decay microbial cells (Hammes *et al.* 2008, 2010; Ramseier *et al.* 2011b), active microbial growth takes place in parallel with BOM consumption in biological filtration, resulting in an increase in microorganisms in the effluent of biological treatment (Hammes *et al.* 2008; Lautenschlager *et al.* 2014). In addition to microbial abundance, specific microbial community structures can be shaped after each treatment (Pinto & Raskin 2012; Li *et al.* 2017). Taken together, ozonation increases microbial regrowth potential by generating BOM, while it decreases microbial abundance. Biological filtration contributes to reduce microbial regrowth potential by eliminating BOM, while it releases more microorganisms in the water that could potentially become seeds of microbial regrowth in the drinking water. Final chlorination also generates BOM, while it reduces viable microorganisms or select chlorine-resistant species (Polanska *et al.* 2005; Luo *et al.* 2021). As changes in BOM and microbial community are complicated during the treatment process, little information is available on how microbial regrowth potential is shaped in the drinking water.

In the present study, we aimed to investigate the changes in microbial regrowth potential in water by operating annular reactors receiving effluents from different treatment steps in a full-scale advanced drinking water treatment plant. While the conditions after each treatment just represent a snapshot of water quality, microbial regrowth is a phenomenon that accompanies the alteration of initial water quality. Annular reactors were used to evaluate such the alternation of initial water quality. We evaluated the AOC consumption and microbial regrowth in each reactor. Moreover, the indigenous microbial communities in finished water were used for assessing specific BOM promoting their regrowth, which was compared to the conventional AOC.

METHODS

Sampling at a full-scale drinking water treatment plant

Water samples were collected from a full-scale drinking water treatment plant in Tokyo. The capacity of the plant was 1.1 million m^3/day. River water is treated by coagulation–sedimentation, followed by ozonation, BAC filtration, and rapid sand filtration (Supplementary Fig. S1). Sodium hypochlorite is injected before and after rapid sand filtration for disinfection. Water samples after each treatment step were collected in carbon-free glass bottles, which had been baked at 550 °C for 6 h to eliminate carbon contamination.

Annular reactor operation

Four annular reactors (MODEL1320LS Laboratory Model, BioSurface Technologies Corporation, MT, USA) were operated at the plant from July 10 to October 15, 2015. Sampling from the reactors was conducted on days 0, 3, 11, 18, 38, 52, 63, 76, 89, and 97 after the operation. The effective volume of the reactor was 1 L. Polycarbonate coupons (1.5 cm × 16.5 cm) were attached to the internal cylinder rotating at 90 rpm. The reactors receiving effluents from coagulation–sedimentation, ozonation, and BAC filtration, as well as the finished water, were designated as AR1, AR2, AR3, and AR4, respectively (Supplementary Fig. S1). The influent flow rate to each reactor was set at 10 mL/min, while water temperature (18.5–28.0 °C in raw water) was not controlled. Sodium thiosulfate solution (44.5 mg/L) was dosed at 0.1 mL/min to the influent of AR4 to quench free chlorine residual (0.2–0.4 mg/L). It was also added to the influents of AR1–AR3 to operate all reactors under the same condition. Influent and effluent of the reactor were collected regularly to determine the AOC concentration and total cell count (TCC). In addition to water samples, coupons were regularly collected from each reactor to determine the TCC attached to the surface of the coupons. The attached cells on the coupon were collected by sterilized scrapers. They were suspended in 10 mL of phosphate buffer at pH 7.0 followed by vortex for 2 min and sonication at 5 W for 2 min for the TCC quantification.

Evaluation of specific BOM promoting the regrowth of microbial communities in finished water

Raw water, effluents from coagulation–sedimentation, ozonation, BAC filtration, and rapid sand filtration, as well as finished water after disinfection were collected on November 25, 2015. The AOC of these samples was determined. In addition, they were filtrated through 0.20-μm polycarbonate membrane filters (Isopore; Merk Millipore,

MA, USA), which were washed by Milli-Q water in advance, to completely remove bacterial cells in the original water samples. The filtrated water samples (50 mL) were dispensed to carbon-free Erlenmeyer flasks. Milli-Q water was treated in the same manner as an operational control. Sodium thiosulfate and inorganic nutrient solution were added to each sample according to the AOC protocol and pasteurized at 75 °C for 30 min. After cooling down, effluent from AR4 was added as a microbial seed so that the initial TCC was 1,000 cells/mL. All samples were incubated at 20 °C in dark without agitation for 6 days to monitor microbial regrowth by flow cytometry. The maximum growth levels were corrected by the result of operational control using Milli-Q water.

Water quality and microbiological analysis

Water samples were filtrated through GF/F glass fiber filters (Whatman, MA, USA) to remove the potential impact of particulate organic matter, which had been baked at 550 °C for 6 h to eliminate carbon contamination. Dissolved organic carbon (DOC) was analyzed as non-purgeable organic carbon with a TOC-L (SHIMADZU, Japan). AOC was determined by the P17-NOX method using *P. fluorescens* P17 (ATCC 49642) and *Aquaspirillum* sp. NOX (ATCC 49643) (Japan Water Works Association 2011). All glassware used for the AOC analysis had been baked at 550 °C for 6 h. Water samples filtrated through the GF/F membranes were supplemented with an inorganic nutrient solution so that only organic matter was regarded as a limiting factor for microbial growth (Kasuga *et al.* 2020). Residual chlorine was quenched by sodium thiosulfate. After pasteurization at 75 °C for 30 min (Japan Water Works Association 2011), P17 and NOX were inoculated and incubated at 15 °C. They were enumerated on an R2A agar medium (Becton Dickinson, NJ, USA) at 20 °C. The growth yields of P17 and NOX used for the calculation of AOC were 4.1×10^6 and 1.2×10^7 CFU/μg acetate-C, respectively (van der Kooij 1992).

For determining the TCC, water samples were stained with 1 × SYBR® Green (Thermo Fisher Scientific, MA, USA) and 5 mM EDTA (Thermo Fisher Scientific) at 37 °C for 10 min. For determining the intact cell counts (ICC), water samples were stained with 1 × SYBR® Green, 1.5 μM propidium iodide (Thermo Fisher Scientific), and 5 mM EDTA at 37 °C for 10 min. The quantification of the TCC and ICC was performed by flow cytometer (Accuri C6, Becton Dickinson). The limit of quantification of the TCC and ICC was 1,000 cells/mL. There are other methods to quantify bacterial abundance such as cultivation and adenosine triphosphate (ATP). However, many bacteria in water are not culturable and ATP might include the large microorganisms other than bacteria, which can result in the underestimation and overestimation of bacterial abundance. Thus, we selected TCC to evaluate bacterial abundance in this study.

RESULTS AND DISCUSSION

Changes in the AOC concentration and microbial abundance in the full-scale advanced water treatment process

Figure 1(a) shows changes in AOC concentration along the water treatment process during the sampling period from July 10 to October 15, 2015. While DOC concentrations steadily decreased from raw water to finished water (Supplementary Material, Figure S2), the AOC concentrations increased and decreased. The average AOC concentration in raw water was 37 ± 4.9 μg acetate-C/L (mean ± SD, $n = 10$). Ozonation increased the AOC to 90 ± 5.5 μg acetate-C/L. Subsequent BAC filtration effectively reduced the AOC to 48 ± 5.7 μg acetate-C/L. A similar trend was reported in the other studies analyzing the drinking water treatment process in Tokyo (Soonglerdsongpha *et al.* 2011; Kasuga *et al.* 2020). Finally, the finished water after disinfection contained 37 ± 6.8 μg acetate-C/L, representing the same AOC level in raw water. The coefficient of variation of the AOC concentration in each sample was only 6.1–18.6%, indicating that the treatment performance was stable during the sampling period.

In raw water, rapid sand filtration effluent and finished water, AOC-P17 and AOC-NOX fractions consisted of 43–51 and 49–57% of the total AOC, respectively. However, the ratios of AOC-NOX in the coagulation–sedimentation, ozonation, and BAC filtration effluents were 72–80%, indicating that chemicals added at coagulation and ozonation generated AOC-NOX. Lower carboxylic acids, such as oxalate, which are major AOC-NOX components, could be generated by ozonation (van der Kooij & Hijnen 1984; Hammes *et al.* 2007; Kasuga *et al.* 2020).

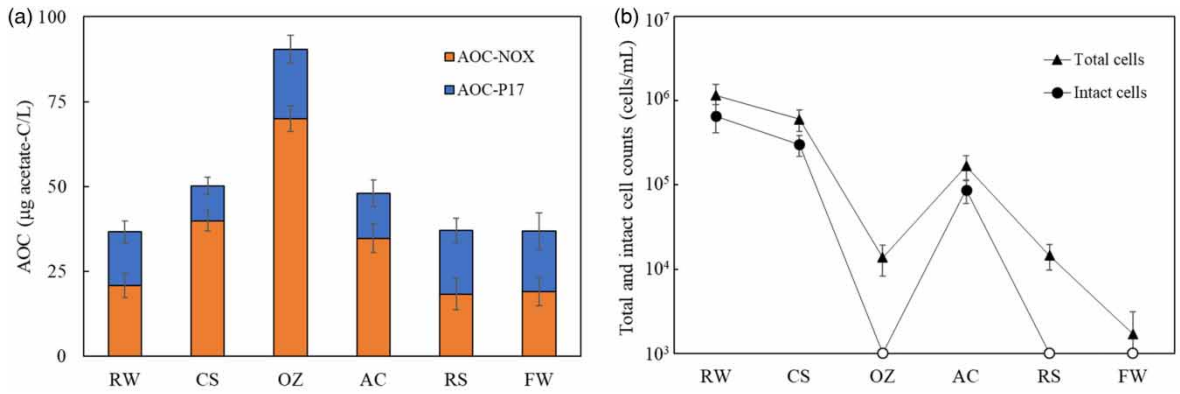

Figure 1 | Changes in the (a) AOC concentration and (b) TCC and ICC along the treatment process during the study period. Open symbols in (b) indicate the levels below the quantification limit of flow cytometry. Error bars indicate standard deviation ($n = 10$). RW, raw water; CS, coagulation–sedimentation; OZ, ozonation; AC, BAC filtration; RS, rapid sand filtration; FW, finished water.

Figure 1(b) shows the changes in TCC and ICC along the treatment process during the sampling period. The TCC in raw water ($1.2 \times 10^6 \pm 4.2 \times 10^5$ cells/mL: mean $\pm$ SD, $n = 10$) was reduced to $1.4 \times 10^4 \pm 5.4 \times 10^3$ cells/mL after ozonation, suggesting that ozonation effectively destroyed microbial cells. However, the TCC recovered to $1.7 \times 10^5 \pm 5.5 \times 10^4$ cells/mL after the BAC filtration due to the active proliferation of BAC-associated microorganisms. Disinfection by sodium hypochlorite before and after the rapid sand filtration reduced the TCC. The TCC in the finished water was $1.7 \times 10^3 \pm 1.4 \times 10^3$ cells/mL. The average $\log_{10}$ reduction value of the TCC was 2.8 in the overall treatment. On average, 56% of the TCC was regarded as ICC in raw water. However, the ICC was below the limit of quantification after ozonation, which was consistent with the changes in the TCC. While 52% of the TCC was estimated as ICC in BAC filtration effluent, the ICC was below the quantification limit in the rapid sand filtration effluent and finished water due to disinfection. Cell membranes were effectively broken by oxidation such as ozonation and chlorination (Ramseier *et al.* 2011b).

AOC consumption and microbial regrowth potential in the annular reactors receiving effluents from different treatment steps

Four annular reactors were operated by supplying effluents from coagulation–sedimentation, ozonation, BAC filtration, and finished water to assess how microbial regrowth potential was shaped during the treatment process. AOC consumption in each reactor was determined by analyzing AOC in influent and effluent (Figure 2). AOC consumption in AR1 increased from 5.5 μg acetate-C/L on day 3 to 17.6 μg acetate-C/L on day 11. After day 18, AOC consumption was almost stabilized. In AR2 and AR3, AOC consumptions increased to 22.3 and 16.4 μg acetate-C/L on day 38, respectively, and those levels were stable until the end of the operation. The AOC consumption in AR2 receiving ozonated water was the largest, which was in good agreement with the highest AOC level in ozonated water (Figure 1(a)), while AR4 demonstrated the smallest consumption (6.7–10.7 μg acetate-C/L) during the operation. AOC-NOX accounted for 87.4, 76.5, 90.1, and 81.8% (mean percentages during days 38–97) of the total AOC consumptions in AR1, AR2, AR3, and AR4, respectively. AOC-NOX was preferentially consumed by microorganisms in the reactors regardless of the effluent type. While AOC-P17 could contain various types of organic matter, AOC-NOX was mainly composed of lower carboxylic acids, such as oxalate, mainly generated by the oxidation process (van der Kooij & Hijnen 1984; Polanska *et al.* 2005). Although the compositional changes in AOC-NOX during the treatment remain unrevealed, the results indicated that AOC-NOX is a primary fraction of BOM during the treatment process.

The net TCC increase in each reactor was determined by comparing the TCC in the influent and effluent (Figure 3). The TCC levels in AR2, AR3, and AR4 were almost stabilized after day 11, while the changes in AR1 fluctuated during the operation. The average net TCC increases during days 11 and 97 were 9.8×10^5 cells/mL (AR2), followed by 7.0×10^5 cells/mL (AR4), 6.4×10^5 cells/mL (AR3), and 2.9×10^5 cells/mL (AR1), respectively. The TCC increases in AR1, AR2, AR3, and AR4 were 1.6, 72.2, 4.9, 410 folds higher than the actual TCC observed in the full-scale treatment (Figure 1(b)). The TCC in the ozonation effluent (influent of AR2) and finished water (influent of AR4) was suppressed by ozone and rapid sand filtration with disinfection.

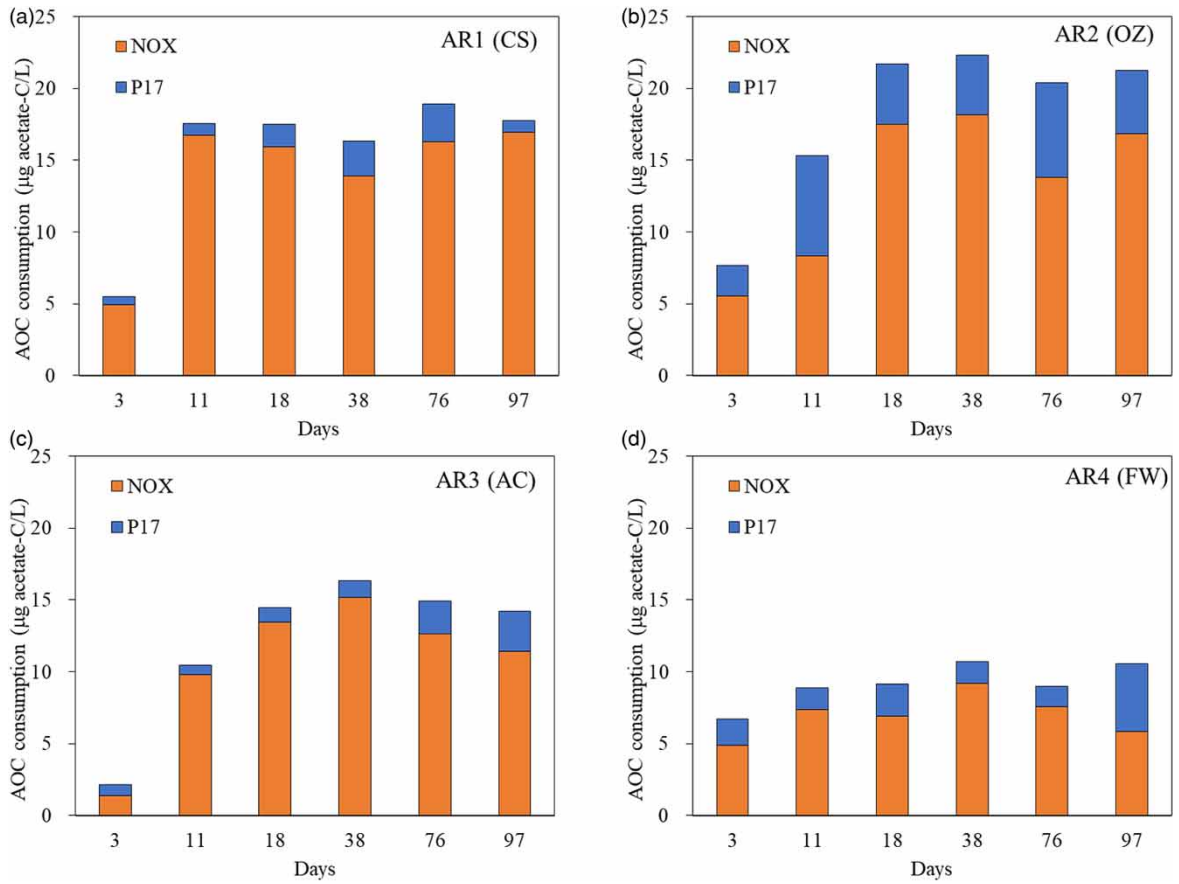

Figure 2 | AOC consumptions in the annular reactors. CS, coagulation–sedimentation; OZ, ozonation; AC, BAC filtration; FW, finished water.

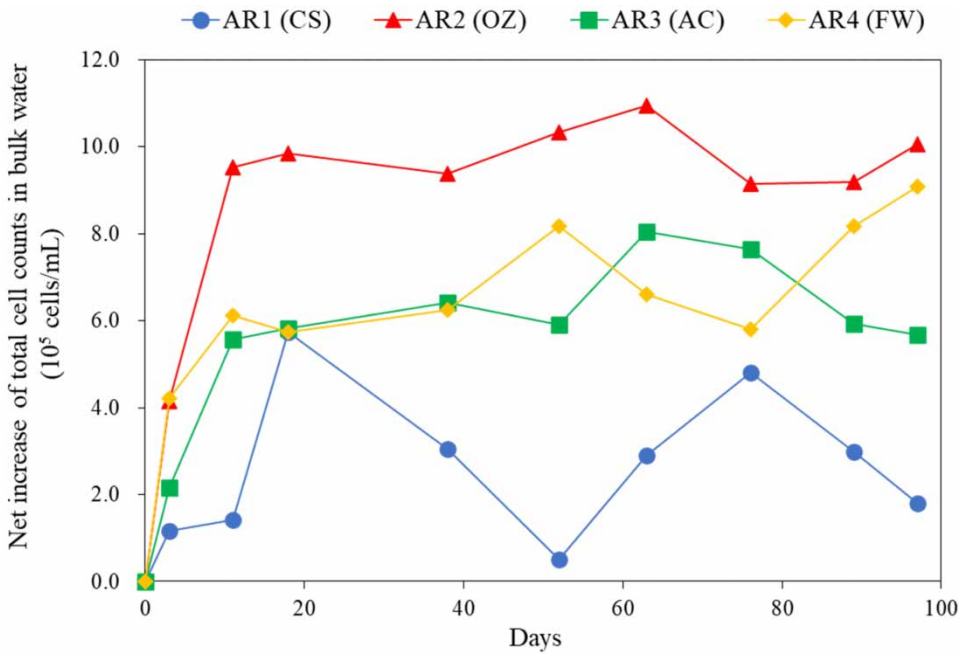

Figure 3 | Net TCC increase in water in the annular reactors. CS, coagulation–sedimentation; OZ, ozonation; AC, BAC filtration; FW, finished water.

However, the residual microbial community in each effluent still had significant regrowth potential based on residual BOM.

In addition to the TCC in bulk water, the TCC on the coupons in the reactors were enumerated (Figure 4). After the rapid attachment of microbial cells by day 11, a steady-state condition was observed in all reactors. The TCC levels in AR1–AR3 during days 11–97 were 1.7×10^6, 2.2×10^6, and 1.6×10^6 cells/cm^2, respectively, indicating that biofilm formation potentials of coagulation–sedimentation effluent, ozonation effluent, and BAC filtration effluent were similar. However, the TCC level in AR4 during days 11–97 was nearly an order of magnitude lower (2.3×10^5 cells/cm^2) than that in the other reactors. Microbial communities selected after disinfection might have less affinity to surfaces of coupons or easily released from actively growing biofilm. In the Netherlands, 1.2×10^5–3.2×10^5 cells/cm^2 of the TCC was observed in biofilm developed in an unchlorinated drinking water distribution system (Liu *et al.* 2014), which is equivalent to the TCC level on the coupons in AR4 receiving quenched finished water.

The relationship between the AOC consumption and net TCC increase in bulk water is compared in Figure 5. The slopes (net TCC increase/AOC consumption) in Figure 5 indicate apparent growth yields (cell number base) of the microbial communities growing in each reactor, while it is important to note that there is uncertainty in the apparent growth yields as cell sizes were not considered. Apparent growth yields of each reactor were AR4 (7.2×10^7 cells/μg acetate-C) > AR2 (4.2×10^7 cells/μg acetate-C) > AR3 (3.6×10^7 cells/μg acetate-C) > AR1 (1.6×10^7 cells/μg acetate-C). These values were more similar to the growth yield of *Aquaspirillum* sp. NOX (1.2×10^7 CFU/μg acetate-C) than that of *P. fluorescens* P17 (4.1×10^6 CFU/μg acetate-C). In particular, the microbial community with the highest apparent growth yield was selected in finished water. The variations in apparent growth yields in different reactors suggest that microbial communities with different cell sizes or substrate utilization patterns were selected by each treatment. Another possibility is that differences in residual BOM composition could affect growth yields. Further study is necessary to reveal compositions of BOM and microbial communities in each reactor. Terry & Summers (2018) reported that AOC accounted for only 30% of biodegradable DOC. In addition, P17 and NOX are unable to assimilate some biodegradable substances (Sack *et al.* 2011; Hijnen *et al.* 2018; Schurer *et al.* 2019). As the TCC could indicate all cells present in the water, the apparent growth yields based on the standard AOC could be potentially overestimated.

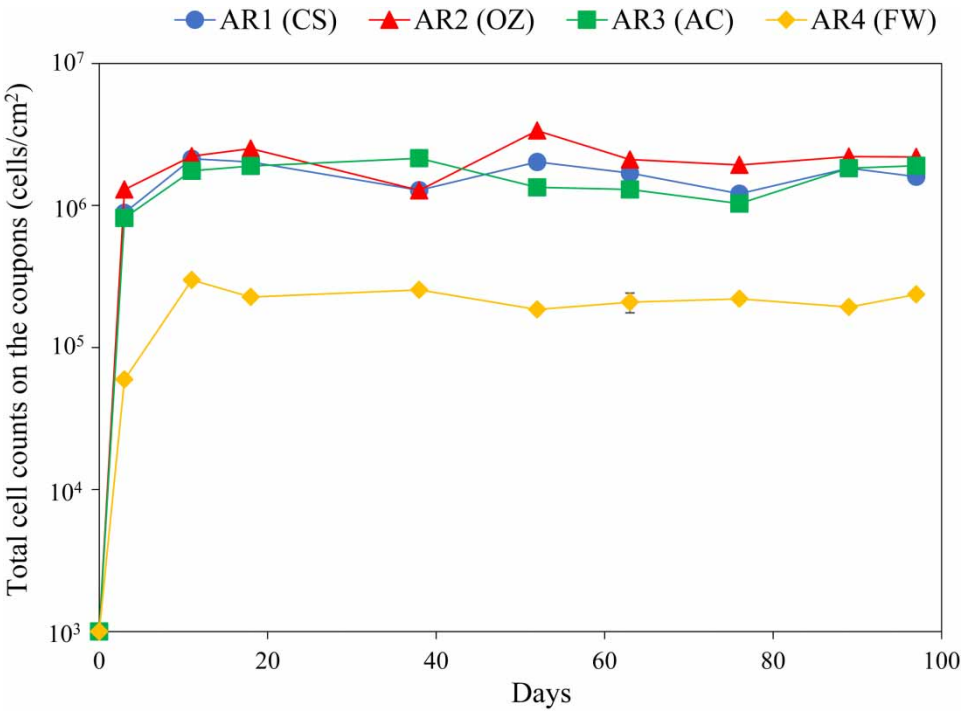

Figure 4 | TCC on the coupons in the annular reactors. Error bars indicate the standard deviation of measurement replicates ($n = 3$). CS, coagulation–sedimentation; OZ, ozonation; AC, BAC filtration; FW, finished water.

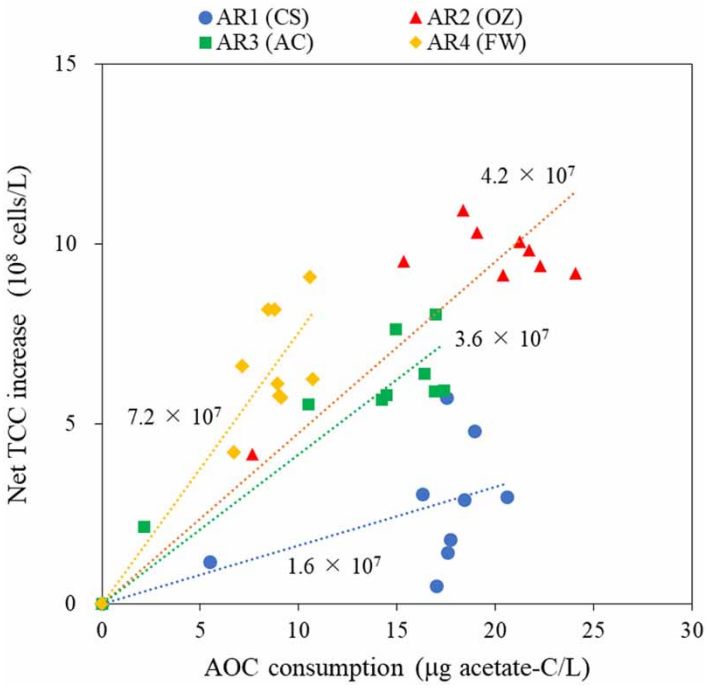

Figure 5 | Relationship between the AOC consumption and net TCC increase in water in the annular reactors. The values indicate the slope of regression lines or apparent growth yields (cells/μg acetate-C). CS, coagulation–sedimentation; OZ, ozonation; AC, BAC filtration; FW, finished water.

Evaluation of specific fraction of BOM promoting the regrowth of microbial community in drinking water

The microbial community in the AR4 effluent was added to the sterilized water samples collected from raw water to finished water in order to assess the regrowth potential of the community. This community growing in AR4 was selected as a seed because it should include seed microorganisms causing microbial regrowth in the distribution system and premise plumbing. Their growth reached the plateau in each sample after 3 days of incubation (Supplementary Material, Figure S3) and the maximum growth levels were obtained (Supplementary Material, Figure S4), which corresponded to BOM quantity specifically supported the growth of the community. The maximum growth level in raw water was 1.4×10^6 cells/mL. It increased to 2.9×10^6 cells/mL in ozonated water and

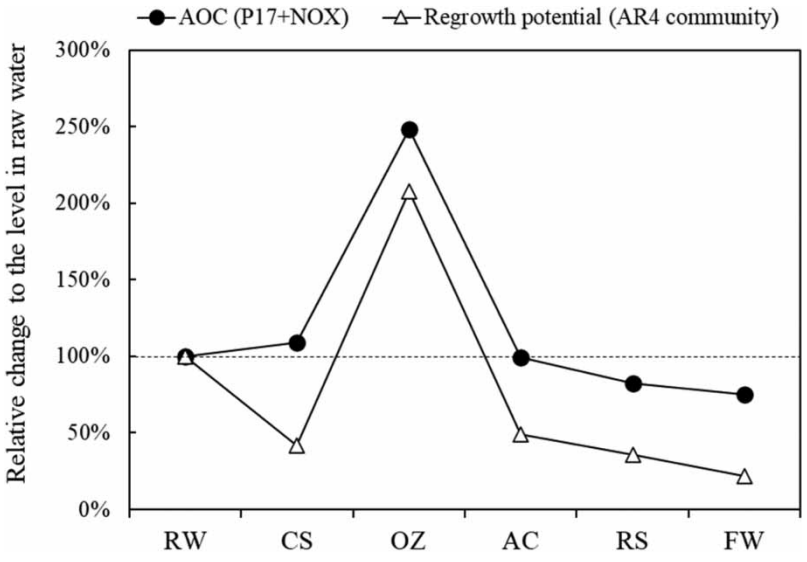

Figure 6 | Comparison between relative changes in AOC concentrations and regrowth potential of the microbial community in AR4. RW, raw water; CS, coagulation–sedimentation; OZ, ozonation; AC, BAC filtration; RS, rapid sand filtration; FW, finished water.

then finally decreased to 3.0×10^5 cells/mL in finished water. The level in finished water was similar to the exact microbial regrowth level in tap water after free chlorine decayed (Rahmatika *et al.* 2020).

The relative changes in AOC concentrations evaluated by the standard strains (P17 and NOX) and regrowth potential evaluated by seed community in AR4 effluent are compared in Figure 6. While AOC concentrations in raw water and coagulation–sedimentation effluent were almost similar, regrowth potential in coagulation–sedimentation effluent decreased to 42% of that in raw water. This indicates that coagulation–sedimentation effectively removed specific substrates for the community. After ozonation, both AOC concentration and regrowth potential increased by more than two folds of those in raw water. BAC filtration reduced 76% of regrowth potential, which was higher than the removal ratio of AOC (60%). However, the levels of AOC concentration and regrowth potential after the BAC filtration were similar to those in the coagulation–sedimentation effluent. It is likely that the BAC filtration just eliminated the additional BOM generated by ozonation and the BOM contained in coagulation–sedimentation effluent might simply pass the BAC filtration. The compositional changes in the BOM promoting microbial regrowth should be investigated (Kasuga *et al.* 2020). The regrowth potential in finished water was only 22% of the initial potential in raw water. On the other hand, AOC concentration decreased to 75% of the initial AOC in raw water, indicating that BOM covered by microbial community in AR4 and the standard strains for AOC analysis might be different. As the relative reduction of regrowth potential was more than AOC in the treatment process, AOC could overestimate BOM contributing to actual microbial regrowth. It is possible that BOM, which can be removed by coagulation–sedimentation, could be important to reduce the potential. The use of indigenous microbial community as a seed is useful to evaluate the actual microbial regrowth potential in drinking water shaped by advanced water treatment.

CONCLUSIONS

In this study, annular reactors were used to evaluate how residual microbial regrowth potential was shaped by different treatment steps in a full-scale advanced drinking water treatment plant. AOC consumption and TCC increase in each reactor demonstrated the shift of regrowth potential during the treatment. Apparent growth yields of microbial community in finished water were higher than those in coagulation–sedimentation effluent, ozonation effluent, and BAC effluent. Indigenous microbial communities in finished water instead of the standard strains for AOC analysis were used to assess the specific fraction of BOM promoting actual microbial regrowth in drinking water. Treatment performance evaluated by indigenous microbial communities was better than that evaluated by AOC. Understanding the impact of each unit treatment on microbial regrowth is informative to optimize the operational conditions for the final production of biologically stable water.

ACKNOWLEDGEMENT

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DATA AVAILABILITY STATEMENT

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

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