12-2014

Microbial Community Shifts Caused by Changes in the Primary Oxidant at a Drinking Water Treatment Plant

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Civil Engineering

by

Connie Moloney
University of Arkansas
Bachelor of Science in Civil Engineering, 2012

December 2014
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

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Abstract

Microbial communities resistant to common oxidants can cause concerns for water treatment plants (WTPs). If a bacterium is not fully oxidized during disinfection, these species can impede upon filtration processes or seed biofilms in the distribution system. In an effort to minimize disinfection by-products (DBP's) that result from the reaction of chlorine (Cl_2) with natural organic matter, water treatment plants have the option to change their primary oxidant to chlorine dioxide (ClO_2). The following study examines the change in microbial communities during the sedimentation process under differing oxidation regimes, specifically chlorine (Cl_2) and ClO_2 at the local water treatment plant (Beaver Water District, Lowell, AR). Both water and biofilm samples taken from the sedimentation basin were investigated using a PCR approach targeting 16S rRNA coupled with denaturing gradient gel electrophoresis (DGGE). It is shown that the biofilm community in this environment exhibits higher diversity indices when compared to planktonic communities. Cl_2 oxidation decreased the diversity index biofilms and basin waters that were previously under ClO_2 treatment. *Pelagibacter ubique*, a common bacteria was observed within the basin biofilm during both Cl_2 and ClO_2 application. *Novosphingobium aromaticivorans* was also observed persistent in basin biofilms under both applications and has been linked to primary biliary cirrhosis if no defense barrier exists in the following treatment steps. This study provided valuable information for WTPs when making the decision to change primary oxidants.

Acknowledgments

Special thanks to my advisor, Dr. Wen Zhang for her guidance and limitless patience. I'd also like extend gratitude to all the graduate students that helped with gathering data as well as the engineers and staff at Beaver Water District for their support during this study.

Dedication

This thesis is dedicated to my husband for getting me on the bus, and riding it to the end with me, love always.

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I. Introduction

Microbial communities in the environment are multi-species assemblages comprised of bacterium that are tolerant to similar physical and chemical conditions. The environmental boundaries of any community have little to do with physical dimension, but rely on geochemical conditions favorable to certain species [1]. These microbial communities can shift in diversity due to selective pressures acting upon their environment. Environmental conditions such as temperature, pH, nutrients or competing biota can act as a selective pressure on a community [1].

More specifically, examination of microbial communities is common in water treatment. The primary goal when treating source water is to address the presence of pathogens present in the water column and remove them to a safe level. Engineers apply common oxidants such as free chlorine or chlorine dioxide within water treatment processes for removal of pathogens through oxidation [2]. The following study was conducted to explore how selective pressures such as differing pre-oxidants impact microbial communities in a water treatment plant. This study is currently under review for publication in the peer-reviewed "*Journal of Applied and Environmental Microbiology*." The article in review contains three authors, and therefore cannot be altered for reformatting.

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ABSTRACT

The use of chlorine dioxide (ClO_2) instead of free chlorine (Cl_2) as a pre-oxidant is an effective option for reducing disinfection by-products (DBPs) in water treatment plants (WTPs). However, due to the dosage limitation of ClO_2 and its tendency to auto-decompose, microbiological overgrowth can happen within the sedimentation basin, potentially impacting subsequent filtration and disinfection processes. The impacts brought by this change in pre-oxidant on plant operations and subsequent treatment processes are not fully understood. To assess the bacterial community response when switching pre-oxidants, water samples drawn and biofilm coupons inserted in the sedimentation basin were collected during the change between ClO_2 and Cl_2 in a local WTP. Biofilm density and bacteria viability changed in response to the pre-oxidant change. A PCR approach targeting 16S rRNA coupled with denaturing gradient gel electrophoresis (DGGE) confirmed shifts in microbial diversity in both basin waters and biofilms. The diversity indices for biofilms were higher than basin water in all tests. The switch to Cl_2 oxidation decreased the diversity index for both biofilms and basin waters. Band sequences revealed the presence of a common bacteria, *Pelagibacter ubique*, within the biofilm during both Cl_2 and ClO_2 application. Furthermore, *Novosphingobium aromaticivorans*, a pre-cursor to primary biliary cirrhosis (PBC), was also observed persistent in biofilms during the oxidation change, which provides evidence that biofilm can harbor pathogens even with the presence of disinfectants. This study provided important insights of microbial growth and diversity induced by pre-oxidant changes, which could help WTPs to make informed decisions regarding disinfection.

INTRODUCTION

Water treatment plants (WTPs) must adhere to increasing regulations aimed at providing safe drinking water. The steps for removal of contaminants such as pathogens and solids in conventional water treatment typically include: primary oxidation, coagulation and flocculation followed by physical settling of particles, depth filtration, and secondary oxidation to provide residual disinfectant within the distribution system. Common oxidants/disinfectants include chlorine (Cl_2), potassium permanganate, ozone, UV, chloramines and chlorine dioxide (ClO_2). The use of a strong oxidant such as Cl_2 on natural organic matter can result in regulated disinfection by-products (DBPs). To curb DBP formation, surface water treatment plants using Cl_2 opt to either change their secondary disinfectant to chloramines, or change the primary oxidant to ClO_2 which can obtain greater bacterial reductions than Cl_2 on a mass-dose basis (1). The use of ClO_2 as a pre-oxidant provides several mechanisms that lead to the inactivation of microorganisms, as well as controlling taste and odor problems. This oxidant inactivates bacteria by readily reacting with amino acids and disrupting protein synthesis (2). In detail, ClO_2 breaks down hydrophobic aromatic organics (i.e. amino acids or all five nucleotides for DNA synthesis) into smaller molecular weight hydrophilic compounds (i.e. sugars) (3).

The dose of ClO_2 in WTPs needs to be closely monitored to control chlorite formation, a regulated DBP resulting from the auto-decomposition of the disinfectant (2). Due to the limitation on dosing and tendency for auto-decomposition, the performance of chlorine dioxide as a primary disinfectant and its subsequent impact on water treatment processes is not fully understood. First, the limited allowable dose and auto-decomposition of ClO_2 may not provide efficient residual disinfection to control microorganism growth, in particular pathogens, within the sedimentation basin prior to secondary disinfection (4). This in turn would negatively impact

subsequent filtration and disinfection processes by the survival of microorganisms (5). Second, biofilms can form in the sedimentation basin and on filtration media prior to the application of secondary disinfectant (5,6). Bacteria within a mature biofilm matrix are protected from fluctuating environments by extracellular polymeric substances (EPS) and can act as a source of microbial contamination within the plant (6). More importantly, infectious agents such as *Escherichia coli* from within the plant would potentially seed the distribution system (7). Third, the biofilm EPS composition can also contribute to higher DBP formation (6). A previous study has shown that separate strains of bacteria produce EPS with differing primary biomolecules, resulting in different DBP formation (6). As a result, it is helpful to understand how the change of pre-oxidants impacts the microbiological activity within the WTPs in addition to DBP control.

Characterization of microbial communities' structure at different locations within WTPs provides valuable information that can be used to improve the efficiency of the WTP (8). A previous study identified communities present from source water to the distribution system (8). It was concluded that a specific bacterial community from within the filtration process was seeding the distribution system, as opposed to the source water (8). Previous studies examining bacterial communities in water treatment were mostly conducted when: (1) pilot plants were used; (2) WTPs applied consistent treatment processes; or (3) the distribution system was solely focused on. Norton and LeChevallier constructed a pilot scale system to examine differences in distribution system biofilms by comparing conventional and biological treatment using fatty acid analysis and heterotrophic plate counts (HPC) (5). In contrast, Pinto et al. applied 454 pyrosequencing to examine these communities in a WTP (8). Hoefel et al. compared HPC with a PCR-DGGE approach on bacteria through a typical water treatment process and distribution

system to identify active bacteria not shown using HPC alone (9). The effectiveness and impact of a changing operation regime on microbial community remains unknown in the WTPs.

The goal of this study is to achieve a clear understanding of the effect of a changing disinfection regime on the microbial community before and after the change at a WTP. Biofilm formation within the sedimentation basins is also studied, which has rarely been done before. The study site is a local WTP (Beaver Water District, Lowell, AR) which employs conventional water treatment processes and has the ability to change pre-oxidants between Cl_2 and ClO_2. From an operational perspective, motivation for changing oxidation regimes includes potential overgrowth issues within the distribution system, or a bloom of microorganisms within the plant. The study captured the plant's pre-oxidant change (ClO_2 to Cl_2 then back to ClO_2) in the summer of 2013 to identify the shifts in microbial diversity in water and sedimentation biofilms using DGGE analysis.

MATERIALS AND METHODS

Study site and sampling. The water treatment plant (WTP) located in Lowell, Arkansas treats surface water from the Beaver Lake Reservoir. This reservoir serves as the drinking water source for 420,000 people in the northwest Arkansas region. The watershed area is 3087.3 km^2 and the lake surface area is approximately 114 km^2 with an average depth of 18.3 m throughout.

Sampling occurred from May 2013 through September 2013. Plastic trays with 48 removable rectangular coupons (3.2 cm by 2.4 cm) of P120 fine grit waterproof sandpaper (3M, Singapore) were suspended in the sedimentation basin to allow biofilm growth for subsequent analysis. During sampling, 2 L of water were taken from the sedimentation basin junction boxes and an additional 2 L from intake headwaters. Triplicate biofilm coupons were harvested from 2

locations within the basin, totaling six coupon replicates per sample event. Water samples were stored in 1 L brown HDPE bottles and transported on ice from the site to laboratory. All water samples were kept at 4ºC for short term storage before filtration. 1 L samples were filtered through 0.45 µm cellulose nitrate membrane filters (GE Healthcare Life Sciences, Buckinghamshire, UK), desiccated, and stored frozen. The remaining 1 L of water samples were used for water quality parameter measurements as described next. Biofilm disks were removed with sterile tweezers and transported in sterile petri dishes on ice. Biofilm and water filters were stored at -20ºC for molecular analysis.

Water quality parameters. All glassware used was washed in phosphorous free laboratory detergent, rinsed three times with tap water, and three times with distilled deionized (DDI) water (Elga Process Water System (18.2 $M\Omega.cm^{-1}$) Purelab flex, Veolia, Ireland). Glassware not used for total solids was air dried. Glassware for total solids was oven baked at 100ºC for at least 1 hour. For sterilization and molecular analysis, all washed glassware was autoclaved (Model 522LS Gravity Steam Sterilizer, Getinge, Rochester, NY). To measure biofilm total solids, a biofilm plate was inserted into a sterile 15 mL centrifuge tube with 10 mL of DDI water and vortexed for 5 minutes to ensure complete solids detachment. This mixture was then transferred to an oven baked, pre-weighed beaker (PMP beakers, Kartell, Italy). The centrifuge tube was rinsed into the beaker three times with DDI water to ensure complete solids transfer. Total solids for biofilm and water samples were measured according to *Standard Method* 2540 B. (10) in triplicate. pH was measured with a pH meter (Thermo-Scientific, Fort Collins, CO). Phosphate and nitrate tests were performed in triplicate with Hach powder pillow kits (PhosVer 3 Phosphate Reagent and NitraVer5 Nitrate Reagent, Hach, Loveland, CO). Turbidity of both intake waters and sedimentation basin water were measured using a turbidimeter (HF Instruments DRT-100

turbidimeter, Fort Myers, FL). TOC was measured with a total organic compound analyzer (Sievers 900 Total Organic Carbon Analyzer, GE Analytical, Boulder, CO).

Bacteria and biofilm analyses. Direct total microbial counts of water samples were enumerated following *Standard Method 9216* B. (10)with the use of nucleic acid stain (DAPI nucleic acid stain, Life Technologies, Grand Island, NY) on an upright fluorescence microscope (Nikon Eclipse N*i*-E upright microscope, Nikon Instruments Inc., Melville, NY). 10 mL were stained and filtered through a Whatman 0.2 µm nuclepore filter (GE Healthcare Life Sciences, Buckinghamshire, UK). Ten randomly selected fields were counted and averaged. Calculation of average number of cells per filter used the following equation:

$$Total\ Cells/_{mL} = \frac{Average\ \#\ Cells}{Picture} * \frac{Pictures}{Filter} * \frac{Dilution\ Factor}{100\ mL} \qquad (1)$$

Confocal laser scanning microscopy (CLSM) with live/dead nucleic acid stain (LIVE/DEAD *bac*Light™ Bacterial viability kit, Life Technologies, Grand Island, NY) was utilized to measure biofilm thickness as well as observe bacterial viability during the change in pre-oxidation. Biofilm thicknesses were averaged from ten measurements, performed in triplicate. Images were obtained with a Nikon 90i upright CLSM using the 100X objective lens (Nikon, Inc., Meville, NY).

DNA extraction. DNA was extracted for subsequent analyses with a soil DNA extraction kit (Power Soil DNA Isolation Kit, Mo-Bio, Carlsbad, CA). The protocol recommended by the manufacturer was followed with additional steps to enhance the extraction efficiency. The cells were lysed in a 60ºC water bath for two hours before the bead beating step. To optimize PCR yields, DNA clean-up (Power Clean DNA Clean-up Kit, Mo-Bio, Carlsbad, CA) was performed following the extraction.

16S rRNA PCR. All plasticware was ordered nuclease free and autoclaved before use. The PCR workstation was sterilized with bleach and UV radiated for 15 minutes prior. PCR reactions were completed using 16S ribosomal DNA universal bacterial primer set 518R, 5'-ATTACCGCGGCTGCTGG-3' and 338F-GC, 5'-ACTCCTACGGGAGGCAGCAG GGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3' (11, 12) . Each 50 µL reaction mixture contained 2X Hotstart Taq master mix (2X Hotstart Taq PCR Ready Master Mix, Amresco, Solon, OH), 200 nM forward primer, 200 nM reverse primer, up to 10 ng DNA template, and nuclease free water as needed. The mixture was UV radiated once more for 15 minutes prior to the addition of DNA template. Thermal cycling (T100™ Thermal Cycler, Bio-Rad Laboratories, Hercules, CA) consisted of initial denaturing at 95ºC for 15 minutes. Then, 35 cycles of the following: denaturing at 94ºC for 30 s, annealing at 56ºC for 30 s, and extension at 72ºC for 45 seconds. A final extension step at 72ºC for 10 minutes was performed. The samples then were held at 4ºC. Each PCR reaction had one negative control which included nuclease free water in lieu of DNA template. Amplified PCR product was verified by 1.5% (w/v) agarose gel electrophoresis.

DGGE. Denaturing gradient gel electrophoresis (DGGE) was performed (Bio-Rad DCode™ Universal Mutation Detection System, Bio-Rad, Hercules, CA) as previously described (12). An 8% (w/v) acrylamide/bisacrylamide (37.5:1; 40% w/v) was cast with a denaturing gradient from 0 – 100% (where 100% is 7 M Urea and 40% (v/v) deionized formamide). Subsequent analysis permitted the authors to narrow the gradient to 45% - 70% denaturant. Duplicate PCR products were combined with a PCR clean up kit to increase concentration as well as eliminate bias (UltraClean PCR Clean-up Kit, Mo-Bio, Carlsbad, CA) and quantified in µg/µL using a microplate reader (Synergy H1 Multi-Mode Microplate Reader, Biotek Instruments, Inc.,

Winooski, VT). Approximately 600 ng of PCR product was loaded into cleaned wells of the polyacrylamide gel with 2X loading buffer (0.25 mL of 2% Bromophenol Blue, 0.25 mL of 2% Xylene Cyanol, 7.0 mL of 100% Glycerol, and 2.5 mL DDI water). The instrument was heated to 56ºC and pre-run at 30V for 20 minutes. Electrophoresis was optimized and run at 74V for 13 hours (13). The polyacrylamide gel was then stained (SYBR Safe DNA Gel Stain, Life Technologies, Grand Island, NY) for 10 minutes, followed by 5 minutes of rinsing with DDI. Images were obtained in a UV light imager (Bio-Rad, Hercules, CA).

Sequencing of Bands. Bands of interest from DGGE profiles were selected and retrieved using the stabbing method, and re-amplified in the same fashion as the original DNA template with identical primer sets without the GC clamp (14). The final PCR product was prepared as required for sequencing at the DNA Resource Center facility (University of Arkansas). Sequences were uploaded and analyzed with BLAST (National Center for Biotechnology Information).

DGGE Analysis. Jaccard and Pearson cluster analysis was performed using GELCOMPARII (Applied Maths, Austin, TX) as previously described (14). Each gel was normalized using GELCOMPARII to an external reference EZ Load Molecular Ruler (Bio-Rad, Hercules, CA) as well as by assigning internal reference points for accurate gel to gel comparison of densitometric profiles.

Statistical Analysis. Principal components were calculated using SAS/IML Studio (Cary, NC, USA). Similarity matrices calculated in GELCOMPARII were subsequently uploaded to JMP statistical software (Cary, NC, USA). Statistical significance calculations (paired student's t-

test) applied to triplicate environmental datasets along with standard deviations were calculated using Microsoft Excel (Microsoft, Redmond, WA).

RESULTS

Change of disinfection regime within the plant. The WTP applied ClO_2 as the pre-oxidant during the sampling events from 5/28-ClO_2 to 7/9-ClO_2. After the sampling on 7/9-ClO_2 the WTP changed its primary oxidant to Cl_2. The next sampling event after this change was 7/16-Cl_2. The plant had changed back to applying ClO_2 by the following sampling event, 7/23-ClO_2 and continued with this oxidant for the remainder of the study. Each sampling event includes the primary oxidant being applied at that time in its abbreviation for clarity throughout the authors' analysis.

Environmental parameter changes. Observed environmental parameters were analyzed with student t-tests to monitor significant changes (Table S1). There was no significant difference in total solids between the non-chlorinated water and the sedimentation basin water on all sample events ($p>0.05$). pH values significantly fluctuated during both oxidation regimes, which can be attributed to the pH variations of the intake water ($p<0.05$). Total bacterial counts of the raw water reflected typical values, ranging from $10^6 – 10^9$ cells/100mL. As expected, total bacterial counts of the basin water were consistently several log values lower than raw water. The basin counts mimicked changes in raw water, regardless of oxidation regime. Phosphate concentrations were observed at or below 0.4 mg/L with no significant spikes ($p> 0.05$). Nitrate concentrations remained unchanged until a significant increase on the last sampling event to 3.31 mg/L from 0.02 mg/L. Temperatures during this time ranged from 24.4 °C to 35 °C. A

complete table of the environmental conditions observed over the study period is available (Table S1).

Biofilm quantification through confocal imaging. CLSM coupled with live/dead staining was used to monitor the biofilm formation and change in thickness (Fig. 1). Green color indicates the bacteria with intact membranes, while red color represents the ones with damaged membranes. For each replicate three z-series stacked images were taken at step sizes no larger than 25 µm. Images captured in this fashion are layered from bottom to top providing a 3D image of that section of biofilm. Although it is not apparent through TS_b or thickness measurements, biofilm viability observed with CLSM exhibited significant changes as the pre-oxidant shifted to Cl_2 ($p<0.05$). Intact membrane averages decreased from 56% to 42%. After switching back to ClO_2, the intact membranes observed increased to 48%. Biofilm thickness was calculated from the 3D images (Table S1). Biofilm data reported begins upon the sixth sampling event to ensure a mature colony has established. Biofilm was the thickest during the final sampling event (73.65 µm), and averaged 52.59 ± 14 µm from the sixth sampling event till the end of the experiment (Fig. 2). However, biofilm total solids (TS_b) measurements did not trend with observed thickness measurements. TS_b were 0.46 mg/cm^2 on 7/2-ClO_2, reflecting little biomass had accumulated on the biofilm (Fig. 2). The biofilm became denser over time. This is shown as TS_b peaked at 5.10 mg/cm^2 with a thickness of 73.7 µm on the last sampling day, 9/30-ClO_2. The sampling event before the WTP changed pre-oxidant shows a decrease in thickness from 67 µm to 35 µm. As the primary oxidant changed from Cl_2 to ClO_2 the biofilm thickness increased from 35 to 67 µm on 7/16-Cl_2. After the switch back to ClO_2 on 7/23-ClO_2, the biofilm continued to become densely populated.

Community profiling during the primary oxidant change. To study the impact of pre-oxidant change on the diversity of bacteria community in both water and biofilms, a PCR-DGGE approach was utilized. Heterogeneity measurements with DGGE included total band counts and the use of the Shannon Index, which measures diversity, taking into account both evenness and abundance of each banded lane (Table 1). The Shannon Index (H') was calculated after correction for exposure background noise and normalization of all acrylamide gels to the reference ladder. A higher diversity index reflects a more diverse community. The intake water diversity was highly variable, showing a range from 0.7105 to 1.1643 with no discernable pattern. The biofilm resulted in higher diversity indices than the basin suspended bacteria in all cases until the final sampling event, indicating biofilm harbors and protects a more diverse community of bacteria than the basin water. During Cl_2 oxidation the biofilm diversity index decreased from 0.8354 to 0.7858 whereas suspended bacteria decreased from 0.7666 to 0.726. It is notable that the suspended bacteria diversity continued to decrease for an additional week under the use of ClO_2, whereas the biofilm diversity index increased to 0.9854, suggesting bacteria in biofilms recover more quickly than in water column.

Each band considered present represents a single group of similar microorganisms, which are referred to as operational taxonomic units (OTUs). The number of OTUs was determined using the auto search function within GELCOMPARII excluding peaks less than 5% intensity (Table 1). Each lane was re-evaluated visually using the image and intensity profile to confirm band presence/absence. The non-chlorinated intake water showed more diversity and more OTUs than the basin water on 7/2-ClO_2 and 7/16-Cl_2. The use of ClO_2 on 7/2-ClO_2 lowered OTUs observed in water to 8 and in the biofilm to 14. The ClO_2 treatment was less effective on the biofilm than on planktonic cells on those sampling events. When applying Cl_2, the biofilm

exhibited 11 OTUs whereas the basin water only resulted in 8 OTUs exhibiting the biofilm's ability to resist changes in the water column. On 9/30-ClO_2, the sedimentation basin showed 19 OTUs, whereas the intake waters had 14 observed OTUs. This increase from source water to basin water may be attributed to the inefficiency of ClO_2 at that time as well as the existence of species in the basin that are no longer prominent within the intake water.

DGGE banding patterns and OTU sequencing. A composite data set was compiled within the GELCOMPARII software in order to complete an overall band matching. The band matching function creates band classes on the normalized set to represent the same point in all lanes. Each class clearly shows the presence/absence of a band by use of markers in the same position within multiple lanes. Bands of interest sequenced from DGGE profiles were assessed to identify the presence of pathogens (Table 2). Gel lanes that included sequenced bands of interest were compared following band matching (Fig. 3). The DGGE gel profiles show that OTU 1, *Novosphingobium aromaticivorans,* was consistently observed in the biofilm during the oxidation change, but not observed in the basin water until 9/30-ClO_2. OTU 2 was present in the water column and biofilm consistently, showing the transfer from basin water to biofilm formation. OTU 3 was present in the biofilm until 7/16-Cl_2, but was not present following 7/16-Cl_2 indicating that although *Gloeobacter violaceus* was able to persist in ClO_2 conditions, changing to Cl_2 oxidation inactivated the population to a point where it was unable to recover. *Sanguibacter keddieii* (OTU 4) was present in the intake and basin waters for most sampling events, however was only present in the biofilm on 7/30-ClO_2 and 9-30-ClO_2. OTU 5 was always present in the intake water. Furthermore, OTU 5 was present in the biofilm for the entirety of July during the oxidation change to and from Cl_2. Interestingly, *Flavobacterium columnare* (OTU 8) was only present in the intake water on 5/28-ClO_2 and 7/09-ClO_2 but was not

present after either oxidation regime on any date, with the exception of 9/30-ClO_2 in the basin water. This indicates *Flavobacterium columnare* has a low tolerance for oxidation of either type and is not as prevalent as the proteobacteria phyla in the reservoir during summer months. Also less prevalent, OTU 9 was only present on three sample events which include intake waters on 7/16-Cl_2 and 9/04-ClO_2, as well as biofilm on 7/02-ClO_2. Overall, the biofilm species sequenced were gram-negative. Cyanobacteria were observed in both biofilm and the water column.

Banding patterns were analyzed using Pearson correlation which calculates a similarity matrix relating each sampling event to all others. A dendrogram was built and 5 clusters were observed (Fig. 3). The majority of biofilm fingerprints grouped into clusters 4 and 5; the exceptions being biofilm on 8/1-ClO_2 8/15-ClO_2, and 7/16-Cl_2. Cluster 1 grouped fingerprints of intake samples 5/28-ClO_2 and 7/9-ClO_2 with the basin water fingerprint on 7/16-Cl_2. Cluster 2 consists of two main nodes showing high similarity between intake fingerprints in July to basin profiles from 8/15-ClO_2 and 7/23-ClO_2. Cluster 3 consists of intake profiles 9/4-ClO_2 and 9/30-ClO_2 sharing a node with the biofilm profile on 7/16-Cl_2. These clusters exhibit a clear difference in community profiles when comparing water column to biofilm communities. However, there is no distinguishable difference between the intake water column and the basin water column. To look further into the relationships between fingerprint profiles, principal components were created from the densiometric profiles of all sampling events (Fig. 4). The biofilm profiles, with the exception of 8/1-ClO_2 fall in the upper left quadrant. The biofilm fingerprint on 7/16-Cl_2 does not group with the other biofilm patterns; component 2 illustrates the variation between 7/16-Cl_2 and the other biofilm fingerprints. Component 1 accounts for 58.4% of the variation and illustrates the difference between water column community profiles

and biofilm profiles. All intake banding patterns fall on the right side of component one, whereas all biofilm except 8/1-ClO_2 fall on the left side of component 1.

DISCUSSION

The motivation behind implementing ClO_2 as a primary oxidant is for reduction of DBPs in the finished water. The use of a strong oxidant such as Cl_2 on natural source waters results in organic DBPs such as trihalomethanes (THMs) and haloacetic acids (HAAs) (15). The United States Environmental Protection Agency (EPA) regulations on DBPs specify a maximum contaminant level (MCL) for total THMs and five HAAs of 0.080 µg/L and 0.060 µg/L, respectively (2). Chronic exposure to these DBPs can lead to neural tube defects, spontaneous abortions, bladder or colorectal cancer (16, 17). It has been shown that applying ClO_2 followed by Cl_2 results in lower THMs and HAAs in the treated water relative to Cl_2 alone (18, 19). On the other hand, ClO_2 does not form organic DBPs; however it does still form chlorite and chlorate. The highest no-observed-adverse-affect-level for chlorite exposure is reported as 3 mg/kg-day regarding neurodevelopmental delay in infants and young children (2). By applying ClO_2 as a pre-oxidant followed by Cl_2 disinfection, THM formation reduction ranged from 13% to 34%, when compared to not using a pre-oxidant (20). Furthermore, DBP formation potential on natural surface waters has been shown to be reduced by up to 45% using ClO_2 before Cl_2 (3). As switching oxidation regime becomes increasingly popular in WTPs, clear understanding is required in terms of how this change impacts the water quality and treatment plant itself. The efficacy of each disinfectant has been studied (2), and the microbial community within the treatment plant has been studied (5, 21, 9, 22–25), but the shift in microbial community is not yet identified when the primary oxidant changes. In addition, biofilm formed within the treatment plant could have significant impact on subsequent plant operations, as pathogens can be harbored

and dispersed into treated water and ultimately affect the health of distribution system and water quality. This study is the first to focus on biofilms formed within a sedimentation basin by investigating the diversity changes under differing primary oxidants at a full size treatment facility. From this study we can conclude that there is a clear reaction by the microorganisms to treatment processes.

Water treatment facilities must provide high quality potable water regardless of fluctuating conditions within the influent water body or inside the plant. The aim of this study was to identify biological issues associated with the oxidation switch in respect to the water column and biofilm bacterial communities. To address this, biofilm coupons were inserted into the WTPs sedimentation basins. The coupons provided undisturbed biofilm samples during the summer months as the facility changed from ClO_2 to Cl_2, then back to ClO_2. Results reflected that after application of a primary oxidant, a biofilm community still persists in the sedimentation basin under differing oxidation regimes. A previous study on microbial communities in distribution systems showed that ClO_2 is effective at decreasing planktonic bacterial cells; however no significant decrease in biofilm bacterial cell counts has been seen (16). This research corroborates with these previous results as applied to biofilms in general. Other studies have characterized microbial communities from source to distribution for specific WTPs (5, 8, 9, 22–25). This study builds upon previous research on microbial activity in water treatment by examining the dynamics of how planktonic and biofilm diversity responds to changes in primary treatment processes.

Biofilm and planktonic species identified in this study have different levels of interest from a water treatment standpoint (Table 2). *Pelagibacter ubique* (OTU 5&6) is the smallest free-living bacterium, however it is found prevalent in both fresh water and seawater (26). This study

observed the presence of *P. ubique* for the month of July, during Cl_2 and ClO_2 application. The resistance to these strong oxidants in the sedimentation basin can hinder further treatment processes (5). *Novosphingobium aromaticivorans* (OTU 1), persistent in the biofilm during the oxidation change, has been linked to primary biliary cirrhosis (PBC), a liver disease resulting from undetected bacterial infections (27). Removal of this pathogen prior to finished water processing will decrease the likelihood of its presence in distribution system biofilms. OTU 9, *Acidothermus cellulolyticus*, a thermophile responsible for decomposing cellulases, was first isolated from hot springs and can be found in decomposing grasses and litter (28).

The transfer from planktonic cells to biofilm formation is the result of the microbial surface attachment and subsequent matrix production (29, 30). Tools for analyzing physical and chemical gradients within biofilms include CLSM, low load compression testing (LLCT), X-ray scanning microscopy, and imaging techniques utilizing fluorescent stains or probes (31). In this study, CLSM allowed for detailed examination of the biofilm milieu as its structure evolved. CLSM showed the formation and aggregation of the biofilm matrix, as well as the disruption correlating with the change in primary oxidant. Although LLCT has been shown to be more accurate when measuring biofilm thickness, studies also reveal that CLSM can provide high quality data for thicknesses up to 70 µm (32).

When examining community shifts caution should always be taken while comparing to previous studies, especially when DGGE is the application in use. Disadvantages with DGGE have previously been examined and discussed in detail (13, 33, 34). In short, PCR bias, co-migration or double banding can all influence densitometric profiles (34). Regardless of these limitations, DGGE provides valuable information regarding community structures during environment changes. Coupled with molecular analysis, DGGE can clearly indicate population dynamics

under selective pressures (35). The absence of OTU 3 after 7/16-Cl_2 showed the inability of *Gloeobacter violaceus* to persist within a stressed environment. This study has shown that a unique microbial community persists under ClO_2 pretreatment. This community includes both planktonic and attached, and responds to pre-oxidants differently. Water treatment plant operations that have the ability to change primary oxidation regimes could benefit from understanding possible effects of each primary oxidant coupled with subsequent chlorination. A previous study observed a clear shift from a mixed population of gram-positive and negative, to predominantly gram-positive after chlorinating raw water with Cl_2 (5). Future work should explore the results of this study to look further into gram negative or gram positive correlations with previous research. Future work can also address changes in microbial communities at other locations in WTPs and distribution systems as the primary oxidant changes. This would aid plant engineers in curbing DBP formation as well as addressing operational problems within the plant.

AKNOWLEDGEMENTS

The authors would like to thank Beaver Water District for providing funding for this project and allowing access within the water treatment plant. Additional thanks to the Arkansas Water Resources Center for supplemental financial support. Confocal images were obtained on an instrument provided by the P3 Center, funded through the Arkansas ASSET Initiative II (EPS-1003970) by the National Science Foundation and the Arkansas Science and Technology Authority.

REFERENCES

1. **Benarde MA, Israel BM, Olivieri VP, Granstrom ML**. 1965. Efficiency of chlorine dioxide as a bactericide. Appl.Microbiol. **13**:776–780.

2. **US Environmental Protection Agency, Office of Water**. 1999. Alternative Disinfectants and Oxidants Guidance Manual. United States Environmental Protection Agency.

3. **Yang X, Guo W, Zhang X, Chen F, Ye T, Liu W**. 2013. Formation of disinfection by-products after pre-oxidation with chlorine dioxide or ferrate. Water Res. **47**:5856–5864.

4. **Berg JD, Hoff JC, Roberts PV, Matin A**. 1988. Resistance of bacterial subpopulations to disinfection by chlorine dioxide. Journal (American Water Works Association) 115–119.

5. **Norton CD, LeChevallier MW**. 2000. A Pilot Study of Bacteriological Population Changes through Potable Water Treatment and Distribution. Applied and Environmental Microbiology **66**:268–276.

6. **Wang Z, Kim J, Seo Y**. 2012. Influence of Bacterial Extracellular Polymeric Substances on the Formation of Carbonaceous and Nitrogenous Disinfection Byproducts. Environ.Sci.Technol. **46**:11361–11369.

7. **Smyk DS, Rigopoulou EI, Bogdanos DP**. 2013. Potential Roles for Infectious Agents in the Pathophysiology of Primary Biliary Cirrhosis: What's New? Curr Infect Dis Rep **15**:14–24.

8. **Pinto AJ, Xi C, Raskin L**. 2012. Bacterial community structure in the drinking water microbiome is governed by filtration processes. Environ.Sci.Technol. **46**:8851–8859.

9. **Hoefel D, Monis P, Grooby W, Andrews S, Saint C**. 2005. Profiling bacterial survival through a water treatment process and subsequent distribution system. J.Appl.Microbiol. **99**:175–186.

10. **APHA (American Public Health Association), American Water Works Association, Water Environment Federation**. 2005. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington, DC.

11. **Boon N, Windt W, Verstraete W, Top EM**. 2002. Evaluation of nested PCR–DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. FEMS Microbiol.Ecol. **39**:101–112.

12. **Muyzer G, de Waal EC, Uitterlinden AG**. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl.Environ.Microbiol. **59**:695–700.

13. **Sigler W, Miniaci C, Zeyer J**. 2004. Electrophoresis time impacts the denaturing gradient gel electrophoresis-based assessment of bacterial community structure. J.Microbiol.Methods **57**:17–22.

14. **Carbonero F**. 2010. Distributions of model microorganisms along an estuarine gradient. University of Warwick.

15. **Rook JJ**. 1976. Haloforms in drinking water. Journal (American Water Works Association) 168–172.

16. **Pressman JG, Richardson SD, Speth TF, Miltner RJ, Narotsky MG, Hunter I, E Sidney, Rice GE, Teuschler LK, McDonald A, Parvez S**. 2010. Concentration, chlorination, and chemical analysis of drinking water for disinfection byproduct mixtures health effects research: US EPA's four lab study. Environ.Sci.Technol. **44**:7184–7192.

17. **Nieuwenhuijsen MJ, Toledano MB, Eaton NE, Fawell J, Elliott P**. 2000. Chlorination disinfection byproducts in water and their association with adverse reproductive outcomes: a review. Occup.Environ.Med. **57**:73–85.

18. **Linder K, Lew J, Carter B, Brauer R**. 2006. Avoiding Chlorite: Chlorine and ClO 2 Together Form Fewer DBPs (PDF). Opflow **32**:24–26.

19. **Rittmann DD**. 1997. 'Can you have your cake and eat it too' with chlorine dioxide? Water Eng. Manag. **144**:30–35.

20. **Yang X, Guo W, Lee W**. 2013. Formation of disinfection byproducts upon chlorine dioxide preoxidation followed by chlorination or chloramination of natural organic matter. Chemosphere **91**:1477–1485.

21. **Pinto AJ, Schroeder J, Lunn M, Sloan W, Raskin L**. 2014. Spatial-temporal survey and occupancy-abundance modeling to predict bacterial community dynamics in the drinking water microbiome. MBio **5**:e01135–14.

22. **Eichler S, Christen R, Holtje C, Westphal P, Botel J, Brettar I, Mehling A, Hofle MG**. 2006. Composition and dynamics of bacterial communities of a drinking water supply system as assessed by RNA- and DNA-based 16S rRNA gene fingerprinting. Appl.Environ.Microbiol. **72**:1858–1872.

23. **Martiny AC, Albrechtsen HJ, Arvin E, Molin S**. 2005. Identification of bacteria in biofilm and bulk water samples from a nonchlorinated model drinking water distribution system: detection of a large nitrite-oxidizing population associated with Nitrospira spp. Appl.Environ.Microbiol. **71**:8611–8617.

24. **Bereschenko LA, Heilig GH, Nederlof MM, van Loosdrecht MC, Stams AJ, Euverink GJ**. 2008. Molecular characterization of the bacterial communities in the different compartments of a full-scale reverse-osmosis water purification plant. Appl.Environ.Microbiol. **74**:5297–5304.

25. **Holinger EP, Ross KA, Robertson CE, Stevens MJ, Harris JK, Pace NR**. 2014. Molecular analysis of point-of-use municipal drinking water microbiology. Water Res. **49**:225–235.

26. **Georgiades K, Madoui M-A, Le P, Robert C, Raoult D**. 2011. Phylogenomic analysis of *Odyssella thessalonicensis* fortifies the common origin of *Rickettsiales, Pelagibacter ubique* and *Reclimonas americana* mitochondrion. PloS One **6**:e24857.

27. **Selmi C, Balkwill DL, Invernizzi P, Ansari AA, Coppel RL, Podda M, Leung PS, Kenny TP, Van De Water J, Nantz MH**. 2003. Patients with primary biliary cirrhosis react against a ubiquitous xenobiotic-metabolizing bacterium. Hepatology **38**:1250–1257.

28. **Tucker MP, Mohagheghi A, Grohmann K, Himmel ME**. 1989. Ultra-thermostable cellulases from *Acidothermus cellulolyticus*: comparison of temperature optima with previously reported cellulases. Nat. Biotechnol. **7**:817–820.

29. **Geesey GG**. 2001. Bacterial behavior at surfaces. Curr.Opin.Microbiol. **4**:296–300.

30. **Fux C, Costerton J, Stewart P, Stoodley P**. 2005. Survival strategies of infectious biofilms. Trends Microbiol. **13**:34–40.

31. **Abee T, Kovács ÁT, Kuipers OP, Van der Veen S**. 2011. Biofilm formation and dispersal in Gram-positive bacteria. Curr.Opin.Biotechnol. **22**:172–179.

32. **Paramonova E, de Jong ED, Krom BP, van der Mei HC, Busscher HJ, Sharma PK**. 2007. Low-load compression testing: a novel way of measuring biofilm thickness. Appl.Environ.Microbiol. **73**:7023–7028.

33. **Lyautey E, Lacoste B, Ten-Hage L, Rols J-L, Garabetian F**. 2005. Analysis of bacterial diversity in river biofilms using 16S rDNA PCR-DGGE: methodological settings and fingerprints interpretation. Water Res. **39**:380–388.

34. **Muyzer G, Smalla K**. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek **73**:127–141.

35. **Kim L, Pagaling E, Zuo YY, Yan T**. 2014. Performance in Biological Aerated Filters Community Structure and Treatment Impact of Substratum Surface on Microbial. Applied and Environmental Microbiology **80**:177.

36. **Maeda S, Omata T**. 1997. Substrate-binding lipoprotein of the cyanobacterium *Synechococcus sp*. strain PCC 7942 involved in the transport of nitrate and nitrite. J. Biol. Chem. **272**:3036–3041.

37. **Figueiredo HCP, Klesius PH, Arias CR, Evans J, Shoemaker CA, Pereira DJ, Peixoto MTD**. 2005. Isolation and characterization of strains of *Flavobacterium columnare* from Brazil. J. Fish Dis. **28**:199–204.

38. **Guglielmi G, Cohen-Bazire G, Bryant DA**. 1981. The structure of *Gloeobacter violaceus* and its phycobilisomes. Arch. Microbiol. **129**:181–189.

TABLES

Table 1 Each DGGE gel lane, normalized through GELCOMPARII showing total OTU counts per lane over time. The Shannon Index, taking into account evenness and diversity, of each banded lane was calculated as data was available. A complete profile is given in Figure 5. OTU quantities and diversity index are given as a function of time with indication of pre-oxidation regime being applied at that time.

		Sampling Date							
Sample Location		7/2-ClO_2	7/9-ClO_2	7/16-Cl_2	7/23-ClO_2	8/1-ClO_2	8/15-ClO_2	9/4-ClO_2	9/30-ClO_2
Intake Water	Qty. of OTU's	16	9	14	9	*	*	19	14
	Diversity Index	1.1138	0.7553	1.0336	0.7105	*	*	1.1643	1.0561
Biofilm	Qty. of OTU's	14	9	11	13	17	17	9	13
	Diversity Index	0.8085	0.8354	0.7858	0.9854	1.0478	1.1251	0.7534	0.9463
Basin Water	Qty. of OTU's	8	*	8	9	*	18	*	19
	Diversity Index	0.7666	*	0.726	0.6522	*	1.1049	*	1.1973

* indicates data unavailable. DNA extractions of these samples were unsuccessful.

OTU: operational taxonomic units

ClO_2: chlorine dioxide pre-oxidation

Cl_2: free chlorine pre-oxidation

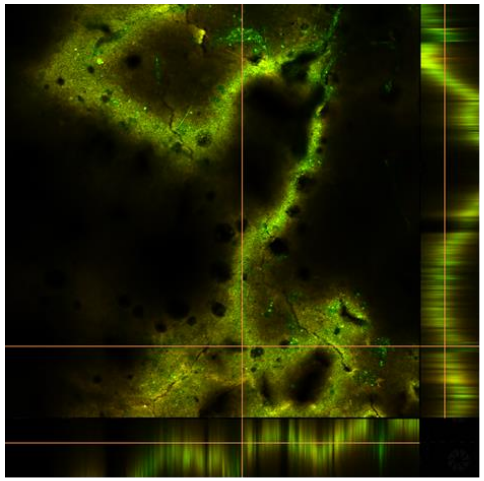
Table 2 Percent similarity 16S rRNA sequences identified from DGGE profiles. OTU designations are also indicated on Figure 5. Sequence information was entered into BLAST (blast.ncbi.nlm.nih.gov).

OTU	Microorganism	Phylogenetic Affiliation	Gram Reaction	Accession Number	Similarity	Coverage	Significance in Water Treatment
1	*Novosphingobium aromaticivorans*	proteobacteria	negative	NC 007794.1	98%	115/117	Pathogenic; causes primary biliary cirrhosis (PBC)
2	*Synechococcus sp.*	cyanobacteria	negative	NC_009482.1	93%	105/113	possible source for cyanotoxins
3	*Gloeobacter violaceus*	cyanobacteria	negative	NC 005125.1	91%	96/106	possible source for cyanotoxins
4	*Sanguibacter keddieii*	actinobacteria	positive	NC 013521.1	88%	111/126	Facultatively anaerobic; versatile
5	*Pelagibacter ubique*	proteobacteria	negative	NC 007205.1	98%	109/111	Highly dominant worldwide in water bodies
6	*Pelagibacter ubique*	proteobacteria	negative	NC_007205.1	98%	112/114	Highly dominant worldwide in water bodies
7	*Novosphingobium sp.*	proteobacteria	negative	NC_015580.1	98%	87/89	*
8	*Flavobacterium columnare*	flavobacterium	negative	NC_016510.2	95%	112/118	Showed a low tolerance for both oxidants
9	*Acidothermus cellulolyticus*	actinobacteria	negative	NC_008578.1	88%	97/110	found in warm cow feces, decomposing hay; thermophilic

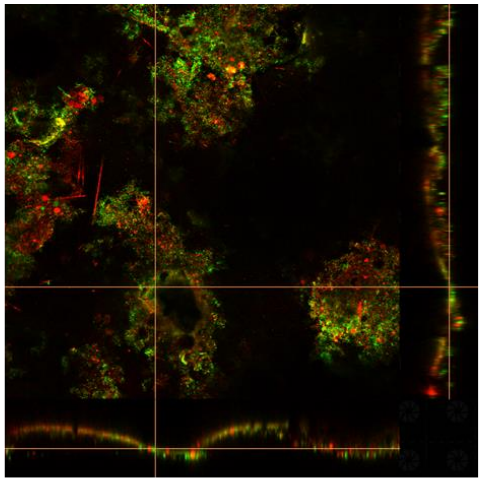
* indicates no conclusion was made

OTU: operational taxonomic unit
References: (26–28, 36–38)

FIGURES



a 6/11-ClO_2



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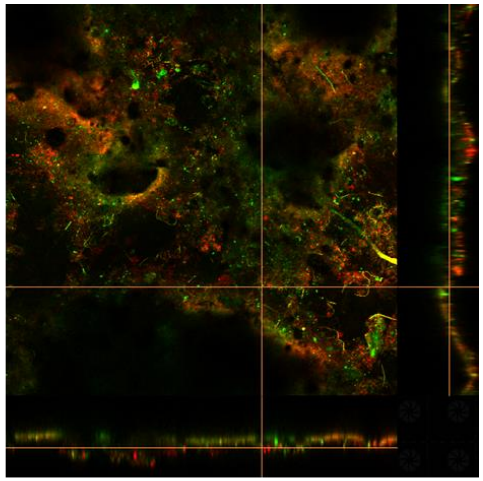
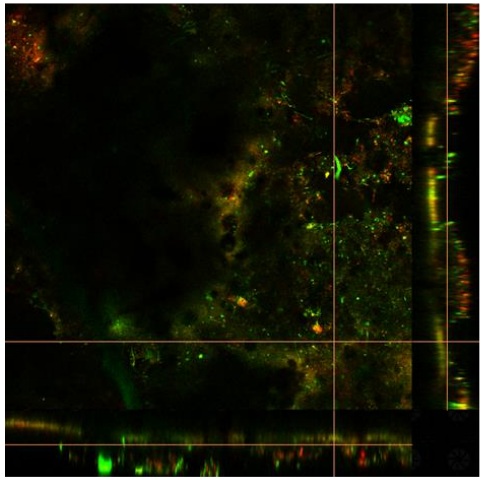


c 7/9-ClO_2



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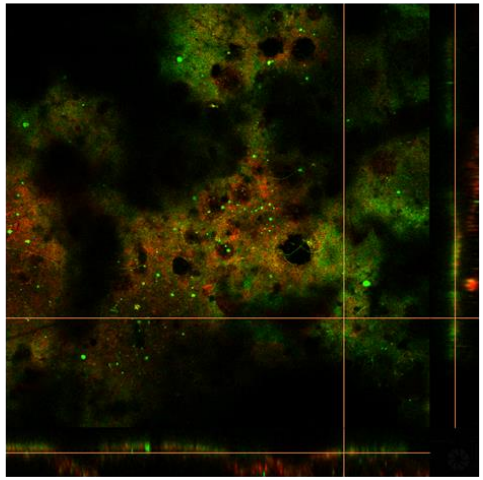
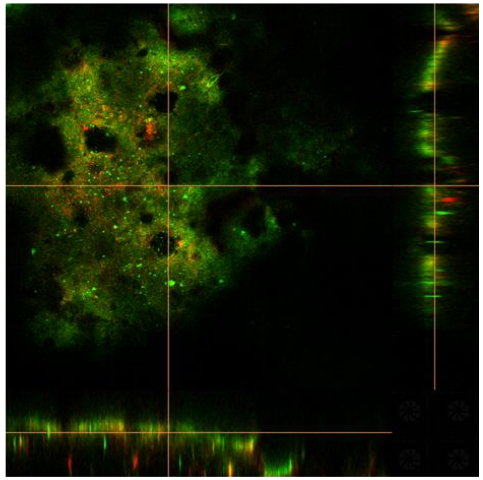


e 7/23-ClO_2



f 8/01-ClO_2

Figure 1 Confocal laser scanning microscopy imaging of the biofilm formation in the sedimentation basin. The biofilm incorporated flocculated particles as they settled in the sedimentation basin resulting in a non-homogenous, complex matrix of ferrous salts and bacterial aggregates. a) Biofilm formation under chlorine dioxide pre-oxidation at a) 2 weeks b) 4 weeks c) 6 weeks d) 4 days after switching pre-oxidation to chlorine e) 1 week after the change, under chlorine dioxide f) 2 weeks after the change.

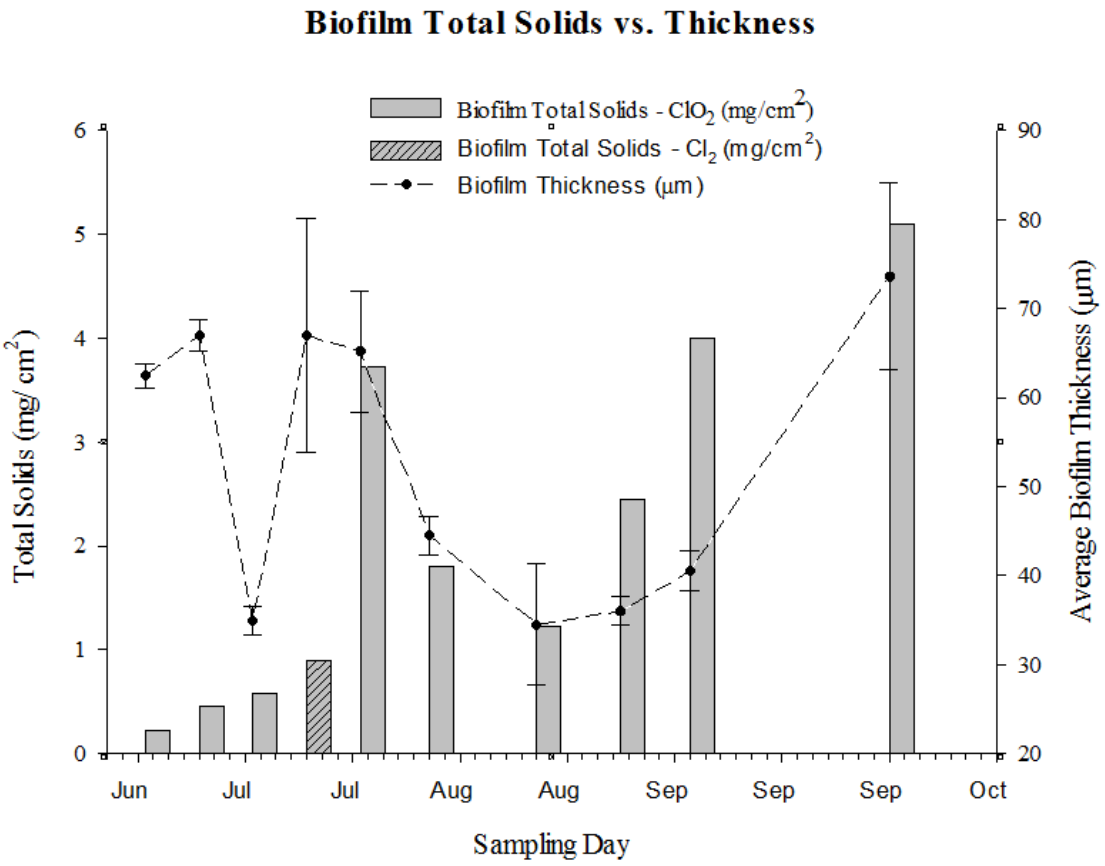


Figure 2 On the primary y-axis, total biofilm solids accumulation (mg/cm^2) on the biofilm plates is shown as an average of two replicates from within the sedimentation basin dependent on time of sampling. The change of pre-oxidant occurred as indicated by the hatched dataset. The secondary y-axis shows average biofilm thickness (µm) including standard error of the mean (n=30) as data points connecting the dashed line, also dependent upon sampling time.

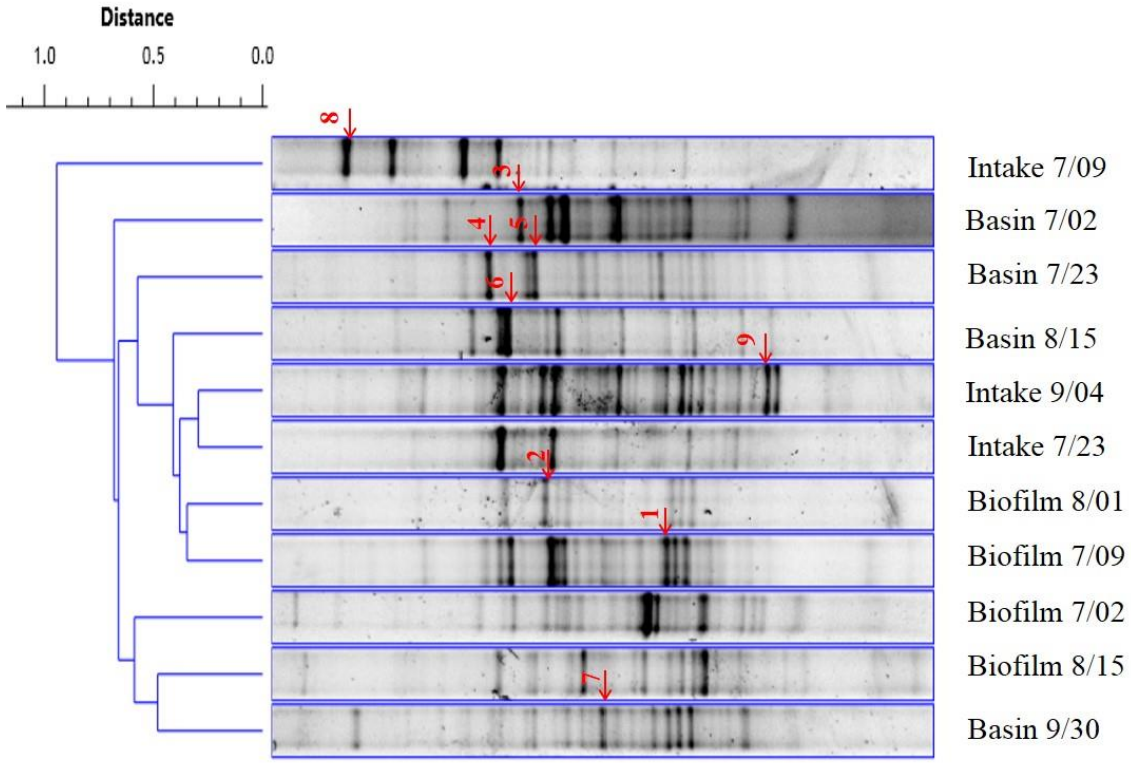


Figure 3 Un-weighted pair group method with arithmetic mean (UPGMA) correlation of sampling dates with sequenced operational taxonomic units. The lanes pictured above are depicted as raw images before normalization. All lanes were normalized to an external reference ladder before band matching was performed; present bands were denoted as 1 and absent bands denoted as 0. The band classes (defined by position in gel lane) derived from intensity profiles were exported into Microsoft Excel to examine presence/absence of sequenced bands in varying classes.

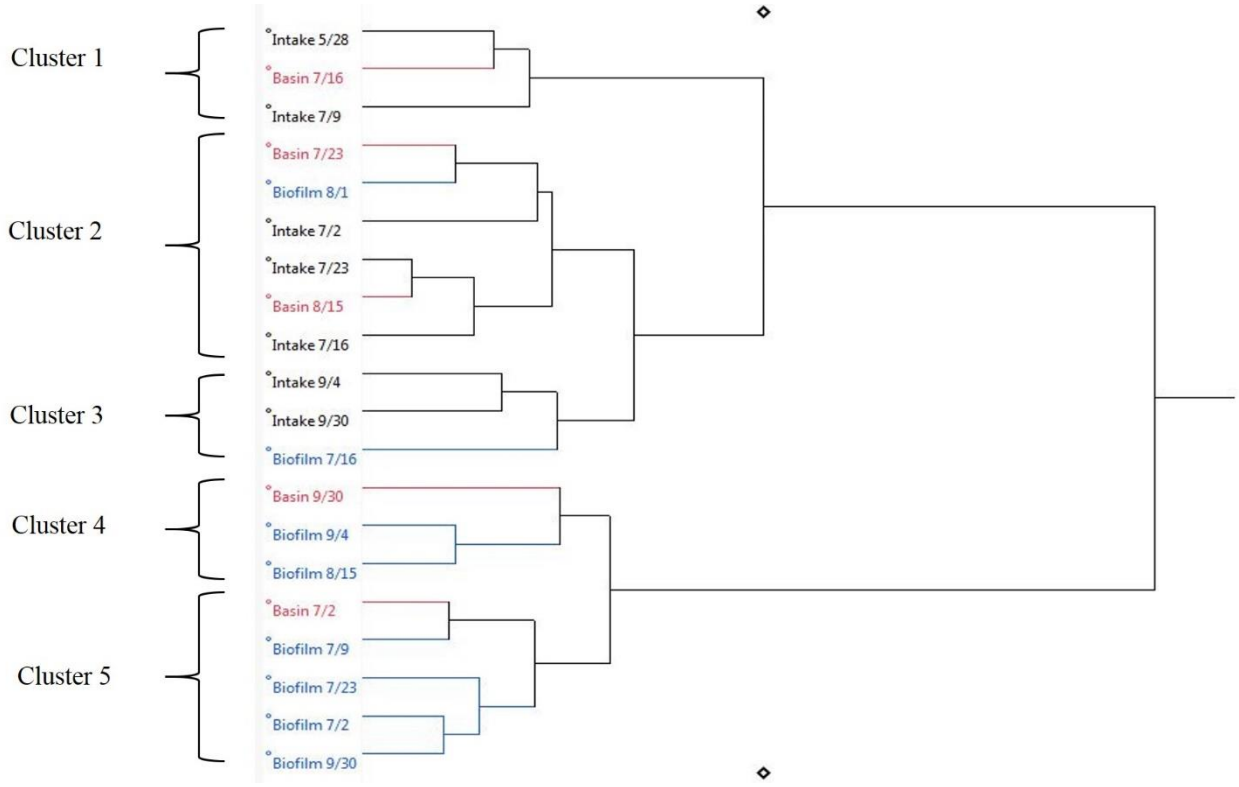


Figure 4 Pearson cluster analysis comparison of non-chlorinated intake waters, basin water and biofilm. Cluster 4 and 5 distance the biofilm densitometric curves from both chlorinated and non-chlorinated samples, with the exceptions of biofilm on 8/1 and 7/16.

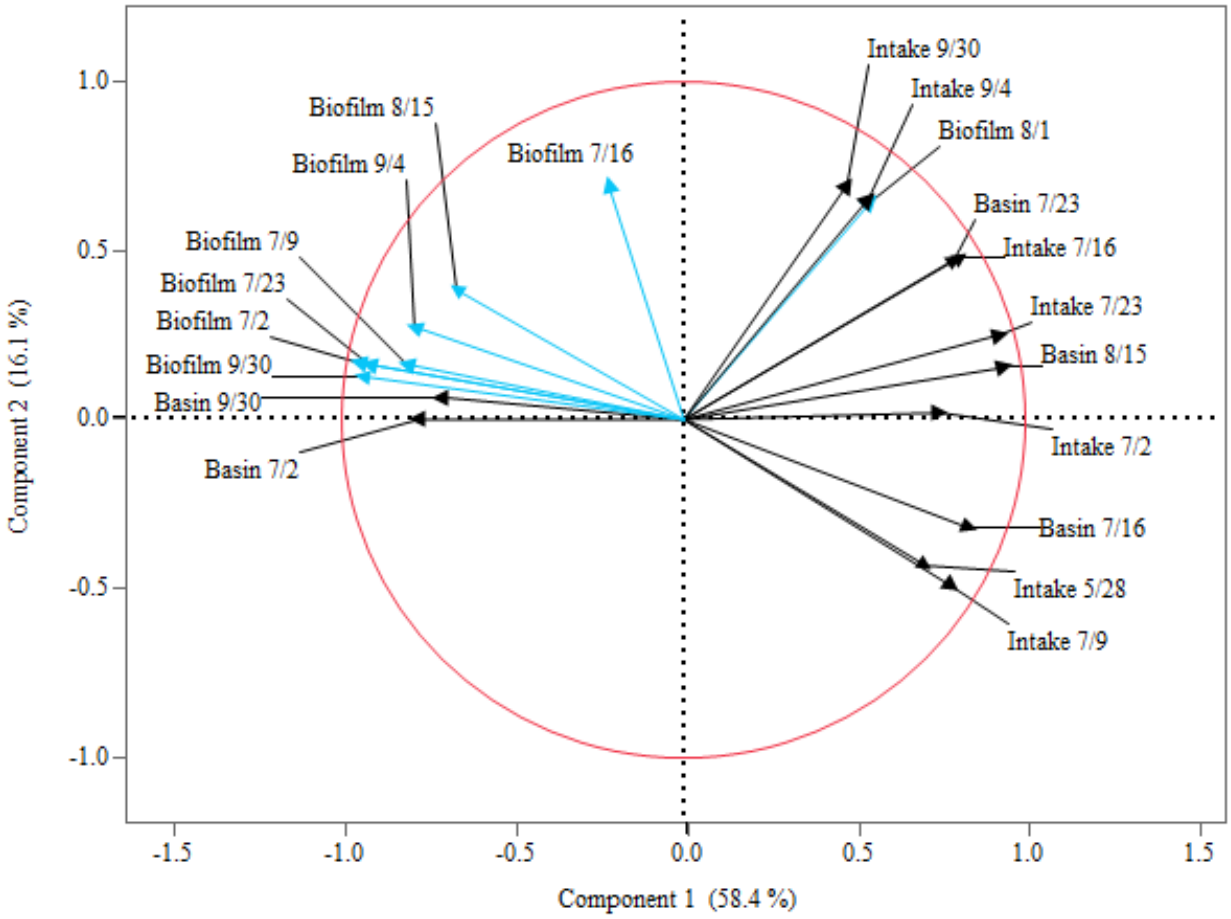


Figure 5 Principal components for all samples created from denaturing gradient gel electrophoresis densitometric curves using un-weighted pair group method with arithmetic mean (UPGMA) clustering. The similarity matrix was uploaded into JMP Pro to calculate principal components.

Table S1. Environmental parameters measured in the sedimentation basin from 6/4 to 9/30.

Samping Date	Week Number	Water Total Solids (mg/L)	Bacterial Count (< 2µm) (cells/100mL)	Organism Count (> 50 µm) (cells/100mL)	pH	PO_4 (mg/L)	NO_3 (mg/L)	Turbidity (NTU)	Organic Carbon (mg/L as C)	High (°C)	Precipitation. (inch)
6/4/2013	3	61	1.81E+02	1.00E+00	6.50	0.04	0.72	0.9	2.18	28.3	0
6/11/2013	4	180	7.91E+06	1.00E+00	6.37	0.07	0.70	0.6	1.93	32.2	0
6/18/2013	5	79	9.30E+06	3.86E+03	6.52	0.06	0.90	0.1	2.11	31.7	0.18
6/25/2013	6	96	2.71E+07	3.86E+03	7.31	0.06	1.09	1.21	2.08	31.7	0
7/2/2013	7	71	2.47E+07	1.93E+04	6.79	0.04	0.78	0.2	2.08	26.1	0
7/9/2013	8	83	2.97E+07	5.10E+05	7.56	0.06	1.02	0.2	2.37	33.3	0
7/16/2013	9	31	2.28E+07	3.48E+04	6.56	0.16	0.85	0.3	2.82	31.1	0
7/23/2013	10	77	5.42E+07	3.86E+03	6.72	0.10	1.12	1	1.96	35.0	1.4
8/1/2013	11	153	1.25E+07	6.96E+04	7.38	0.39	0.78	0.4	1.8	30.6	0
8/15/2013	12	165	7.70E+06	3.48E+04	7.24	0.10	0.75	0.5	1.91	25.0	0
8/26/2013	13	53	4.52E+09	1.04E+07	6.76	0.03	0.41	1.0	2.11	32.2	0
9/6/2013	14	69	4.13E+06	2.71E+04	8.15	0.13	0.02	0.2	2.01	30.0	0
9/30/2013	15	252	4.97E+06	1.16E+04	7.01	0.03	3.31	0.2	1.75	24.4	0

Conclusion

This study shows the ability of biofilm to harbor pathogens under extreme selective pressures such as strong oxidants including Cl_2 and ClO_2. There are advantages and disadvantages to using either Cl_2 or ClO_2 as a primary oxidant. Although ClO_2 is a stronger oxidant on a mass:dose basis, it is not as effective as Cl_2 on disinfection within biofilm communities. However, since the use of Cl_2 results in organic DBP's, ClO_2 is a safer alternative if the resulting chlorite is properly quenched. In the future, development of strategies to overcome biofilm formation in the sedimentation basins would aid primary oxidation efficiency and protect further treatment processes, and microbial quality within the treatment plant should be considered when switching pre-oxidants for DBP reduction purposes.

References

(1) **A. Konopka**. 2009. "What is microbial community ecology?" *The ISME journal.* vol. 3. no. 1223-1230.

(2) **United States Environmental Protection Agency**. 1999. Alternative Disinfectants and Oxidants Guidance Manual. United States Environmental Protection Agency.