Water Research 154 (2019) 377–386

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Using discrete and online ATP measurements to evaluate regrowth potential following ozonation and (non)biological drinking water treatment

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ARTICLE INFO

Article history:
Received 7 December 2018
Received in revised form
28 January 2019
Accepted 11 February 2019
Available online 14 February 2019

Keywords:
Biostability
Regrowth
Adenosine Triphosphate (ATP)
Biomass production potential
Chlorine
Biofiltration

ABSTRACT

Water utilities must control microbial regrowth in the distribution system to protect public health. In this study, an adenosine triphosphate (ATP)-based biomass production potential test using indigenous bacterial communities were used to evaluate regrowth potential following ozonation with either biofiltration (BF) or sustained chlorination (SCl_2). Two full-scale water treatment plants with different upstream processes (i.e., WTP-BF: ozonation, coagulation/flocculation, biofiltration, UV irradiation, chlorination; and WTP-SCl_2: ozonation, chlorination, coagulation/flocculation, filtration, chlorination) were compared. Characterization of indigenous bacteria using 16S rRNA gene sequencing, qPCR, and cellular ATP (cATP) showed microbial diversity changes across treatment, biomass sloughing from biofilters (effluent cATP = 30 ± 1 ng/L), and disinfection by chlorine (cATP < 1 ng/L). For both WTPs, 14-day cumulative biomass production ($CBP_t = \sum_{t=0}^{t} ATP(t) \times \Delta t$) was highest for ozonated water samples ($CBP_{14} = 1.2 \times 10^3$–$3.0 \times 10^3$ d ngATP/L). CBP_{14} further increased with increasing ozone dose due to production of more biodegradable carbon. Growth promotion by carbon was confirmed from the consumption of ozonation byproducts (carboxylic acids, aldehydes) and the increase in CBP (9.5×10^2 –2.9×10^3 d ngATP/L) after addition of 50–300 μgC/L acetate. Ozone followed by sustained chlorination (WTP-SCl_2) effectively controlled biomass growth across the treatment process (CBP_{14} <10 d ngATP/L). In contrast, ozone followed by biofiltration (WTP-BF) reduced regrowth potential by 30% (biofilter influent $CBP_{14} = 1.3 \times 10^3$ d ngATP/L; biofilter effluent $CBP_{14} = 9.3 \times 10^2$ d ngATP/L). After adding chlorine to the biofilter effluent, CBP_{14} was reduced to <10 d ngATP/L. Lastly, online ATP measurements confirmed the discrete measurements and improved identification of the cATP peak and growth phases of indigenous bacteria.

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1. Introduction

Strategies for controlling microbial growth in distributions systems vary globally to protect public health against waterborne pathogens. In some European countries, where use of oxidant residuals are less common, source water protection and advanced multibarrier treatment are strictly practiced to minimize the amount of biodegradable organic carbon entering the distribution system, preventing growth of organisms in case of insufficient inactivation or regrowth due to inappropriate plumbing materials (Prest et al., 2016; Semenza and Menne, 2009; van der Kooij and van der Wielen, 2014). The United States Environmental Protection Agency (USEPA), on the other hand, requires systems using surface waters to have greater than 0.2 mg/L of chlorine at the point of entry and a detectable residual in the distribution system (US EPA, 1999). In either case, the amount of biodegradable organic carbon must be properly measured and managed to minimize the risk of biofouling in the distribution system (Rosario-Ortiz et al., 2016).

Water utilities aim to achieve biological stability throughout the water network. Biological stability refers to a condition where drinking water does not promote changes in bacterial concentration and composition (Prest et al., 2016; Rittmann and Snoeyink, 1984). A biostable water would have the same microbial quality in the distribution system and the consumers' taps as the water coming out of the treatment facility (Prest et al., 2016). Several

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https://doi.org/10.1016/j.watres.2019.02.006
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variables may impact biostability including changing seasons and high temperature (e.g., drought), during water main breaks, diminished oxidant residuals, increased nutrient loading from runoff events, and fluctuating hydraulic conditions (e.g., stagnation, biofilm detachment) (Prest et al., 2016; van der Kooij and van der Wielen, 2014).

To meet bacteriological water quality standards in distribution systems, water utilities monitor residual disinfectants in addition to total coliforms and *E. coli* according to the Revised Total Coliform Rule which aims to prevent incidences of fecal contamination and exposure to waterborne pathogens (US EPA, 2013). If the system is more vulnerable to contamination (e.g., absence of chlorine residuals) additional assessments are performed and corrective actions are required. To assess the potential of water to grow heterotrophic bacteria, several studies proposed measuring growth-limiting constituents such as inorganic nutrients (e.g., phosphorus and nitrogen) and assimilable organic carbon (AOC) and biodegradable organic carbon (BDOC) formed from oxidation of natural organic matter (Hammes et al., 2006; Yavich et al., 2004). van der Kooij et al. (1989) demonstrated that biostable waters should not have AOC greater than 10 μg/L. Others have reported regrowth concerns (reported as coliform occurrence) with >50 μg/L AOC (LeChevallier et al., 1991). Recently, LeChevallier et al. (2015) recommended a threshold value of 120 μg/L AOC, 1.8 mg/L dissolved organic carbon (DOC), and 0.8 mg/L phosphate as practical indicators of biostability for chlorinated systems. In terms of BDOC, Niquette et al. (2001) showed that a value < 0.25 mg/L BDOC at the outlet of the plant would be sufficient to limit bacterial growth in distribution systems. Others recommended to limit BDOC to less than 0.15 mg/L to have biologically stable waters (van der Kooij and van der Wielen, 2014). These methods, however, rely on certain bacterial strains (e.g., AOC: *P. fluorescens* P17 and *Spirillum* NOX) (Table S1) and seed bacteria (e.g., BDOC: bacteria fixed on sand) that may not be representative of the indigenous organisms present in the actual water sample (van der Kooij and van der Wielen, 2014).

Other methods for biostability have therefore focused on direct measurements of growth of indigenous bacteria (i.e. adenosine triphosphate (ATP), flow cytometry (FCM), cell counts) following nutrient addition and incubation. ATP has received significant attention in recent years due to its simplicity and fast analysis time (Delahaye et al., 2003; Hammes et al., 2010; van der Kooij et al., 2017). It can serve as a suitable surrogate for bulk viable biomass as evident from its good correlation with AOC (van der Kooij et al., 2017) and intact cell counts (Hammes et al., 2010; Nescerecka et al., 2014). A recent study of van der Kooij et al. (2017) employed periodic ATP measurements to determine biomass production potentials (BPP) in water supplies in Netherlands employing slow sand filtration. That study involved direct incubation (25 °C) of water samples with nitrogen and phosphorus and subsequent measurement of cumulative ATP concentrations. Farhat et al. (2018) also proposed a bacterial growth potential assay based on FCM and ATP measurements after inoculating various samples (e.g., non-chlorinated tap waters, seawater, river water, wastewater effluent) with indigenous bacterial community.

Upstream treatment processes have a direct influence over the resulting microbial quality of treated waters (Pinto et al., 2012). For example, ozonation can inactivate organisms while also producing biodegradable compounds from its reaction with organic matter (von Sonntag and von Gunten, 2012). Biofilters, which are primarily applied to control biodegradable carbon and prevent regrowth (Urfer et al., 1997), can leak certain organisms affecting the effluent quality (Pinto et al., 2012) and possibly acting as seed bacteria for regrowth downstream. UV treatment can induce bacteria into its dormant state which can later revive and proliferate (Zhang et al., 2015). As a final barrier, disinfection by chlorine is widely used. However, similar to other oxidants, chlorine oxidation can increase AOC (van der Kooij, 2003). Some organisms (e.g., gram-positive bacteria) were also found to survive after chlorination (Norton and LeChevallier, 2000) and combined with AOC could promote bacterial growth. With non-optimized chlorine dose, regrowth might occur due to fast chlorine decay and diminished ability to inactivate microorganisms (LeChevallier et al., 1996; Nescerecka et al., 2014). All these scenarios suggest the need to investigate the response of microorganisms with various upstream treatment and disinfection processes. Ensuring that bacterial growth is suppressed within the facility is an important step towards a more biostable drinking water quality.

This study aims to determine synergies that exist among unit treatment processes (i.e. ozonation, chlorination, and biofiltration) with respect to biostability as determined from growth-based methods involving indigenous bacterial community (i.e., ATP proliferation, BPP). The effect of full-scale treatment with sustained chlorination and biological filtration on microbial quality was first evaluated using molecular methods such as 16S rRNA gene sequencing, quantitative polymerase chain reaction (qPCR), and intracellular ATP (cATP). Regrowth of indigenous microorganisms in water samples subjected to various upstream treatment and biodegradable carbon concentrations was further assessed by measuring temporal cATP changes. Lastly, an online ATP method that can continuously measure bacterial growth phases was reported for an improved BPP evaluation.

2. Experimental

2.1. Water samples

Water samples were collected from Big Bend (BBWTP) and Alfred Merritt Smith (AMSWTP) water treatment plants, Nevada, USA. Both treatment plants take source waters from the Colorado River with DOC = 2.6 ± 0.1 mg/L, pH = 8.0 ± 0.1 (Table S2). Fig. 1 shows the treatment processes employed in each WTP demonstrating differences in chlorine application (i.e., terminal versus distributed). Water in BBWTP is treated using ozonation, coagulation/flocculation, biological anthracite filtration (BF), UV irradiation, and final chlorination (WTP-BF). Treatment at AMSWTP involves ozonation, chlorination, coagulation/flocculation, non-biological anthracite filtration, and final chlorination (WTP-SCl_2). The absence of chlorine residuals in BBWTP filter influent resulted in having biological filters while those used in AMSWTP (chlorinated) operate as conventional filters (i.e., designed for particle removal). These utilities also represent plants with standard chlorine CT (±standard deviation, n = 151) from typical final chlorination (CT at BBWTP = 247 ± 71 $mgCl_2 \cdot min/L$) and plants with high chlorine CT from sustained chlorine application (e.g., pre-filter + final) (CT at AMSWTP = 398 ± 81 $mgCl_2 \cdot min/L$). The difference in chlorine dosing points may lead to differences in microbial community across treatment. The selected WTPs (i.e., sharing similar source water quality) therefore provide a full-scale comparison of the effect of upstream treatment and varying disinfection levels on the overall microbial quality of drinking waters.

Water quality characteristics were measured using the following methods. Dissolved organic carbon was measured using a Shimadzu V analyser (Standard Method 5310B). Ammonia was analysed on a Seal AQ400 Discrete Analyzer (Standard Method 4500), while nitrite and nitrate were analysed using a Dionex ICS 5000 (EPA Method 300.0). Orthophosphate was measured using a Segmented Flow Analyzer (Standard Method 4500). Assimilable organic carbon (AOC) of finished waters were determined using the method reported by Weinrich et al. (2009).

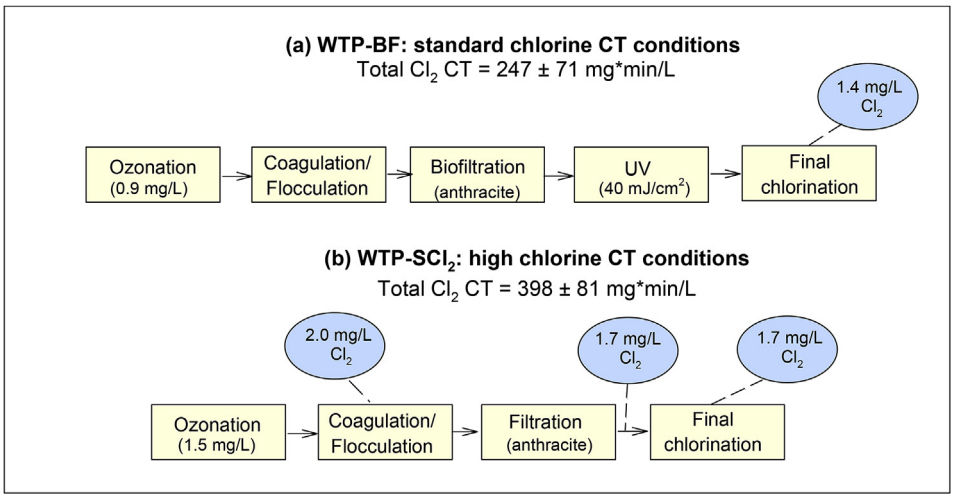


Fig. 1. General unit processes across water treatment plants with (a) biofiltration (WTP-BF) and (b) sustained chlorination (WTP-SCl_2). In circles are chlorine residual concentrations. Error bars correspond to standard deviation (n = 151). Data obtained from Jan–May 2018.

2.2. Indigenous bacteria characterization

Water samples (150 mL) and filter media (50 g) from BBWTP were shipped in dry ice to RTLGenomics (Lubbock, TX, USA) for DNA extraction and further analyses. Quantitative polymerase chain reaction (qPCR) of the 16S rRNA gene was conducted to determine the total bacterial concentration in each water sample. 16S qPCR was performed as follows: 2.5 μL of sample DNA extract was loaded into 10 μL Quanta Perfecta Tough Mix (QuantaBio) and run on a Roche 480 LightCycler® with one cycle at 50 °C for 2 min, one cycle at 95 °C for 10 min, 35 cycles at 95 °C for 15 s and 60 °C for 1 min, and finally, one cycle at 40 °C for 30 s, with the limit of detection being above 30 cycles. The following primers and probe were used: 16S Forward: CCATGAAGTCGGAATCGCTAG, 16S Reverse: GCTTGACGGGCGGTGT, 16S Probe: TACAAGGCCCGGGAACGTATTCACCG. Microbial community profiling was also conducted using DNA extracts amplified using the following primers: 515 yF: GTGYCAGCMGCCGCGGTAA and 926pfR: CCGYCAATTYMTTTRAGTTT. After Illumina MiSeq sequencing, data analysis was performed using the standard pipeline of RTLGenomics described by de Vera et al. (2018). Data processing was conducted using Quantitative Insights into Microbial Ecology (QIIME2) (version 2018.6) (Caporaso et al., 2010). Alpha and beta diversity indices were calculated after rarefaction at a sampling depth of 8118 to include all samples. Bray-Curtis similarity indices were calculated using PAST (Hammer et al., 2001). Taxonomy assignment was achieved with a naïve Bayes classifier trained on the 97% majority SILVA (release 132) database (Quast et al., 2013). All raw sequence data were deposited in the Sequence Read Archive (SRA) under accession number SRP155731.

The active microbial biomass of water samples was also analyzed by measuring intracellular ATP through a luminescence-based Quench-Gone Aqueous (QGA) test kit, LuminUltra Technologies Ltd., Canada.

2.3. Biomass production potential (BPP) of indigenous bacteria

Biomass production potentials (BPP) of water samples from WTPs employing biofiltration (WTP-BF) and sustained chlorination (WTP-SCl_2) were measured based on the method reported by van der Kooij et al. (2017). Briefly, 600 mL water samples (n = 2) contained in autoclaved flasks were incubated in the dark at 25 °C for at least 14 days in presence of NH_4Cl (0.07–0.2 mgN/L) and KH_2PO4 (0.02–0.3 mgP/L). N and P were added at a minimum molar ratio of 100C:10N:1P (Lauderdale et al., 2012; LeChevallier et al., 1991) to allow biomass growth during incubation and achieve uniform N and P conditions across different samples. To achieve the target C:N:P ratio, waters with 0.3 mg/L (25 μM) bioavailable carbon (typical for both WTP-BF and WTP-SCl_2) would require at least 0.04 mg/L NH_3–N (2.5 μM) and 0.008 mg/L PO_4^{3-}-P (0.25 μM). In experiments assessing regrowth of indigenous bacteria in full-scale treated waters, no bacterial inoculum was added. Chlorine-containing water samples were first quenched with 0.1 mM $Na_2S_2O_3$. Further details about the BPP experiments are provided in Table S3. All spiked solutions were filter-sterilized using Nalgene SFCA membrane filters (0.2 μm). An ultrapure water blank with nutrients was also used as a negative control. Intracellular ATP was measured at different days to determine the cumulative biomass production (van der Kooij et al., 2017), i.e., $CBP(t) = \sum_{t=0}^{t} ATP(t) \times \Delta t$ (refer to Fig. S2 and supplementary information for example calculations). cATP-based estimation of viable biomass was preferred to capture natural communities in both WTPs (i.e., unlike cultivation-based methods) and because it was found to be a suitable alternative to conventional AOC test (LeChevallier et al., 1993; van der Kooij et al., 2017). It also accounts for all complex factors affecting microbial growth under various water quality conditions which may not be covered using other surrogate measures for bioactivity (e.g., UV absorbance, fluorescence, nutrients, turbidity, particle counts).

2.4. Organic carbon challenge testing

Experiments to evaluate the response of indigenous bacteria with varying bioavailable carbon concentrations were also carried out. The first part investigated the effect of ozone dose and subsequent oxidation byproduct formation on BPP. Ozonation was conducted in batch mode at doses of 0, 0.25, 0.5, and 1 mgO_3/mgDOC using filter-sterilized raw water samples from WTP-SCl_2 (total volume = 500 mL). Ozone stock solution (1 mM O_3) was generated from pure oxygen (99.995%) with an Ozonia CFS-1A ozone generator and standardized spectrophotometrically at 260 nm ($\varepsilon = 3200\ M^{-1}cm^{-1}$) (von Sonntag and von Gunten, 2012). Once all ozone has fully reacted, 0.02 mg/L PO_4^{3-}-P and 0.07 mg/L NH_3–N were added into each water sample, followed by spiking of

2-mL aliquot of unfiltered raw water. The spiked raw water aliquot (~40 microbial equivalents/mL) served as an inoculum of a diverse indigenous community. The initial filter sterilization step (98.8% bacterial removal efficiency) and addition of raw water aliquot ensured having equal starting microbial concentrations, as certain strains are expected to be oxidized/disinfected at different degrees depending on the ozone dose. Microbial consumption of carboxylic acids (i.e., formate, acetate, and oxalate) and aldehydes (i.e., formaldehyde, acetaldehyde, propanal, butanal, hexanal) produced after addition of 1 mgO_3/mgDOC was also measured at days 0, 3, 7, and 14 along with changes in cATP. Aldehydes and carboxylic acids were analyzed using EPA Method 556 (Eurofins Eaton Analytical, CA) which involved derivatization of analytes to their corresponding pentafluorobenzyl oximes, followed by extraction with hexane, and analysis by gas chromatography mass spectrometry (GCMS).

The second part involves sodium acetate addition to confirm the dependence of biomass production on biodegradable carbon and the sensitivity of the BPP method. Acetate was added as sole carbon source to 600 mL ultrapure water at the following concentrations: 0.05, 0.1, 0.2, and 0.3 mg-C/L. This was followed by addition of nutrients (0.02 mg/L PO_4^{3-}-P and 0.07 mg/L NH_3–N) and 2 mL unfiltered raw water into each sample. ATP production at higher acetate concentrations (0.5 and 1.2 mg-C/L) were also tested using WTP-BF raw water.

The last part of this section involved testing the activity of the WTP-BF's indigenous bacteria to utilize dihaloacetic acids (DHAAs), a known biodegradable pollutant in drinking water systems (de Vera et al., 2016). 14 μg/L of each analyte (bromochloro-, dibromo-, dichloroacetic acid) was added to 600 mL raw water and filter effluent samples containing 0.05 mg/L PO_4^{3-}-P and 0.4 mg/L NH_3–N and incubated in dark for 14 days at 25 °C. 60 mL aliquots were taken at days 0, 3, 7, and 14 for HAA extraction, derivatization with methanol, and analysis by GCMS (EPA Method 552.3) (Eurofins Eaton Analytical, CA). Sodium azide (10 mg/L) was added as a preservative into each vial to quench microbial activity at the time of sampling (Singer et al., 2002).

2.5. Regrowth evaluation using an online ATP analyzer

This study also reports an automatic ATP analyzer method to evaluate regrowth of indigenous bacteria in drinking water samples online. The schematic diagram of the set-up is shown in Fig. S3. The online ATP method is based on the chemiluminescent reaction using luciferin and luciferase (i.e., complying with ASTM D4012-81) and was applied to operate in batch mode to measure ATP changes in water samples every 6 h. The instrument (EZ-ATP® Microbial Load ATP Analyzer, AppliTek-Hach, USA) was calibrated using ATP-free Tris HCl buffer and 200 pg/mL ATP standard. During analysis, 100-mL aliquot from 10 L sample (25 °C, pH = 8) was pumped through Norprene and Teflon tubings washed with sequential 1M HCl, 1M NaOH, and ultrapure water. The bioluminescent reaction happens in a measuring cell (controlled at 25 °C) and detected with a photomultiplier tube (PMT). Extracellular ATP is first measured followed by cell lysis using an ultrasonic probe to release intracellular ATP and calculate for total ATP. One full analysis cycle (i.e., flushing, sampling, luciferin/luciferase addition, ultrasonic treatment, measurement) and data processing takes 7 min. To demonstrate proof-of-concept, the following water samples obtained from WTP-SCl_2 were subjected to online ATP analysis: raw water, raw water +0.5 mg/L acetate-C, ozonated water, and final chlorinated water samples. The samples were incubated in the dark at 25 °C and spiked with 0.05 mg/L PO_4^{3-}-P and 0.2 mg/L NH_3–N.

3. Results and discussion

3.1. Characterization of indigenous bacteria across treatment

The quantity, activity, and diversity of indigenous bacteria in the biofiltration plant (WTP-BF) were investigated following varying levels and types of disinfection processes. The results of the 16S rRNA gene sequencing showed that most changes in bacterial community occurred prior to filtration, with ozonation having the greatest impact. Alpha diversity indices (Table S4) were found to increase after ozonation (i.e., Simpson (evenness) = 0.75 (raw) – 0.89 (ozone), Shannon (richness and evenness) = 3.02 (raw) – 4.67 (ozone), Chao1 (richness) = 114.0 (raw) – 219.6 (ozone)). This observation can be attributed to the vulnerability of raw water organisms towards oxidation, potential ozone-tolerance of certain organisms, and/or presence of more growth-promoting factors (e.g., biodegradable carbon) that allow a diverse group of organisms to proliferate. After filtration, alpha diversity decreased (e.g., Shannon (filter effluent) = 1.19) and only a few types of bacteria dominate at the effluent. With respect to beta diversity, these effluent bacteria appeared to be different (Table S5) to those in the raw water (Bray-Curtis (BC) similarity = 0.05) and bottom layer of the filter media (BC similarity = 0.48) but were very similar with bacteria at the top of the filter bed (BC similarity = 0.84). These results suggest that populations near the filter surface are more prone to sloughing, shaping the biological quality of the filtrate.

The taxonomic differences contributing to the diversity differences above are described below. The major bacterial taxa present in the samples are shown in Fig. 2 and summarized in Table S6. WTP-BF raw water was dominated by Proteobacteria (55%) and Actinobacteria (35%), consistent with the phyla commonly reported in drinking water systems (Lautenschlager et al., 2014; Li et al., 2017; Pinto et al., 2012). Actinobacteria, however, was found to be highly susceptible to ozone (dose setpoint = 0.90 mg/L) as shown in the decrease in relative abundance from 35% to 1%, similar to those observed by Li et al. (2017). Ozone could have also altered the substrate being utilized by Actinobacteria leading to its decreased abundance. In contrast, ozonation had non-lethal effects to the genus *Hydrogenophaga* (9%), *Flavobacterium* (phylum Bacteroidetes) (7%), and other unknown Proteobacteria (28%). After coagulation/flocculation, *Hydrogenophaga* (26%), *Flavobacterium* (5%), and unknown Proteobacteria (12–24%) dominated the filter influent. This trend appeared to carry over the filter surface suggesting that the filter influent seeds the bacteria colonizing the biofilters. At the top of the filter media, *Hydrogenophaga* (28%) and *Flavobacterium* (42%) outcompeted other organisms and growth was further promoted due to nutrient-rich conditions (i.e., AOC from ozonation) and the biofilm support by the media. Filter depth was found to impact the microbial structure. It was only at the bottom layer of the filter bed where *Hyphomicrobium* (15%) became abundant. Alphaproteobacteria, like *Hyphomicrobium*, have been described to be competitive in low nutrient settings (Lautenschlager et al., 2014), such as those in deep filter zones. After filtration, the effluent and UV-treated waters consistently contained *Hydrogenophaga* (42–52%) and *Flavobacterium* (29–50%) which are also the genus most prominent at the filter surface. Biomass sloughing is therefore evident particularly for bacteria residing at the top of the filter bed. No sequencing data was available for the chlorinated water sample due to low biomass concentrations (i.e., insufficient DNA in the extract).

The total bacterial concentrations in WTP-BF water samples were also assessed using 16S rRNA qPCR (Fig. S3). Raw water bacterial concentrations were observed to have 6.4×10^3 copies/mL (~1.6×10^3 cells/mL, assuming 4 gene copies/cell (median value in 11278 genomes (Center for Microbial Systems University of

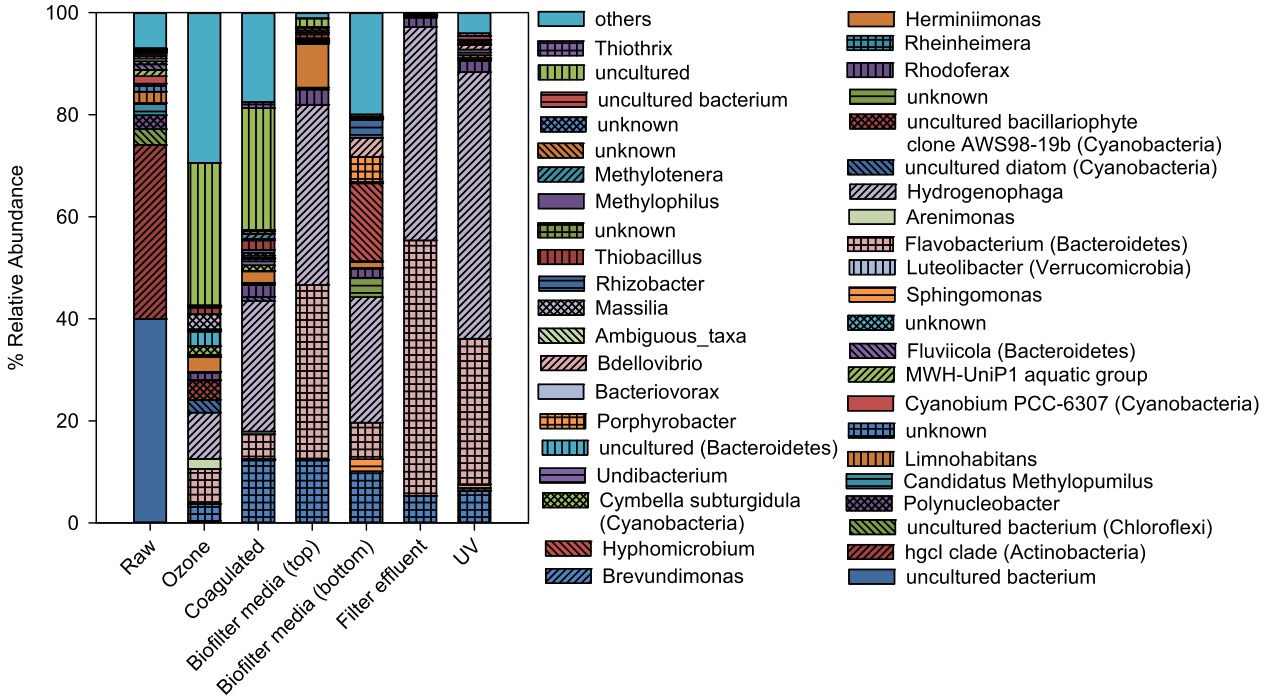


Fig. 2. Relative abundance of each genus for the amplified 16S rRNA gene of indigenous bacteria for water samples and filter media from WTP-BF. Majority of the genus belong to the phylum Proteobacteria, unless otherwise stated in parentheses.

Michigan, 2017)). Due to potential DNA extraction efficiency issues, this value was lower than typical bacterial concentrations in source waters as measured by flow cytometry (e.g., Lake Zurich = 1×10^6 cells/mL (Hammes et al., 2008)). After ozonation at a dose of 0.90 mg/L O_3, bacterial concentration decreased to 1.5×10^3 copies/mL. This concentration then doubles at the filter influent (3.0×10^3 copies/mL) possibly due to sloughing of bacteria that have grown in the walls of the floc basin in the absence of an oxidant. The highest bacterial concentration was observed at the filter effluent (8.8×10^3 copies/mL), consistent with biomass sloughing as discussed above. This concentration decreased to 3.8×10^3 copies/mL after UV irradiation (40 mJ/cm^2) which may be due to DNA damage or dimerization preventing amplification. Only final chlorination eliminated viable bacteria in the water sample.

The qPCR results were also compared with cATP measurements. At WTP-BF, apparent cATP of the water samples decreased as follows: filter effluent > raw > coagulated > UV > ozone > final Cl_2. These results agree well with the trend observed for qPCR. Plotting cATP versus 16S rRNA gene copies (Fig. S4) resulted in a direct linear relationship ($R^2 = 0.98$, slope = 3.5×10^{-6} ng ATP/16S rRNA gene copy), demonstrating suitability of cATP as a surrogate for total bacterial concentrations in water. If each bacterial cell is assumed to have four 16S rRNA gene copies, the correlation above suggests that each cell has an estimated ATP of 1.5×10^{-5} ng (equivalent to 3.0×10^{-8} nmol ATP/cell) which is 1–2 orders of magnitude higher than previously reported values (Deininger and Lee, 2001; Hammes et al., 2010). This overestimation could be due to a more efficient extraction of cATP than DNA for qPCR and varying counts of 16S rRNA gene among different species in each sample.

Using the cATP assay, biomass concentrations of water samples from WTP-BF were compared to those obtained from WTP-SCl_2 (Fig. 3). At WTP-SCl_2, the inhibitory effect of chlorine on biomass growth (cATP = 2 ng/L) was apparent for the chlorinated coagulated water sample. cATP values remained barely detectable after filtration (1.1 ng/L), which is lower than the effluent of WTP-BF

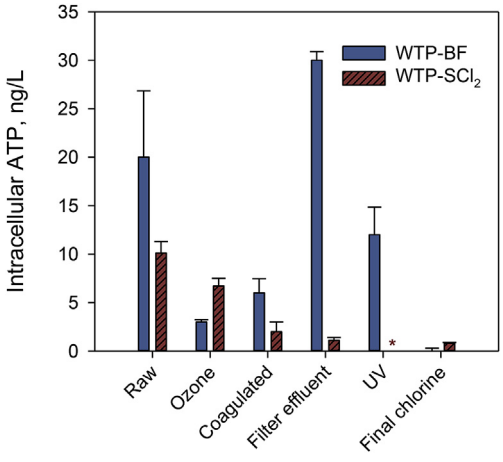


Fig. 3. Comparison between intracellular ATP of water samples from WTP-BF and WTP-SCl_2. Upstream chlorination (2 mg/L Cl_2) is applied at the filter influent of WTP-SCl_2. Error bars in WTP-BF and WTP-SCl_2 correspond to standard error (n = 3) and absolute deviation (n = 2), respectively.

(30 ng/L). The results suggest that sustained chlorine residuals inhibited colonization of the filter media and prevented subsequent sloughing from the filter biomass. The suppressed biomass growth in disinfected filters was also observed in another full-scale facility (River Mountains (RM) Water Treatment Plant) utilizing the same source water and treatment processes as WTP-SCl_2 (de Vera et al., 2018). Similarly, the presence of chlorine residuals in the anthracite filter influent (1.8 mg/L) and effluent (1.0 mg/L) at RMWTP led to non-detectable effluent cATP. The constant exposure of the filter media to chlorine led to low filter bioactivity and presence of *Undibacterium*-like taxon (45–68%) (de Vera et al., 2018), which is a genus also found abundant in other chlorinated waters (Jia et al., 2015).

3.2. Biomass production potentials

To further elucidate the effect of these treatment steps on bacterial inactivation and recovery of process-selected communities, the use of cATP was extended to determine the biomass production potentials (BPP) of water samples using indigenous bacteria identified previously as seed organisms for CBP_{14}.

3.2.1. Biofiltration plant (WTP-BF)

CBP_{14} of WTP-BF waters decreased as follows: coagulation, ozone > filter effluent > UV > raw > final chlorine (Fig. 4a, see also Fig. S6 for the cATP data). Highest CBP was observed for coagulated/ozonated waters due to greater biodegradable organic carbon generated from organic matter oxidation. This was confirmed from ΔDOC measurements after the 14-day incubation period (Fig. S5). Raw water DOC remained the same while ozonated and coagulated waters had ΔDOC of 0.25–0.4 mgC/L, respectively (equivalent to 10–16% of raw water DOC). While ΔDOC can be useful to indirectly assess regrowth of organisms, this approach can be highly dependent on the analytical capabilities of a laboratory (i.e., method detection limits). cATP measurement is more advantageous in this regard due to its high sensitivity to microbial growth despite minor changes in biodegradable DOC (slope = 2.0×10^3 d ngATP/mg DOC) (Fig. S5). In addition, other studies showed that biomass production is not solely dependent on carbon but also on other inorganic nutrients (e.g., phosphorus). In such cases, cATP can be used to capture dynamics of biomass concentrations resulting from a variety of growth-limiting factors, while ΔDOC would only apply to scenarios where growth depends on biodegradable carbon.

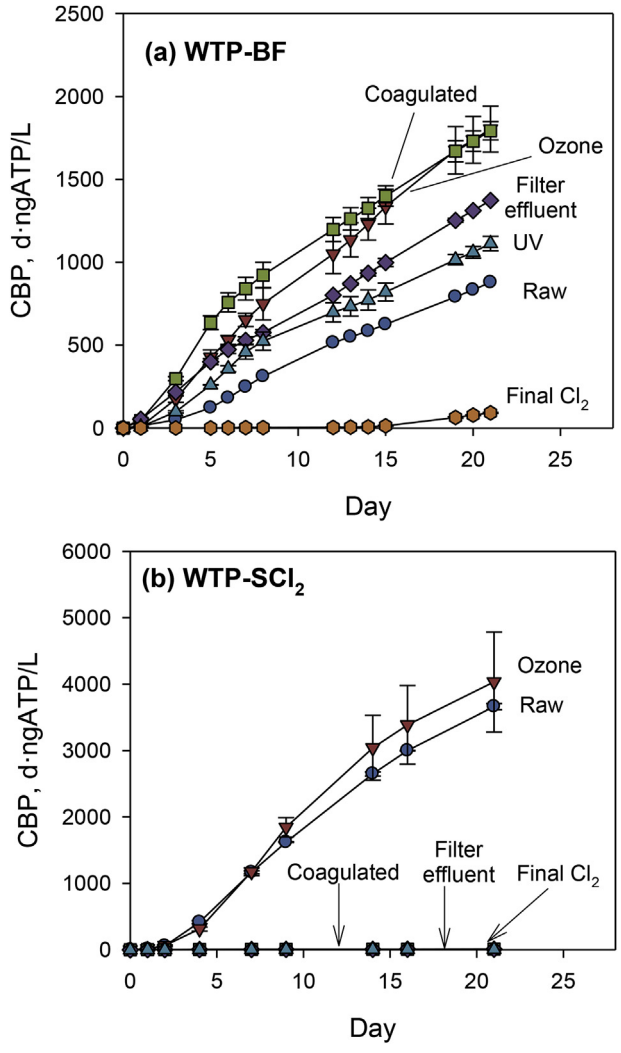


Fig. 4. Cumulative biomass production (CBP) across treatment in (a) WTP-BF and (b) WTP-SCl_2. Error bars correspond to absolute deviation (n = 2, 25 °C). Conditions: WTP-BF: DOC = 2.6 mg/L, PO_4^{3-}-P = 0.3 mg/L, NH_3–N = 0.2 mg/L, NO_3^--N = 1.2 mg/L; WTP-SCl_2: DOC = 2.7 mg/L, PO_4^{3-}-P = 0.05 mg/L, NH_3–N = 0.2 mg/L, NO_3^--N = 0.4 mg/L.

The effect of ozone on CBP was of great interest. As a strong oxidant, it was not surprising to observe low cATP (2 ng/L, day 0) after ozonation that was designed to achieve 1–2 log *Cryptosporidium* inactivation. However, after 3 days of incubation cATP peaked to 153 ng/L (Fig. S6) suggesting that certain organisms could have survived and tolerated oxidative conditions resulting in abundant biomass production (CBP_{14} = 1229 d ngATP/L). This CBP slightly increased at the filter influent (Fig. 4a) (CBP_{14} = 1326 d ngATP/L) possibly due to a different set of bacteria (Table S6) that utilizes the biodegradable carbon from the ozone contactor and floc basin. After coagulation/flocculation, the CBP14 was reduced by 30% during biofiltration (CBP_{14} = 934 d ngATP/L). Regrowth after biofiltration could be attributed to slowly biodegradable compounds utilized by the sloughed biomass in the effluent. Although CBP decreased after UV (CBP_{14} = 772 d ngATP/L), regrowth was still observed despite DNA damage by UV. Growth after UV irradiation may be caused by UV tolerance of certain organisms and/or non-optimized irradiation conditions in the reactor (e.g., matrix absorbance). Significant inhibition of biomass production (CBP_{14} = 7.6 d ngATP/L) was only achieved at the final chlorinated water sample due to chlorine disinfection (CT = 247 ± 71 mg min/L Cl_2). Certain synergies could also exist with other upstream treatment processes. For example, previous studies demonstrated that sequential UV-Cl_2 disinfection scheme was more effective in controlling suspended heterotrophic bacteria and *E. coli* than without UV pretreatment (Dykstra et al., 2007; Rand et al., 2008). These studies proposed that higher bacterial log reductions were achieved because cells become more susceptible to chlorine after UV irradiation.

3.2.2. Sustained chlorination plant (WTP-SCl_2)

Fig. 4b shows the CBP of water samples obtained from WTP-SCl_2. Unlike those observed in the biofiltration plant, biomass production at WTP-SCl_2 was only observed at raw and ozonated water. The CBP values for raw and ozonated waters at WTP-SCl_2 were almost twice than WTP-BF which can be explained by seasonal/temperature differences, consistent with a previous study (van der Wielen and van der Kooij, 2010). Higher bioactivity was expected during summer (WTP-SCl_2 sampling: May 2018, raw water temperature = 15.4 °C) as opposed to winter (WTP-BF sampling: February 2018, raw water temperature = 11.2 °C). Repeating BPP experiments in May 2018 (15.8 °C) using raw, ozonated, and effluent water samples of WTP-BF gave 2–2.4 times higher CBP values, confirming the effect of sampling period on bioactivity (Table S8). Nevertheless, the CBP results were consistent in showing that highest biomass production is achieved after ozonation.

At WTP-SCl_2, no increase in CBP was observed for the coagulated water, filter effluent, and final disinfected water. This can be attributed to sustained disinfection by chlorine across the plant. The chlorine added in the rapid mix chamber (2 mg/L Cl_2) together with the coagulant carries over to the filter influent. Influent chlorine is then partially consumed by the filter (e.g., demand caused by reactions with filtered particles) having a residual of 1.7 mg/L at the effluent. The calculated filter loading chlorine CT (±standard deviation) at this plant is equal to 197 ± 41 mg min/L. Additional CT (201 ± 41 mg min/L) is credited at the final chlorine

boosting step to maintain a residual of 1.7 mg/L Cl_2 for distribution. The sustained presence of chlorine across the plant resulted in an overall inhibition of microbial growth from the influent to the subsequent unit processes. The use of pre-ozonation (1.5 mg/L O_3) could have also contributed in this enhanced control for microbial growth. Previous studies showed synergistic effects with sequential ozonation and chlorination (Rennecker et al., 2000). In this approach, ozone causes microbial inactivation by first damaging the cell surface making the organisms vulnerable for intracellular attack by chlorine. A study using *E. coli* showed that ozone primarily attacks the cell wall components evident from release of proteins, lipid peroxidation, and change in cell permeability (Cho et al., 2010). In contrast, as a weaker oxidant, experiments with chlorine demonstrated that it can diffuse through the cell wall and attack the inner cell components without severely damaging the cell surface (Cho et al., 2010). The mechanisms described above, combined with high chlorine CT (398 ± 81 mg min/L), ensured controlled microbial growth after ozone-chlorine treatment.

3.2.3. Carbon utilization by indigenous bacteria

The effect of biodegradable organic carbon on biomass production was further confirmed by conducting experiments with increasing ozone doses, quantifying consumption of known ozonation byproducts (i.e., carboxylic acids and aldehydes), and spiking model biodegradable compounds (e.g., acetate, dihaloacetic acids).

Increasing the ozone dose resulted in increased CBP_{14} (Fig. 5a; 2050 d ngATP/L (no ozone) – 2810 d ngATP/L (1 mgO_3/mgDOC)) due to production of more carbon substrate for indigenous bacteria. Ozonation of raw water from WTP-SCl_2 at 1 mgO_3/mgDOC generated 154 μg-C/L total carboxylic acids (formate, acetate, and oxalate) and 19 μg-C/L total aldehydes (formaldehyde, acetaldehyde, propanal, butanal, hexanal) (Day 0, Fig. 5b). When the ozonated sample was subjected to the BPP test, carboxylic acids were biodegraded much faster than aldehydes. At day 2 of incubation, >85% of the total carboxylic acids were consumed by the indigenous bacteria while aldehydes decreased by only 10%. Day 2 cATP was at 2 ng/L which rapidly increased to 100 ng/L at day 4. Complete consumption of carboxylic acids and aldehydes by the indigenous bacteria occurred at day 7 where cATP peaked at 324 ng/L. Beyond this period, cATP decreased (day 14 cATP = 82 ng/L) indicating bacterial cell death due to starvation conditions.

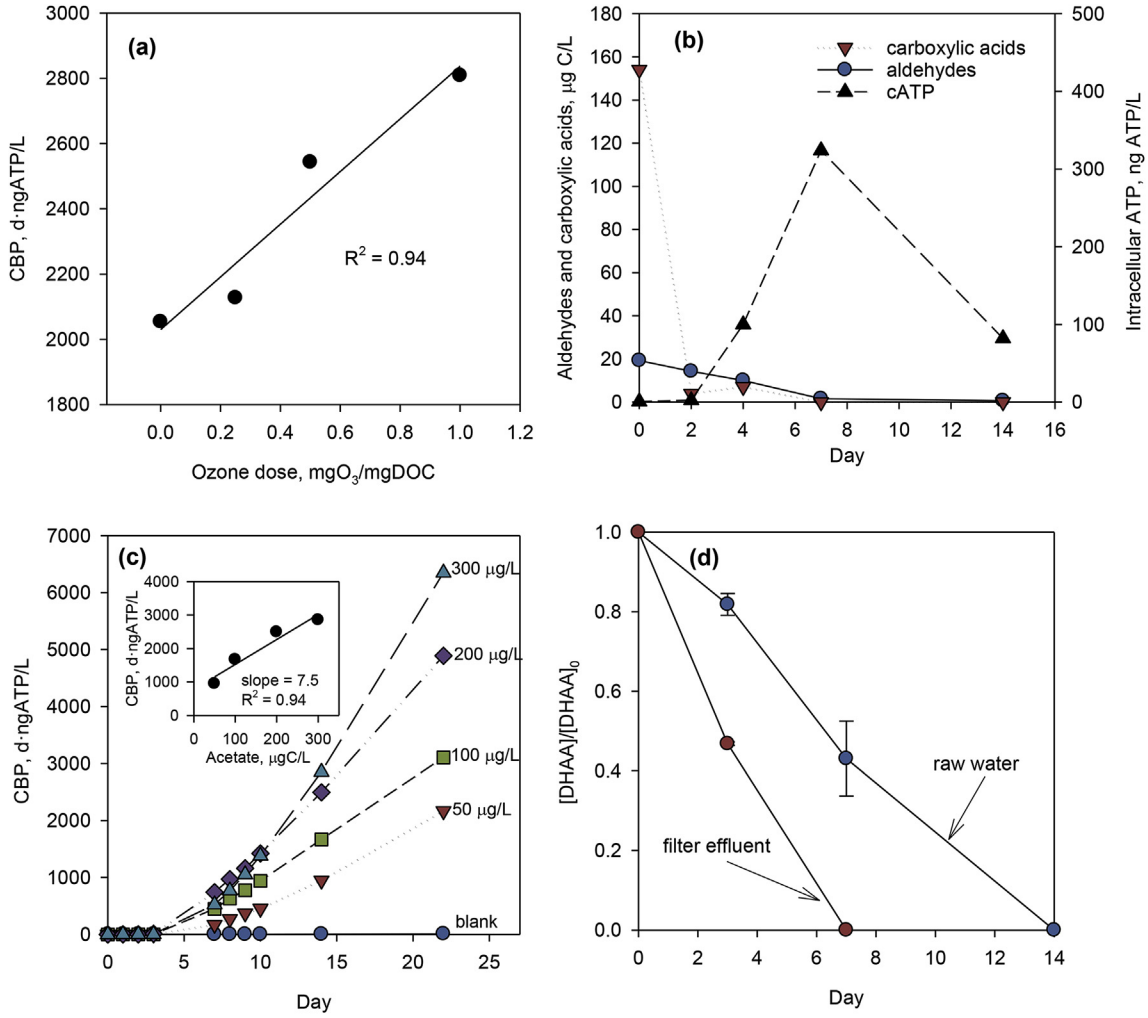


Fig. 5. Carbon utilization during biomass production. (a) Effect of ozone dose on 14-day cumulative biomass production (CBP_{14}); (b) Removal of aldehydes (sum of formaldehyde, acetaldehyde, propanal, butanal, hexanal) and carboxylic acids (sum of formate, acetate, oxalate) and corresponding cATP changes in ozonated water sample (1 mgO_3/mgDOC); (c) CBP response with increasing acetate concentrations (inset shows the direct relationship between acetate concentration and CBP_{14}); (d) Biodegradation of dihaloacetic acids (DHAA) by indigenous bacteria of raw and filter effluent water samples. DHAA = sum of concentrations of bromochloro-, dibromo-, and dichloroacetic acid. (measured initial DHAA = 36 μg/L). Refer to Table S3 for experimental conditions.

The indigenous bacteria also responded proportionally to spiked biodegradable compounds. Fig. 5c shows that utilization of 50 μg/L acetate-C resulted in a CBP_{14} of 950 d ngATP/L which increased to 2852 d ngATP/L when acetate was dosed at 300 μg C/L. Plotting CBP_{14} versus acetate-C concentrations gave a direct linear correlation ($R^2 = 0.94$) and a slope of 7.5 d ngATP/μg acetate-C. This slope is within the range of CBP_{14}/AOC ratio (6.5–10.6 d ngATP/μg acetate-C equivalents) reported in the study by van der Kooij et al. (2017). The observed ATP response to biodegradable C of <300 μg/L confirmed the sensitivity of the BPP assay even to small changes in available substrate. Raw water samples from WTP-BF were also spiked with acetate but at higher concentrations (500 and 1200 μg-C/L) (Fig. S7). As expected, CBP_{14} increased with increasing acetate-C (raw + 500 μg-C/L = 3639 ± 345 d ngATP/L, raw + 1200 μg-C/L = 4806 ± 201 d ngATP/L).

In a separate experiment, dihaloacetic acids (DHAA) were added as a model biodegradable pollutant to raw and filter effluent samples of WTP-BF (Fig. 5d). DHAAs were consumed by the indigenous bacteria at different rates depending on the water type. Faster DHAA biodegradation was observed at the filter effluent (7 days) compared to using raw water (14 days). The CBP_{14} for these samples was at the same order of magnitude (raw water = 1423 ± 76 d ngATP/L; filtered water = 1141 ± 2 d ngATP/L) suggesting that removal of a specific compound is more influenced by the type of microbial community than the extent of biomass production. Since DHAAs are known to be degraded by filter biomass (Grigorescu and Hozalski, 2010; Zhang et al., 2009), the higher degree of DHAA removal at the effluent can be ascribed to the sloughed bacteria from WTP-BF biofilters. Some species of *Flavobacterium* (see section 3.1) have been reported to degrade chlorinated organic compounds (Rasul Chaudhry and Chapalamadugu, 1991).

3.3. Online ATP monitoring for BPP testing

Previous studies raised concerns about limitations of ATP monitoring for assessing regrowth potential as key features of bacterial cell growth (e.g., cATP peak) might be missed with less frequent ATP analyses (Farhat et al., 2018). To address this, we demonstrated an online ATP method for continuous cATP determination.

The results of the online ATP analyses (6 h interval) (Fig. 6) agreed well with the results obtained from discrete ATP measurements: raw water + acetate > ozonated water > raw water > final chlorinated water, providing a proof-of-concept that biomass production can be successfully monitored using an online automated approach. Again, the final chlorinated water sample from WTP-SCl_2 did not promote biomass growth as much as the raw water spiked with acetate and ozonated water. The difference in occurrence of cATP peak between raw + acetate and ozonated samples may be caused by the type of organisms present in the samples. Ozone could have killed off strains of bacteria that are rapid consumers of acetate. Communities that survived oxidation then utilized the ozonation byproducts but required more time to establish (~4 days), as shown in the delayed cATP peak. During the 6-day test period, the online ATP analyzer generated well-defined cATP curves (lag phase, exponential phase, cATP peak, and stationary phases) that can differentiate various water samples. The method was also able to capture the rapid spike in ATP when a simple carbon like acetate was added, showing the sensitivity of the method to sudden changes in biomass production. Integrating the area under the continuous cATP curve thus provides more accurate CBP values, avoiding under/over-estimation that is commonly encountered with offline cATP monitoring.

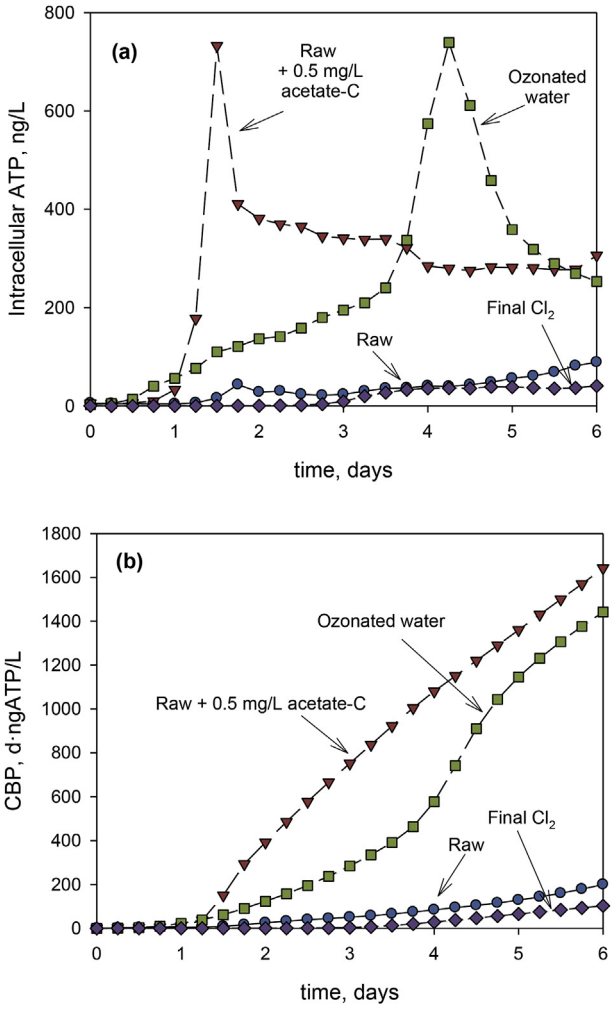


Fig. 6. Biomass production potential of raw water, raw water with 0.5 mg/L acetate-C, ozonated water, and final chlorinated water measured using the online ATP method. (a) Changes in intracellular ATP during incubation at 25 °C. (b) Cumulative biomass production calculated using the online ATP results. Water sample source: WTP-SCl_2. Conditions: DOC = 2.7 mg/L, PO_4^{3-}-P = 0.05 mg/L, NH_3–N = 0.2 mg/L, NO_3^--N = 0.4 mg/L.

3.4. Implications

This work highlights the changes in microbiological characteristics and biomass production potentials brought about by each unit treatment process. Sustained chlorination and possible synergies with other upstream processes were found useful in ensuring minimal CBP at the final treated water. For plants without intermediate chlorination, the biofilter effluent showed the highest total bacterial concentrations due to biomass sloughing. As shown in this study, these bacteria may subsequently promote biomass production downstream. Bacterial growth after oxidation (chlorine, ozone, UV, monochloramine) warrants further investigation to confirm tolerance of certain populations towards oxidants and understand the factors and mechanisms leading to their survival. Characteristics of these surviving populations should be studied especially in the context of pathogen proliferation.

The complete inactivation of indigenous bacteria by chlorine resulted in having biostable waters, even in presence of nutrients and growth substrate (e.g., AOC). Fig. S8 shows the historical AOC data of finished waters in WTP-SCl_2 (580–632 μg/L) and WTP-BF (489 μg/L). Due to bioactivity in WTP-BF filters, lower AOC was observed at WTP-BF than at WTP-SCl_2 employing conventional

(chlorinated) filtration. In both plants, no regrowth was observed in the finished waters despite AOC values that are well-above the recommended AOC threshold of 120 μg/L and 180 μg/L for chlorinated and chloraminated systems, respectively (LeChevallier et al., 2015). These results suggest that the presence of high AOC may not be representative of regrowth especially for utilities that implement secondary disinfection with chlorine (e.g., United States). Limiting AOC for regrowth control is particularly important in countries without residual disinfection (e.g., Europe) due to presence of viable biomass in their system. A study in Netherlands, for example, reported that slow-sand filtrates with AOC <50 μg/L can have CBP_{14} of ~200 d ngATP/L (van der Kooij et al., 2017). In the current study, final chlorinated waters only had $CBP_{14} = 6–8$ d ngATP/L, despite elevated AOC levels.

This study systematically investigates microbial dynamics in full-scale treatment plants using an integrated approach that explores bacterial composition and diversity (16S rRNA gene sequencing), abundance (qPCR), viability (cATP, offline and online), and growth promotion involving natural bacterial community (biodegradable C monitoring, BPP tests). The direct on-site cATP measurement and the online ATP method can be useful for rapid assessment of viable biomass and impact of each unit process on bacterial abundance. As a bulk water parameter, it offers a more accurate way to assess bacterial viability than surrogate parameters (e.g., UV absorbance, fluorescence, turbidity, particle counts) which may lack sensitivity and suffer from interferences from the matrix components. With more data collection, cATP can be incorporated in hydrologic models to better understand microbial dynamics in the water network. It can further serve as a good early warning parameter to signal heightened biological activity prompting immediate corrective actions and further investigations of contamination sources, bioactivity hotspots, and biofouling in the distribution system.

In addition, the BPP method can complement methods such as flow cytometry (Farhat et al., 2018), microbial fuel cell-based biosensors (ElMekawy et al., 2017), and the time-consuming cultivation-based techniques (e.g., HPC, coliforms, AOC) (van der Kooij and van der Wielen, 2014). It further addresses the limitations of the widely-used AOC method such as use of pure strains that may not be representative of the natural community, underutilization of potential substrates in water used by those strains, inability to capture autotrophic growth, and organic matter alterations due to sample preparation (e.g., pasteurization) (Prest et al., 2016; van der Kooij et al., 2017). The assay also considers the complex factors affecting microbial growth of a specific water source that may not be achieved using indirect, predictive approaches (e.g., nutrient monitoring). Thus, the BPP method can provide a more accurate description of the growth potential of the indigenous bacteria that can guide treatment engineers in selecting adequate treatment and improving current processes.

4. Conclusions

This study investigated the microbial characteristics and biomass production from water samples of two full-scale treatment plants. The associated effects of treatment processes (ozonation, filtration, chlorination) on the microbiological quality of the treated waters (abundance, viability, composition, growth promotion) were investigated using 16S rRNA gene sequencing, qPCR, intracellular ATP, and analysis of biodegradable organic carbon and biomass production potentials. The following conclusions can be drawn from this study:

- Direct on-site intracellular ATP measurements of indigenous bacteria were useful in monitoring biomass concentrations of water samples.
- Treatment processes shape the microbial community within the treatment plants. Abundance of Proteobacteria and Bacteroidetes increased after ozonation and dominated the biofilter media and filter effluent.
- Cumulative biomass production of water samples using the indigenous bacteria identified via 16S rRNA gene sequencing is greatly influenced by the type of treatment process. Highest CBP was observed after ozonation which increased with increasing ozone dose and biodegradable carbon (e.g., carboxylic acids, aldehydes). The differences in seed bacteria for CBP_{14} testing demonstrate the role of unit processes not only for production of bioavailable carbon but also for bacterial selection.
- Biofiltration alone did not prevent bacterial regrowth as shown in the results from WTP-BF. Biofiltration may therefore be more important to utilities where carbon limitation is used to control regrowth (e.g., Netherlands) versus utilities that implement residual disinfection with chlorine (e.g., United States).
- An online ATP approach confirmed results from discrete ATP measurements. The continuous measurement of intracellular ATP led to more accurate interpretation of microbial regrowth and enhanced identification of cATP peak and growth phases of indigenous bacteria (e.g., lag, exponential, stationary).

Declaration of interests

None.

Acknowledgments

The authors would like to acknowledge the following: James Park, Mary Murphy, Yesika Otano-Alonso, and Shandra Staker for laboratory assistance; Julia Lew, Stan Van Wagenen, Wilbur Frehner, Jeff Wauffle, Rocio Navarro, Daniel Gerrity, and Katerina Papp for useful discussions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.02.006.

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