Organic Carbon Generation Mechanisms in Main and Premise Distribution Systems

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ABSTRACT

Assimilable organic carbon (AOC) is a suspected contributor to growth of microbes, including pathogens, in plumbing systems. Two phases of research were completed to improve knowledge of AOC and other forms of organic carbon in premise plumbing. In the first phase, the AOC Standard Method 9217B was compared to a new luminescence-based AOC in terms of time, cost, convenience, and sources of error. The luminescence method was generally more accurate, as it better captured the peak growth of the test organisms. It was also less expensive and less time-consuming. A few approaches to improving the accuracy of the method and detect possible errors were also presented.

In the second phase of research, the possibility of AOC generation in premise plumbing was reviewed and then tested in experiments. It has been hypothesized that removal of AOC entering distribution systems might be a viable control strategy for opportunistic premise plumbing pathogens (OPPPs), but if AOC was generated in premise plumbing systems this approach would be undermined. Possible sources of AOC creation in premise plumbing, which is herein termed "distribution system derived biodegradable organic carbon (DSD-BDOC)," include: leaching of organic matter from cross linked polyethylene (PEX) pipes, autotrophic oxidation of H_2 generated from metal corrosion (e.g. sacrificial magnesium anode rods and iron pipes), rendering of humic substances more biodegradable by sorption to oxides such as $Fe(OH)_3$, and accumulation of AOC on filters and sediments. The potential for various plumbing and pipe materials to generate AOC was compared in controlled simulated water heater experiments. Under the worst-case condition, generation up to 645 µg C/L was observed. IT was not possible to directly confirm the biodegradability of the generated organic carbon, and there were generally no correlations between suspected generation of organic carbon and either heterotrophic plate counts (HPC) or of bacterial 16S rRNA genes. DSD-BDOC was also explored in a simulated distribution system with two disinfectant types (chlorine and chloramine) and three pipe materials (PVC, cement, and iron). TOC increased with water age, probably due to leaching of organics from PVC and possibly the aforementioned DSD-BDOC due to autotrophic reactions of nitrifiers and iron-related bacteria. As before, relationships between the higher levels of organic carbon and either HPC or 16S were not observed.

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List of Abbreviations

Abbreviation	Definition
AOC	Assimilable organic carbon
APC	Autotrophically produced carbon
ATP	Adenosine triphosphate
BDOC	Biodegradable dissolved organic carbon
CA	Chloramine, referring to the chloramine simulated distribution system
CFU	Colony forming units
DSD-BDOC	Distribution system derived biodegradable dissolved organic carbon
DO	Dissolved oxygen
FC	Free chlorine, referring to the free chlorine simulated distribution system
GAC	Granular activated carbon
HPC(s)	Heterotrophic plate count(s)
MSB	Mineral salt buffer
NOM	Natural organic matter
NOX	*Spirillum* strain NOX, an organism used for measuring AOC
NTM	Non-tuberculous *Mycobacteria*
OPPP(s)	Opportunistic premise plumbing pathogen(s)
P-17	*Pseudomonas fluorescens* strain P-17, an organism used for measuring AOC
PEX	Cross-linked polyethylene
PTFE	Polytetrafluoroethylene
PVC	Polyvinyl chloride
SWH	Simulated water heater
TOC	Total organic carbon

Chapter 1. A Practical Comparison of Two Assimilable Organic Carbon Methods

Amanda Martin, Lauren Weinrich, Amy Pruden, and Marc Edwards

1.1 Abstract

This study compared the AOC Standard Method 9217B to a new AOC method developed by Weinrich et al. (2009) that utilizes genetically modified bioluminescent strains of the traditional AOC test organisms, NOX and P-17. The methods were compared in terms of time, cost, convenience, and sources of error. Although both methods have some inherent sources of error, the luminescence method was generally more accurate because it better captures the peak growth of the test organisms. Additionally, the luminescence method is less expensive and less time-consuming. A few additions to the luminescence assay can be adopted to sometimes improve the accuracy and reliability of the method, including the addition of minerals, normalizing for background luminescence, and streaking plates to check for contamination.

1.2 Introduction

Growth of bacteria in drinking water distribution systems can cause taste and odor problems, induce microbial corrosion, violate drinking water standards, and cause waterborne disease (Silvey et al. 1950; Morris et al. 1963; Zhang et al. 2009; CDC 2008; Alary et al. 1991; Falkinham et al. 2008). Bacteria are present in biofilms throughout distribution systems, utilizing nutrients derived from the water, other bacteria, and distribution system materials (Characklis 1988). Organic carbon is often a critical nutrient that limits regrowth and therefore its accurate quantification in drinking water is of engineering and scientific importance.

The different types of organic carbon present vary markedly in their amenability to be assimilated by distribution system bacteria and the vast majority is considered to be non-biodegradable (van der Kooij et al. 1982). Consequently, methods have been developed to quantify fractions of organic carbon that are readily available to microbes of interest, which have been termed Biodegradable Dissolved Organic Carbon (BDOC) and Assimilable Organic Carbon (AOC). BDOC is the concentration of organic carbon consumed by the diverse ambient microbiota present in the sample over a week in dark, room temperature conditions (Servais et al. 1987). AOC has been determined by various bioassay procedures

using an inoculum of one to four species of bacteria, but the Standard Method defines AOC as the carbon consumed by two test organisms, *Pseudomonas fluorescens* strain P-17 and *Spirillum* strain NOX, to reach growth to maximum density (SM 9217B). Previous studies have found AOC to be 0.1 – 10% of total organic carbon (TOC) and BDOC to be about 15% of TOC (Hammes et al. 2005; Williams et al. 2011). AOC has been useful in establishing rough thresholds of nutrients limiting heterotrophic regrowth in water distribution systems (van der Kooij 1992; Camper et al. 2003; LeChevallier et al. 1991). Levels less than 10 μg/L are reported to be sufficient to limit many problems of microbial growth in water distribution systems with little or no disinfectant, and AOC levels greater than 100 μg/L have been associated with problems attributed to bacteria in main distribution systems (LeChevallier et al. 1991).

Currently, there are no easily-measured chemical surrogates for AOC, so its concentration is determined via biological growth assays. In a bioassay, the net change in initial biomass can be used to estimate the level of limiting nutrients that were assimilated. Although many versions of the AOC bioassay have been described, this study directly compares practical performance of the AOC Standard Method (9217B) and a new luminescence-based method (Weinrich et al 2009). The AOC Standard Method (9217 B) originally developed by van der Kooij is a bioassay utilizing two bacterial species, *Pseudomonas fluorescens* strain P-17 (P-17) and *Spirillum* strain NOX (NOX), which were selected for their nutritional diversity (van der Kooij 1988). A small inoculum of both organisms in batch culture is allowed to reach maximum growth density in the water of interest. The two organisms are purposefully inoculated into the same batch reactor to represent a range of physiological capabilities and capture a broad range of AOC. Because P-17 and NOX do not assimilate all types of organic matter , probably underestimates the total quantity of AOC for the diversity of microbes in potable water and is correspondingly lower than BDOC estimates (Prevost et al. 1990), some versions of the AOC bioassay utilize up to four species of with the intention of measuring a broader extent of AOC constituents (van der Kooij et al. 1982; Kenney et al. 1988; Nedwell 1987; van der Kooij et al. 1983; van der Kooij and Hijnen 1984).

The organisms are then enumerated by the spread plate method for heterotrophic plate counts (Standard Method 9215C) three times over the course of the incubation period. The numbers of the cells in colony forming units (CFU) is converted to AOC by an empirically derived yield factor obtained from the growth of P-17 and NOX on acetate-carbon and

oxalate-carbon, respectively. These yields are assumed to be equal to the yield of NOX and P-17 with naturally-occurring AOC, and the concentration of NOX and P-17 at stationary phase is assumed to be the maximum number of organisms that can be supported by the nutrients in the sample.

Standard Method 9217B is acknowledged to be time-consuming and costly for routine use in a laboratory or utility setting (Weinrich et al. 2009; Haddix et al. 2004; Leddy and Bold 2011), and many modifications have been suggested to the original assay to ease the burden of measuring AOC. For example, LeChevallier et al. (1993) attempted to simplify the method by measuring adenosine triphosphate (ATP), but this technique was not widely adopted due to problems with commercial ATP substrate (Haddix et al. 2003). Weinrich et al. (2009) developed a method using genetically modified versions of the standard test bacteria, NOX and P-17, that express inducible bioluminescence. This enables monitoring of bacterial growth using a luminometer rather than a spread plate. This study reports on extensive practical comparisons between the Weinrich et al. (2009) and Standard Methods in terms of time, cost, compatibility, and accuracy. Some insights regarding limitations of each method and possible improvements in QA/QC are provided as part of this evaluation.

1.3 Materials and Methods

Standard Method 9217B and the luminescence AOC method according to Weinrich et al. (2009) were used as described below.

1.3.1 Glassware

Graduated, 125 mL borosilicate glass bottles with black polypropylene caps welded to a polytetrafluoroethylene-silicone (PTFE) liner (Kimax; Kimble-Chase, Vineland, NJ) were used as the incubation vessels for both the standard and luminescence methods. Both bottles and caps were washed in a glassware washer (Lancer, Lake Mary, FL) with an acid cycle. Acid was 25% acetic acid by volume in a solution of deionized water (Lancer, Lake Mary, FL) and the detergent was 23.5% of a 45% potassium hydroxide solution or 16% of pure KOH by weight, 12% sodium silicate, 0.5% EDTA, and 64% deionized water (Lancer, Lake Mary, FL). Caps were then autoclaved and stored in sterilization pouches until needed. Bottle openings were covered in aluminum foil and bottles were baked in a muffle furnace at 550°C for 5.5 hours.

1.3.2 Bacteria, Stocks, and Media

Bioluminescent strains of the standard AOC test bacteria, P-17 and NOX, were obtained from American Water (Voorhees, NJ). Stocks were stored at – 80°C in 20% glycerol-2% peptone solution. Bacteria were recovered by streaking onto R_2A agar (Difco, Lawrence, KS) and incubating at room temperature for 3 to 5 days. Stock solutions of NOX and P-17 were created by picking single colonies from the R_2A plates, inoculating them into a 2 mg/L acetate-carbon solution in 1X mineral salt buffer (MSB), and growing for 7 days at room temperature. Bacterial densities of stock cultures were then monitored by spread plating onto R2A agar. Stock cultures were stored at 4°C for up to 40 days to be used for inoculation of water samples.

A 1,000X mineral salt buffer stock was prepared by adding the following chemicals to 1 L of nanopure water from a Nanopure™ TOC-UV (Thermo Scientific Barnstead, Marietta, OH): 7.0 g K_2HPO_4, 3.0 g KH_2PO_4 , 0.1 g $MgSO_4 \cdot 7H2O$, 1.0 g $(NH_4)_2SO_4$ (anhydrous), 0.1 g NaCl (anhydrous), and 1.8 mg $FeSO_4 \cdot 7H_2O$. The stock was mixed thoroughly and then autoclaved for sterility. 1X MSB were created by adding 1 mL of 1,000X MSB to 999 mL of nanopure water and autoclaving. A 200 mg/L acetate-carbon stock solution was prepared by adding 113 mg of sodium acetate to 100 mL of nanopure water. The acetate-carbon stock was then sterile-filtered into a baked bottle using a 0.22 µM Millex® -GV syringe filter (Millipore, Tullagreen, Carrigtwohill, CO) and a 30 mL Luer-Lok-tip syringe (BD, Franklin Lakes, NJ). A 2 mg/L acetate-carbon stock in 1X MSB was prepared by adding 10 mL of 200 mg/L sodium-acetate to 990 mL of 1X MSB. All solutions were adjusted to pH 7.2 ± 0.2 with 0.1 N NaOH or 0.1 N HCl prior to autoclaving.

1.3.3 Standard Method Plate Counts

The plate count AOC method was followed according to Standard Method 9217B. Water samples were collected into sterile, baked borosilicate Kimax bottles with PTFE-lined, autoclaved caps which contained sodium thiosulfate to quench residual disinfectant. Samples were stored at 4°C if not immediately analyzed. Prior to analysis, the sample pH was checked and adjusted to 7.2 ± 0.2 if necessary using a 1 N sodium hydroxide solution or carbon dioxide. Each sample was then split into two 100 mL aliquots and pasteurized for 30 min at 70°C in a water bath. After pasteurization, the cooled samples were inoculated with P-17 and NOX into the same reactor with approximately 10^4 CFU/mL of each organism. The 10^4 CFU/mL inoculum was an adjustment to the original 500 CFU/mL inoculum recommended by the Standard Method, as suggested in LeChevallier et al. (1993). The

larger initial inoculum decreases the time needed to reach peak growth (LeChevallier et al. 1993). Bioassays were stored in the dark at room temperature. On days 5, 7, and 9 of growth, serial dilutions were performed in sterile dilution tubes using 1X MSB and dilutions were spread-plated in duplicate by adding 0.1 mL aliquots onto R_2A agar. Plating was performed in a laminar flow hood (1300 Series A2, Thermo Fisher Scientific, Marietta, OH) to minimize potential for contamination. After 5 days of growth at room temperature on the R_2A plates, colonies numbering 30 to 300 per plate were enumerated. Plate counts were expressed as the means of duplicate plates and were converted to acetate-carbon equivalents by using the standard conversion factors: $4.1x10^3$ P-17 CFU/mL equals 1 g P-17 AOC/L and $1.2x10^4$ NOX CFU/mL equals 1 g NOX AOC/L (Standard Method 9217B). AOC by the plate count method was reported as the sum of values obtained for P-17 and NOX.

1.3.4 Luminescence Method

Luminometer. A high-sensitivity, automated, photon-counting luminometer (SpectraMax L; Molecular Devices, Sunnyvale, CA) was used to monitor the growth of the bioluminescent bacteria strains in the bioassays. Three-hundred microliters of sample were transferred to 96-well microplate wells in triplicate. White polystyrene microtiter plates (Thermo Scientific Corporation, Milford, MA) were used to minimize light interference. Luminescence over 30 sec intervals was measured. Readings were reported in relative light units (RLU), which were defined as 10,000 times the integral of the photon count versus the time-reaction curve. Counting began immediately following injection of 30 µL of 0.2% *n*-decanal, an artificial luminescence substrate which induces luminescence, directly into the microplate well. The substrate solution was prepared as 0.2% (vol/vol) *n*-decanal in a volumetric flask by delivering 500 µL of *n*-decanal (MP Biomedicals, Solon, OH) into 250 ml of 200-proof ethyl alcohol. This solution was then transferred into the injection reservoir on the luminometer for auto-injection.

Standard curves. Standard curves for the luminescence method were developed to relate luminescence in RLU/mL to AOC (acetate-C eq./L). Standard curve assays were developed using the mineral salt buffer (MSB) described in Weinrich et al. (2009) with acetate carbon concentrations ranging from 0 to 1,000 ppb by adding the requisite volume of acetate stock to sterile 1X MSB. Carbon standards were inoculated separately with 10^4 cells of P-17 or NOX per mL. Samples were swirled to mix and 300 µL aliquots were removed and added to microplates two or three times daily. To maintain sterility, transfer of the samples was completed within a laminar flow hood (1300 Series A2, Thermo Fisher

Scientific, Marietta, OH). Peak luminescence values were considered to be the maximum density of cells and this value in RLU/mL was related to the corresponding AOC value (acetate-C eq./L) to create the standard curves. Using peak luminescence values is one of two methods outlined by Weinrich et al. (2009), which also applied Monod modeling to develop more refined estimates of NOX and P-17 growth curves and AOC. Six sets of standard curves were conducted over the course of the experiment. For each acetate standard concentration, at least three separate replicate tests were conducted and the average coefficient of variation among the test results was 16% for NOX and P-17. Regression analysis of the five-point standard curve between luminescence (RLU) and acetate-carbon levels produced R^2 values of 0.99 and 0.92 for NOX and P-17, respectively (Figure 1.1). The intercept of both regressions was set to zero.

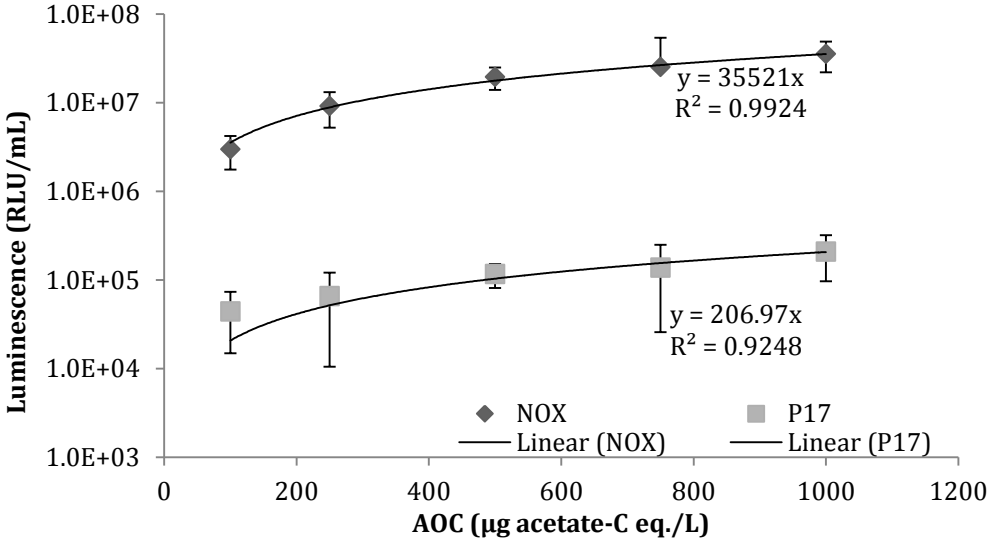

Figure 1.1. Luminescence AOC Standard Curves for NOX and P-17. Error bars represent 95% confidence intervals of *n* independent replicate tests, where *n* was between 3 and 6.

Measuring AOC of samples. One-hundred milliliter water samples were collected into sterile, baked borosilicate Kimax bottles with PTFE-lined, autoclaved caps which contained sodium thiosulfate to quench any residual disinfectant. Samples were stored at 4°C for up to two weeks if not immediately analyzed. Prior to analysis, the sample pH was checked and adjusted to 7.2 ± 0.2 using a 1 N sodium hydroxide solution or 1 N hydrochloric acid solution if necessary. Each sample was then split into two 100 mL aliquots and pasteurized for 30 min at 70°C in a water bath. After pasteurization, samples were allowed to cool to room temperature and inoculated separately with P-17 and NOX with approximately 10^4 CFU/mL. A negative control consisting of 1X MSB and a positive control consisting of 500

ppb acetate carbon in 1X MSB were also inoculated separately with NOX and P-17. Samples were gently swirled and monitored by adding 300 µL aliquots to the sample microplates in triplicate. Measurements were made immediately post-inoculation and then two or three times per day until peak luminescence was reached, usually in four to seven days. Samples were stored in the dark at room temperature. The regressions determined from the standard curves could then be used to relate the peak luminescence value to the level of AOC in each sample. AOC values are calculated for each test organisms and the sum of both AOC values is considered the total AOC in the sample.

1.4 Results and Discussion

1.4.1 Time and Cost Comparisons

The luminescence assay was developed with the purpose of decreasing the amount of time and expense needed to obtain AOC results (Weinrich et al. 2009). Because the time and cost to analyze samples does not have a linear relationship to the number of samples, three representative numbers of samples were selected for time and cost estimates (Table 1.1) including 3 samples, a dozen samples, and the maximum number of samples that could be analyzed by a single analyst during a normal work week using each approach (24 when using the standard method and 60 when using the luminescence method). The maximum weekly number of samples using the luminescence method was calculated based on our experience that running the luminometer was a limiting factor. Sixty samples requires almost four 96 well plates for one data point collection, and at about 50 minutes per plate, 3 hrs 20 mins are needed to read the four plates, or 6 hours and 40 minutes to collect two data points per sample each day. Estimates are based on the cost of all consumables and do not consider overhead or a profit margin. Additionally, our time estimates for the luminescence method do not include data analysis to use Monod modeling on the bacterial growth curves, which provides additional insights and parameters that are not provided with the Standard Method (Weinrich et al. 2009).

Table 1.1. Time and Cost Estimate Comparison for the AOC Standard Method and Luminescence Method. Cost of labor used in calculations is $30/hr.

No. of Samples	Standard Method		Luminescence Method	
	Time per sample (hrs)	Cost per sample (US$)	Time per sample (hrs)	Cost per Sample (US$)
A few (3)	5.83	$185	4.17	$129
A dozen (12)	2.42	$77	1.50	$49
Max Weekly (24 Standard, 60 Lumin.)	1.67	$54	0.67	$23

Without considering the capital costs of purchasing the luminometer for the luminescence method (approximately $25,000), the luminescence method is more economical and less-time consuming. The time and cost per sample decreases as more samples are analyzed when using either method. When analyzing 3 samples, the luminescence method is about 30% cheaper and less time-consuming than the standard method, whereas for the maximum weekly samples the luminescence method is about 60% cheaper and less time-consuming.

Figure 1.2 illustrates the processes to obtain AOC values using both Standard Method 9217B and the luminescence method. While the number of days required to reach peak luminescence varies between samples, results can usually be obtained in four to seven days, whereas the Standard Method typically takes twelve or more days to obtain results. Thus, the luminescence approach also has a 170-300%faster turn-around time.

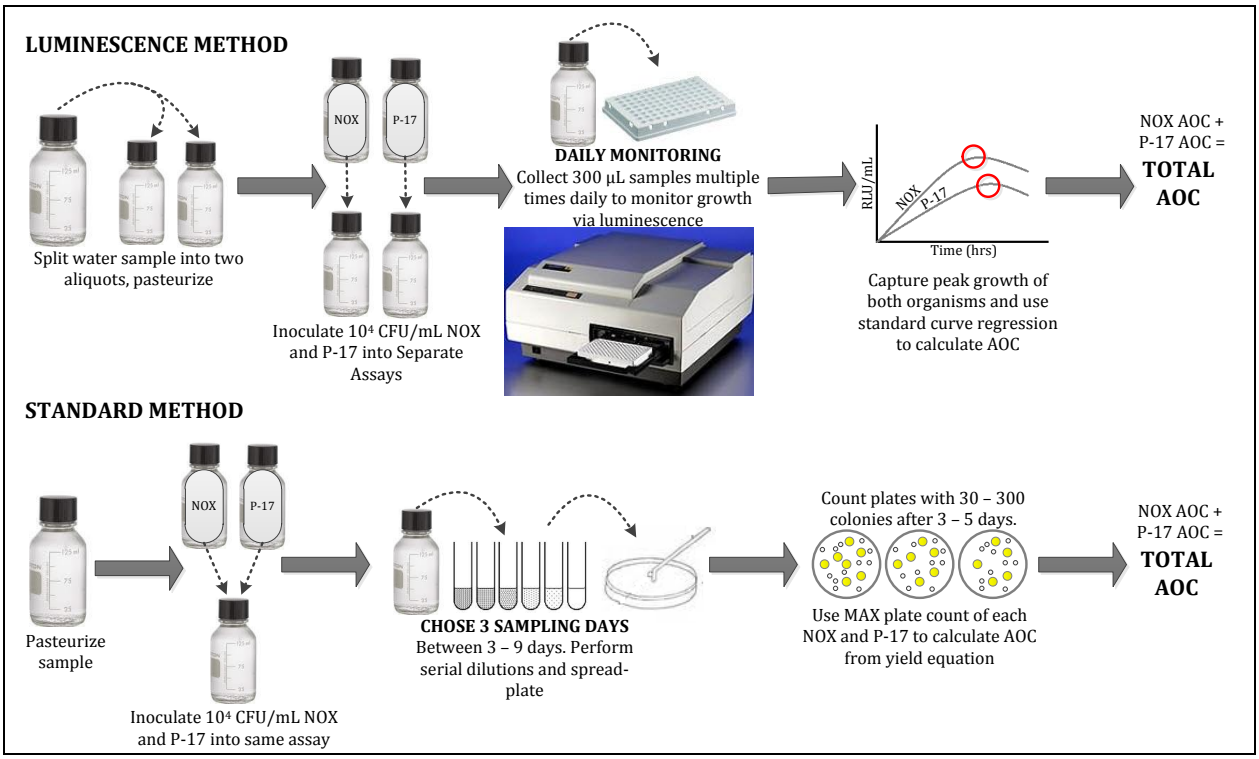

Figure 1.2. Schematic of Luminescence vs. Standard Method 9217B AOC Measurement Processes

1.4.2 Comparability of the Two Methods

A head-to-head analysis of both the Standard Method and the luminescence method was performed on a total of 35 water samples from various sources. The initial expectation was that the two approaches would be nearly identical in their results, but the comparison indicated a very poor correlation. It was observed that the luminescence method yielded a much higher estimate of AOC than the Standard Method. In the most extreme case, the luminescence method produced an AOC value 3053% higher than the corresponding AOC value from the Standard Method. Due to the large observed difference, further experiments sought to elucidate and quantify possible sources of discrepancy and error.

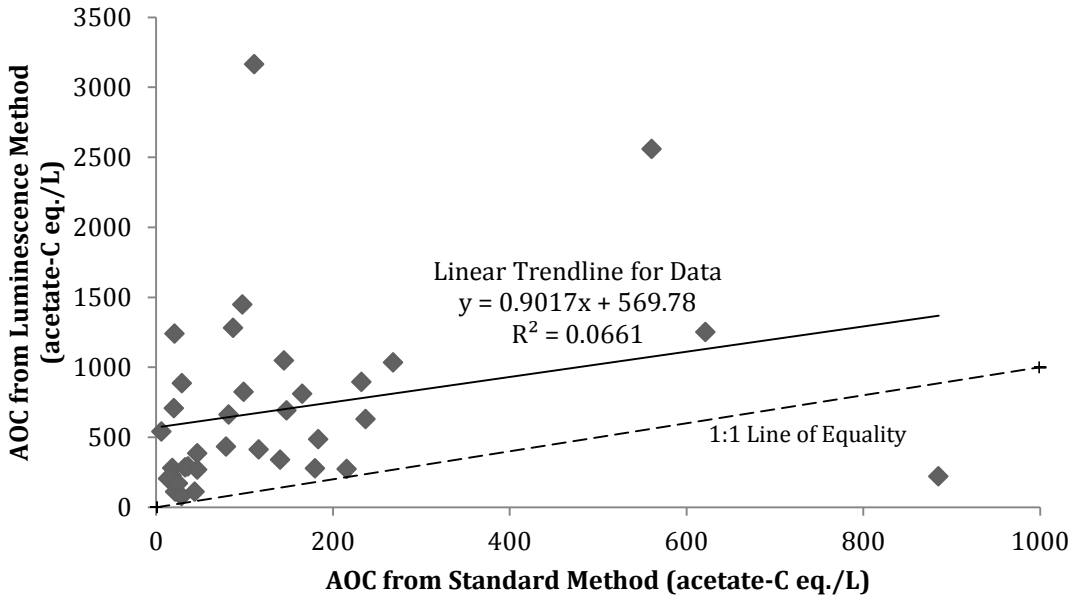

Figure 1.3. Comparison of AOC Determined by Standard Method and Luminescence Method. A linear trendline is fit to the data points. The line of equality represents equivalent results for the two methods.

1.4.3 Sources of Error with the Standard Method

It was determined that for the samples investigated herein, the major source of error associated with the standard method is a failure to catch the peak growth of the test organisms. NOX and P-17 are grown in the bioassays for up to nine days, and samples are collected from the assays for plating at three points during the growth period. Standard Method 9217B suggests that plating occur on days 7, 8, and 9, but these dates can be varied based on the water being analyzed and practical experience, as NOX and P-17 will reach stationary phase much more rapidly in some waters than in others. No matter which sampling dates are chosen using Standard Method, it is likely that only three data points will be collected because of the large cost and time associated with spread plate counting. In contrast, the luminescence method monitors the growth of the test organisms several times each day, and therefore has a much higher likelihood of detecting the actual peak growth of the organisms.

To illustrate, Figure 1.4 plots the growth curves of five different water samples, named samples A through E, illustrating the variation in the amount of time required to reach peak growth in the luminescence assay for a range of samples. For example, Curve B peaks at 72 hours (3 days) while Curve E peaks at 190 hours (8 days).

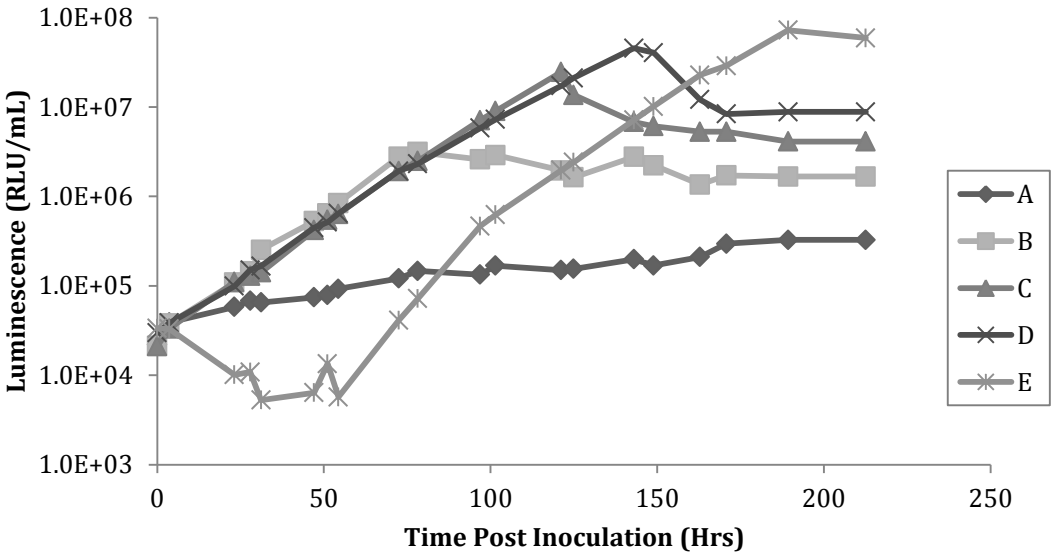

Figure 1.4. AOC Luminescence Growth Curves demonstrating the large variation in the time required reach to peak luminescence as a result of different water types A - E.

If the samples in Figure 1.4 were plated according to the Standard Method on days 7, 8, and 9, the peak growth of Sample E would be captured but the peak growth of the other samples would be missed. A factor of error caused by missing the peak can be calculated by dividing the actual peak luminescence by the luminescence on any sample day. For example, Sample E reaches peak growth on Day 8 around 190 hours, where the peak luminescence is 72,392,247 RLU/mL. If the sample had been plated according to the Standard Method on Day 7 instead of Day 8, when the luminescence was 28,883,210 RLU/mL, this would result in a factor of error of 3X (72,392,247(RLU/mL) /28,883,210(RLU/mL) = 3). Therefore, the AOC value obtained from plating on Day 8 is three times higher than the AOC value that would have been obtained on Day 7 for Sample E.

The percent recovery of AOC in a sample on Days 3 to 8 from the samples in Figure 1.4., is calculated (Percent recovery = AOC on a select day/AOC on day of peak luminescence **x** 100%) is presented in Figure 1.5. Sample E peaks on Day 8 of the test and therefore has 100% recovery on Day 8, and has less than 40% recovery of the AOC in the sample on every other day. Conversely, Sample C has 100% recovery on Day 5 and less than 30% recovery on every other sampling day. As an example, if Days 6, 7, and 8 were selected for plating according to Standard Method 9217B, the peak growth of Sample E would be obtained on Day 8 and 100% recovery would be obtained, but Sample C results would only be 28% on Day 6, 22% on Day 7, and 17% on Day 8.

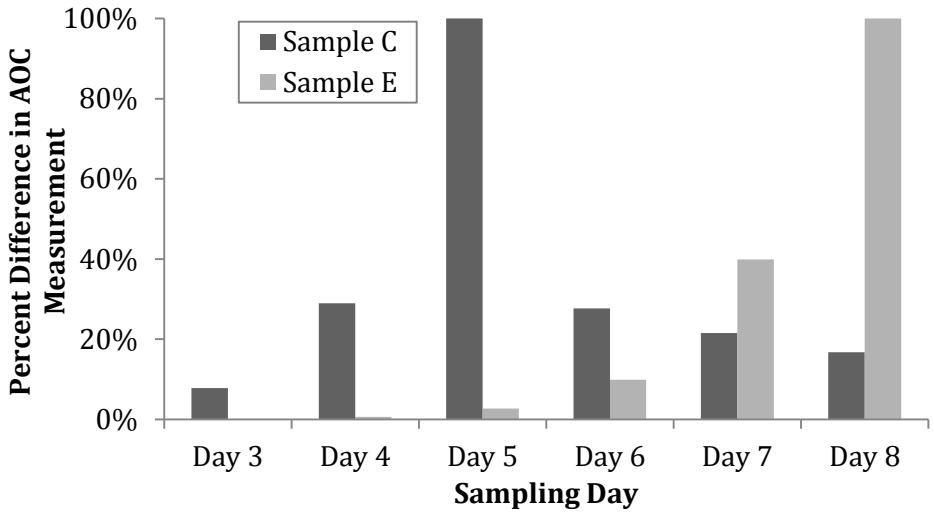

Figure 1.5. Percent recovery of AOC on various sampling days using two sample waters (C and E) relative to peak AOC values.

1.4.4 Sources of Error with the Luminescence Method

While the luminescence assay provides better accuracy by capturing peak growth as previously noted by Weinrich et al. (2009), the approach has a few sources of smaller error including potential double-counting of some AOC and background luminescence. In the Standard Method, NOX and P-17 are inoculated into the same batch reactor, allowed to reach peak growth, and then enumerated by plate counts. The two strains can be visually differentiated in the plate counts as P-17 colonies are larger, yellow colonies than the small, white NOX colonies. The cell densities of both NOX and P-17 are then converted to AOC concentrations by a yield factor and the two AOC concentrations are added to determine the total. It is appropriate to add these two numbers because the NOX and P-17 cells have grown in the presence of each other and the same AOC substrate could not be used twice.

Conversely, in the luminescence-based AOC assay, NOX and P-17 are each inoculated into separate batch reactors. AOC is calculated from peak luminescence for each NOX and P-17 using standard curves, and these two numbers are added to calculate total AOC in the sample. Because NOX and P-17 are inoculated separately, adding the NOX AOC value and the P-17 value to calculate total AOC could hypothetically result in up to an error of 2X, because growth of each individual organism may be representing precisely the same fractions of AOC. For instance, if only 5 µg/L acetate were present, a total of 10 µg/L AOC

would be quantified (5 µg/L from NOX and 5 µg/L from P-17)., since both NOX and P-17 are both known to grow on acetate as is illustrated by the standard curves.

Prior work indicated that there may be relatively little overlap in the naturally-occurring organic matter typically metabolized by both NOX and P-17, based on comparisons of Standard Method results to ATP-based luminescence and also on known differences in compounds metabolized by P-17 and NOX (LeChevallier et al. 1993; van der Kooij 1979; van der Kooij 1990; van der Kooij and Hijnen 1984). In our study, it was initially considered possible that some of the higher AOC values obtained from the luminescence method versus the Standard Method, could be due to double counting of carbon sources, but the analysis that follows indicates that this was probably not the case. The upper bound to potential double counting decreases as the difference in the amount of AOC determined from each strain in the test increases. For example, if only P-17 grew on AOC in a sample and NOX did not grow at all, there is no double-counting. Conversely, the upper bound to possible double counting occurs if the AOC determined by each strain is the same. Practically, analysis of the maximum possible error for a majority of data points collected in this research fall into a range that produces a relatively small possible factor of error (Figure 1.6). This distribution of data points collected in a frequency distribution curve indicates that most AOC was determined from P-17. The median value was 93% of AOC from P-17 and the average value was 86% of AOC from P-17. Only 33 data points out of 195 fell into the range of having greater than 1.5X possible error due to double-counting AOC. Hence, analogous to conclusions in LeChevalier et al. (1993), although double counting cannot be excluded as a factor, it is clear that it cannot explain the large discrepancies reported herein between Standard Method 9217B and the luminescence method.

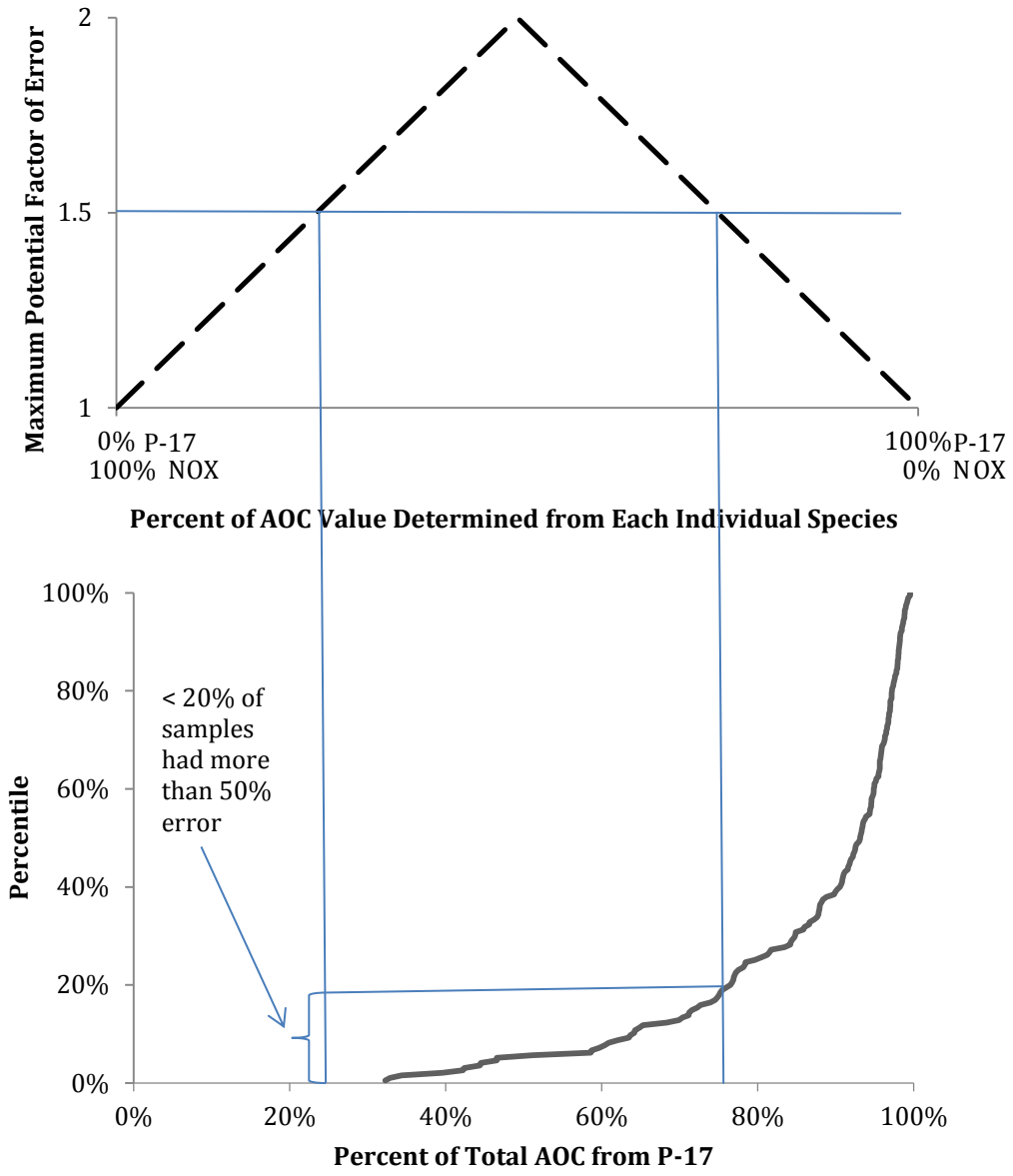

Figure 1.6. Maximum possible factor of error associated with overlap of AOC utilization between NOX and P-17, with a frequency plot of percent of total AOC from P-17 from all AOC data obtained. This analysis assumes 100% overlap in actual AOC consumed, which is the upper bound of possible overlap.

Additionally, background luminescence can be present when using the luminescence method. The luminescence of eight different types of water were measured. Samples were granular activated carbon (GAC)-treated tap water from Blacksburg, VA, filtered GAC-treated water, pasteurized GAC-treated tap water, water from a distribution pipe with iron, filtered iron-pipe water, pasteurized filtered iron-pipe water, nanopure water, and 1X MSB. Each water was read in triplicate with and without 0.2% v/v *n*-decanal. AOC test organisms

NOX and P-17 were not inoculated into the waters. A t-test (two-sample assuming unequal variances, two-tail) indicated that there was no statistical difference between the samples with and without n-decanal solution ($p = 0.32$), so the data were treated as one large set of background luminescence values.

Luminescence values as high as 7,581 RLU were obtained and the average luminescence was 2,494 RLU/mL. Using the standard curve regression for P-17, the average luminescence would provide 14 ppb AOC from P-17 and the maximum luminescence provide 39 ppb AOC from P-17. Alternatively, background luminescence values below 8000 RLU equate to 0 ppb AOC from NOX. Figure 1.7 displays a cumulative distribution plot of "false" AOC from background luminescence values. NOX provides 0 ppb of false AOC at these levels of luminescence, while AOC can be inflated by up to 39 ppb from P-17 AOC. Roughly 55% of the tested waters produced P-17 AOC between 10 and 20 µg acetate-C eq./L. This possible source of error is easily corrected as noted in later text.

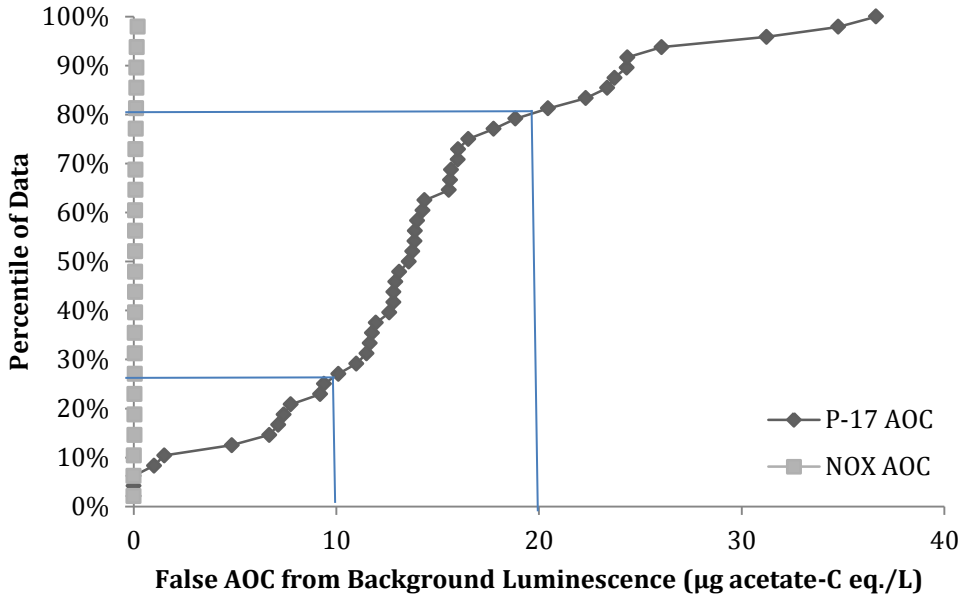

Figure 1.7. Cumulative Distribution of "False" AOC from Background Luminescence. Background luminescence values of select waters were a maximum of around 8,000 RLU/mL, which produced NOX AOC values of zero and P-17 AOC values up to 39 µg acetate-C eq./L. N = 48.

A further point of clarification is that AOC is sometimes regarded as "regrowth potential," not as actual carbon, as in Weinrich et al. (2009). Regrowth potential indicates the maximum growth of a heterotrophic community in a certain water based on all available

nutrients, including nitrogen, phosphorous, and others. However, if the AOC assay is being used to measure actual carbon, one of the underlying assumptions is that all other nutrients are present in excess and that carbon is the limiting factor (Standard Method 9217B). In this case, a baseline of nutrients could be added to the water samples to ensure carbon is the limiting growth factor. To explore this concept, 7 samples were analyzed for AOC content with and without the addition of a MSB spike. One-hundred µL of 1,000X MSB were added to 100 mL of sample to create a nutrient baseline equal to the 1X MSB solution. Figure 1.8 illustrates that adding nutrients to the AOC assay increases the measurable carbon in the sample. In fact, six of the seven samples increased with the addition of nutrients, indicating that the majority of the water samples were at least slightly limited by nutrients other than organic carbon. If AOC is being measured to determine actual carbon concentrations, nutrients should be added to the bioassay to ensure the sample is carbon-limited, whereas the nutrients should not be added if the method is a regrowth potential test.

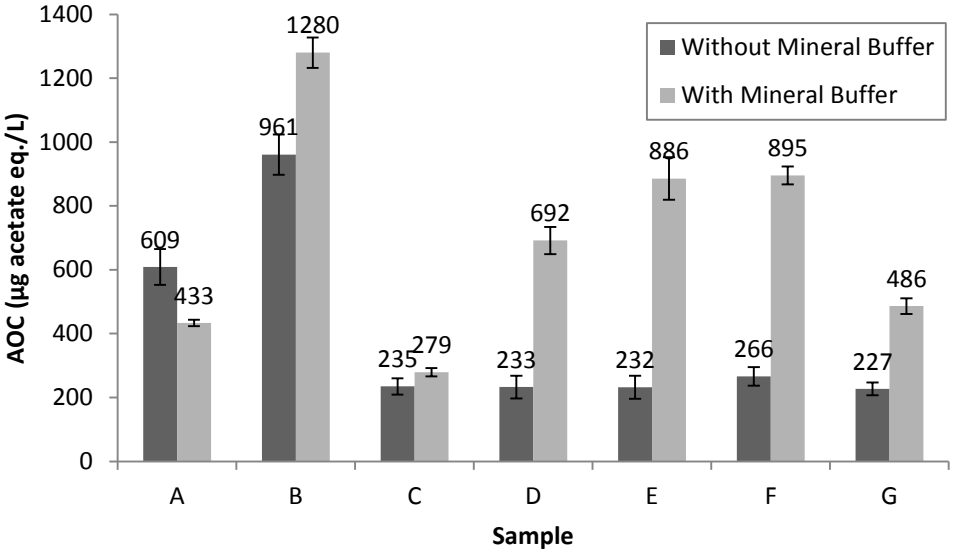

Figure 1.8. AOC With and Without Added Nutrients. Samples were analyzed using the luminescence method.

1.4.5 Possible Modifications to the Luminescence Assay

Several small modifications to the luminescence assay were considered as possible improvements dependent on the needs of the user.

Streaking plates. When using the AOC standard method, contamination by non-test organisms is easily detected by visual detection of microbial colonies uncharacteristic of NOX and P-17. P-17 colonies are yellow and 3 to 4 mm in diameter, and NOX colonies are

small white dots 1 to 2 mm in diameter. When using the luminescence assay, however, contamination by foreign microbes may not be noticeable since no plating is involved. If the luminescence bioassays are contaminated, NOX and P-17 will not grow the maximum possible densities and AOC values will be falsely low, which was obviously not the key concern in our experience. Nonetheless, a simple way to check for contamination that is less time consuming than spread plating is streaking samples onto R_2A agar with a sterile loop at the end of the incubation period. Plates can be stored at room temperature for 3 to 5 days, then checked for contamination by organisms other than NOX and P-17. Measured AOC concentrations of samples containing organisms other than NOX and P-17 can then be invalidated. Although streaking is usually applied to isolated colonies, not liquid media, this method is suggested to save time compared to performing spread-plating to check for contamination. Checking for contamination using the spread plate method (Standard Method 9215C) is another option, but is more time-consuming than the loop method. Future research should explore simple, time-efficient methods for checking the luminescence assay for contamination.

Mineral buffer spike. Dependent on goals of the test and the research, a MSB spike can be added to create an assay that attempts to measure only carbon limitations, instead of regrowth potential dependent on other possible limiting nutrients. An aliquot of the 1,000X MSB described in Section 1.2 can be added to the samples to create a nutrient level equivalent to the 1X MSB. As demonstrated in Figure 1.8, AOC values sometimes increased with the addition of the MSB, and results were then more consistent with expectations based on actual carbon limitations in the water. Addition of MSB is consistent with the rest of the method because it is used for the positive and negative controls and is used in the development of the standard curves. Adding a nutrient spike will also provide a uniform baseline of nutrients in every sample. But in other situations researchers may desire to determine regrowth potential with other possible limiting nutrients.

Background luminescence normalization. Some waters can have "background" luminescence that could inflate measured AOC values. To account for this discrepancy, the luminescence of a small aliquot of sample water can be measured prior to inoculation with NOX and P-17, and the resulting "background" luminescence value can converted to AOC using the regression equations from the standard curve. At this time it seems appropriate to subtract this value from the total AOC measured at the end of the test, although additional research would be needed to confirm the validity of this approach.

1.5 Conclusions

Although both the AOC Standard Method and luminescence-based method have possible sources of error, in our experience the the luminescence method was generally more accurate because it better captures the peak growth of the test organisms. While the luminescence method could potentially double-count some forms of AOC, this is a relatively small source of error in our experience and in that of others, and the possibility of missing the peak when using the Standard Method results in a much more serious source of error. Additionally, the luminescence method was found to be up to 60% less time consuming and costly.

Analysts wishing to measure AOC should assess the benefits and constraints of both methods, and make necessary additions and adjustments to fit their individual research needs. Carbon is the reference source across all forms of AOC tests to provide a basis of comparison. Unless AOC results were being compared to analytical results based on carbon, practical applications of measuring sources of drinking water, surface water, reclaimed water, or ground water would likely be concerned with the potential for regrowth, as measured using AOC assay without addition of MSB. However, if the assay is being used for carbon analysis, nutrient addition may be necessary. Additionally, subtracting background luminescence of a sample water from the end AOC results will ensure that the AOC value is not artificially inflated. Although contamination is easily detectable when using the Standard Method, contamination of the luminescence assay is not necessarily detectable.

Acknowledgements

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Appendix A. Supplemental Tables

Table A1. Comparison of AOC Test Organisms, NOX and P-17

Metabolic or Physiological Trait	P-17	NOX
Geneology	*Pseudomonas fluorescens* strain P-17	*Spirillum* strain NOX (species?)
Colony Description	Circular, 3 – 4 mm in diameter, with diffuse yellow pigmentation (SM 9217B)	Small white dots, 1 – 2 mm in diameter (SM 9217B)
Yields on Acetate and Oxalate	4.1×10^6 CFU P-17/µg acetate-C at 15°C No yield on oxalate (SM 9217B)	1.2×10^7 CFU NOX/ µg acetate-C 15°C 2.9×10^6 CFU NOX/ µg oxalate-C 15°C (SM 9217B)
Maximum Cell Density in MSB	$(4.65 \pm 2.26) \times 10^5$ (LeChevallier et al. 1993)	$(6.43 \pm 1.62) \times 10^5$ (LeChevallier et al. 1993)
Forms of Carbon Metabolized	Wide variety of compounds: amino acids, carboxylic acids, hydrocarboxylic acids, alcohols, carbohydrates NOT Oxalic acid, polysaccharides (LeChevallier et al. 1993; van der Kooij et al. 1982)	Carboxylic acids NOT Carbohydrates, alcohols, aromatic acids, does not assimilate amino acids when grown on mixtures of compounds (LeChevallier et al. 1993; van der Kooij et al. 1984)
Slope for Luminescence produced/µg acetate	y=206.97x	y=35521x
Relationship between ATP luminescence units and viable counts	log(y)=0.9915(logx)-3.680 Where: y = ATP luminescence units x = P-17 viable count/mL (LeChevallier et al. 1993)	log(y)=0.903(logx)-4.069 Where: y = ATP luminescence units x = NOX viable count/mL (LeChevallier et al. 1993)

Chapter 2. Assimilable Organic Carbon Generation in Premise and Main Plumbing

Amanda Martin, Amy Pruden, and Marc Edwards

Abstract

Assimilable organic carbon (AOC) is a suspected contributor to growth of microbes, including pathogens, in plumbing systems. Removal of AOC entering distribution systems might has been proposed as a control strategy for opportunistic premise plumbing pathogens (OPPPs), but AOC generation in premise plumbing systems may undermine this possibility. This study compares AOC produced in simulated glass water heaters by premise plumbing deficiencies, including leaching of organic matter from cross linked polyethylene (PEX) pipes, autotrophic oxidation of H_2 generated from metal corrosion (e.g. sacrificial magnesium anode rods and iron pipes), rendering of humic substances more biodegradable by sorption to oxides (e.g., $Fe(OH)_3$) and accumulation of AOC on filters and sediments. Distribution system-derived biodegradable dissolved organic carbon (DSD-BDOC) generation of up to 645 ug C/L were quantified and all test conditions with plumbing deficiencies showed TOC levels significantly greater than that of the influent when sampled at the bottom of the water heater reactor. DSD-BDOC generation was also explored in a simulated distribution system with two disinfectant types (chlorine and chloramine) and three pipe materials (PVC, cement, iron). TOC increased with water age, probably due to leaching of organics from PVC and possibly autotrophic reactions of nitrifiers and iron-related bacteria. This study demonstrates that DSD-BDOC occurs in premise and main distribution systems.

2.1 Introduction

Control of pathogens in potable water leaving treatment plants is recognized as one of the greatest engineering achievements of the 20th century (NRC 2006). However, the Center for Disease Control (CDC) recently acknowledged a higher incidence of waterborne disease caused by pathogens that grow in premise plumbing systems, versus pathogens escaping water treatment plants (CDC 2008). Premise plumbing refers to the portion of the potable water distribution system located within schools, hospitals, businesses, and private housing. Characteristics of plumbing systems such as surface area to volume ratio, stagnation periods, corrosive plumbing materials (e.g. copper, iron, magnesium), and

disinfectant residuals vary markedly from the main water distribution system when compared to that present in consumers' homes. Many of these factors can contribute to amplification and regrowth of pathogenic bacteria (CDC 2008).

Two opportunistic premise plumbing pathogens (OPPPs) of concern are *Legionella pneumophila* and *Nontuberculous Mycobacteria (NTM).* In general, these pathogens tend to cause disease in immunocompromised persons such as the elderly, HIV infected persons, or hospitalized patients. The CDC estimates that 8,000 to 18,000 people contract Legionnaires' disease annually in the United States and that 5 to 30 percent of these cases are fatal (CDC 2008; Marston et al. 1997). From 2004 to 2006, *L. pneumophila* was responsible for more than 40% of U.S. waterborne disease outbreaks and for a majority of waterborne disease deaths (CDC 2008). The EPA considers *L. pneumophila* to be a health concern, but current regulations apply only to water leaving treatment plants and do not address the more important concern of growth in premise plumbing systems (EPA 2006).

Human infection by *NTM* is known to cause severe pulmonary disease and Cervical Lymphadentis in children (CDC 2005). There are about currently 30,000 *NTM*-infected patients in the United States (Marras et al. 2007) and *MAC* accounts for over 75% of these cases. The National Institute of Allergy and Infectious Diseases (NIAID) recently announced that *NTM* pulmonary lung disease is on rise in the United States, at rates of +3.2% for men in Florida, +6.5% for women in Florida, and +4.6% for women in New York (NIAID 2009). Risk and exposure factors include those mentioned for *L. pneumophila,* as well as recently identified genetic factors (Kim et al. 2005; Rodman et al. 2005; Chan et al. 2007; Thomson et al. 2007).

These pathogens can grow in shower heads, faucets, biofilm of pipe walls, or water heaters, whereas conventional pathogens are present in source water and do not multiply in the water itself. Studies conducted at the households of *NTM* patients have provided conclusive evidence that premise plumbing was a source of the *NTM* disease because DNA samples of *MAC* isolates in tap water and showerhead biofilm were clonally related to the *MAC* isolates from a pulmonary disease patient (Falkinham et al. 2008). Human infection in showers can occur through inhalation of water containing *L. pneumophila* or aspiration (NRC 2006). In a few observational studies conducted to date, *Legionella* has been reported to colonize between 22-60% of hot water systems surveyed, although incidence dropped to as low as 4% a few months after a switch to chloramine disinfection (Alary et al. 1991; Borella et al. 2004; Flannery et al. 2006).

Although there are not currently any simple and universally successful control strategies for pathogen growth in plumbing systems, some interventions are sometimes effective. Water temperatures above 60°C are believed to stop growth of *MAC* and *Legionella pneumophila* (WHO 2004), although there are concerns about potential impacts of this practice on incidence of scalding (U.S. Consumer Product Safety Commission 2005). The Environmental Protection Agency also recommends lower temperatures to save energy (EPA 1997). An additional concern is that microbes killed thermally at the top of an electric water heater can settlet to the bottom where temperatures can be more amenable to pathogen growth and limiting nutrients such as iron can be present in abundance. Temmerman, et al. (2007) recently demonstrated that L. pneumophia can rapidly amplify in the presence of this dead microbial biomass through necrotrophic growth.

Another important factor has been proposed to limit pathogen growth is assimilable organic carbon (AOC). AOC is the fraction of organic carbon that can be used most readily for microbiological growth (Haddix et al. 2004) and has been established as major limiting nutrient for pathogen growth in water distribution systems (van der Kooij 1992; Camper 2003; LeChevallier et al. 1991). Previous studies have found AOC to be 0.1 – 10% of total organic carbon (TOC) (Hammes et al. 2005). Levels less than 10 μg/L are believed to limit growth, and levels greater than 100 μg/L have been associated with heterotrophic regrowth problems in main distribution systems (LeChevalier et al. 1991). Controlling microbial growth by removing AOC at treatment plants has been considered to be effective for water mains, and recent work has explored whether very low levels of organic carbon in water flowing to premise plumbing could reduce growth of premise plumbing pathogens there (Williams 2011a, 2011b). However, the possibility that AOC can actually be generated in premise plumbing systems has not been explored, and to the extent it occurs, it would undermine potential effectiveness of AOC control at the treatment plant. Possible sources of AOC generation in premise plumbing include cross linked polyethylene (PEX) pipes, nitrification, corrosion of sacrificial magnesium anode rods and iron pipes, humic sorption to $Fe(OH)_3$ sediments, and concentration of AOC on filters and sediments. These mechanisms are illustrated in Figure 2.1 and elaborated on in the following sections. Carbon generated due to these mechanisms in this research is henceforth referred to as distribution system derived biodegradable dissolved organic carbon (DSD-BDOC) to indicate the nature of generation of the carbon.

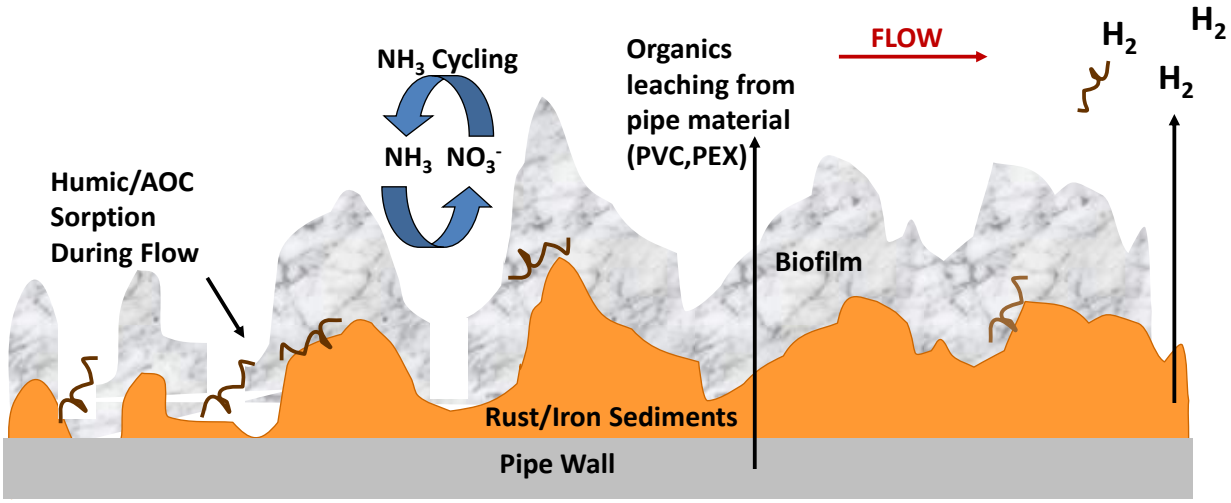

Figure 2.1. Schematic of potential AOC generation mechanisms occurring in consumer plumbing systems: PEX pipes, nitrification processes, H_2 evolution from corroding materials, and humic sorption to iron sediments.

2.1.1 Leaching from PEX Pipes

Certain building plumbing systems have a large fraction of PEX, PVC, and HDPE in addition to copper materials. Rogers et al. (1994) demonstrated that high levels of TOC (>0.6 mg/L) can leach from new PVC pipe. On the other hand, a similar study by van der Kooij et al. (2005) in a different water did not demonstrate an increased likelihood of *Legionella* occurrence in PEX plumbing systems. Investigations have also revealed that low weight organic materials can permeate into potable water at levels up to 200 µg/L though PEX pipes under extreme circumstances, such as when the external surfaces of pipes contact solvents (Skjevra et al., 2003; Durand et al., 2007; Heim et al., 2007). Still other studies have demonstrated that phosphate and other nutrients present in the pipe material can also leach to water from PEX (Lehtola and Miettinen, 2001; Lehtola et al., 2002a, b, 2004). If potable water usage is lowered by prolonged stagnation as commonly occurs in premise plumbing, these impacts might be much more significant per unit volume of tubing (Zhang et al., 2009).

2.1.2 Nitrification

Nitrifying bacteria convert ammonia to nitrite and then nitrate to generate new biomass from inorganic carbon. Ammonia-oxidizing bacteria (AOB) use ammonia-N as an electron donor and oxygen as an electron acceptor, and then nitrite-oxidizing bacteria (NOB) use

nitrite-N as an electron donor and oxygen as an electron acceptor. Nitrification can occur in premise plumbing when microbes utilize naturally occurring ammonia or added ammonia from chloraminated systems. Zhang et al., 2009 calculated an upper bound of 87 µg of organic carbon created per mg NH_3 consumed, although cycling reactions with iron and other metallic materials could potentially increase organic carbon yields per mg NH_3 consumed. In some circumstances nitrification may be more prevalent in premise plumbing than in the main distribution system due to higher temperature, greater stagnation periods, and low disinfectant residual concentrations.

2.1.3 Humic Sorption to Iron and Rust sediments

Humic and fulvic acids are naturally occurring organic compounds that can be subject to oxidation and polymerization reactions in water treatment plants and in nature (Edwards et al., 1993; Camper et al., 2004). Studies have shown that sorption of humic substances to iron oxides may increase overall biodegradability of the organic material (Butterfield et al., 2002a, 2002b), which effectively increases the concentration of biodegradable organic carbon (BDOC). Logical extrapolation of these results would suggest that humic substances sorbed to rusts on galvanized iron pipe, iron sediment trapped in a whole house filter, or sediments at the bottom of a hot water heater tank may also increase the effective concentration of AOC in plumbing. Camper et al. (2004) suggested that these conditions could support higher rates of microbial growth.

2.1.4 Hydrogen Oxidation from Magnesium, Aluminum, or Iron

Another possible mechanism of AOC generation in premise plumbing systems results from electrochemical generation of H_2 in storage water heaters. A typical storage water heater includes a sacrificial aluminum or magnesium anode rod to prevent corrosion of the water heater. Accumulation of H_2 gas from anodes is a well-known issue, and can even cause catastrophic explosions of residential water heaters in extreme cases (Cook 2004). This is of interest because some bacteria, known as hydrogenotrophs, can use molecular H_2 as an electron donor, enabling them to fix carbon into new biomass (Bowien and Schlegel 1981; Schegel and Lafferty 1971; Igarashi 2001). A typical magnesium anode rod is 44" long, 0.7" wide, weighs 490g, and lasts 5 years. By stoichiometric calculation, corrosion of the anode by H_2 generation would create 20 moles of H_2 over the anode lifespan. This H_2 could produce up to 160 µg/L day of organic carbon over a five-year lifespan in an 80 gallon heater according to the 0.2 mg carbon produced/mg H_2 utilized for autotrophs considered

by Morton et al. (2005). Of course, only a fraction of the anode corrosion that occurs would be via H_2 evolution, but even 1-5% of the 160 µg/L per day cited above could be highly significant. Morton et al. (2005) also indicated that H_2 is evolved from iron surfaces in plumbing, and it is reasonable to expect H_2 generation from galvanized iron plumbing, as well.

Hydrogen-oxidizing bacteria are taxonomically diverse, comprising 28 species belonging to 15 genera, indicating that hydrogen-oxidation occurs in many unrelated bacteria and in a wide range of environments, therefore presumably certain potable water heaters (Bowien and Schlegel 1981). High concentrations of heterotrophic microbes have been found living on sacrificial anode rods; however, H_2 oxidizers were not specifically examined and may have occurred at relatively high concentrations (Bagh et al. 2004). In addition to concerns that autotrophic H_2 oxidizers could undermine AOC control of microbial and pathogen growth in water heaters, certain types of *NTM* are capable of direct hydrogen-oxidation and autotrophic growth (Aragno and Schlegel 1992; Gomila et al. 2008). Thus, *NTM* pathogens might grow on hydrogen evolved from anode rods without any additional organic carbon source.

2.1.5 Possible Reservoirs of AOC in Scale, Whole House Filters, and Sediments

Capture of AOC in whole house filters or in water heater sediments might create an important reservoir of AOC. In a typical home, a large percent of water is utilized during continuous flow events (showering, toilet flushing or washing machine use). If surfaces are present that can sorb AOC from the flowing water, high levels of AOC could be concentrated on and stored on surfaces and used as a carbon source during stagnation events. A similar concentration effect can occur for particulate and soluble NOM captured on whole house GAC or other filters. Technically, no AOC or BDOC is created in this process, although it could explain the presence of much higher levels of microbial growth than would be expected based on bulk water concentrations. Additionally, microbes killed thermally at the top of water heaters may settle to the bottom of the tank where temperatures can be more conducive to pathogen growth and nutrients, such as iron, can be present in abundance (Rogers et al., 1994). Temmerman et al. (2007) recently demonstrated that *L. pneumophia* can rapidly amplify in the presence thermally inactivated biomass through necrotrophic growth.

2.1.6 Challenges of Tracking APC or BDOC Production

There is no simple means by which autotrophically produced carbon (APC) or BDOC derived from premise plumbing can be directly monitored. Certainly, any sustained and significant increase in the concentration of total organic carbon, AOC, or BDOC as water passes through a water system provides evidence of organic carbon production source, but the converse is not true because the rate at which heterotrophic bacteria convert organic carbon to CO_2 might equal or exceed the rate at which autotrophic bacteria fix CO_2. Hence, careful analysis and mass balances must be conducted to move beyond circumstantial evidence of APC or BDOC derived from a given premise plumbing system.

2.1.7 Goals

The goal of this research was to examine the possible generation of organic carbon under controlled lab conditions representative of the above scenarios. Relative production of AOC by various plumbing conditions is important to inform decision making with respect to use of AOC removal at water treatment plants as a potential control strategy for OPPPs. The information is also valuable to help explain variability in OPPP occurrence from one building plumbing system to another based on design and materials use. While there is no known prior research or discussion on this subject, authors of a 2005 Danish study using very low-AOC water reported an increase in AOC from 3 to 16 µg/L as water passed through an electric water heater. The authors did not highlight this fact or otherwise attempt to explain the observed >500% increase, although it is noteworthy that *L. pneumophila* were amplifying to very high levels in water within these water heaters, despite the very low AOC in the influent water (van der Kooij 2005). With this increase in mind, AOC generation was explored in simulated water heaters (SWHs) containing many of the aforementioned premise plumbing deficiencies which could generate organic carbon. The possibility that AOC could be generated in distribution system mains was also explored in simulated distribution systems (SDS) with two disinfectant types (chlorine and chloramine) and three pipe materials (PVC, cement, and iron).

2.2 Materials and Methods

2.2.1 Simulated Water Heater Reactors

The experimental design utilized simulated water heaters (SWH) of 120 mL French square glass bottles (Quorpak) with polytetrafluoroethylene (PTFE) caps. The total

calculated surface area to volume ratio for the SWH in this experiment was 1.24 cm^{-1} versus approximately 0.05 cm^{-1} for a typical 40 gallon residential water heater. Before use in experiments the bottles were acid washed, rinsed in reagent grade nanopure water and baked in a muffle oven at 550°C for 5.5 hours.

Waters with two different levels of TOC were tested: "low-TOC" and "high-TOC" Blacksburg, VA tap water. The high-TOC Blacksburg tap water was break-point chlorinated using a sodium hypochlorite solution to remove chloramine. Three mg/L of sodium thiosulfate was added to remove the trace chlorine residual. High-TOC water varied in concentration from 1.2 to 1.5 mg/L TOC. To create low-TOC water, break-point chlorinated tap water was biofiltered using a granular activated carbon (GAC) filter (GE Undercounter Standard Filter Unit, Model GX1S01R, General Electric, Fairfield, CT) for at least 24 hours and more commonly much longer. Low-TOC tap water typically had 0.2 to 0.8 mg/L TOC and had an undetectable level of BDOC (0.157±0.146 mg/L).

An array of "premise plumbing deficiency" conditions were created in the SWHs to simulate different conditions found in premise plumbing that could lead to AOC generation. Controls for the low- and high-TOC waters consisted of French square bottles with no "deficiencies." Glass beads (1 mm diameter; Avogadro's Lab Supply, Inc., Miller Place, NY) were added to the bottom of the reactors to provide extra surface area for biofilm attachment. Each SWH series was run in triplicate with a total of 42 SWH (6 SWH (3 at high-TOC; 3 at low-TOC) x 6 experimental conditions + 6 controls (3 at high-TOC; 3 at low-TOC) = 42 reactors total). The following deficiencies were examined:

- **Mg-ANODE SWH**: A 1.4 cm piece of a Mg anode rod was mounted to the bottom of SWH with a surface area to volume ratio of 3.33 cm^{-1}. The Mg-anode is a potential source of H_2-derived AOC.
- **$Fe(OH)_3$ SEDIMENT SWH**: A freshly precipitated $Fe(OH)_3$ sediment layer of a 1 mm depth served as an iron nutrient source and sorbed organic carbon reservoir. $Fe(OH)_3$ sediments were created by dissolving ferric chloride in distilled water, raising the pH to 7 using a 1M sodium hydroxide solution, allowing the sediments to settle, and decanting the supernatant and rinsing with distilled water multiple times.
- **IRON COUPON SWH**: A crescent shaped cast iron coupon was mounted to the bottom of the iron SWH with a surface area to volume ratio of 0.5 cm^{-1}. The iron

coupon served as an H_2 source, an iron nutrient source, and sorbed organic carbon reservoir

- **PEX PIPE SWH**: Ten cm of a 3/4" diameter PEX pipe was taken and subsequently cut into 2 cm pieces and mounted to the bottom of the SWH (PEX-derived AOC). The surface area to volume ratio was calculated to be 0.48 cm^{-1} and 4.20 cm^{-1} for the outer and inner pipe diameters respectively. Given an initial SWH surface area of 1.24 cm^{-1}, the total surface area equates to 5.92 cm^{-1}.
- **COMBINATION SWH**: A Combination of factors present at the same time including: Mg anode rods (Mg SWH) and cast iron coupons (Iron SWH).

SWHs were stored in a 32°C incubator to represent the temperature of the bottom of an electric water heater and were operated for approximately two years before the beginning of this study. These conditions will be referred to with the abbreviated names shown in Table 2.1. Eighty percent, bi-weekly water changes were performed to simulate a low-use water system and prolonged stagnation events. The water level was filled to the top of the SWH to leave no airspace at the top of the reactor. Dissolved oxygen (DO), pH, and bacterial densities (as heterotrophic plate counts (HPCs) and 16s rRNA) were monitored. DO and pH were monitored weekly, HPCs were measured six times over a four-month period, and 16S rRNA data was taken once.

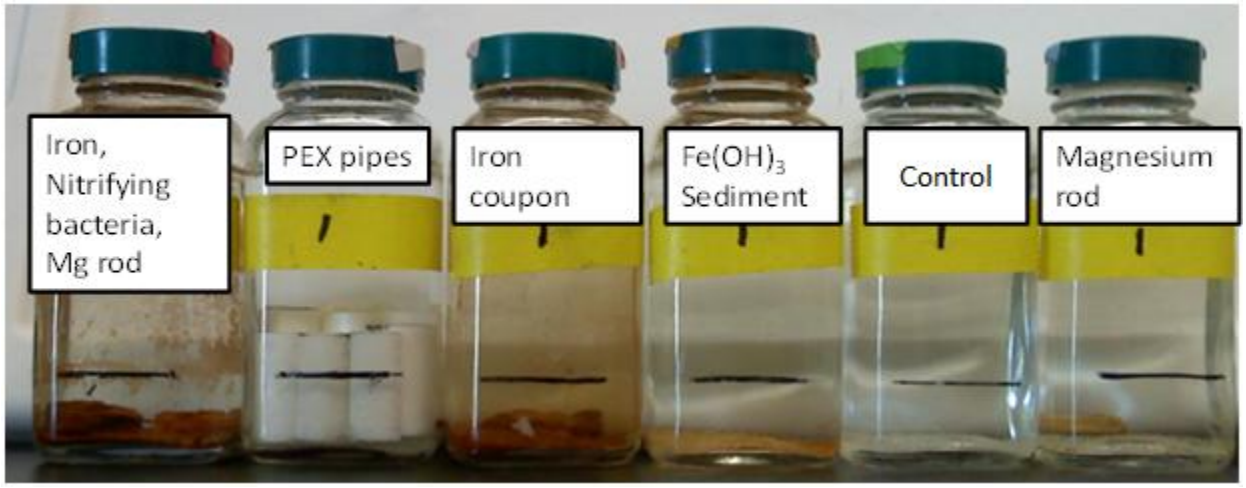

Figure 2.2. Simulated water heaters.

Table 2.1. Abbreviated Names of SWH Reactors.

Abbreviation	SWH Reactor Condition
IRON L	Iron Coupon SWH with Low-TOC Water
IRON H	Iron Coupon SWH with High-TOC Water
FeOH L	$Fe(OH)_3$ Sediments SWH with Low-TOC Water
FeOH H	$Fe(OH)_3$ Sediments SWH with High-TOC Water
COMB L	Combination SWH with Low-TOC Water
COMB H	Combination SWH with High-TOC Water
PEX L	Cross-Linked Polyethylene SWH with Low-TOC Water
PEX H	Cross-Linked Polyethylene SWH with High-TOC Water
MG L	Mg Anode Rod SWH with Low-TOC Water
MG H	Mg Anode Rod SWH with High-TOC Water
CONTROL L	Control SWH with Low-TOC Water
CONTROL H	Control SWH with High-TOC Water

2.2.2 Simulated Distribution System

As water passes through a distribution system, changes occur that can impact water quality, including disinfectant decay, pH changes, and development of various redox zones that could support different microbes. This phase of the study assessed the changes in TOC and AOC that occurred throughout a distribution system. A simulated distribution system (SDS) was created that examined two types of disinfectants (chlorine and chloramines) and three types of typical distribution system materials: concrete, cast iron, and polyvinyl chloride (PVC) (Figure 2.3). Each system was tested in triplicate, making 18 distribution systems total. PVC pipes with 1 ½ inch diameters were used to make main line of each pipe. Cement, iron, or PVC coupons were placed inside the pipes, which were ¼'' in height and width and ran the entire length of the pipe. The surface area:volume ratio of coupons was 0.30 in^{-1}, which is about the same surface area:volume level found in an 13.46 in diameter pipe of the indicated material. The conditions are referred to as PVC, PVC-iron, and PVC-cement since the main distribution pipe of each condition is PVC.

Influent water consisted of break-point chlorinated Blacksburg, VA, tap water that had been re-dosed with 2.5mg/L free chlorine or 4mg/L chloramine. Water was pumped into the system using a peristaltic pump at a flow rate of 0.40mL/min, allowing an approximate 10 day retention time. Sample ports in the simulated distribution system allowed sample collection at 1, 2.3, 3.5, 5.7, and 10.2 days of water age. Four distinct sections were created using threaded check-valves to eliminate the possibility of a particle moving back and forth throughout the length of the simulated systems. Pipe sections were disinfected by washing each section in a 500mg/L chlorine solution and then rinsing three times with deionized water. Each sleeve was soaked in a pH 12 solution of sodium hydroxide for half an hour,

which acted as a degreasing agent before the materials were inserted. TOC and AOC were monitored on a weekly basis according to the methods described below.

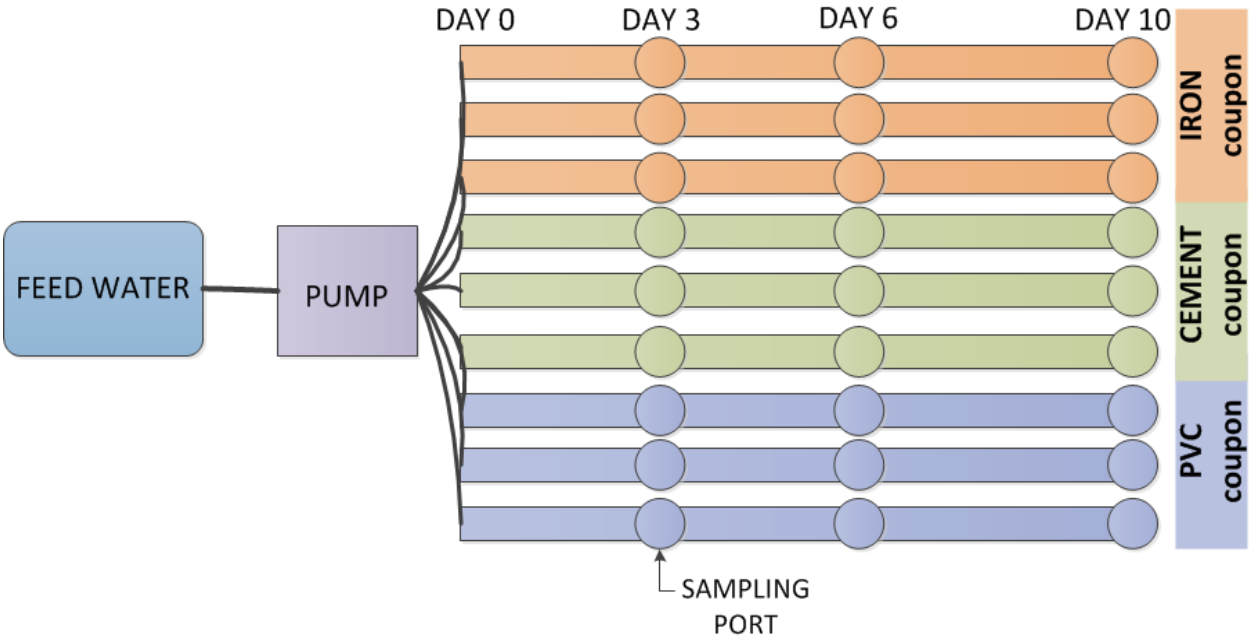

Figure 2.3. Diagram of Simulated Distribution System with Iron, Cement, and PVC Conditions. Each pipe system was created in triplicates and two total pipe systems were used, one with chlorine disinfectant and one with chloramine disinfectant. Total detention time in the system was 10.2 days. All conditions received the same influent water with either chlorine or chloramine disinfectant. Day 1 and 2.3 sample ports are not displayed in the diagram.

2.2.3 Experimental Methods

AOC. AOC was measured according to the method of Weinrich et al. (2009). Bioluminescent strains of the standard AOC test bacteria *P. fluorescens* P-17 (P-17) and *Spirillum* strain NOX (NOX) were obtained from American Water (Voorhees, NJ). Water samples were collected into sterile, baked borosilicate Kimax bottles with PTFE-lined, autoclaved caps which contained sodium thiosulfate to quench any residual disinfectant. Samples were stored at 4°C if not immediately analyzed. Glassware was rendered carbon-free by baking in a muffle furnace for 5 hours at 550°C. Prior to analysis, the sample pH was checked and adjusted to 7.2 ± 0.2 if necessary. Each sample was then split into two 100 mL aliquots and pasteurized for 30 min at 70°C in a water bath. After pasteurization, the cooled samples were inoculated separately with P-17 and NOX with approximately 10^4 CFU/mL. A negative control consisting of 1X MSB and a positive control consisting of 500 ppb acetate carbon in 1X MSB were also inoculated separately with NOX and P-17. A high-sensitivity,

automated, photon-counting luminometer (SpectraMax L; Molecular Devices, Sunnyvale, CA) was used to monitor the growth of the bioluminescent bacteria strains in the bioassays.

Samples were gently swirled and monitored by adding 300 µL aliquots to the sample microplates in triplicate. Transfers were made immediately post-inoculation and then two or three times per day until peak luminescence was reached, usually in four to seven days. Standard curve regressions were used to relate the peak luminescence value to the AOC in each sample.

Hydrogen measurements. A Trace Analytical™ KAPPA-5/E-002 Gas Chromatograph with a Reduced Gas Detector (RGD) were used to determine hydrogen concentrations. The RGD measures minute concentrations of H_2 and other gaseous constituents at parts per billion (ppb) concentrations. The detector uses the strong absorption of UV light by mercury vapor. The change in absorption is directly proportional to the concentration of the reducing compound (H_2) within the sample (Ametek 2009).

Heterotrophic plate counts (HPC). Heterotrophic bacteria were monitored by heterotrophic plate counts (HPC) according to Standard Method 9215 using the spread plate method and R_2A media.

Total organic carbon (TOC). Persulfate-ultraviolet detection using a Sievers Model 5300C with an autosampler were utilized according to Standard Method 5310 C to measure total organic carbon.

Deoxyribonucleic Acid (DNA) extraction. For bacterial 16S rRNA gene quantification of microorganisms in the SWH effluent, composite samples from the triplicate SWH were collected in a 250 mL sterile nalgene bottle. 200 mL of was concentrated onto 47 mm diameter 0.22 µm mixed cellulose ester filters (Whatman) using vacuum filtration. The filter membranes were then torn into 6 pieces using sterile tweezers and added to Lysing Matrix A extraction tubes from the FastDNA kit. DNA extraction proceeded utilized as detailed by FastDNAR SPIN Kit protocol (MP Biomedicals, Solon, OH) as recommended, except the FastPrepR (MP Biomedicals) instrument was set at a speed setting of 4.0 for 20 seconds to reduce the possibility of DNA shearing. Following the extraction process, DNA was re-suspended in 100 µL of DNA Elution Solution (DES) and preserved in a -22 °C freezer prior to quantification by qPCR.

Quantitative Polymerase Chain Reaction (qPCR) and 16S rRNA Assay. Bacterial16S rRNA genes were quantified using a Bio-Rad CFX96 real time system (Hercules, CA). A SsoFast supermix R (Bio-Rad, Hercules, CA) qPCR assay was developed for the 16S rRNA

gene (Smith et al., 2006) using forward primer 1396F (CGGTGAATACGTTCYCGG) and reverse primer 1492R (GGWTACCTTGTTACGACTT). The 16S rRNA gene Taqman Probe (CTTGTACACACCGCCCGTC) was labeled at the 5' end with FAM reporter dye and TAMRA quencher located at the 3' end, respectively. Each real-time PCR reaction contained 10 µl of 2×SsoFast supermix (Bio-Rad), 250 nM of each primer, 93.75 nM of probe, and 1 µl DNA template for a total volume of 20 µl. The thermal cycle profile consists of initial incubation for 2 minutes at 95°C, followed by 40 cycles of 5 seconds at 95°C (denaturing) and 10 sections min at 60°C (annealing and extension, plate read). Negative controls (consisting of template DNA replaced by molecular grade nanopure water) and 10-fold serial dilutions of known amounts of positive control DNA are included in triplicate for each qPCR assay, and each sample is tested in triplicate.

2.3 Results and Discussion

2.3.1 TOC Results from the Simulated Water Heater Reactors

Total organic carbon from the influent and effluent of the simulated water heaters was intensively monitored over a four-month period, and results from high-TOC influent and low-TOC influent reactors are discussed separately below.

High TOC reactors. Influent water into the high-TOC SWHs had an average of 1.73 mg/L TOC (Figure 2.4) and TOC dropped significantly in the all glass control SWH reactor by an average of 345 ppb ($p = 0.001$ paired t-test two tail). In other words, the BDOC utilized after 48 hours in the all-glass reactor averaged 345 ppb, consistent with expectations there is no biogrowth and no known mechanism of BDOC generation in this reactor. The general trend showed SWH conditions with potential BDOC generation mechanisms had less TOC loss than this control, but only the effluent from the Mg H SWH was significantly higher than the control (t-test, paired two-samples for means; $p = 0.004$). All other conditions did not have significantly more TOC than the control (p consistently > 0.05). Overall, there was not strong evidence that in waters with relatively high TOC and BDOC, autotrophicaly or distribution system-derived BDOC (DSD-BDOC) outweighs mineralization of inorganic carbon by respiration of heterotrophic bacteria. DSD-BDOC formation may be implied by the lower loss of organic carbon for conditions with potential BDOC generation mechanisms versus the glass control.

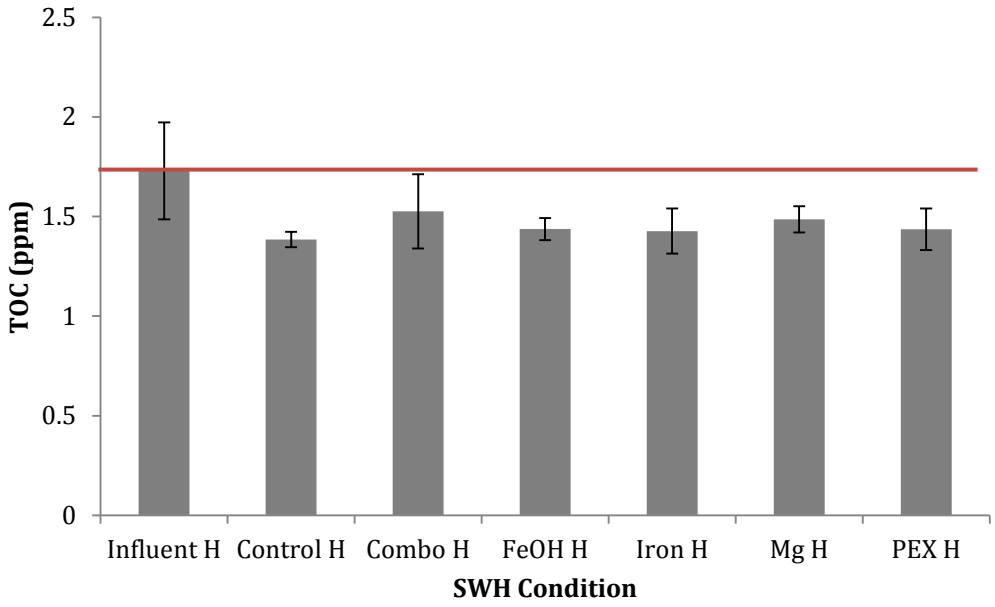

Figure 2.4. TOC in effluent of high-TOC SWH Reactors. The red line represents the average TOC concentration in the influent water. Error bars represent 95% confidence intervals of *n* samples over the study, where *n* varies between 9 and 15, depending on the condition.

Low TOC reactors. TOC generation was apparent in the low-TOC SWHs (Figure 2.5). As an extra check, TOC was monitored in the low-TOC SWHs at both the top and bottom of the reactor, to account for any settling of TOC in particles or biogrowth. The low-TOC influent had an average of 0.47 mg/L TOC and the organic matter present was not subject to settling because presumably only dissolved species were present, due to pre-filtration of the water through a 0.45 μm filter. After stagnation and presumed microbial growth, all conditions showed TOC levels significantly greater than that of the influent when sampled at the bottom of the reactor (p consistently < 0.03). The magnitude of the increase was in the range of 0.45-0.65 mg/L.

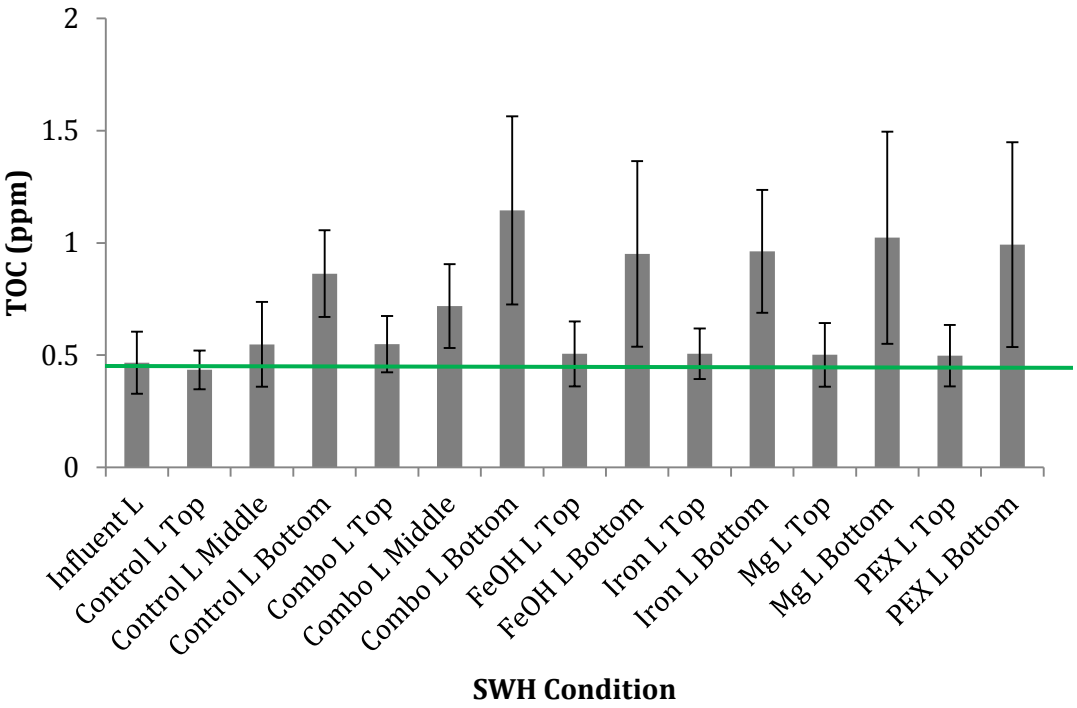

Figure 2.5. TOC in the Effluent of Low-TOC SWH Reactors. The green line represents the average TOC concentration in the influent water. Error bars represent 95% confidence intervals of *n* samples, where *n* is between 15 and 57, depending on the condition.

Samples collected from the middle or top of the reactors contained between 75 ppb less TOC to 219 ppb more TOC than the influent, but these differences were never significant at > 95% confidence. However, conditions with premise plumbing deficiencies all had significantly higher TOC than the corresponding control reactor when sampled at the top or middle of the reactor (p consistently < 0.03). The magnitude of the higher TOC versus the control condition ranged from 73-171 ppb. Overall, the results are consistent with net DSD-BDOC generation in the Low-TOC SWHs, and this generation is most noticeable when sampling from the bottom of the SWH.

2.3.2 Heterotrophic Plate Counts and 16S rRNA Genes in Simulated Water Heaters

Heterotrophic plate counts (HPCs) showed all SWH conditions had roughly the same level of heterotrophic bacteria, between 10^5 and 10^6 CFU/mL (Figure 2.6). The control condition had higher HPCs on average than the other conditions, with the exception of PEX H, which was 2.18×10^4 CFU/mL greater than Control H on average but was not significantly different than Control H (paired t-test, p = 0.764). The Combo H, Iron H, and Mg H conditions had lower HPCs than Control H on average by 4.91×10^5, 5.93×10^5, and 3.61×10^5

CFU/mL, respectively ($p = 2.37x10^{-4}$, $1.47x10^{-4}$, and $5.26x10^{-4}$, respectively). HPCs from FeOH H and PEX H were not different from the control within 95% confidence according to a paired t-test. FeOH L, Iron L, and PEX L had significantly lower HPCs than Control L on average by $4.48x10^{-5}$, $5.34x10^{-5}$, and $4.02x10^{-5}$ CFU/mL, respectively ($p = 0.045$, 0.042, and 0.047, respectively). HPCs from Combo L and Mg L were not different from the control within 95% confidence according to a paired t-test.

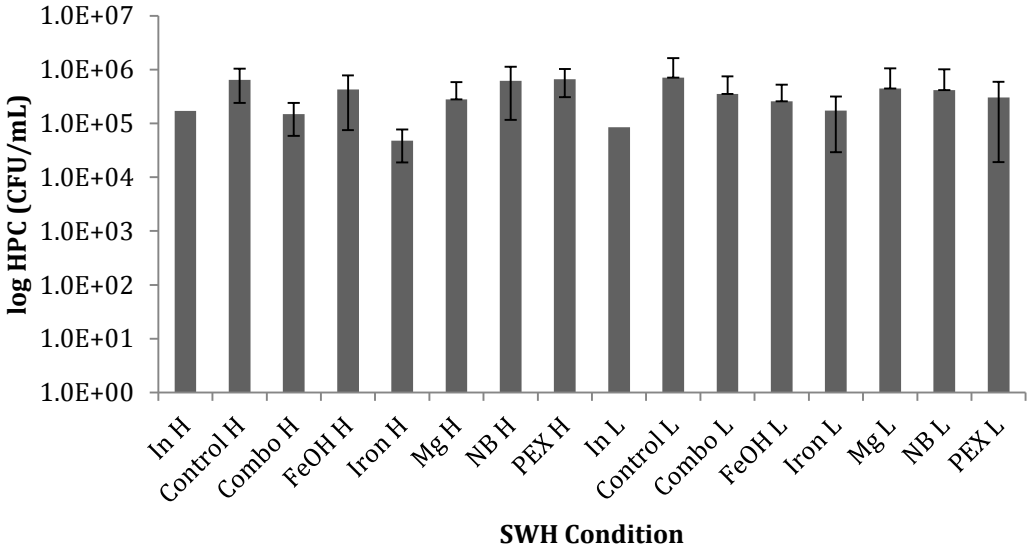

Figure 2.6. Heterotrophic plate counts of SWH reactors. Error bars represent 95% confidence intervals of 6 samples. Error bars are not present on influent samples because only one data point was collected. FeOH L, Iron L, and PEX L had significantly lower HPCs than Control L on average, while Combo H, Iron H, and Mg H conditions had lower HPCs than Control H on average.

Because only a small fraction of waterborne microorganisms (approximately 0.01%) are thought to be culturable heterotrophic bacteria, and even the newest HPC techniques yield as low as 2% of the total bacteria. Bacterial 16S rRNA genes were also measured using qPCR (Figure 2.7) (Exner et al. 2003; Bagh et al. 2004). All conditions yielded lower 16S rRNA (gene copies/mL) than their respective control, with the exception of Combo H, which was not significantly higher than Control H (t-test, two sample assuming equal variance; $p = 0.068$). Iron H, Mg H, and PEX H had lower 16S rRNA than Control H by $5.00x10^{6}$, $1.21x10^{7}$, and $1.18x10^{7}$ gene copies/mL, respectively ($p = 0.020$, $5.00x10^{-6}$, and $3.16x10^{-7}$, respectively). The Iron H and Mg H 16S rRNA results are consistent with the HPC results, as both were found to be significantly less than Control H. 16S rRNA in the Combo H and FeOH

H conditions were not different from Control H within 95% confidence (t-test, two sample assuming equal variance; p = 0.068 and 0.282, respectively).

In the low-TOC reactors, FeOH L, Iron L, Mg L, and PEX L all had lower 16S rRNA (gene copies/mL) than Control L, by $5.75x10^6$, $4.59x10^6$, $4.85x10^6$, and $4.90x10^6$ gene copies/mL, respectively (p = 0.015, 0.043, .036, and 0.036, respectively). Combo L was not different from Control L within 95% confidence (t-test, two sample assuming equal variance; p = 0.187).

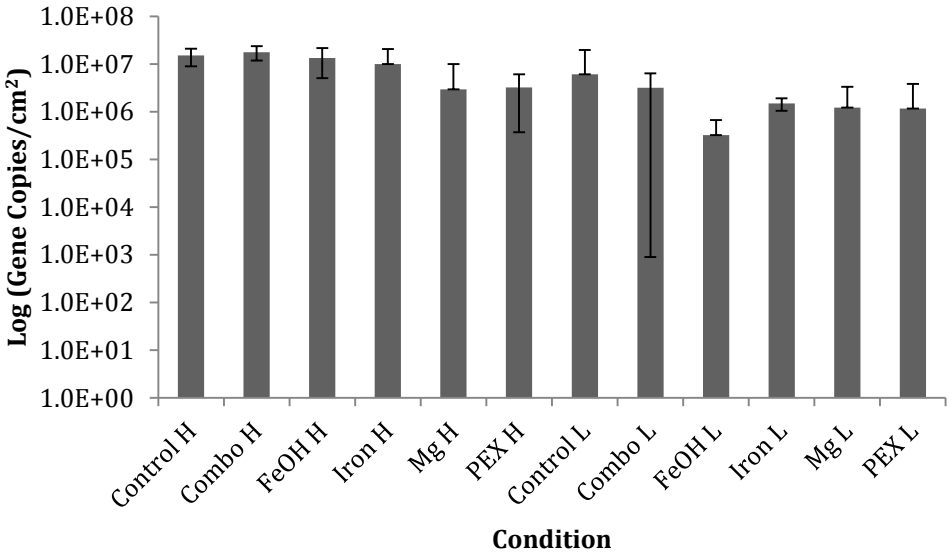

Figure 2.7. Bacterial 16S rRNA genes from biofilm in SWH reactors by qPCR detection at day 5.7. Error bars represent 95% confidence intervals.

In summary, the control condition had the highest bacterial counts of all the SWHs according to HPCs and 16s rRNA analysis. At first glance this seems to be inconsistent with expectations that higher organic carbon and higher microbial biomass would be present in the conditions with potential formation of organic matter in premise plumbing. But on the other hand, the microbial ecology might be supporting biomass that does not have a 16S gene such as protozoan and yeasts, or much of the created organic matter might not have been incorporated into biomass.

2.3.3 Dissolved Oxygen and pH of the Simulated Water Heaters

The pH of the influent water to the SWHs was adjusted to 7.5. The pH of each of the reactors tended to remain between 6.5 and 7.0, except for the Mg-anode containing

conditions (Mg L, Mg H, Combo L, Combo H), which had average effluent pHs around 8.5 – 9.5 (Table 2.2).

Influent water had an average of 6.60 mg/L DO. DO in the effluent was highly variable, ranging from 3.47 to 9.33 mg/L. DO tended to remain higher in the Control reactors than in the other conditions, with the Combo H, Combo L, Iron H, and Iron L conditions have the largest drop in DO consistently to below 2 mg/L (Table 2.2). The Mg, PEX, and FeOH conditions also tended to have lower DO than their respective controls and the High-TOC conditions had lower DO than the corresponding Low-TOC conditions.

Table 2.2. Dissolved oxygen and pH data from the SWH Reactors.

Sample	pH			Dissolved Oxygen (mg/L)		
	Average	Min	Max	Average	Min	Max
Influent	7.5	-	-	6.60	3.47	9.33
Control H	6.62	6.09	7.56	4.40	1.41	7.54
Combo H	9.05	8.72	9.22	1.55	0.23	5.36
FeOH H	6.63	5.97	7.60	3.77	1.04	6.10
Iron H	6.57	5.86	7.08	1.24	0.46	4.82
Mg H	9.50	9.34	9.67	4.09	2.12	5.77
PEX H	6.52	5.90	7.07	4.60	2.27	6.45
Control L	6.71	4.68	7.82	6.90	4.88	9.01
Combo L	8.58	6.71	9.12	1.91	0.71	5.19
FeOH L	6.82	4.97	7.92	6.57	3.26	8.48
Iron L	7.04	5.89	7.86	1.32	0.56	4.53
Mg L	9.00	8.63	9.23	5.41	2.64	7.13
PEX L	6.73	4.73	7.84	6.56	4.76	7.86

2.3.4 TOC Results from the Simulated Distribution System

In the free chlorine system, TOC increased approximately 300% in the first three days in for all three materials (Figure 2.8). The TOC leveled off after day three of retention time. As chlorine residual was only present in the reactors until day 3, this indicates that the TOC did not increase in the free chlorine simulated distribution system once a residual was no longer present. Bacterial levels, as indicated by HPC and 16S rRNA, and dissolved oxygen data help explain these results (see 2.4.5 and 2.4.6). Regression analysis of the normalized data indicates that TOC is increasing in all three systems, as the slope greater than zero ($p = 9x10^{-8}$, $7.56x10^{-7}$, and $3.86x10^{-9}$ for PVC, PVC-cement, and PVC-iron, respectively). However, if the day zero (influent) TOC data is omitted from the regression analysis, the slope for

days greater than 3.5 is no longer significantly greater than zero for any of the conditions ($p > 0.05$ for all conditions).

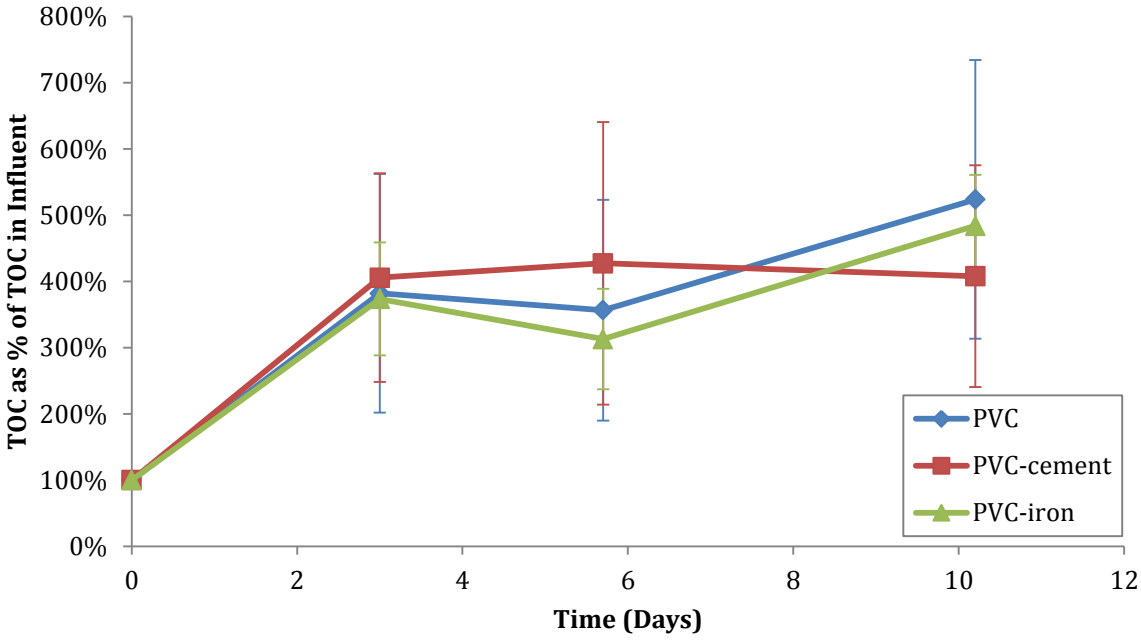

Figure 2.8. TOC over Detention Time in Simulated Distribution System with Chlorine Disinfectant. Error bars represent 95% confidence intervals of *n* samples, where *n* is between 7 and 9 experimental replicates. Data were normalized to show the % increase in TOC from the influent TOC.

At day 10, a single-factor ANOVA indicated that the mean TOC of the three conditions were statistically different ($p = 0.002$). The TOC in the PVC system was 0.79 mg/L TOC higher than the cement system ($p = 0.024$) and was 1.40 mg/L higher than the PVC-iron system ($p = 0.002$). Additionally, TOC in the PVC-cement condition was 0.62 mg/L higher than TOC in the PVC-iron system ($p = 0.010$), indicating that the three pipe materials do have different TOC levels at the end of the system.

TOC in the chloramine simulated distribution system had little or no decrease at the beginning of the system (Figure 2.9). The PVC-cement condition produced the highest levels of TOC consistently, with PVC-iron initially producing the lowest amount except on day 10. Overall, the trend was approximately the same for all three conditions although the average quantities of TOC varied slightly. Regression analysis of the normalized data indicates that TOC is increasing in all three systems, as the slope was greater than zero ($p < 0.01$ for all conditions).

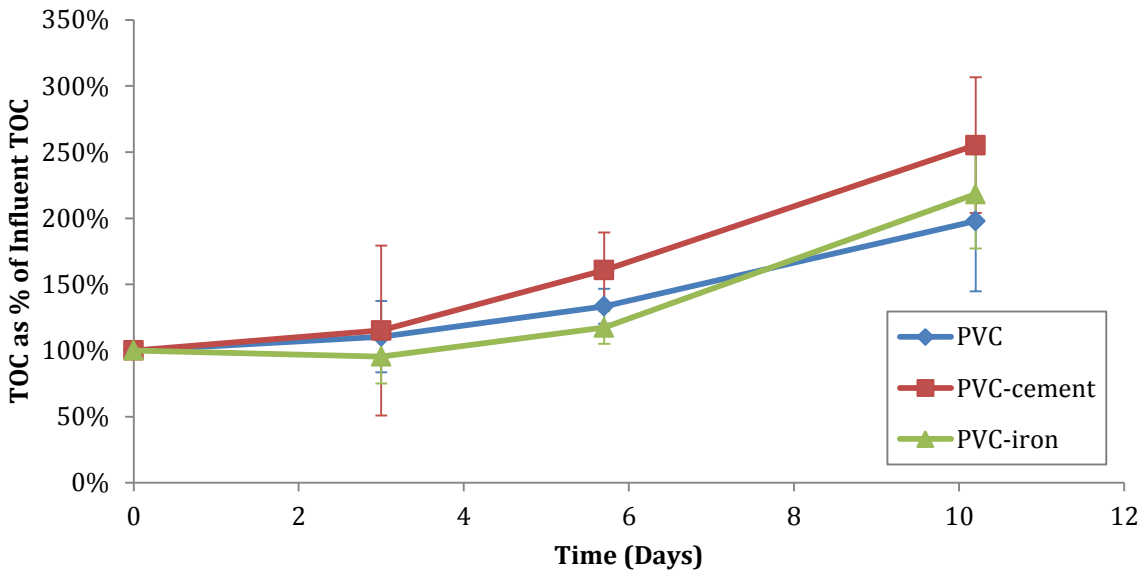

Figure 2.9. TOC over Detention Time in Simulated Distribution System with Chloramine Disinfectant. Error bars represent 95% confidence intervals of *n* samples, where *n* is between 4 and 8. Data were normalized to show the % increase in TOC from the influent TOC.

When comparing the quantities of TOC between systems at day 10, the TOC in the PVC-cement system was 0.74 mg/L TOC higher than the PVC system (paired t-test; $p = 0.04$) and 0.86 mg/L TOC higher than the PVC-iron system (paired t-test; $p = 0.04$). However, the average TOC in the PVC system on day 10 was not found to be significantly higher than in the PVC-iron system ($p > 0.05$).

2.3.5 AOC Results from the Simulated Distribution System

The AOC in the SDS at day 5.7 was lower in each condition than the influent, indicating that AOC is being consumed in the system (Figure 2.10). However, none of the conditions had significantly lower AOC than the influent due to large variations between duplicate measurements (t-test, paired two-sample for means; $p > 0.05$).

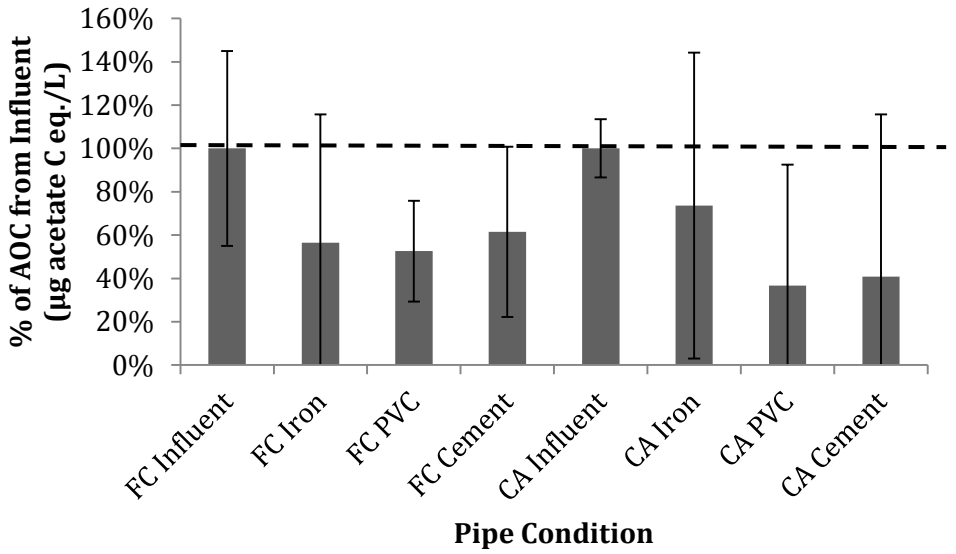

Figure 2.10. Normalized AOC Results from the Simulated Distribution System. FC represents the free chlorine distribution system and CA represents the chloramine distribution system. Error bars represent standard deviation of duplicate samples of two measurements (n=4). Results are normalized to be a percent of the influent.

2.3.6 Heterotrophic Plate Counts and 16S rRNA from the Simulated Distribution System

To monitor bacterial growth in the SDS, HPCs were collected from the day 5.7 sample port (Figure 2.11). In the free chlorine system, the influent consistently had no heterotrophic growth, which is consistent with expectations since disinfectant was present. The free chlorine PVC-iron system had an average of 1.91×10^5 CFU/mL and PVC-cement had 2.43×10^5 CFU/mL, which were not significantly different from one another (paired t-test; $p = 0.519$) but were both significantly higher than the influent ($p = 2.56 \times 10^{-10}$ for PVC-iron and 7.59×10^{-4} for PVC-cement). However, the PVC condition had significantly lower HPCs than PVC-iron and PVC-cement at day 6 in the free chlorine system, at an average of 3.39×10^2 CFU/mL (paired t-test; $p < 0.01$), and was not significantly higher than the influent (paired t-test; $p = 0.087$).

In the chloramine system, although the influent typically had 0 CFU/mL of HPCs, one instance of elevated HPCs (255 CFU/mL) gave an overall average influent HPC of 51 CFU/mL. At day 5.7, the PVC-iron, PVC-cement, and PVC systems all had higher HPCs than the influent (paired t-test; $p < 0.0001$ for all conditions). PVC-cement had the highest HPCs at 5.14×10^5 CFU/mL, which was 3.16×10^5 CFU/mL higher than the PVC system (paired t-test; $p = 0.039$) and 3.88×10^5 CFU/mL higher than the PVC-iron system (paired t-test; p =

0.048). The PVC system had the second largest HPC at 1.98×10^5 CFU/mL, which was 7.20×10^4 higher than the PVC-iron system (paired t-test; p = 0.039).

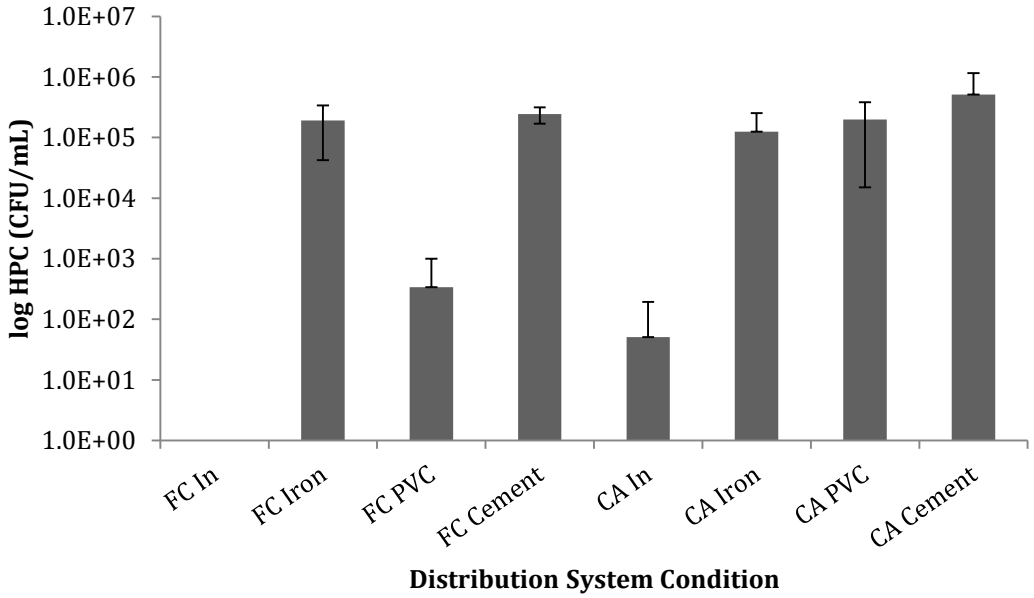

Figure 2.11. Heterotrophic plate count from simulated distribution system water at the day 5.7 sample port. Error bars represent 95% confidence intervals of 5 sampling events.

16S rRNA was also measured from the days 1, 2.3, 3.5 and 5.7 sample ports of the SDS. Influent 16S rRNA samples were not collected. In the free chlorine system, all three pipe materials had relatively low bacteria levels for days 1 through 3.5, between 10^3 and 10^4 gene copies/mL (Figure 2.12). By day 5.7, bacteria levels had risen to 3×10^5 gene copies per mL in the PVC-cement and PVC-iron system, but remained low in the PVC system around 5×10^3 gene copies/mL, consistent with HPC results. Overall, 16S rRNA gene analysis at day 6 of the distribution system is consistent with HPC data. The free chlorine PVC system again had the lowest levels of bacteria at 5.07×10^3 gene copies/mL, which was 3.50×10^5 gene copies/mL less than the free chlorine PVC-iron system (t-test, two-sample assuming unequal variances; p = 0.001)(Figure 2.12). However, the free chlorine PVC-cement condition did not have significantly different 16S rRNA concentrations than PVC or PVC-iron at day 6 due to large deviation between the triplicate samples (t-test, two-sample assuming unequal variances; p = 0.353 for PVC and 0.961 for iron).

Bacterial data confirms the trend in TOC data from the free chlorine distribution system. As a chlorine residual is present in the system until day 3, microbial growth is suppressed

and TOC levels are high. After day 3, when the chlorine residual is gone, microbial growth increases substantially and the TOC in the pipe levels off. It is unclear whether this leveling in TOC is due to bacteria reducing the rate of organic carbon increase due to consumption or whether TOC leaching rates are actually reduced. Disinfectant often remained present in the PVC condition longer than the PVC-cement and PVC-iron, which might explain why microbial levels are much lower at day 5.7 in the PVC pipe. Dissolved oxygen (DO) decreases from day 0 to day 5.7 in the free chlorine cement and iron systems, indicating respiration (Figure 2.13). DO decreases the least amount in the PVC system, which had the lowest HPC and 16S rRNA, the highest disinfectant, and the highest TOC. The free chlorine system had consistently higher disinfectant residuals at day 3.5 in the PVC and PVC-cement system than the chloramine system (Figure 2.14). The high chlorine residual in the free chlorine PVC system explains the low levels of HPC and 16S rRNA in the system.

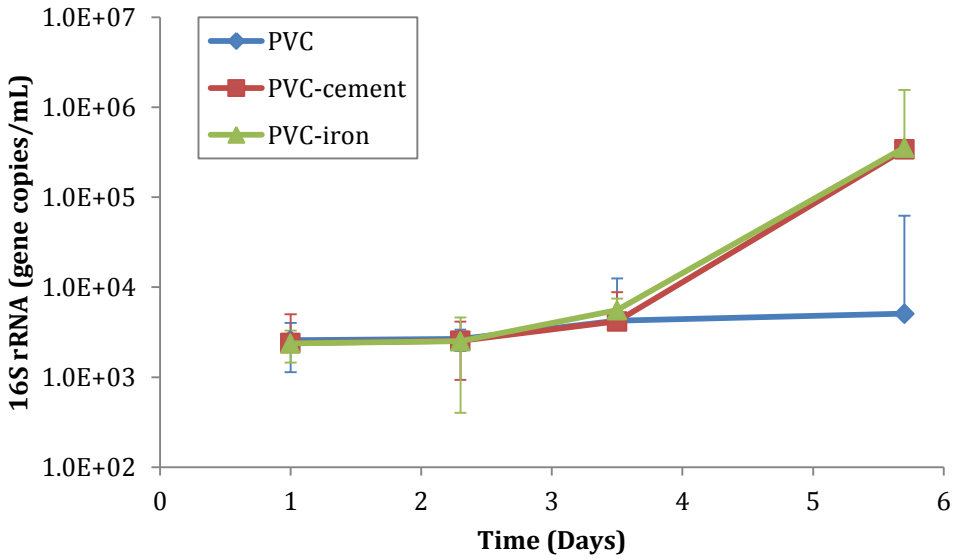

Figure 2.12. 16S rRNA from the free chlorine simulated distribution system from days 1 through 5.7 sample ports. Error bars represent 95% confidence intervals of triplicate pipes. Only one sampling for 16S rRNA was performed.

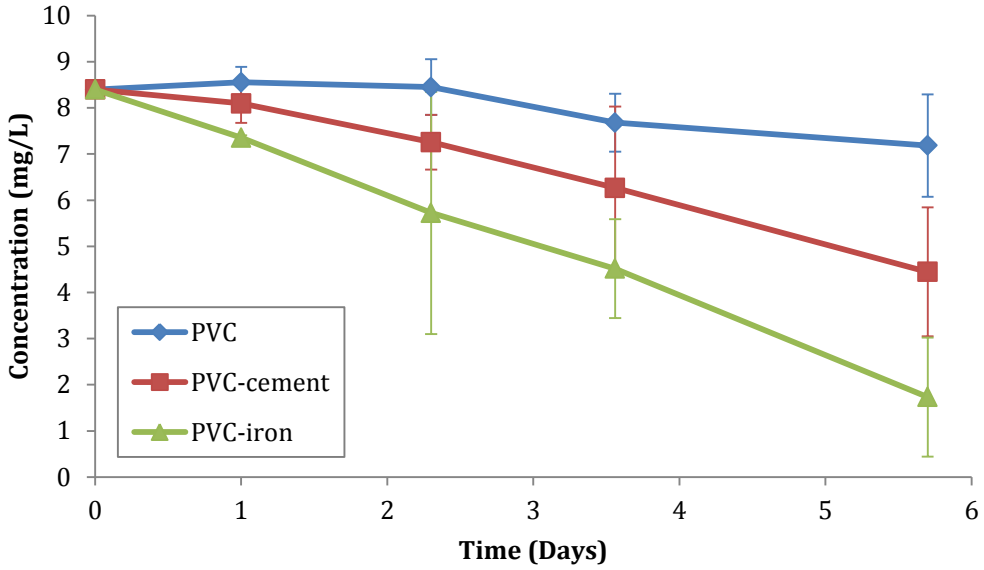

Figure 2.13. Dissolved oxygen in the free chlorine simulated distribution system. Error bars represent 95% confidence intervals of triplicate pipes.

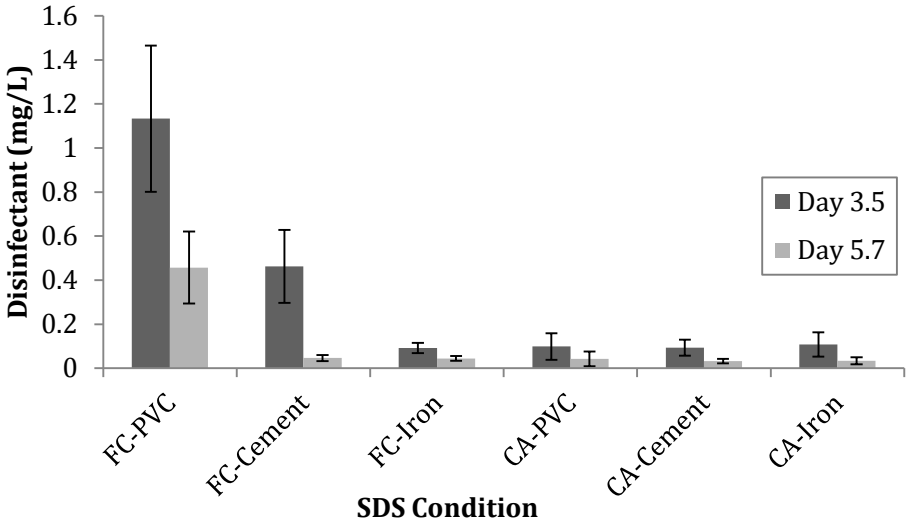

Figure 2.14. Average disinfectant concentrations in the free chlorine (FC) and chloramine (CA) simulated distribution systems. Error bars represent 95% confidence intervals of *n* samples, where n varies between 34 and 36.

In the chloramine system, microbial levels increase rapidly until day 3.5 and then level off in each material. 16S rRNA indicates around 10^4 gene copies/mL on day 1 in all three pipe materials, with more than 10^5 gene copies/mL by day 3.5 and almost 10^6 gene copies/mL on day 5.7 in all three conditions (Figure 2.15). At day 5.7, the three materials did not have different levels of 16S rRNA within 95% confidence (single-factor ANOVA; $p = 0.163$). Dissolved oxygen decreases rapidly in the first few days, and then levels off in all

three systems, indicating that respiration is occurring at a higher rate on days 0 through 3.5, consistent with 16S rRNA levels (Figure 2.16). The chloramine residual in the system was typically depleted by day 2.3 in all three systems, due to nitrification occurring the system, as nitrifying bacteria were confirmed to be present by a Nitrifying Biological Activity Reaction Test (N-BART). Disinfectant levels were consistently lower in the chloramine SDS than in the free chlorine SDS (Figure 2.14).The lack of disinfectant in the system allowed microbes to proliferate more quickly and to higher levels than in the chlorine system. The cause behind the plateau in 16S rRNA genes from days 3.5 to 5.7 was not determined. Possible causes considered included nutrient limitations, a lack of TOC that was assimilable to the microbes present, and amoeba grazing on biofilm present in the system. Microbiological data confirms the TOC trend in the chloramine SDS. TOC levels steadily increase in the system because TOC is being consumed by organisms near the beginning of the system, and TOC appears to increase throughout the system because microbes are producing organic carbon through autotrophic growth.

It is also possible that the difference in microbial levels in the chloramine SDS and free chlorine SDS is due to differences in AOC generation. Using yield for AOC test strains NOX and P-17 cited in SM 9217b (4.1×10^6 CFU/µg AOC for P-17 and 1.2×10^7 CFU/µg AOC for NOX), and assuming each bacterium has one gene copy/mL, consumption of only 58 – 380 ppb AOC could account for the difference of 1.45×10^6 to 7.01×10^5 gene copies/mL.

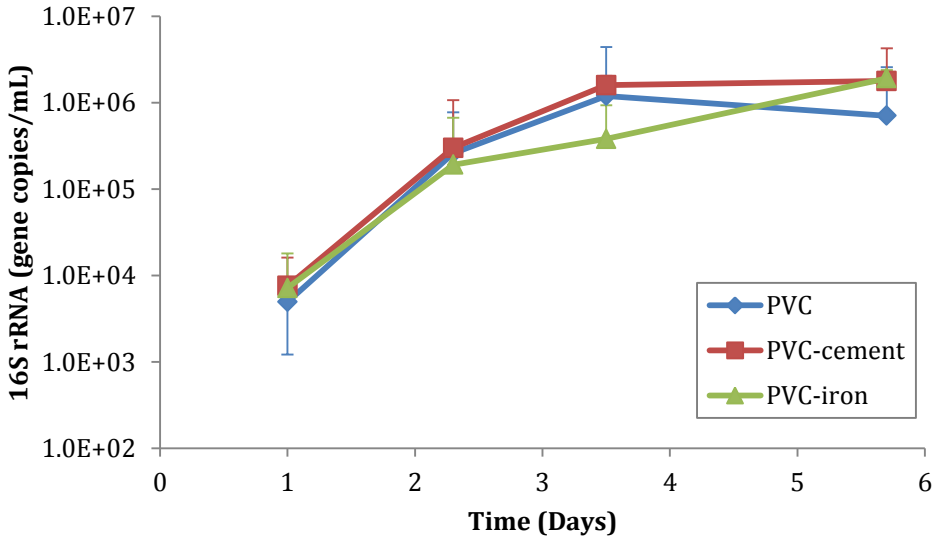

Figure 2.15. 16S rRNA from the chloramine simulated distribution system from days 1 through 5.7 sample ports. Error bars represent 95% confidence intervals of triplicate pipes. Only one sampling for 16S rRNA was performed.

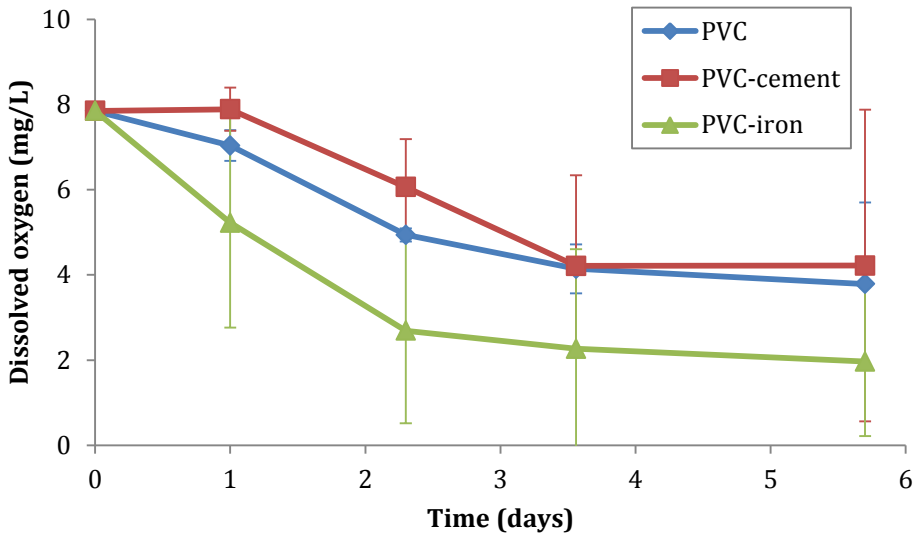

Figure 2.16. Dissolved oxygen in chloramine simulated distribution system. Error bars represent 95% confidence intervals of triplicate pipes.

2.4 Conclusions

The hypothesis that significant DSD-BDOC generation could occur under conditions found in water main and premise plumbing was verified in some circumstances. The ability to detect DSD-BDOC improves as the level of organic carbon (BDOC, AOC, TOC) of the influent water is reduced. In some cases such as stagnation found in water heaters, the DSD-BDOC will not be distributed throughout the entire volume of the water, but ultimately is detected preferentially at the bottom sediments due to settling.

Under the conditions studied the DSD-BDOC did not translate to significantly higher levels of bacteria. Even at the lowest levels of organic carbon tested in the influent water, carbon levels were sufficient to support very high levels of microbial growth with DSD-BDOC for the long stagnation and warm water conditions found in premise plumbing systems without disinfectant, or under simulated distribution system conditions after disinfectant disappeared. It is likely that predator-prey relationships and other factors, rather than organic carbon limitations, exert a key control on the absolute levels of microbes under such circumstances.

Free chlorine induced a particularly high release of organic carbon from PVC pipe if a residual was present, but relatively low additional levels of TOC were released when free chlorine was absent. In contrast, TOC levels from PVC pipe increased steadily in systems with chloramine, when the residual was present or absent. Differences in microbial levels in

the free chlorine SDS and chloramine SDS seem most likely due to differences in the disinfectant residuals.

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