Characterization of the Bacterial Population in RO Distribution Systems and their ability to form Biofilms on Pipe Surfaces

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Abstract

The Water Authority-Cayman (WAC) supplies drinking water to approximately 11,000 customers in Grand Cayman, Cayman Islands. The WAC water system in the capital George Town consists of two reverse osmosis (RO) plants and nearby pumping facilities capable of producing a total of 7.9 million litres/day. A third RO plant is located at Lower Valley, approximately 16 kilometres east of George Town. The two George Town plants are linked to three storage reservoirs with a combined storage capacity of 12.1 million litres. Water is produced from both plants via RO, by pumping saline groundwater from 30 – 60 m deep wells through a series of prefilters to remove silt before circulation through a series of RO membranes. The feed water to both plants contains approximately 1-2 mg/L H_2S. The product water is passed through a counter flow degasifier to oxygenate and is treated with acid to reduce the pH to 6.0 or less. Water is disinfected with Ca hypochlorite, Zn orthophosphate is added to inhibit corrosion and NaOH is added to control the pH.

A pilot study was conducted over a 14-month period comparing the type and quantity of bacteria present in the WAC distribution system using a combination of HPC culture methods and fatty ester methyl ester (FAME) analysis techniques. The study was divided into two distinct phases: in the first phase HPC bacteria in the bulkwater were isolated from inlet (feedwater) to outlet (treated distribution water) and further characterized down to genus or species depending on an available database match, a pipe-loop system was constructed for the second phase of the study to examine the rate of biofilm growth on different pipe surfaces (PVC, ductile iron, steel, PE, brass, copper) and to compare the type of biofilm organisms to those in the bulk fluid.

Keywords: *biofilm, pipe-loop, fatty acid methyl ester analysis*

1 Introduction

The purpose of the 14-month study is to characterize the type and quantity of bacteria present through the RO treatment process and to understand the factors that influence bacterial growth along the WAC distribution system.

Internal surfaces of drinking water distribution systems are always colonized by microorganisms mostly in the form of single cells or microcolonies but sometimes also as dense biofilms. It is estimated that 95% of the overall biomass in a distribution system is located on drinking water pipe walls, while only 5% occurs in the water phase (Flemming et al., 2002). This paper will illustrate that planktonic and biofilm populations in this tropical RO system behave in a similar manner.

Heterotrophic plate count (HPC) analysis in conjunction with fatty acid methyl ester (FAME) analysis is useful in culturing a small proportion of bacteria in the system indicating a low nutrient status and low physiological activity. FAME analysis results suggest that halophilic organisms originating in saline feedwater are eliminated after RO treatment while other organisms, some of which may originate from the RO process are carried through the system. The organisms isolated from feedwater will be shown to be characteristically different from those isolated from the RO and treated distribution water. Total cells determined by epifluorescence and acridine orange direct count are several log times greater than for HPC.

Several factors influence the growth of HPC bacteria in this unique distribution system: sample location, distribution system temperature, incubation temperature, culture media type and other factors. Due to a nutrient starved environment some bacteria have specially adapted to their environment as is evident by the presence of capsulated bacteria such as *Novosphingobium capsulatum* and *Rhodobacter capsulatus.* Although R2A bacteria is the recommended media for the growth of HPC bacteria in most pilot studies on biofilms, this study shows that both R2A and mHPC media give comparable HPC results.

Numerous studies have been conducted on the factors controlling biofilm development on various types of pilots however comparison of the data produced is difficult due to many different approaches used for studying fixed biofilm on various materials. To date this is the first study conducted on an RO distribution in a tropical environment.

This pipe loop study although still in an initial phase of development, investigates the quantity of bacteria present on different pipe surfaces or coupons installed in an experimental pipe loop system located at the George Town plant, Grand Cayman.

The main sections of WAC distribution system pipework are constructed of PVC apart from customer service connections, which are composed of PE and internal pipe metal fittings composed of steel, ductile iron (cement lined), brass or copper. The pipe loop study was initiated in July 2005 to investigate the growth of biofilm on different surfaces served by treated RO water. Coupons of PVC, PE, copper, brass, cement lined ductile iron, and steel were exposed to chlorinated and non-chlorinated water in a pipe loop system. For practical reasons the coupons were installed in-line with a continuous feed of water from the distribution system so as to distribution system conditions. The coupons were incubated for a 30-day period and analysed for HPC and epifluorescence to determine the extent of biofilm growth.

The Water Authority-Cayman (WAC) water distribution system consists of two RO plants and pumping facilities at Red Gate Road capable of producing a total of 7.9 million litres/day. Water is produced from the Red Gate and Lower Valley plants via reverse osmosis. The product or raw water from each plant is disinfected by the WAC using a calcium hypochlorite solution. Zinc orthophosphate is added to inhibit corrosion and sodium hydroxide is added to control the pH.

2 Methods

The first phase of the study consisted of a characterization of the distribution system in terms of microbiological and chemical analysis of selected sampling points within the George Town distribution system for a 14-month period between September 2003 and November 2004. The sample locations were chosen so as to represent a broad crossection of the distribution system from input to output. Figure 1 shows a map of the sampling point locations.

Figure 1 Study area showing the location of sampling points in the GTWS system

Deleted Figure 1.

Table 1 Parameters monitored

Sample Origin	FEEDWATER			UNTREATED RO			TREATED RO								
Site	RG Feed	NS Feed	LV Feed	OCL RG	OCL NS	LV RO	GTR	LVR	#3	#4	#7	#11	#13	#20	#21
Chemical Parameters															
pH units	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
EC µS/cm	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
TDS mg/L	nd	nd	nd	√	√	√	√	√	√	√	√	√	√	√	√
Temperature ^{o}C	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Sulphide mg/L	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Free Chlorine mg/L	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Total Chorine mg/L	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Orthophosphate mg/L	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Zinc mg/L	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Microbiological Parameters															
HPC cfu/mL (mHPC)	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
HPC cfu/mL (Modified HPC)	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
HPC cfu/mL (R2A)	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Total Direct Count cfu/mL	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√

Notes: On-site parameters include: pH, EC, Temperature, Total & Free Chorine and Sulphide.

Samples for chemical analysis were collected in pre-cleaned Nalgene 500ml polypropylene (PP) bottles after running the tap for a 5-minute period. Feedwell samples were run for a shorter period of approximately one minute due to the constant flow of water through the pipe. Samples collected close to the pipe end (Sample taps # 20 and 21) were run for a 15-minute period before sample collection in order to flush out stagnant water in the system due to longer retention periods.

Electrical conductivity (EC), Total Dissolved Solids (TDS) and pH analysis were performed on site with a Myron 6P Ultrameter in accordance with Standard Methods, 20th Edition (1998), Method 2510B for EC/TDS and Method 4500-H^{+}B for pH. Due to the high salinity of feedwells, conductivity was measured using a WTW Model LF 330i Conductivity Meter calibrated with a 50% sodium chloride reference standard. Total and free chlorine analysis was performed on-site by colorimetric analysis using a Hach Chlorine Pocket Colorimeter in accordance with Standard Method 4500-Cl G. On-site sulphide analysis was performed using a Hach DR700 spectrophotometer according to the Hach Methylene Blue Method 8131.

Samples for zinc and orthophosphate analysis were performed in the laboratory on the same day of collection using a Hach DR 4000 Spectrophotometer.

Samples for HPC analysis were collected in sterilized 1L Nalgene PP bottles and analysed using the membrane filtration technique according to Standard Method

9215D with the following exceptions: samples were incubated for a 7-day period and cultured on three different types of media: mHPC, R2A and Modified HPC. These media types were chosen on the basis of the results of a comparative media study performed from April–June 2003 which showed that feedwater organisms grow best on modified mHPC media at 35^{o}C±0.5^{o}C and that R2A at 26^{o}C±0.5^{o}C was the more appropriate media for growth of HPC although there was not a significant difference in counts obtained from R2A and mHPC.

Biofilm samples were collected on demand by scraping a small area of approximately 4.5 to 10.5cm^{2} of pipe or coupon surface into a sterile sampling bag containing 10-20ml of sterile buffered water. The sample was sonicated for 3 minutes using a Fisher Scientific Sonicator Model FS14. A portion of the sample was transferred to a sterile plastic 6cm tube for total bacterial count analysis overseas and the rest of the sample was diluted as necessary and analysed for HPC using the spread plate technique. Bacterial colonies were subcultured to obtain pure cultures and subsequently analysed down to genus or species using FAME analysis by an overseas ISO 17025 accredited laboratory.

The second phase of the distribution study consisted of a pipe loop study to investigate the extent of biofilm growth on different pipe surfaces and to determine whether organisms found in the initial 14-month distribution characterization study were similar to those isolated from pipe loop coupons.

An experimental pipe loop was constructed at the Red Gate Water Treatment building in George Town Grand Cayman. The pipe loop system consisted of two separate wall-mounted pipe loops (chlorinated and unchlorinated) made from 1.9 – 2.5 centimetre PVC piping as in Figure 2.

Figure 2 Pipe Loop Experiment

Coupons of the various materials were installed in-line for an incubation period of approximately 30 days. Treated distribution water was fed into both loops via a separate flow meter and pressure recorder. For the unchlorinated pipe loop an activated carbon filter (Model C Max PB-975) was installed in-line for chlorine removal. Each loop was serviced by two sample taps, one located before the coupons and one after. This type of pipe loop system is inexpensive and more practical to use and has used for the study of biofilm in various drinking water systems by Servais *et al*., (1995), Laurent *et al*., (1999) and Niquette *et al*. (2001).

The pipe loop system was monitored four times a week for pH, temperature, TDS, EC, free chlorine and sulphide and weekly for EPI and HPC counts using the same methods as for the characterization study: For biofilm analysis the coupons were removed separately by placing the whole coupon in a sterile sample bag containing approximately 20mL of water from the pipe loop and sonicated in place for 1-2 minutes. Due to large dimensions of the cement-lined ductile iron fixture, a measured cm^2 area was scraped into the sterile bag and sonicated to promote mixing. Isolated colonies were subcultured and further identified down to genus using FAME analysis.

3. Results and Discussion

3.1 *Chemical analysis*

The water quality of the treated distribution water at Red Gate is similar to that found at the Lower Valley plant since the same RO technology is used to produce water at both locations. For this reason a summary of the water quality at Red Gate only is summarized in Table 2.

Table 2 Average values for Red Gate RO Feed, RO Product and Treated Distribution

Water for the period September 2003 – November 2004.

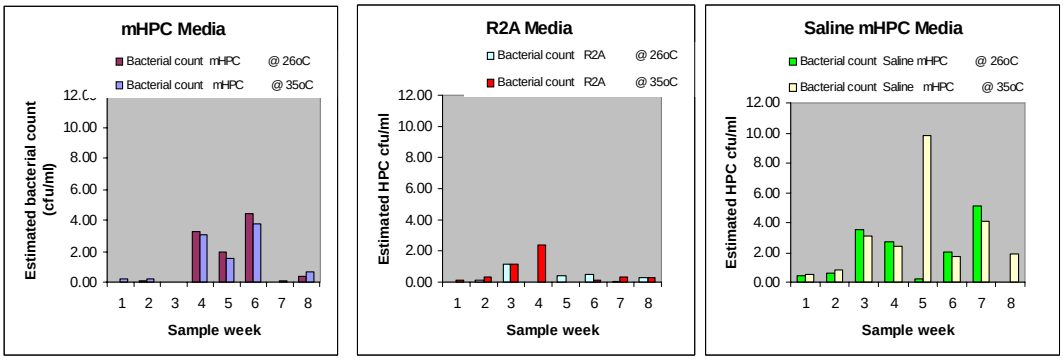
Parameter	Units	Red Gate Feed	Red Gate RO Product	Treated Water
EC	µS/cm	51,049	285.2	292.6
TDS	mg/L	n/a	137.2	139.6
pH	units	7.32	5.94	7.54
Temp	^{o}C	25.7	26.6	27.5
Total Chlorine	mg/L	n/a	n/a	0.43
Free Chlorine	mg/L	n/a	n/a	0.41
H_2S	mg/L	1.7	0.034	0.000
Zinc	mg/L	0.022	0.060	0.607
Orthophosphate	mg/L	0.171	0.093	1.919

Note: n/a: not applicable
EC ± 20µs/cm, pH ± 0.05 units, Chlorine ± 0.02 mg/L, Zinc ± 0.005 mg/L, Ortho ± 0.005 mg/L, Sulphide ± 0.002 mg/L (using Hach DR700) and ± 0.05mg/L using the Chemetrics Colour Comparator Kit for feedwells.

3.2 *Comparative media study*

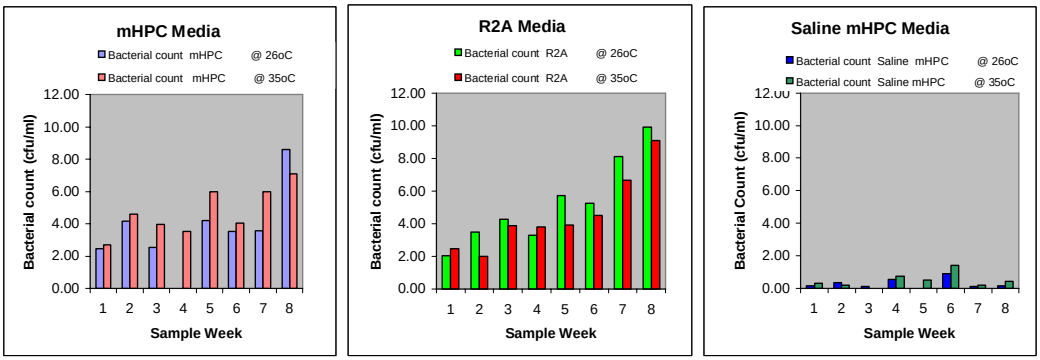
A comparative media study was carried out from April-June 2003 to determine the optimal growth requirements for RO organisms in a tropical climate. Samples from highly saline feedwater, untreated RO and treated RO water were collected and analysed for HPC using three types of media R2A, mHPC and Modified (Saline) HPC media. The organisms were grown at two different temperatures 26±0.5 ^{o}C and 35±0.5 ^{o}C and incubated for a 7-day period. Results are summarized for feed, RO and treated water in Figure 3 – 5 below.

Figure 3 Feedwater



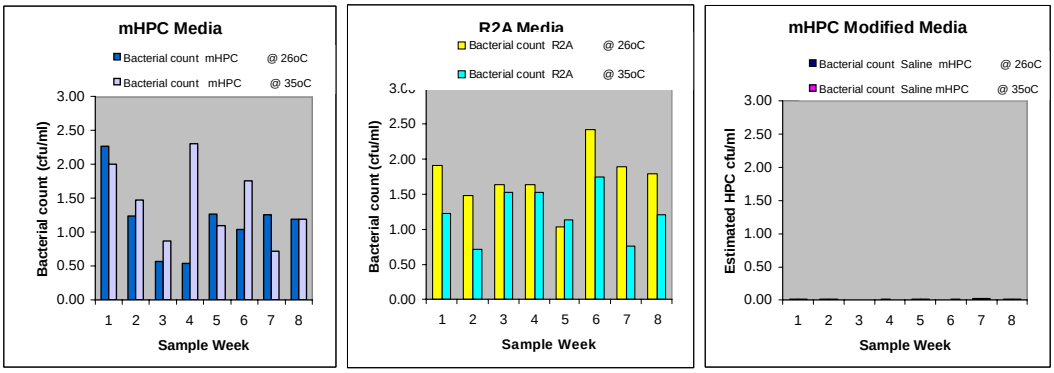
Saline mHPC media is best suited for the growth of feedwater organisms.

Figure 4 RO Water



Organisms from RO product water grow well on R2A and mHPC media. The optimal growth temperature is shown to be 35^{o}C.

Figure 5 Treated Distribution Water



Organisms from both RO product water and RO treated water grow well on R2A and HPC with little variation in counts. The optimal growth temperature for R2A is 26^{o}C.

Historically the Water Authority-Cayman laboratory has used a 48-hour incubation time for the growth of heterotrophic bacteria on mHPC media in accordance with Standard Methods, 20th Edition (1998). In this study higher HPC counts have been observed at both 35^{o}C and 26^{o}C using an extended incubation period of seven days. Although Maki *et al.,* (1986) reported that the optimum temperature for the growth of many heterotrophic bacteria is somewhat less than 30^{o}C. It is evident that tropical RO systems display different heterotrophic growth characteristics to other drinking water distribution systems.

3.3 *HPC vs Total Direct Count using Epifluorescence*

It has been reported that 95% of the overall biomass in a distribution system is located on drinking water pipe walls, while only 5% occurs in the water phase (Flemming *et al*., 2002). This is supported by the difference in HPC counts observed in water compared to those in the biofilm. HPC counts for WAC Treated Distribution Water illustrated in Table 3 are several log times lower than those observed for WAC Biofilm as shown in Table 4. Table 3 and 4 also illustrates that enumeration of bacteria using the epifluorescence technique gives higher bacterial counts in comparison to the standard HPC culture method.

Table 3 HPC vs. EPI Counts for WAC Treated Distribution Water

Date & Time Collected	GTR		LVRes		ST 3		ST 20		ST 21	
	EPI cfu/ml	HPC cfu/ml	EPI cfu/ml	HPC cfu/ml	EPI cfu/ml	HPC cfu/ml	EPI cfu/ml	HPC cfu/ml	EPI cfu/ml	HPC cfu/ml
21-Feb-04 @ 7:04am	7.30E+03	0.75	3.60E+02	0.13	n/d	8.00	6.20E+03	0.74	n/a	n/a
13-Mar-04 @ 4:57pm	1.20E+04	0.49	n/d	0.22	4.20E+03	2.64	3.70E+03	3.04	n/a	n/a
4-Apr-04 @ 5:10pm	8.20E+03	0.30	n/d	0.08	9.60E+02	8.00	3.00E+03	1.44	n/d	0.74
24-Apr-04 @ 10:34am	n/d	0.30	1.20E+03	0.17	1.20E+03	3.64	2.60E+03	0.80	4.00E+03	5.84
30-May-04 @ 3:30pm	n/d	1.60	4.80E+03	0.11	4.20E+02	1.80	5.90E+03	3.44	2.10E+04	1.36
19-Jun-04 @ 6:12pm	9.60E+02	2.04	7.20E+02	0.07	8.40E+02	2.56	3.70E+03	1.56	1.50E+03	4.80
10-Jul-04 @ 6:21am	7.20E+02	0.20	1.20E+03	0.02	n/d	3.04	7.20E+02	3.52	2.20E+03	1.68
1-Aug-04 @ 6:19am	1.90E+04	0.06	1.10E+03	0.01	1.10E+03	0.29	6.00E+02	1.20	1.80E+04	1.44
17-Oct-04 @ 8:05am	6.10E+03	0.08	7.70E+03	2.12	3.20E+05	0.28	n/d	n/d	n/d	n/d
27-Dec-04 @6:05am	1.50E+03	0.12	4.30E+02	0.21	1.00E+03	1.64	n/d	n/d	n/d	n/d

Note: n/d – not done, n/a – not applicable
GTR – Sample Tap at George Town, Red Gate production facility
LVRes – Sample Tap at Lower Valley Reservoir production facility
ST 3 – Sample Tap # 3 Courts Road (close to Red Gate production facility)
ST 20 – Sample Tap # 20 East End Post Office (close to end of line)
ST 21 – Sample Tap # 21 Colliers, East End (end of line)
EPI counts provided by Clancy Environmental Consultants, USA.

The bacteria isolated from RO feedwater were characteristically different from those isolated from other parts of the distribution system. The halophile *Pseudomonas nautica* grown only on Modfied HPC agar was found in the RO feedwater but was eliminated in the RO process. *Novosphingobium capsulatum* originates in RO water prior to chlorination however manages to survive the chlorination process and persist in the distribution system. Five different types of *Pseudomonas* species were isolated from the RO system: *Pseudomonas (Ps.) flourescens, Ps. putida Biotype B, Ps. paucimobilis, Ps. huttiensis, Ps. maltophila* prior to treatment indicating that operational factors within the RO process may have contributed to a prevalence of this type of bacterium in the system. *Bacillus cereus* and *Bacillus –GC Group 22* sp. were isolated from treated distribution water and feedwater but were not found in the untreated RO. *Bacillus lichenformis* commonly isolated from soil was found in the treated distribution water only.

3.4 Pipe loop study

Under chlorinated conditions cement-lined ductile iron produced the lowest HPC cfu/cm^2 while mild steel produced the highest counts. For the non-chlorinated loop biofilm growth on each of the surfaces was several log times higher with the exception of mild steel, indicating the importance of chlorine in the control of biofilm. A summary of the microbiological results is in Table 4.

Table 4 HPC Counts cfu/cm^2 for WAC Pipe Loop Biofilm Samples collected from Different Pipe Surfaces August 2005.

Pipe Coupon	Date & Time Collected	Incubation Time	cm^2 Sampled	Chlorinated Loop HPC cfu/cm^2	Unchlorinated Loop HPC cfu/cm^2
Brass	9-Aug-05 @ 4:00pm	30	6	8.30E+03	4.82E+05
Copper	9-Aug-05 @ 4:13pm	30	6	7.43E+02	3.54E+05
Ductile Iron Cement Lined	9-Aug-05 @ 5:20pm	30	4.5	0.32E+01	9.31E+03
Mild Steel	9-Aug-05 @ 4:20pm	30	6	5.00E+04	1.76E+04
PVC	9-Aug-05 @ 4:36pm	30	10.5	2.85E+02	1.69E+05
PE	9-Aug-05 @ 4:52pm	30	10.5	9.51E+02	2.48E+05

The carbon filter installed in-line removed chlorine effectively during the 30-day coupon incubation period however after approximately three weeks the flow to the unchlorinated pipe section was reduced. Table 5 below shows average values for TDS, EC, temperature, pH, and free chlorine for the period 8 July – 9 August 2005.

The HPC count in cfu/ml for the chlorinated loop remains stable at <0.05 cfu/ml over the 30-day period however the HPC for the unchlorinated loop increased significantly. The pH of the unchlorinated loop is lower than that in the chlorinated loop after 30 days. The reduced flow may have influenced the pH drop. In all cases however the HPC counts in the pipe loop water are much smaller than those for biofilm. This supports the findings of Block, (1992) and Servais *et al* .(2004).

Table 5 Pipe loop water quality after 7 & 30 days

	After 7 Days				After 30 days			
	Chlorinated		Unchlorinated		Chlorinated		Unchlorinated	
Parameter	AC	BC	AC	BC	AC	BC	AC	BC
TDS mg/L	176.6	176.4	176.7	176.3	181.4	181.7	181.7	181.6
EC µS/cm	367.9	367.5	368.1	367.4	377.9	378.4	378.4	378.2
Temperature ^{o}C	28.3	28.3	28.3	28.3	28.7	28.7	28.8	28.7
Free Chlorine mg/L	0.64	0.66	0.01	0.01	0.49	0.51	0.01	0.01
pH units	7.59	7.60	7.35	7.29	7.28	7.31	7.08	6.99
HPC cfu/ml	0.04	0.02	0.46	0.19	0.01	0.02	3.77	4.23

Note: BC = before coupons, AC = after coupons

1 Conclusions

Both R2A at 26^{o}C and HPC at 35^{o}C are suitable for the study of biofilms in tropical RO water and Modified HPC media has proven to be the best for isolating halophiles in saline groundwater. Some bacteria survive the RO process and are carried through into the distribution system indicating that some organisms are resistant to the chlorination process and adapt to the surrounding environment. An example of this is the presence of capsulated bacteria *Novosphingobium capsulatum* in treated water. Other bacteria *Ps. nautica.* will only grow in a saline environment and are killed off in the RO treatment process.

Mild steel influenced corrosion in this RO system as the chlorinated coupons exhibited more corrosion than the unchlorinated coupons. Biofilm growth generally decreased with chlorination. Chlorination is therefore a controlling factor in biofilm growth. Cement-lined ductile iron helps to reduce biofilm growth as was evident by the lower HPC cfu/cm^2 counts in both the chlorinated and unchlorinated loops.

The temperature changes between the wet and dry Cayman seasons does not seem to be significant enough to produce a change in HPC counts although residence time in the pipe at the end does influence bacterial counts.

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