Biostability In Drinking Water Distribution Systems Study At Pilot-scale

2004

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BIOSTABILITY IN DRINKING WATER DISTRIBUTION SYSTEMS
STUDY AT PILOT-SCALE

by

MICHAEL LE PUIL

MS (Environmental Engineering) ESIP – France, 2001

A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
in the Department of Civil and Environmental Engineering
in the College of Engineering and Computer Science
at the University of Central Florida
Orlando, Florida

Summer Term
2004

Major Professor: Andrew A. Randall
ABSTRACT

Biostability and related issues (e.g. nitrification) were investigated for 18 months in 18 pilot distribution systems, under various water quality scenarios. This study specifically investigated the impact of steady-state water changes on HPC levels in chlorinated and chloraminated distribution systems. Chlorination was more effective than chloramination in reducing HPC levels (1-2 log difference). There was a rapid increase in HPC corresponding to the change in steady-state water quality, which was observed in all PDS. Modeling effort demonstrated that HPC levels reached a maximum within five days after water quality change and return to initial level ten days after the change. Since alkalinity was used as a tracer of the steady-state water quality change, time to reach maximum HPC was related to a mixing model using alkalinity as a surrogate that confirmed alkalinity transition was complete in approximately eight days.

Biostability was assessed by HPC levels, since no coliform were ever detected. It was observed that HPC levels would be above four logs if residual droped below 0.1-0.2 mg/L as Cl₂, which is below the regulatory minimum of 0.6 mg/L as Cl₂. Therefore bacterial proliferation is more likely to be controlled in distribution systems as long as residual regulatory requirements are met. An empirical modeling effort showed that residual, pipe material and temperature were the most important parameters in controlling HPC levels in distribution systems, residual being the only parameter that can be practically used by utilities to control biological stability in their distribution systems. Use of less reactive (i.e. with less chlorine demand) pipes is recommended in order to prevent residual depletion and subsequent bacterial proliferation.
This study is investigated biofilm growth simultaneously with suspended growth under a wide range of water quality scenarios and pipe materials. It was found that increasing the degree of treatment led to reduction of biofilm density, except for reverse osmosis treated groundwater, which exerted the highest biofilm density of all waters. Biofilm densities on corrodible, highly reactive materials (e.g. unlined cast iron and galvanized steel) were significantly greater than on PVC and lined cast iron. Biofilm modeling showed that attached bacteria were most affected by temperature and much less by HRT, bulk HPC and residual. The model predicts biofilms will always be active for environments common to drinking water distribution systems. As American utilities do not control biofilms with extensive and costly AOC reduction, American utilities must maintain a strong residual to maintain biological integrity and stability in drinking water distribution systems.

Nitrite and nitrate were considered the most suitable indicators for utilities to predict onset of a nitrification episode in the distribution system bulk liquid. DO and ammonia were correlated to production of nitrite and nitrate and therefore could be related to nitrification. However since ammonia and DO consumptions can be caused by other phenomena than nitrification (e.g. oxidation by disinfectant to nitrite and reduction at the pipe wall, respectively), these parameters are not considered indicators of nitrification.

Ammonia-Oxidizing Bacteria (AOB) densities in the bulk phase correlated well with nitrite and nitrate production, reinforcing the fact that nitrite and nitrate are good monitoring tools to predict nitrification. Chloramine residual proved to be helpful in reducing nitrification in the bulk phase but has little effect on biofilm densities. As DO has been related to bacterial proliferation and nitrification, it can be a useful and inexpensive option for utilities in predicting biological instability, if monitored in conjunction with residual, nitrite and nitrate. Autotrophic
(i.e. AOB) and heterotrophic (i.e. HPC) organisms were correlated in the bulk phase and biofilms.
To Maman, Soizick, Nicolas, and Mamy

In memory of Kevin Lee Young
ACKNOWLEDGMENTS

I wish to thank my PhD Committee Dr Andrew A. Randall (Committee chair), Professor James S. Taylor, Dr John D. Dietz, Dr Debra R. Reinhart and Dr Linda C. Malone for supporting and pushing me along this difficult and sometimes painful educational path. I would like to express my profound gratitude to my advisor Dr Randall for believing in me even when I had doubts and for giving me autonomy in my work and directions in my life.

I wish to thank Dr Young C. Chang, for his friendship, support and teachings that kept me going even through the longest nights in the lab.

I would like to thank all of my colleagues and UCF graduate program fellow companions who worked on this project, days after days, nights after nights, rain or shine. I extend my gratitude to Oz Wiezner and Dennis Marshall whose help and support on site was instrumental to the success of this project.

It was an honor to be part of such a large and challenging project and to work with such sharp, supportive and driven individuals. I will be forever greatful to have got the chance to work within such a diverse and amazing team. In many different ways each of you has made my stay at UCF the most enriching experience of my life.

I would like to thank the American Water Works Association Research Foundation (AWWARF), Tampa Bay Water (TBW) and Pinellas County, Hillsborough County, Pasco County, Cities of Tampa, New Port Richey and St. Petersburg and University of Central Florida (UCF) for their financial support of the three-year Tampa Bay Water-AWWARF tailored collaborative project.
I would like to thank all my friends and especially Indira Carlo, who held me in the
darkest hours and whose support and care, helped me walk the last steps on this path.

I would like to express my immense gratitude to my late friend, student and colleague
Kevin Lee Young. His hard work was crucial in obtaining the data presented here and his
friendship was an unvaluable support. His passion for environmental science and life will
forever be a source of inspiration.

Finally I am eternally greatful to my mom Nicole, my sister Soizick, my brother Nicolas
and my grand-mother Marie for their unconditional love and support in any of my endeavours.
The only thought of you always kept me strong, wherever I decided to go.
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<tbody>
<tr>
<td>AOB</td>
<td>Ammonia-Oxidizing Bacteria</td>
</tr>
<tr>
<td>AOC</td>
<td>Assimilable Organic Carbon</td>
</tr>
<tr>
<td>AWWARF</td>
<td>American Water Works Association Research Foundation</td>
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<tr>
<td>BAC</td>
<td>Biologically Activated Carbon</td>
</tr>
<tr>
<td>BAC</td>
<td>Biologically Activated Carbon</td>
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<tr>
<td>BAT</td>
<td>Best Available Technology</td>
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<td>BDOC</td>
<td>Biochemical Dissolved Organic Carbon</td>
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<td>BFHPC</td>
<td>Biofilm Heterotrophic Plate Count</td>
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<td>BOM</td>
<td>Biodegradable Organic Matter</td>
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<tr>
<td>CCW</td>
<td>Cypress Creek Wellfield</td>
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<td>CDWQ</td>
<td>Comprehensive Disinfection Water Quality</td>
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<tr>
<td>Cfu</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal Laser Scanning Microscopy</td>
</tr>
<tr>
<td>CPU</td>
<td>Chloropatinate Unit</td>
</tr>
<tr>
<td>CSF</td>
<td>Coagulation-Settling-Floculation</td>
</tr>
<tr>
<td>CT</td>
<td>Disinfectant concentration times contact Time</td>
</tr>
<tr>
<td>D/DBP</td>
<td>Disinfectants/Disinfection By-Products</td>
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<td>Disinfection By-Products</td>
</tr>
<tr>
<td>Diam.</td>
<td>Diameter</td>
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<tr>
<td>DO</td>
<td>Dissolved Oxygen</td>
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<td>DWQM</td>
<td>Dynamic Water Quality Model</td>
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<td>EBCT</td>
<td>Empty Bed Contact Time</td>
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<td>ESEI</td>
<td>Environmental Systems Engineering Institute</td>
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<tr>
<td>FC</td>
<td>Fecal Coliform</td>
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<td>FISH</td>
<td>Fluorescent In-Situ Hybridization</td>
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<td>G</td>
<td>Galvanized Steel</td>
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<td>GAC</td>
<td>Granulated Cativated Carbon</td>
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<tr>
<td>GPD</td>
<td>Gallon Per Day</td>
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<td>Ground Water</td>
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<td>Ground Water Rule</td>
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<tr>
<td>HPC</td>
<td>Heterotrophic Plate Count</td>
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<td>MEANING</td>
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</tr>
<tr>
<td>HRT</td>
<td>Hydraulic Residence Time</td>
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<td>Interim Enhanced Surface Water Treatment Rule</td>
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<td>LCI</td>
<td>Lined Cast Iron</td>
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<td>LLβN</td>
<td>L-Leucine β-Naphtylamide</td>
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<tr>
<td>LSI</td>
<td>Langelier Saturation Index</td>
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<td>LT1ESWTR</td>
<td>Long-Term 1 Enhanced Surface Water Treatment Rule</td>
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<td>MCL</td>
<td>Maximum Contaminant Level</td>
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<td>MCLG</td>
<td>Maximum Contaminant Level Goals</td>
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<tr>
<td>MGD</td>
<td>Million Gallons per Day</td>
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<td>MRDLG</td>
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<tr>
<td>NOM</td>
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<tr>
<td>NPDOC</td>
<td>Non-Particulate Dissolved Organic Carbon</td>
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<td>NTU</td>
<td>Nephelometric Titration Unit</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffer Solution</td>
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<tr>
<td>PDS</td>
<td>Pilot Distribution System</td>
</tr>
<tr>
<td>PEPA</td>
<td>Potential of Exo-Proteolytic Activity</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinyl Chloride</td>
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<tr>
<td>PWTS</td>
<td>Pilot Water Treatment System</td>
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<td>Quality Assurance</td>
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<td>RO</td>
<td>Reverse Osmosis</td>
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<tr>
<td>RPDP</td>
<td>Relative Percentage Difference</td>
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<tr>
<td>rRNA</td>
<td>Ribosomal Ribonucleic Acid</td>
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<tr>
<td>RSD</td>
<td>Relative Standard Deviation</td>
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<td>SEM</td>
<td>Scanning Electron Microscopy</td>
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<tr>
<td>SW</td>
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<td>TC</td>
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CHAPTER ONE: GENERAL INTRODUCTION

This dissertation entitled “Biostability in Distribution Systems: Study at Pilot-Scale” is submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Civil and Environmental Engineering in the College of Engineering and Computer Science at the University of Central Florida (UCF), Orlando, Florida. The research work published in this document is deemed original work and consist in part of:

- three years of research work in the Department of Civil and Environmental Engineering of the University of Central Florida (UCF) on a tailored collaborative project between American Water Works Association Research Foundation (AWWARF), Tampa Bay Water (TBW) and UCF to evaluate the effects of blending significantly different source waters on distribution water quality;
- extensive review of literature on biostability, bacterial regrowth and water quality modeling in drinking water distribution systems and related issues (e.g. nitrification);
- investigations and modeling of biostability and factors influencing bacterial regrowth in distribution systems using the pilot distribution systems created for the project.

This dissertation is divided into several chapters:

- Chapter 1 provides a general introduction, overview of Tampa Bay water and the challenges faced by their member governments, and a problem statement and research objectives for both the project and this dissertation.
• Chapter 2 provides an extensive literature review on biostability, bacterial regrowth in distribution systems and related issues.

• Chapter 3 describes in detail the project experimental plan.

• Chapters 4 to 9 are insertion of formatted journal articles intended for publication:
  
  ▪ Chapter 4 evaluates AOC and BDOC as potential indicators of biostability in distribution systems.
  
  ▪ Chapter 5 presents a modeling effort on impact of steady-state change in water quality on HPC levels.
  
  ▪ Chapter 6 investigates heterotrophic organisms proliferation in the bulk phase and its causes and provides a non-linear empirical model for suspended bacterial regrowth in distribution systems.
  
  ▪ Chapter 7 investigates heterotrophic organisms proliferation in the biofilm and its causes and provides a non-linear empirical model for attached bacterial regrowth in distribution systems.
  
  ▪ Chapter 8 describes the onset of a nitrification episode under optimum controlled conditions and provides an assessment of the most suitable indicators for nitrification occurrences.
  
  ▪ Chapter 9 describes attached and suspended growth of nitrifying bacteria, (detected by Fluorescent In-Situ Hybridization), and their relationships with water quality parameters and each other.
• Chapter 10 provides conclusions and recommendations based on the results discussed in chapters 4 to 9.

Problem Statement and Research Objectives

As populations increase, so does the need for water supply and more advanced water treatment, in response to the associated increase of human-made pollutants in the source waters. Since water supplies are not limitless, it is crucial to design master waterplans that both preserve the actual resources and guarantee sufficient provision of biologically safe drinking water to these populations. This concern has been raised in the Tampa Bay area by Tampa Bay Water (TBW) due to a predicted shortage of water supply in a near future. To better understand the situation, an overview of TBW and the problems they are facing is provided.

Tampa Bay Water

Overall Description

TBW is a special district agency created by interlocal agreement among member governments -- Hillsborough County, Pasco County, Pinellas County, St. Petersburg, New Port Richey and Tampa. It provides wholesale water to the member utilities, which in turn provide water to nearly 2 million people in the tri-county area. TBW was created in 1998, with assistance from the Florida Legislature and Governor, by restructuring the West Coast Regional Water Supply Authority.
TBW provides an average of 176 million gallons of water to their members every day. Currently, that water is produced from 12 regional groundwater facilities. TBW also owns and operates two water treatment facilities and one surface water augmentation facility.

**Master Water Plan – Planning for the Future**

*From Chlorine to Chloramines*

TBW has switched from chlorine to chloramines as its primary disinfectant and secondary residual for treatment of drinking water. While TBW already complied with all current water quality regulations, this change was made in advance of new, more stringent health standards, which the United States Environmental Protection Agency (USEPA) will phase in over the next few years. Chloramines were selected as the method of choice because they are the most cost-effective way of maintaining compliance under the new regulations. The use of chloramines is 20 percent less expensive than alternative methods of treatment, and at the same time reduces the formation of suspected cancer-causing compounds. And, by switching to chloramines, it is hoped that there will be a more consistent water quality will be maintained throughout Tampa Bay Water's wholesale system.

The change to chloramines will reduce the level of some regulated disinfection by-products formed when chlorine mixes with trace quantities of naturally occurring organic substances found in water. This is particularly significant because the use of chloramines will reduce the formation of trihalomethanes (THMs) and haloacetic acids (HAAs), two types of by-products suspected to cause cancer with prolonged exposure.
**Protecting the Water Resources**

TBW is committed to preserve water supplies and in particular the Floridian Aquifer which served as its main and almost only drinking water supply in the past. By 2003, it reduced groundwater production at 11 interconnected facilities in Pasco and northern Hillsborough counties from a formerly permitted level of 158 million gallons per day to 121 million gallons per day. By 2008, pumping from existing facilities will be further reduced from 121 million gallons per day to 90 million gallons per day, in order to allow the Aquifer to recover from years of unmonitored extractions. New water supplies must be found to accommodate these pumping reductions while still meeting its members’ needs. That is why TBW has a Master Water Plan (Figure 1) that combines new sources like desalinated seawater and surface water with limited additional groundwater and aggressive conservation. This Master Water Plan should ensure adequate supplies for the Tampa Bay region through the year 2010 and beyond.

**Problem Statement**

However the implementation of such a plan brings several challenges that need to be overcome in order to keep supplying similar quality water to the populations of Tampa Bay area. The new sources or blends that are considered are significantly different from the actual sources in use by TBW as raw water sources. Therefore appropriate water treatment for these new sources has to be determined. Impact of these new finished waters on distribution system water quality has to be taken into account also, as disruption of films and pipe scales are expected upon blend change. In anticipation of the implementation of this Master Water Plan and for the reasons cited above, TBW decided to finance (in collaboration with AWWARF) a 3 million
dollar tailored collaborative project (TCP) entitled “Required Treatment and Water Quality Criteria for Distribution System Blending of Treated Surface, Ground and Saline Sources” to study the effect of blending on distribution system water quality by University of Central Florida (UCF) Environmental Systems Engineering Institute (ESEI) structure.

![Actual TBW Blend](image)

![Future TBW Blend](image)

Figure 1. TBW Master Water Plan
Research Objectives

This project dictated design, construction, and continuous operation (for a 2-year period, 24 hours a day, 7 days a week, 365 days a year) of a pilot-plant consisting of 18 independent Pilot Distribution Systems (PDS) and capable of producing and blending seven different water sources into the PDS. The design of the PDS allowed for sampling at influent, effluent and internal ports along the lines. An overview of the experiment site is presented in Figure 2. The general goal of the TCP was to gain a better understanding of the impact of blending different water sources on water quality in the distribution system. Specifically, the objectives were:

- Determination of seasonal changes in aesthetic and regulated water quality from variations of blending finished waters from significantly different sources, and

- Determination of methods to mitigate adverse seasonal changes in aesthetic and regulated water quality due to blending of finished waters from significantly different sources.

In order to gain such information, intensive analytical monitoring was required and was divided among microbiological, surface characteristics and chemical analyses.
**Dissertation Scope and Objectives**

While blending of different water sources is a common practice of water utilities in the United States, its impact on water quality in the distribution system has yet to be elucidated. In order to avoid any detrimental consequences of blending, it is of critical importance to fundamentally understand changes in water quality resulting from various blending scenarios. None of previous studies that investigated and tried to predict water quality of blended water explored blending with waters processed by various advanced water treatment technologies such as membranes (RO and/or NF), GAC, and ozonation. Each of these treated waters has unique physical and chemical characteristics quite different from conventionally treated water.

The impact of blending on biostability in distribution systems, despite its importance, has also yet to be investigated. Another issue encountered by many utilities using post-
chloramination is nitrification in their distribution system. Since this phenomenon is not desirable due to severe adverse effects on distribution system water quality (depletion of disinfectant residual and subsequent bacterial proliferation with potential coliform regrowth), nitrification is usually studied at full scale while an episode is on-going, but rarely is nitrification studied under controlled conditions. Finally, simultaneous study of attached and suspended growth in distribution system under a wide range of water quality scenarios and environmental conditions has not been reported. For these reasons, the main objectives of the research discussed in this dissertation were:

- To conduct an extensive literature review on biostability in distribution systems, accounting for significant parameters influencing bacterial proliferation and extended to related issues such as nitrification.

- To investigate attached and suspended growth simultaneously, to model both proliferation with water quality parameters, and to attempt to elucidate the relationship between the two phenomena.

- To study biostability in the context of on-going, controlled nitrification episode and investigate most suitable indicators of nitrification and relationships between nitrifying populations and water quality parameters.
CHAPTER TWO: LITERATURE REVIEW

Drinking Water Regulations

Modern disinfection practices are largely driven by the dual requirements of disinfection accompanied by avoidance of Disinfection By-Product (DBP) production. These driving forces have lead to radically different approaches in the United States versus Europe. In Europe the focus is on removing natural organic matter (NOM) that serves as precursors for both DBPs and drives bacterial growth, prior to primary disinfection. An additional difference is that secondary disinfectant residuals are not required in some European countries (Hydes, 1999), and in some countries low ammonia standards effectively eliminate the use of chloramine residual (White, 1999). In the United States current disinfection practices are driven largely by the Total Coliform Rule or TCR, the Surface Water Treatment Rule or SWTR, and the Maximum Contaminant Level or MCL for total trihalomethanes or TTHMs. The approach developed in the United States, instead of relying on removal of, or low, NOM concentrations, relies on alternate primary disinfectant practices when source water NOM is high, and increasingly less use of free chlorine for secondary residuals as well. One significant aspect of this has been a resurgence of chloramine use to maintain a secondary residual, and in fact since 1980 chloramines have also been an approved primary disinfectant as well. This contrasts significantly with European practice where limits on organic content, ammonia, and aesthetic considerations have resulted in far less frequent use of secondary residuals, and even when they are used free chlorine or chlorine dioxide residual rather than chloramines is preferred (van der Kooij et al., 1999).

Currently USEPA standards for microorganisms are the result of the SWTR and the TCR. Some of the contaminants have set MCLs, while others have treatment technique requirements.
The TCR pertains to both groundwaters and surface waters alike, and sets an MCL of zero for Total Coliforms, Fecal Coliforms, and *E. coli* (Pontius, 2000). The Best Available Technology (BAT) for meeting these MCLs is defined as disinfection. There are also many additional details for actually meeting the MCL with respect to the regulatory requirements. For example in some types of systems the Total Coliform requirement may be met if no more than 5% of the samples per month are positive, or no more than 1 sample positive per month for less than 40 samples per month (U.S.E.P.A., 2000).

The SWTR establishes MCLs for *Giardia lamblia*, *Legionella* (bacteria), and viruses (Pontius, 2000; U.S.E.P.A., 2000). In addition treatment technique requirements are established for meeting HPC and turbidity criteria. Under the SWTR both parameters with and without MCLs have filtration and disinfection defined as the BAT required. There are specific criteria making it possible not to use filtration in some cases however. The Enhanced Surface Water Treatment Rule (ESWTR) contains a proposed MCL of zero for *Cryptosporidium* (protozoal cysts) which will add this parameter to the list of regulated microbiological contaminants (Pontius, 2000).

In many European countries there are distribution system standards for maximum heterotrophic plate counts, unlike the United States where a BAT instead of an MCL is required (Hydes, 1999; Pontius, 2000; Van der Kooij et al., 1999). However an HPC < 500 cfu/mL can be used in lieu of measurable chlorine residual to meet SWTR regulations concerning maintenance of a secondary residual in the distribution system (U.S.E.P.A., 2000). In Europe Fecal *Streptococci* and sulphite-reducing *Clostridia* (surface water only) are also included in the microbiological standards for drinking water (Hydes, 1999; Van der Kooij et al., 1999) while these are not regulated in the U.S.A.
The Disinfectants and Disinfection By-products rule also affects disinfection practice. It contains maximum residual disinfectant level goals (MRDLGs) for disinfectants that are likely to become enforceable requirements in the near future. The MRDLGs are 4 mg/L as Cl₂ for Chlorine and Chloramines, and 0.8 mg/L as ClO₂ for Chlorine Dioxide (Pontius, 2000; U.S.E.P.A., 1999). In addition there is an MCL of 1.0 mg/L for chlorite, a byproduct of chlorine dioxide use, with an MCL goal (MCLG) of 0.8 mg/L. Chlorite in low levels (0.05 mg/L to 0.8 mg/L) can be used to inhibit nitrification (e.g. such as that occurring with use of chloramines) but in higher concentrations is a public health concern, which can cause anemia (Haas, 2000).

Current US regulations for disinfection pertain to surface waters and groundwaters designated as under the direct influence of surface water (SWTR). These regulations are largely based on the concept of CT, the product of disinfectant concentration (mg/L) and contact time (minutes). Minimum CT products have been defined from prior studies for inactivation of the microbiological contaminants regulated in the US.

For determining the effectiveness of disinfection, the concept of inactivation ratio is used:

\[
\frac{CT_{\text{calc}}}{CT_{99.9}}
\]

where CT₉₉.₉ is the CT value required for 99.9 percent (3 log) inactivation of *Giardia lamblia* cysts in this particular example. CT.calc is the actual observed disinfectant/contact product in the system being assessed. CT₉₉.₉ values are published by the USEPA (2000) and an example is shown for Free Chlorine at 15.0° C in Table 1:
Table 1. CT Values for Three Log Inactivation of Giardia Lamblia By Free Chlorine at 15.0° C

<table>
<thead>
<tr>
<th>Free Residual (mg/L)</th>
<th>pH</th>
<th>6.0</th>
<th>6.5</th>
<th>7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤0.4</td>
<td></td>
<td>49</td>
<td>59</td>
<td>70</td>
</tr>
<tr>
<td>0.6</td>
<td></td>
<td>50</td>
<td>60</td>
<td>72</td>
</tr>
<tr>
<td>0.8</td>
<td></td>
<td>52</td>
<td>61</td>
<td>73</td>
</tr>
</tbody>
</table>


CT values for Giardia lamblia have been found to result in at least a 4 log inactivation of viruses, and thus the EPA Giardia lamblia tables imply inactivation of viruses. So if Giardia lamblia CT requirements are met, this is presumed to be in excess of CT requirements for other monitored microbiological contaminants under the SWTR. In addition separate tables are published for chlorine dioxide, ozone, and chloramine inactivation of Giardia lamblia (USEPA, 2000). The CT table for chloramines is only valid for virus disinfection if chlorine is added prior to addition of ammonia so there is some free chlorine contact time (USEPA, 2000). Specific guidelines for calculating aggregate inactivation ratios for plants with multiple disinfectant addition points are also described by the USEPA (2000). The contact time is the time exceeded by 90% of the fluid, or t₁₀ (Haas, 2000).

Treatment technique requirements pertaining mainly to a) meeting specified CT times, and b) filtration, can sometimes be used in lieu of actual measurements of the maximum contaminant levels (MCLs) for Giardia lamblia, Legionella, viruses, and for meeting turbidity and heterotrophic plate count requirements also (USEPA, 2000). However specific monitoring requirements for turbidity and other measurements are also outlined, depending on how the plant
is categorized according to the regulations. Unless certain criteria are met, filtration is required for treatment of water systems supplied by surface water or surface water influenced groundwater.

Under the SWTR effective filtration is assumed to achieve (credited with) 2.5 log removal of *Giardia* and 2 log removal of viruses. Disinfection is required for the remainder of the removal-inactivation (Haas, 2000).

A number of forthcoming regulations will also exert an influence on disinfection practices. The Stage 1 and Stage 2 Disinfectants/Disinfection By-products Rule (Stage 1 and 2 D/DBP Rule); the Interim Enhanced Surface Water Treatment Rule (IESWTR) and Long-term 1 Enhanced SWTR (LT1ESWTR); and the Ground Water Rule (GWR) will all significantly impact disinfection practices in the U.S.A. For example the GWR is likely to establish a formal multiple barrier approach, and also strict criteria, which, if not met, would require disinfection (Pontius, 2000).

Modern disinfection practice has also recognized that chlorine resistant pathogens, such as protozoans, exist. This has led to a multiple barrier approach that does not rely solely on chlorine or other oxidants to protect public health for source waters where protozoans or other disinfectant resistant pathogens may be present (Haas, 1999). Other treatment processes such as filtration become important aspects of the overall production of biologically safe drinking water. In addition design and operation of treatment processes prioritizing for the concept of robustness rather than peak performance is an important aspect of insuring the safety of the consumer. Practices such as covering reservoirs and other source protection techniques, maintaining positive pressure and corrosion control in distribution systems, use of flushing, pigging, are all
significant aspects of eliminating pathogens and maintaining a biologically stable system (Trussel, 1999).

Secondary disinfectant residual is also an important aspect of practice in the U.S.A. The use of a residual is based on three arguments: 1) suppression of bacterial regrowth, 2) disinfection of exogenous intrusions, 3) as a sentinel to detect intrusions or breaches (Haas, 1999). In Europe elimination of the presumed limiting nutrient, carbon, is often seen as a superior way of suppressing bacterial regrowth (Van der Kooij, 1999; White, 1999), but this point of view is questioned by Haas (1999) who cites several studies in which phosphorus, not carbon, was the limiting nutrient, and he suggests other inorganic nutrients may be limiting in some systems as well. Other practitioners have reported full scale systems where phosphorus rather than carbon was limiting (e.g. Haas et al., 1988).

Volk and LeChevallier (2000) found that coliform occurrences in distribution systems were a function of temperature, disinfectant residual, and Biodegradable Organic Matter (BOM) levels. Threshold values were 15° C, Assimilable Organic Carbon (AOC) > 100 µg/L, and dead-end disinfectant levels < 0.5 mg/L for free chlorine or 1.0 mg/L for chloramines.

**Bacterial Nutrients in Drinking Water Distribution Systems**

The bacterial growth in water distribution systems is due to the natural presence in soils and the waters of bacteria. Most of them are participating in one of the following elementary cycles: 1) the carbon cycle, 2) the nitrogen cycle or 3) the sulfur cycle. Iron oxidizing and iron respiring bacteria can also be significant. The bacteria involved in the carbon cycle are heterotrophic organisms, which are very diverse in terms of their metabolism. They can use several electron acceptors (i.e. oxygen, nitrates, nitrites, sulfates, ferric ions). The autotrophs
(such as nitrifiers) can initiate the colonization of the inner surface of the distribution systems.

So, by converting inorganic carbon to organic carbon the autotrophs produce a nutrient source for the heterotrophs. Then the autotrophs are the primary initiators of a more complex food chain and make the colonization by heterotrophs possible even under low carbon level conditions. For that reason, a multi-nutrient monitoring is necessary. The limitation of carbon source only cannot control bacterial growth in a distribution system if other nutrients such as ammonia nitrogen are abundantly present. However since carbon is widely considered as the limiting nutrient, several methods for the measurement of biodegradable or assimilable organic carbon are used to evaluate biological growth in distribution systems.

There are several methods to quantify the potential of bacterial regrowth in water samples. Those methods are divided into two different types: 1) those which directly quantify a limiting nutrient (usually biodegradable dissolved organic carbon, BDOC), and 2) those which directly quantify the bacterial growth in the sample (e.g. AOC).

The methods which quantify a limiting nutrient often assume carbon as the limiting nutrient for growth (i.e. BDOC). In addition there are methods that directly measure heterotrophic bacterial growth in a way implying that carbon is the limiting nutrient (i.e. AOC). However it can be found in the literature examples of distribution systems for which other nutrients, such as phosphorus, were limiting. Haas(1999) observed a greater growth with phosphorus addition than with carbon addition, which could be significant since several corrosion inhibitors are based on ortho- and poly-phosphates. In addition, it has been suggested in the literature that the global effect of those inhibitors to prevent the biofilm development is associated with the saturation of adsorption sites at the inner surface of the pipes. This blocks simultaneously the adsorption of natural organic matter (NOM) – which could be used as
substrate for fixed biomass – and the adsorption of microorganisms at the surface (Haas, 2000). Certain methods have been specifically developed to determine the effect of phosphorus as major limiting nutrient (Lehtola et al., 1999). Moreover, certain methods such as AOC sometimes give relatively high results when the samples receive inorganic nutrients (Charnock and Kjonno, 2000) indicating that carbon is not the only limiting nutrient of interest for bacterial regrowth.

Other nutrients are also significant since they can lead to the production of organic carbon from inorganic carbon in a distribution system biofilm. For example, if ammonia and oxygen are present, nitrifying biofilms and planktonic bacteria can develop. The nitrifying bacteria are autotrophs, thus converting carbon dioxide/bicarbonates into organic carbon, they produce new biomass and eventually new soluble microbial products that can be released in the system.

**Bacterial Growth in Distribution Systems**

**Definition**

Biostability is a concept that addresses the overall tendency of the water to promote or suppress microbial proliferation, and can be viewed as an assessment of overall distribution system quality with respect to microorganisms. It pertains to the proliferation of microorganisms in the water distribution system and does not address the ecology of opportunistic or other pathogens or coliforms. Biostability describes aggregate proliferation and does not address the fate of specific subpopulations. As a result biological instability may or may not favor the proliferation of coliforms or a specific pathogen(s) depending on a host of additional factors.
Brazos and O’Connor (1985) proposed specific definitions for two terms that have been synonymously used to describe the unexplained occurrence of blooms or high bacterial population in potable water distribution systems: “regrowth” and “aftergrowth”.

Regrowth is the recovery of disinfectant-injured cells which have entered the distribution system from the water source or treatment plant, while aftergrowth is growth of microorganisms native to a water distribution system. These definitions do not clearly discriminate between the two primary mechanisms by which the microorganisms appear in the distribution system, i.e. breakthrough in the treatment plant and growth within the distribution system (van der Wende and Characklis, 1990).

Breakthrough is the increase in bacterial numbers in the distribution system resulting from viable or injured bacteria passing through the disinfection process, which is only meant to suppress pathogenic organisms. Injured cells have the ability to recover and as well as viable cells can inoculate the biofilms and/or reproduce in the bulk water. Growth is the increase in viable bacterial numbers in the distribution system (either in biofilms or in the bulk water) resulting from bacterial growth downstream of the disinfection process (van der Wende and Characklis, 1990).

**Bacterial Enumeration and Identification**

Bacterial growth defined as the increase in the mass or number of microbial cells in a population can occur in distribution systems either in the bulk liquid phase or at the pipe wall. Different techniques apply to evaluate this growth. One should be aware of the limitations of each of these techniques before implementing them in an experimental plan.
Techniques Applied to Suspended Bacteria

Microscopy

Light microscope (in bright-field, phase-contrast or fluorescence configuration) can be used to observe and enumerate bacteria present in water samples, so called direct microscopic count. It has however limitations: (i) dead cells are not distinguished from living cells, unless an advanced staining technique is used (i.e. live-dead staining technique), (ii) small cells may be difficult to visualize, and (iii) contaminants (e.g. inorganic corrosion products) makes cell counting difficult (Ollos, 1998).

Studying detailed structure of cells is performed using electron microscopes. Transmission electron microscopes (TEM) are used to study the internal structure of cells. In the TEM, electrons are used instead of light rays, and electromagnets function as lenses. Scanning electron microscopes (SEM) are used to observe external features of an organism. Intact whole cells can be examined using SEM. However only the surface of a specimen can be observed with SEM. The new application of confocal laser scanning microscopy (CLSM) to environmental engineering provides accurate high resolution of living, fully hydrated microbial biofilms. It allows, for the first time, three-dimensional imaging of intact biofilms.

Standard Plate Count Procedures

The most common application of plate count in monitoring of microbiological water quality is heterotrophic plate count. The cell suspension is plated in duplicate or triplicate on non-selective, low-nutrient R2A agar. After plating by either spread, pour or streak plate methods (Brock et al., 1994), the plates are typically incubated for seven days in the dark at 25℃ and the colonies are enumerated after incubation (APHA, 1995). In order to obtain an accurate
count, it is recommended that the number of colonies developing on the plates should not be too large or too small. Therefore serial ten-folds dilutions are performed, if necessary, before plating in order to obtain counts falling in a 30-300 cfu/mL range, which is commonly selected for proper enumeration.

The use of media for cultivation leads to a major limitation of these techniques. A given media type is by essence selective of a given metabolic group. Therefore plate counts do not provide with an accurate understanding of the diversity of the microbial population present in a water sample. Moreover, since only viable, culturable organisms can be cultivated, a significant fraction of viable but non-culturable organisms are not accounted for with these techniques. For example, McFeters et al. (1986) reported that injured coliforms were largely undetected by the use of an analytical media. Stewart et al. (1994) postulated that plate counts may seriously overestimate biocide efficacy if the culture technique fails to detect injured organisms.

**Fluorescent In-Situ Hybridization (FISH)**

Oligonucleotides (short strands of nucleic acids – usually 15-30 nucleotides in length), complementary to 16S rRNA sequence regions with an intermediate degree of conservation and characteristic for phylogenetic entities like genera, families, and subclasses, have been used successfully for rapid identification of bacteria. The oligonucleotides are able to enter fixed bacterial cells and once inside the cells, they may form stable associations (hybrids via hydrogen bonding between complementary nucleotides) with the 16S rRNA in the ribosomes. If the complementary sequence for the nucleotide is not present in the 16S rRNA in the ribosome, stable hybridization does not occur and the oligonucleotide is washed from the bacterial cell. Thus the “targets” for the oligonucleotides are the ribosomes of which there are up to $10^4$ per
actively growing bacterial cell. In order to observe when hybridization occurs, the oligonucleotides also contain a “reporter” molecule or label, which is often a fluorochrome. Cells in which the fluorescently-labeled oligonucleotide has hybridized with the 16S rRNA in the ribosome can be directly visualized by epifluorescence microscopy. This technique is called fluorescence in situ hybridization (FISH) or whole cell probing (Blackall, 2002).

FISH can be used, for example, to accurately detect and quantify in a timely manner ammonia-oxidizing bacteria (AOB) in drinking water samples or biofilms, as compared to the traditional cultivation technique that require three weeks of incubation.

**Techniques Applied to Biofilms.**

Historically most methods to quantifying biofilm inventories have relied on detaching the biofilm, suspending and homogenizing the cells, and then enumerating and/or isolating the organisms using selective media (e.g. agars). This allows quantification and/or identification of the organisms, but only of the culturable population for the chosen selective media. Moreover the detachment step is delicate and may damage the cells and affect their viability and/or culturability. Sonication has been used as a mean for removing biofilm from a supporting surface, such as coupons (Mathieu et al., 1993). A minimum of 80% removal efficiency and “guaranteed” variability were reported. In the other hand, Stewart et al. (1994) reported that the scraping procedure removed 95 to 98% of biofilm organisms. After homogenization (Camper et al., 1985), the total cells can be enumerated by plate count or microscopy techniques described previously.

A variety of approaches to quantify the biofilm inventory or activity without disruption due to detachment or the bias of selective media have been developed over the years. One basic
approach is the combination of advanced microscopy with molecular staining. Advanced microscopy alone (e.g. SEM) is unable to consistently differentiate cells and inorganic material based on morphology alone. Molecular stains specific to DNA, respiration, or membrane components and other molecules unique to biotic material seem to provide a mechanism to achieve this. However many stains (e.g. DAPI) can be non-specific when corrosion products, precipitates, and pipe material are present. Debris or corrosion products, commonly present in actual distribution systems, have a real detrimental effect on the measurements, since they are detached with the cells and interfere with microscopic techniques, especially iron oxides (Hobbie et al., 1977; Porter and Feig, 1980; Lisle et al., 1998). In addition some pipe materials cause high background when using epifluorescent microscopy. Chang et al. (2003) investigated the coupling of Ramen spectroscopy and molecular staining with a specific DNA-probe for determination of biofilm inventories on PVC, unlined and lined cast iron and galvanized steel coupons, without detachment of the cells. Their results showed that background (also referred to as “noise”) from inorganic materials was significantly reduced thanks to the emission wavelength of the chosen probe. Fluorescent intensity of the probe was successfully correlated to traditional plate count results performed after scraping and resuspension of the biofilm cells.

Another approach for non-destructive techniques is to use the enzymatic properties of bacteria. The Potential of ExoProteolytic Activity (PEPA) assay described by Billen (1991) and Servais (1995) can be used to quantify global activity of the biofilm on supporting materials without detaching the cells. Exoproteolytic activity has been shown to correlate with microbial biomass (Somville and Billen, 1983; Laurent and Servais, 1995). No significant difference was observed in the ratio PEPA/biomass for the bacteria from natural water samples and for bacteria detached from solid supports (Billen, 1991). As a result this assay has been used to quantify both
suspension and fixed biomass inventories (Billen, 1991; Laurent and Servais, 1995; Butterfield et al., 2002).

**Biological Instability of Potable Waters along their Distribution.**

The potable water in distribution systems transports an extremely diversified microbial flora (Block et al., 1997; Doggett, 2000; Percival et al., 2000) and complex organic matter (Croué et al., 2000). A relatively significant fraction is biodegradable (Block et al., 1992; Servais et al., 1992a). Even when the organic fraction is considered to be small: so called oligotroph ground waters (<0.5 mg DOC/L) or nanofiltered waters (<0.2 mg DOC/L), bacterial growth is observed (Crissot-Laruade et al., 1999; Devender, 1995; Levi et al., 1992; Sibille et al., 1997). Three remarkable facts explain the difficulty of managing this situation:

- In most potable water distribution systems, the water/material interface is a favored site for cells and organic matter accumulation and for multiplication of bacteria (Block et al., 1993; Servais et al., 1992b and c, 1995a; Van der Wende et al., 1989). This proliferation is followed by the detachment (Ascon et al., 1995) or by their displacement from the pipe surface to the bulk liquid (Rittman, 1989) and by their transport in the bulk liquid.

- This bacterial population is adapted to the oligotroph environment of potable waters (less than 2 mg/L of biodegradable organic matter in most of the cases). It is very difficult to destroy this microbial ecosystem, and even when the nutrient flux is reduced (less than 200 µg/L of biodegradable organic matter), the bacterial biomass is only slightly reduced (Block, 1998, personal communication).
• The imported bacterial biomass (breakthrough) and bacteria that grow in the system (regrowth) represent the starting point of a complex trophic chain.

Potable water distribution systems work as continuously fed reactors in which physico-chemical (corrosion, chlorine consumption, flocculation, particles sedimentation, etc) and biological reactions (biological growth) are carried out in the bulk and at the interface bulk/surface. These reactions are often misunderstood and are always difficult to control. Once the chemical and biological deposits (biofilms) form in the system, pseudo-equilibrium takes place between the water column and the inner walls of the pipes, leading to water with a relatively stable quality and complying with regulations. However, when this equilibrium is upset by changes in the water quality (seasons, storage time, blends), these deposits can be redissolved which implies a new equilibrium. This redissolution can generate release of matter, which can result in a failure to comply with the regulations.

Control of the Microbiological Quality of Waters during their Distribution

The fact that biomass enters the distribution system and can potentially grow there raises at least two related issues in terms of public health:

• The system is constantly sowed by unknown organisms (saprophytes but also opportunistic pathogens) with a high probability of presence of non-cultivable coliforms (estimated to be 0.1 to 1% of the global biomass) which, on certain occasions, can find in the system favorable conditions for their survival and growth, potentially leading to a violation of existing regulations. (Block, personal communication)
A fraction of the microorganisms introduced in the system (and multiplying in the biofilm) may represent a danger for the consumer, have been linked to a relatively high frequency of occurrence for gastro-enteric symptoms (diarrhea and vomiting). The epidemiological surveys published by Paynent et al. (1991, 1997) show a rate of 0.1 occurrence of gastro-intestinal trouble per person and per year resulting from the consumption of water conforming to current regulations. In a different context, for a group of kids more susceptible, Gofti et al. (1999) report even greater values up to 4 digestive troubles/person.year and 1 diarrheic occurrence/person/year. These epidemiological data have been confirmed in a radically different environment (rural area, waters sources simply chlorinated, in compliance with regulations, 2000 kids from 7 to 11 years old, relative risk 1.4;(Zmirou et al., 1995). Most of those epidemiological events with small public impact (i.e. not classified as an epidemic due to a global contamination or an accident) are not identified by the medical community unless a specific study is carried out on exposed populations.

Considering that 10% of the adults that have episodes of illness lose 1 work day, the economical consequences are not negligible (Paynent, 1997 ; Garthright et al., 1988). Moreover the image of the product “potable water”, and public confidence in it suffers from documented cases of contamination. As a consequence, limiting biological instability in water distribution systems and the growth of organisms in the biofilm is critical for both consumers and producers.

Since organic carbon has been widely considered to be a limiting nutrient for bacterial growth in distribution systems, assessment of biostability in such systems often relied on
biodegradable organic matter (BOM) levels in finished waters. Biodegradable Organic Carbon (BDOC) and assimilable organic carbon (AOC) (either individually or in conjunction) have been used to characterize biostability of drinking water in previous studies. Heterotrophic density in distribution water supplies has been significantly correlated to AOC levels (van der Kooij, 1982; LeChevallier et al., 1987). Van der Kooij (1982) showed that heterotrophic bacteria in a non-chlorinated system did not increase when AOC was lower than 10 µg/L. In systems maintaining a 3-6 mg/L chlorine residual, LeChevallier et al. (1987) suggested that coliform regrowth may be limited by AOC levels less than 50-100 µg/L. It has been recommended in the literature that no biodegradable organics should be present after water treatment to limit bacterial regrowth in distribution systems (Block et al., 1993). Biological stability, i.e. no consumption of BDOC within the distribution system has been associated with a BDOC concentration of 0.16 mg/L or less in the finished water, with or without residual (Servais et al., 1993). Volk et al. (1994) proposed 0.15 mg/L and 0.30 mg/L biostability thresholds for BDOC at 15°C and 20°C respectively. The above criteria for biostability of distributed drinking water are summarized in Table 2.

Table 2. Biostability Criteria for Treated Drinking Water

<table>
<thead>
<tr>
<th></th>
<th>No Residual</th>
<th>With Residual</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOC Threshold</td>
<td>10 µg/L as acetate-C (van der Kooij, 1992)</td>
<td>50-100 µg/L as acetate-C (LeChevallier et al., 1987)</td>
</tr>
<tr>
<td>BDOC Threshold</td>
<td>0.16 mg/L C (Servais et al., 1993)</td>
<td>0.15 mg/L C at 20°C (Volk et al., 1994)</td>
</tr>
</tbody>
</table>
Biofilms in Drinking Water Distribution Systems

Organization of Biofilms

The structure of the biofilms in potable water distribution systems is not well described and uncertain (dispersed colonies at the inner surface of the pipes or thick films as deep as a few micrometers?) due to the great difficulty to explore the fixed biomass without disturbing it. Debris, corrosion products, mineral deposits as well as the formation of corrosion clumps (offering new sites or surfaces to be colonized by biomass) complicate this struture (Allen et al., 1980; Sly et al., 1988; Flemming et Geesey, 1990; LeChevallier et al., 1987; Ridgway et Olson, 1981; Stolzenbach, 1989; Tuovinen et al., 1980).

Most of the descriptors (thickness, porosity, density, fractal dimensions) have been generally studied with thicker biofilms, generated in laboratory and therefore are undoubtedly partially not adapted to potable water. However by analogy system biofilms can be described using several approaches:

- The biofilm is composed of a mixture of microorganisms with variable activities, as a function of their position in the aggregate (Rittmann and Manem, 1992). Even microcolonies (50 cells) may represent an association of several genuses (Manz et al., 1993).

- When the biofilm (or the microcolonies) are thin (< 40µm), the oxygen-and-nutrient transfer would not be limited and the parameters describing the activity of the global biofilm would be the same as the one used for bacteria in suspension (Bakke et al., 1984). When the biofilm (or the colony) is thick (>80µm) the respiratory activity in
the deepest layers is reduced (de Beer et al., 1994b). This explains that a fraction of the fixed biomass is less active (Kalmbach et al., 2000; Zhang and Bishop, 1994).

The discontinuity and the heterogeneity of the biofilm structure leads to a non-uniform dispersion at the surface of the materials in contact with water (van der Kooij and Veenendaal, 1992). The aggregates that are distinct from each other in the biofilm are surrounded by canals that occupy up to 50% of the volume of the film and in which there is circulation of water, particles, protozoa, etc (de Beer et al., 1994a et b; Devender, 1995; Gjaltema et al., 1994; Massol-Deyá et al., 1995; Stewart et al., 1993, 1995; Stoodley et al., 1994).

- Then the accumulation of biofilms at the surface of the materials takes place in a zone where the water circulation is slowed by friction on the wall (hydraulics specialists call it the viscous layer, which can be up to 70 µm deep in a cast iron concrete lined pipe with a flow rate equal to 1 m/s). The transfer of molecules (oxygen, disinfectant, nutrients) is limited by their diffusion velocity.

- The total microbial count on those surfaces in contact with drinking water is generally high (ranging from $10^6$ to $10^8$ cells per cm$^2$) (Donlan and Pipes, 1988; Lévi et al., 1992; Mathieu et al., 1992; Pedersen, 1990). The organic carbon content of this biomass is low: from 0.1 to a few µg organic carbon per cm$^2$ (Niquette et al., 2000; Fass et al., 2001).

- The stationary phase is undoubtedly never reached in a real distribution system, due to frequent discontinuities: variation in the hydraulic regime, changes in the nature and concentration of nutrients and disinfectants, introduction of new microorganisms.
The recent results from Zacheus et al. (2000) show that there is a slow but continuous accumulation of biomass after 5 months of exposure to potable water. Percival et al. (1998) observed a switch in the dominant bacterial populations from the “pioneers” which colonize a steel pipe in less than one month to the species existing after 5 months of immersion in the water. Finally by analogy with the actual concepts of differentiation of biofilms during their ageing (Allison et al., 2000), there is also reorganization of physical biofilm system as it develops there (formation of microaggregates, canals, etc.).

**Biofilm Activity**

Even if the density of fixed microorganisms is up to $10^6 – 10^8$ cells per cm$^2$ in most distribution systems, the species diversity and their activity are highly variable and not well known due to obvious technical limitations.

First, having a precise knowledge of the existing species is currently limited by the difficulty of cultivating these microorganisms (Byrd et Colwell, 1991; Colwell et Grimes, 2000; Rozack et Colwell, 1987). The Heterotrophic Plate Count method (HPC-R2A USEPA Standard Methods) still remains a reference technique, widely used (Reasoner, 1990), which can be correlated to enumeration techniques based on respiratory activity (INT or CTC marking) (Coallier et al., 1994; Rodriguez et al., 1992; Yu et McFeters, 1994), and total direct count (Saby et al., 1997).

Plus, the cell production can vary in a large range. For example, by incorporation of thymidin (Servais, 1988), Servais et al. (1992) measured bacterial production from 0.001 to 0.008 $\mu$g C cm$^{-2}$ h$^{-1}$ in systems under study. Block et al. (1993), using the formalism and the
hypotheses of van der Wende et al. (1989) (growth in the biofilm largely greater than in the bulk liquid, low decay without disinfectant) calculated times for doubling the fixed biomass from a few days to a few months according to the nutrient content of the waters. Donlan et al. (1994) demonstrated biofilm growth rates from 0.1 to 5.5 log cells cm\(^{-2}\) d\(^{-1}\) due to seasonal effect.

**Parameters Controlling the Accumulation of Biofilm**

The accumulation and proliferation of fixed bacteria under the form of biofilms is usually controlled by a large number of parameters, more or less well studied at bench scale and more rarely in the field at full scale (hydraulics, temperature, nature and concentration of nutrients, bacterial density and species introduced in the system, nature of pipe materials, predators)(Figure 3).

![Figure 3. Scheme of Several Mechanisms Taking Place in the Accumulation of Biofilms on a Surface in Contact with Potable Water.](image-url)
It has not been demonstrated yet that the concentration of biodegradable organic matter in distributed water directly controls the bacterial density in the biofilm (Block, personal communication).

The biofilm appears to be a metastable system, in one hand “fed” by the entrance of cells from the bulk (the velocity of deposit/fixation of cells on the surface of materials is correlated to the cell density of moving waters) and by the multiplication of bacteria, and in the other hand stabilized by the constant pulling up and release of cells from the biofilm to the bulk.

Under these conditions, the organization of the biofilm and the structure of the settlements mainly depend on several other factors:

- Bacterial species introduced in the system from the treated or non-treated source. An astonishing example is the case of acidic boreal waters, characterized by a high content of humic substances and acido-resistant bacteria. Once treated, these sources allow the growth of biofilm with classical cell density (about $10^7$ cells/cm$^2$) but with $10^3$ Mycobacterium/cm$^2$ (Iivanainen et al., 1999).
- Competition and advantage given to certain species due to their nutritional requirements. As an example, the presence of ammonium in the system supports the development of a strong autotrophic nitrifying population (Lipponen et al., 1998).
- Nature of the materials used for potable water distribution systems. All of them are largely colonized by microorganisms (Niquette et al., 2000; Zacheus et al., 2000) but
the supporting materials play a significant role in the selection of biomass and its organization (Kerr et al., 1999; Kielemoes et al., 2000; Pedersen, 1990; Rogers et al., 1994; Van der Kooij et al., 1995). Indeed material determines the adsorption efficiency of the “pioneers” and can be source of nutrients or growth factors. Analysis of biofilms adsorbed on glass or polyethylene showed a different proportion of bacteria detected on each material (4 and 26% respectively) by oligonucleotidic probe ALF 1b (Kalmbach et al., 2000). In another situation, polyethylene, PVC, steel and copper display similar cell density but on copper the bacterial activity was a lot less due to the toxicity of Cu ions (Schwartz et al., 2000). Finally, in the case of materials susceptible to corrosion, it has been clearly demonstrated that the presence of iron corrosion products enhances the activity and the production of heterotrophic biomass (Appenzeller et al., 2001).

- Resistance of fixed biomass to oxidants (Morin et al., 1999) can be partially explained by the reducing ability of the biofilm, of the fixed organic matter and sometimes of the supporting material, as well as poor diffusion of the oxidant (de Beer et al., 1994a; Stewart and Raquenas, 1995). The exposure of the biofilm bacteria to sub lethal oxidative stresses (Storz et Zheng, 2000) leads to a remarkable cellular defense (surproduction of intracellular glutathion) (Saby, 1999) and an increased resistance of the bacteria to oxidants (Saby et al., 2001).
Chloramination and Nitrification

Originally the addition of ammonia and chlorine in combination to form chloramines was used for taste and odor control and became widely adopted during the 1920s and 1930s (White, 1999). However the discovery of breakpoint chlorination in 1939, and the need of ammonia for the war effort during the early 1940s caused the process to be largely abandoned (White, 1999). By the early 1980s, driven by disinfection by-product (DBP) regulations, chloramines were allowed first as a residual disinfectant, and then as a primary disinfectant by the EPA. Over the years even more stringent DBP regulation has driven many utilities to switch to chloramines as secondary and primary disinfectant. The D/DBP rule (USEPA, 1998) states levels of THMs not to exceed 80 µg/L and levels of HAAs not to exceed 60 µg/L. Chloramines reduce the potential for DBPs formation with respect to free chlorine (Brodtmann and Russo 1979; Norman et al. 1980; Mitcham et al. 1983). Generally preammoniation (addition of ammonia prior to chlorine) leads to the lowest formation of DBPs such as trihalomethanes (White, 1999). However, no clear consensus exists on the best method of chemical addition (pre, simultaneous, or post ammoniation; (Haas, 2000). The 3 log tables for Giardia inactivation only apply for 4 log inactivation of viruses when chlorine is added first, however (USEPA, 2000), and with other types of addition strategies the plant must demonstrate 4 log inactivation through other means.

The USEPA accepted chloramines as a secondary disinfectant in 1978, and as a primary disinfectant in 1983 (White, 1999). However a longer contact time is required to obtain similar disinfection levels with chloramines as compared to free chlorine. The D/DBP Rule limits average distribution system concentrations to 4 mg Cl₂/L or less (USEPA, 1998). Chloramines require extreme concentration times contact time (CT) in order to comply with regulations for
pathogen inactivation, which severely limited its use as primary disinfectant (Malcolm Pirnie, Inc. and HDR Engineering, Inc. for USEPA, 1990). In addition chloramines, since they contain ammonia, can potentially act as a food source for autotrophic bacteria associated with the nitrogen cycle. These autotrophs then act as a primary producer for heterotrophic bacterial growth. This effect has been observed with blending of different source waters in the Metropolitan Water District (Los Angeles, California), resulting in nitrification in water reservoirs (White, 1999). In addition problems with dialysis machines occurred there with the use of chloramination since it gave no obvious visual signs of activated carbon exhaustion as the free chlorine residual did. Chloramine residuals must be neutralized by GAC or ascorbic acid as they enter dialysis machines.

Odell et al. (1996) found that two thirds of US systems using chloramination experienced problems with nitrification in the distribution system. Effective control methods included periodic breakpoint chlorination, reducing the available ammonia, increasing chloramine residual levels, cleaning/flushing/pigging the distribution systems, and decreasing system hydraulic retention times. Some strategies were superior for short term problems whereas others reduced the long term potential for nitrification. Nitrification associated with long storage times, or increasing drawdown elevation, has been observed so storage is an important factor in storage tanks and covered reservoirs (Odell et al., 1996). Nitrification was accompanied by a decrease in ammonia concentrations below 0.2 mg/L as N, an increase in nitrite levels from 0.01 to > 0.1 mg/L as N, and no noticeable change in nitrate at about 1.5 mg/L as N. In other systems another main indicator of nitrification was a loss of chloramine residual in dead ends of the system. However there was sometimes a significant lag between the depleting of the chloramine residual and the onset of ammonia to nitrite conversion in both lab and full scale observations. Studies
showed that a too low residual (below 0.1 mg/L as Cl₂) was conducive to nitrification whether ammonia levels were low or high and was an important prerequisite for the acceleration of nitrification events. pH decreases and increased HPCs also accompanied nitrification. However they noted that in other episodes no increase in HPC was observed. A decrease in dissolved oxygen often accompanied nitrification episodes.

Odell et al. (1996) found from their survey of full scale utilities that systems with low dissolved oxygen concentrations were less likely to experience nitrification problems. Nitrification was also possible even in systems with low ammonia levels if other conditions were right. High temperatures (above 25°C) facilitated nitrification. High chloramine residuals inactivated nitrifying bacteria, but once a nitrification event began even high levels were ineffective and quickly degraded in the presence of nitrite. Thus chloramine levels were good for preventing nitrification but not good for controlling an event already under way. Removal of Natural Organic Material (NOM) prevents the conversion of inorganic chloramines to less bactericidal (but normally undistinguishable) organic chloramines in the distribution system. Thus NOM removal can result in a more biologically stable water not only with respect to heterotrophic growth, but also with respect to autotrophic nitrification. This tends to support Whites (1999) contention that the European approach of removing NOM rather than relying on disinfectant residual, produces a superior, albeit more expensive finished water. However there is even more widespread disagreement with this point of view (Haas, 1999; LeChevallier, 1999) for other reasons. Odell et al. (1996) note that treatment plant processes, which remove NOM or promote complete nitrification in the treatment process (e.g. biologically active filters) reduce the potential for nitrification in the distribution system.
Odell et al. (1996) rated the various control methods for nitrification for both episode control, and in terms of long term benefit (Table 3). Breakpoint chlorination is often used once or twice yearly for periods of one week to one month. Chloramine residuals of at least 2 to 3 mg/L throughout the distribution system are thought to be needed for nitrification prevention. One study showed AOB could survive 2 mg/L of chloramines for 60 minutes (Wilczak et al., 1996). Biofiltration is practiced in Europe for NOM removal and sometimes nitrification, but no study has been conducted on biofiltration to control nitrification.

Table 3. Evaluation of Control Methods for Distribution System Nitrification

<table>
<thead>
<tr>
<th>Control Method</th>
<th>Episode Control</th>
<th>Long-term Improvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breakpoint Chlorination</td>
<td>Superior</td>
<td>Fair</td>
</tr>
<tr>
<td>Reduction of available ammonia</td>
<td>Fair</td>
<td>Good</td>
</tr>
<tr>
<td>Or increase Cl:N ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increase Chloramine Residual</td>
<td>Fair</td>
<td>Good</td>
</tr>
<tr>
<td>Remove NOM</td>
<td>Good</td>
<td>Unknown</td>
</tr>
<tr>
<td>Clean Distribution System</td>
<td>Good</td>
<td>Good</td>
</tr>
<tr>
<td>Improve Distribution System</td>
<td>Good</td>
<td></td>
</tr>
<tr>
<td>Detention Times/Hydraulics</td>
<td>Good</td>
<td>Good</td>
</tr>
</tbody>
</table>

Source: adapted from Odell et al., 1996

Improving system detention times refers to a variety of improvements including reduced storage times, increased drawdowns, looping dead-end mains, recirculation or rechlorination facilities on standpipes and elevated storage, reservoirs with baffles preventing short circuiting, and increased reservoir turnover.
Wilczak et al. (1996) noted that no one water quality parameter was a good indicator of nitrification. Nitrite or alternately nitrate were sometimes the predominant form of oxidized nitrogen observed. Typically it was nitrite, probably because nitrite reacts so rapidly with chlorine/chloramine residual. Thus the kinetics of nitrite consumption are probably more rapid than the kinetics for nitrite oxidation to nitrate in many systems. However in a few systems nitrate was observed, and thus must be monitored also.

Other parameters that are useful in assessing nitrification are chloramine dosage and residual, ammonia, pH, heterotrophic plate counts, and dissolved oxygen (Wilczak et al., 1996). HPC and AOB numbers have been observed to correlate with each other above HPC counts of 350 cfu/mL in a Metropolitan Water District study (cited in Wilczak et al., 1996). Ammonia, alkalinity, pH, and TOC were not found to be very sensitive or accurate indicators of nitrification in the same full scale survey by Wilczak et al. (1996).

Another aspect of quantifying chloramine residual is that chloramines formed from organic rather than inorganic nitrogen will be measured by conventional methods as residual but are actually far less effective as disinfectants (Haas, 2000; White, 1999). This is one reason why systems with apparently sufficient combined chlorine residual to inhibit nitrification may experience it (Odell et al., 1996). These organic chloramines are measured by forward titration using either the amperometric or the DPD-FAS method and appear in the dichloramine fraction (White, 1999). Thus the measured chloramine residual may be very high, but deceptively so, when organic nitrogen has reacted with chlorine or chloramine residuals (Wilczak et al., 1996; White, 1999). In some systems increases of nitrate and nitrite were observed even with 3 to 6 mg/L chloramine residuals (Wilczak et al., 1996), however it was not reported whether this was due to organic chloramine presence or other factors.
Method detection limit is a concern for oxidized nitrogen forms since changes in nitrite and nitrate associated with nitrification can be significant as low as 50 micrograms/L (Wilczak et al., 1996) although changes can be as high as 1 mg/L as N (possibly exceeding the MCL for nitrite; the current MCL for nitrate is 10 mg/L, nitrite is 1 mg/L, and combined nitrate + nitrite is 10 mg/L as N, Pontius, 2000). In one case nitrate MDL for the automated cadmium reduction method was lowered by increasing cell-path length, increasing electronic gain on the colorimeter, increasing sample-reagent mixing, sample-to-wash ratio, and sample-to-reagent ratio (Wilczak et al., 1996). Odell et al. (1996) noted that a significant fraction of utilities did not notice nitrate increases during nitrification probably because they exclusively focused their monitoring efforts on nitrite. Wilczak et al. (1996) noted from a full scale survey that measurement of both nitrite and nitrate was absolutely necessary since either or both could be an important indicator of a nitrification event.

Nitrification is caused by the growth of ammonia-oxidizing bacteria (AOB), and nitrite oxidizing bacteria (NOB), in the distribution system. AOB use ammonia as an energy source, converting ammonia to nitrite. Nitrite is known to accelerate monochloramine decay through an abiotic oxidation-reduction reaction which liberates ammonium, chloride, and nitrate as end products. The subsequent disappearance of the disinfectant residual along with production of ammonium can lead to the proliferation of AOBs, producing more nitrite and consuming more chloramines in a residual “death spiral”. In addition the disappearance of residual can lead to an increase of heterotrophic plate count (HPC) bacteria in the distribution system and may result in violation of the disinfectant residual requirements in the Surface Water Treatment Rule. One method of controlling nitrification is the addition of free chlorine for brief periods of time. However some utilities use this as a last resort due to concern over non-cancer end points.
associated with free chlorine such as spontaneous abortions. However other utilities practice periodic chlorination more routinely to control nitrification (Odell et al., 1996) but they have found this also results in high coliform positive samples.

The chlorine to ammonia-nitrogen ratio is seen as a fundamental way of controlling nitrification in a chloraminated system. However even with a 5:1 ratio a small amount of free ammonia is always present in chloraminated systems (McGuire et al., 1999). McGuire et al. (1999) propose the use of chlorine dioxide as primary disinfectant, resulting in small concentrations of the disinfectant byproduct chlorite, which results from degradation of chlorine dioxide. Then chloramines could be used as the secondary disinfectant with chlorite inhibiting ammonia oxidizing bacteria.

Chlorine to ammonia ratio (Cl-to-N) is a significant parameter for ammonia oxidizing bacteria (AOB) growth since the presence of free ammonia is one of the roots of AOB colonization of distribution systems. AOBs tend to be in greatest abundance in sediments and in storage reservoirs, particularly when temperatures are from 77 to 82°F and detention times are significant (White, 1999). Chloramines can inactivate AOBs at sufficiently high concentrations but this has been found to be temperature dependant (Lieu et al., 1991 and 1993) and they recommend a ratio > 5:1 for chlorine:nitrogen. At 15° a residual of 2 to 2.5 mg/L coupled with a 5:1 or greater ratio was sufficient to prevent nitrification. Norton and LeChevallier (1997) noted that one full scale utility monitors free ammonia and adjusts the Cl:N ratio accordingly to minimize the free ammonia concentrations entering the distribution system (usually below 0.1 mg/L). It is generally believed that chloramines are superior to free chlorine for controlling biofilms (Haas, 2000). Odell et al. (1996) noted that sunlight can inhibit nitrification in uncovered reservoirs.
An alternate method of nitrification control that uses neither breakpoint chlorination (i.e. free chlorine residual) or conventional Cl:N ratio control is breakpoint ammonia reduction treatment (BART; Murphy et al., 1998). In this strategy enough chlorine is added to go partway to destroying the combined chlorine residual (i.e. to the downward slope of the breakpoint curve), but without reaching the breakpoint. The desired point is where the free ammonia concentration as close to zero as possible, but to stop while there is still significant combined chlorine residual remaining. By using this technology in a full scale system Murphy et al. (1998) were able to maintain a higher chloramine residual within the distribution system even in areas where maintenance had been difficult prior to using BART. This rapid change was followed by a slow decrease in oxidized nitrogen forms over the following 2 month period. The plant was able to reduce disinfectant applications and eliminate a booster station as well. However there was still some seasonal variations in nitrate and nitrite levels indicating that nitrification had been reduced but not eliminated. The main disadvantage the authors noted was that it was somewhat more difficult to maintain consistent chloramine residuals in the region of the breakpoint curve they were trying to attain.

Recent studies have also shown an apparent connection between nitrification and copper corrosion (Haas, 2000). Norton and LeChevallier (1997) noted that high Assimilable Organic Carbon (AOC) levels corresponded to pitting corrosion suggesting that heterotrophic bacteria (e.g. some sulfate reducing bacteria, iron respirers, aerobes and denitrifiers) may influence corrosion as well. They also noted that in some distribution systems nitrification problems may not occur for several years after the switch to chloramines, but that in some cases it may take only a number of months.
**Water Quality Modeling**

It is essential to understand, describe and model the various phenomena, which lead to the evolution of water quality during distribution. Mathematical modeling is necessary in order to take all parameters into account in view of the complexity of the different phenomena involved.

**Single Species Models**

The early water quality models were steady-state models that describe reactions using independent, single species mass balances typically using first-order kinetic terms. These models evolved to be capable of dynamic (i.e., non steady-state) solutions. The most significant model of this type is the Dynamic Water Quality Model (DWQM) documented by Grayman, Clark, and Males (1988). DWQM is the basis of the original water quality module contained in the widely used EPANET hydraulic and water quality model. Later versions of EPANET add a pipe wall demand to simulate constituents reacting at pipe surfaces, such as chlorine loss at iron pipe surfaces (Rossman, Clark, and Grayman, 1994).

Additionally, many other commercially available models have incorporated single species, first-order kinetics interfaced with a graphical interface, including WaterCAD, H2ONET, Synergee, and PICCOLO-Chlorine. Because of their simplicity, single species models are rapidly solved for full-scale distribution networks and the results of field studies have shown that they can be adjusted to fit the propagation of disinfectant residuals and fluoride tracers in real distribution systems (Grayman, Clark, and Males, 1988; Clark *et al.*, 1992; Rossman, Clark, & Grayman, 1994; Vasconcelos, *et al.*, 1997).
Although single species models can fit distribution system data, they are limited in the water quality processes they can model. Unless a process can be represented as an independent species, it is beyond the scope of these models. For example, the chlorine demand in the bulk water actually depends on reactions with organic and inorganic compounds. These reactions depend on the concentration of chlorine and the reactive species (e.g., organics matter or nitrite), which change within the distribution system.

Therefore, single species models do not allow a deep understanding of the trends that influence water quality in distribution systems. Because the kinetic parameters are site-specific fitting parameters, the single species models cannot predict results for other systems or for the same system when significant changes are made to operation or input quality. To help overcome these limitations, some researchers have developed the multispecies models described in the next section.

**Multi-Species or Next Generation Models**

Multi-species models more accurately describe microbial metabolism and disinfectant decay by using sets of interdependent, multi-species, mass-balance equations based on fundamental processes. The first multi-species model designed for drinking water systems is the SANCHO model described in Servais *et al.* (1995). The SANCHO model contains mass-balance equations describing microbial synthesis, BOM utilization, chlorine reactivity with organic matter, and disinfection processes. Also, SANCHO calculates biomass concentrations in the bulk water and attached to pipe surfaces. The SANCHO model is limited to the analysis of straight pipes of decreasing diameter. However, SANCHO was recently applied to full-scale distribution
systems by using detention times calculated by a hydraulic model to project the SANCHO water quality solution to the distribution system (Laurent et al., 1997). The SANCHO model has proven to be a useful research and analysis tool.

Another major multi-species model is described by Dukan et al. (1996). This model contains similar processes to those contained in the SANCHO model, but these models differ in how some of these processes are represented. For example, the Dukan et al. model contains a complex, multi-level biofilm growth and disinfection submodel. Furthermore, it accounts for important chlorine loss by reactions with pipe surfaces. The Dukan et al. model was originally calibrated to a pilot-pipe system. Later, it was revised to be solvable for, and was field-tested with, a full-scale distribution system networks as the PICCOBIO model (Piriou, et al., 1998).

Another multi-species model called the Comprehensive Disinfection and Water Quality Model (CDWQ) has been developed. The CDWQ model addresses special issues of systems where chloramines are used for disinfection. CDWQ contains a detailed chloramine and free chlorine chemistry subroutine to accurately model chloramine and chlorine decay and heterotrophic and nitrifying bacterial processes. The CDWQ model will be commercially available as the PICCOBIO-Chloramine model planned to be available by July of 2002.

The CDWQ model is a great example of how computer models can be used to analyze water quality issues and develop water quality improvement plan for a full scale distributions system.

As an example, PICCOBIO is described below to gain a better understanding of the approaches involved in water quality and biofilm accumulation modeling.
PICCOBIO is a determinist model that has been developed by Piriou et al. (1996, Lyonnaise des Eaux-CIRSEE) to predict bacterial variations (viable and total bacteria) during distribution. The model takes into account:

- The fate of available nutrients consumed for the growth of suspended and fixed bacteria;
- The influence of temperature on bacterial dynamics;
- The natural mortality of bacteria by senescence and grazing;
- The mortality resulting from the presence of chlorine in water (HClO/ClO) depending on pH on the mortality rate;
- The deposition of suspended bacteria and the detachment of fixed bacteria;
- The chlorine decay kinetics under the influence of temperature, hydraulics and pipe materials.

The modeling of the fixed biomass as a layer uniformly distributed over the pipe surface, expressed as an equivalent thickness of carbon, has been adopted. By this way, a differentiation between the mathematical expression of the free and that of the fixed biomass was made in the model. This means it is possible to distinguish between phenomena depending on their locations: reactions in solution, reaction at the water/biofilm surface interface and within the biofilm.

This model proposes also an original approach for chlorine bactericidal action on suspended and fixed biomass. To model the action of chlorine on the fixed biomass and its stronger resistance compared with the free biomass, the diffusion of the chlorine through the boundary layer and the biofilm has been taken into account. This calculation of the average penetration depth of the chlorine front into the biofilm enables the identification of two layers: a
chlorinated layer and a layer not attained by the chlorine which provides a material indication of the better resistance of the fixed biomass.

As detachment is a key phenomenon in the modelling of bacterial dynamics in distribution systems, the influence of different formulas of detachment kinetics on the mathematical expression of model variables were determined by solving model equations.

The model has been interfaced with PICCOLO software, the SAFEGE hydraulic calculation model. It is constructed by using hydraulic results previously generated by PICCOLO and a numerical scheme to predict bacterial count at each node and on each link of a network. Installed on a PC type computer, the model uses the graphic interface of PICCOLO and provides an effective and easy way to visualise on a computer screen water quality variations in the network, using a colour code for bacterial count, nutrient concentration and chlorine residual.

The first model calibration was done using data from our pipe loop pilot under various operating conditions. The model has been also used to simulate a variety of distribution systems of different sizes and levels of details and a validation of the model has been carried out by means of measurement campaigns on different distribution systems.

Animating and visualising variations of bacteria counts in distribution system is an unique approach to study the changes in water quality.

This tool is helpful to propose strategies for the management of distribution systems and treatment plants and define the different zones of bacterial regrowth in relation with hydraulics conditions (Piriou et al., 1996).
CHAPTER THREE: METHODS AND PROCEDURES

Pilot Distribution System (PDS) Design

The pilot distribution system was designed to mimic water quality changes resulting from single sources and blends of seven significantly different source waters in old distribution pipe systems. The feed water sources represented both the extreme and typical water chemistry likely to be experienced by utilities. The existing distribution systems traditionally received conventionally treated groundwater. Care was taken to maintain the internal film physical and chemical structures during pipe excavation, transportation and construction of the PDSs. Both the physical systems and pipe geometries selected represent typical scenarios experienced in a real distribution system.

The PDS was composed of 18 different distribution lines. Lines 1 to 14 are hybrid lines that have four different materials: PVC, lined cast iron, unlined cast iron and galvanized steel pipes. The PDS was constructed of aged pipes that were obtained from existing utility distribution systems. The pipes were removed from the Member Government’s distribution networks, wetted, capped and transported to the pilot site at CCW (Cypress Creek Wellfield). Once onsite, the pipes were assembled and allowed to equilibrate with TBW (Tampa Bay Water) groundwater over a period of 4 months. After equilibrium was established, different blends were introduced into the PDS. The project was divided into 6 phases; each of three months duration. Similar blends were used in alternate phases to evaluate the effect of seasonal conditions on the PDS and related water quality.
Pilot Distribution System Components

Relevant characteristics of the individual lines include pipe identification, material of construction, pipe length and diameter and feed rates. The pilot distribution systems were identified sequentially (PDS01 to PDS18). The pilot distribution lines were operated to maintain either a five-day or two-day hydraulic residence time.

Pilot distribution systems 01 to 14 were composed of four materials, laid out sequentially as:

- Approximately 20 feet (6.1 m) of 6-inch (0.15 m) diameter polyvinylchloride (PVC) pipe,
- Approximately 20 feet (6.1 m) of 6-inch (0.15 m) diameter lined cast iron (LCI) pipe,
- Approximately 12 feet (3.7 m) of 6-inch (0.15 m) diameter unlined cast iron (UCI) pipe,
- Approximately 40 feet (12.2 m) of 2-inch (0.05 m) diameter galvanized iron (G) pipe

PDS 15 to 18 were composed of a single material each as follows:

- PDS15: Eight reaches of approximately 12 feet (6.1 m) of 6-inch (0.15 m) diameter cast iron each,
- PDS16: Four pipe reaches of approximately 20 feet (6.1 m) of 6-inch (0.15 m) diameter lined cast iron plus 10 feet of 6-inch lined cast iron,
- PDS17: Five pipe reaches of approximately 20 feet (6.1 m) of 6-inch (0.15 m) diameter PVC each,
PDS18: Six pipe reaches totaling 135 feet (41.1 m) of 2-inch (0.05 m) diameter galvanized iron pipe.

All pilot distribution systems were provided with a sampling port after each pipe reach to allow an assessment of water quality changes associated with each pipe reach. Pictures of the structures build for the field study are shown in Figure 4 to Figure 15. As shown in Figure 4, a truck and a stainless steel food grade trailer was used to haul surface water. The surface water was collected as shown in Figure 5 from an intake built by TBW specifically for this project. The raw surface water was stored in two 7000 gallon storage tanks as shown in Figure 6 before treatment. The large process area used to prepare the finished waters is shown in Figure 7 and was covered by a 4400 ft² of 6" cement pad and hurricane rated roof. The five trailers shown in Figure 8 from front to back contained an ozone pilot plant, a reverse osmosis pilot plant, a nanofiltration pilot plant, a storage facility and a field laboratory.

The annular reactors shown in Figure 9 were used for biofilm studies on the various finished waters produced for this investigation. The peristaltic pumps used to control the blends of the various finished waters are shown in Figure 10 with the feed stream standpipes used to regulate flow through the pilot distribution systems (PDSs), which can also be seen in Figure 11. The PDSs are described in detail later in the text, but were made of actual membrane government (MG) pipe taken from MG distribution systems.

The PDSs were followed by coupon cradles as shown in Figure 12 and Figure 13. The cradles were six inch PVC pipes that housed four inch PVC pipes, which had been cut in half and supported pipe coupons for surface characterization studies. Coupon holder and cradle are
presented on Figure 16 and Figure 17. Finally the PDS effluent was directed to a corrosion shed as shown in Figure 14 that contained eighteen looped copper pipes and lead coupons as shown in Figure 15 for the copper and lead corrosion study.

Figure 4. Truck and Stainless Steel Trailer used to Haul Raw Surface Water

Figure 5. Collection of Raw Surface Water using TBW Project Intake

Figure 6. Raw Surface Water Storage

Figure 7. Covered Tanks for Process Treatment
Figure 8. Field Trailers

Figure 9. Annular Reactors

Figure 10. Peristaltic Pumps and Feed Standpipes

Figure 11. Pilot Distribution Systems
Figure 12. Cradles for Housing Coupons
Figure 13. Mounted Coupons

Figure 14. Corrosion Shed
Figure 15. Copper Lines Containing Lead Coupons

Figure 16 Coupons for Biological Characterization Affixed on Coupon Holder
PVC Coupon holder, diam. 3”

PVC Cradle, diam 4”

Figure 17 Description of Coupon Cradle.

**Pilot Plant Operation**

**Finished Waters**

The following is a description of the finished waters produced by pilot water treatment systems (PWTS) located at the TBW Cypress Creek water treatment facility (WTF) project site. All finished waters discharged to the pilot distribution systems (PDSs) were produced at the project site. The seven PWTS and the finished water goals for the project are identified in Table 4 and Table 5.
Table 4. PDS Source Water Descriptions

<table>
<thead>
<tr>
<th>Symbol</th>
<th>System Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>Ground water source. Treatment by aeration, disinfection by free chlorine with a</td>
</tr>
<tr>
<td></td>
<td>residual of 4 mg/L after a 5 minute contact time, 4.0 mg/L combined chlorine</td>
</tr>
<tr>
<td></td>
<td>residual.</td>
</tr>
<tr>
<td>G2</td>
<td>Ground water source. Treatment by lime softening to total hardness of 120 mg/L</td>
</tr>
<tr>
<td></td>
<td>CaCO₃ disinfection by free chlorine with a residual of 4.0 mg/L after a 5 minute</td>
</tr>
<tr>
<td></td>
<td>contact time, 4.0 mg/L combined chlorine residual.</td>
</tr>
<tr>
<td>G3</td>
<td>Blend of finished G1, S1 and RO water source. Treatment by lime softening to total</td>
</tr>
<tr>
<td></td>
<td>hardness of 120 mg/L CaCO₃ or alkalinity of not less than 50 mg/L, 4.0 mg/L</td>
</tr>
<tr>
<td></td>
<td>combined chlorine residual.</td>
</tr>
<tr>
<td>G4</td>
<td>Blend of finished G1, S1 and RO water source. Treatment by membrane nanofiltration</td>
</tr>
<tr>
<td></td>
<td>aeration, 4.0 mg/L combined chlorine residual.</td>
</tr>
<tr>
<td>S1</td>
<td>Surface water source. Treatment by ferric sulfate coagulation flocculation settling</td>
</tr>
<tr>
<td></td>
<td>filtration disinfection by ozonation biologically activated carbon filtration,</td>
</tr>
<tr>
<td></td>
<td>4.0 mg/L combined chlorine residual.</td>
</tr>
<tr>
<td>S2</td>
<td>Surface water pretreated by ferric sulfate coagulation, flocculation and sedimentation, followed by nanofiltration, aeration and disinfection by free chlorine with a residual of 4 mg/L after a 5 minute contact time, 4.0 mg/L combined chlorine residual.</td>
</tr>
<tr>
<td>RO</td>
<td>Ground water source. Treatment by membrane reverse osmosis aeration disinfection</td>
</tr>
<tr>
<td></td>
<td>by free chlorine with a residual of 4 mg/L after a 5 minute contact time, 4.0 mg/L</td>
</tr>
<tr>
<td></td>
<td>combined chlorine residual.</td>
</tr>
</tbody>
</table>

Table 5. Pilot Plant Finished Water Treatment Goals

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Standard</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH units</td>
<td>7.4 min.</td>
<td>0.2 above pHs</td>
</tr>
<tr>
<td>Alkalinity (mg/L as CaCO₃)</td>
<td>40 min</td>
<td>50</td>
</tr>
<tr>
<td>Calcium (mg/L as CaCO₃)</td>
<td>50 min, 250 max</td>
<td>60 mg/L</td>
</tr>
<tr>
<td>Total Chlorine (mg/L Cl₂)</td>
<td>4.5 mg/L</td>
<td></td>
</tr>
<tr>
<td>Na (mg/L)</td>
<td>80 max</td>
<td>&lt;80</td>
</tr>
<tr>
<td>Cl (mg/L)</td>
<td>100 max</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Sulfate &amp; Chloride Sum.</td>
<td>3.8 meq/L max</td>
<td>&lt;3.8 meq/L</td>
</tr>
<tr>
<td>TDS (mg/L)</td>
<td>500 max</td>
<td>&lt;500</td>
</tr>
<tr>
<td>Fe (mg/L)</td>
<td>0.15 max</td>
<td>&lt;0.15</td>
</tr>
<tr>
<td>Color (CPU)</td>
<td>15 max</td>
<td>&lt;15</td>
</tr>
<tr>
<td>TOC (mg/L)</td>
<td>3.6 max avg./6.5 max</td>
<td>&lt;3.6</td>
</tr>
<tr>
<td>Ammonia (mg/L as N)</td>
<td>1 max</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>At filter 0.3 max/ 0.1 (95%), 0.25(100%)</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td></td>
<td>Finished 1 max avg.</td>
<td>&lt;0.3</td>
</tr>
</tbody>
</table>
Groundwater Pilot Plant

The groundwater, G1, pilot unit was designed to simulate the finished water of member governments that utilize conventional treatment of ground water. The G1 unit used the Cypress Creek well field as a raw water source. The main unit processes were aeration, disinfection and pH stabilization. Before disinfection, G1 was aerated to reduce hydrogen sulfide, produce an aerobic water supply and stabilize the water with respect to calcium carbonate. Aeration was achieved in G1 by pumping the raw water to the top of the finished water tank through a nozzle that sprayed the water inside the tank. This same aeration technique was used to stabilize G2 and G3.

Sodium hypochlorite was used as the source of free chlorine for primary disinfection and was dosed to provide a 4 mg/L residual after a 5 minute contact time. Afterwards, ammonium chloride was added to produce a 4 to 4.5 mg/L monochloramine residual. Ammonia was added in the form of NH₄Cl at a 4:1 to 5:1 ratio. The NH₃:Cl₂ ratio was initially 4:1 to protect against DBP formation. This ratio was increased to 5:1 in Phase III to reduce free ammonia. Five thousand gallons of G1 were produced every week.

Lime Softening Pilot Plant

The lime softening groundwater treatment pilot system that produced G2 used raw groundwater from the Cypress Creek well field. Three to four meq/L of CaO were added to the raw groundwater to achieve a settled total hardness of 120 mg/L as CaCO₃. G2 Alkalinity following groundwater softening was always greater than 50 mg/L as CaCO₃ and did not require alkalinity addition to meet the alkalinity for finished water.
Ca(OH)₂ slurry at 111,000 mg/L was used for lime feed. The slurry fed to the raw water during rapid mix. The water overflowed into a tank where flocculation was achieved using a submersible pump that suspended and recirculated the floc until softening was achieved. After the tank was full, the pump operated for 1 hour and then the softened water settled for 24 hours. The settled water was pumped out of the settling tank and filtered using 1 micron bag filtration followed by filtration with 0.35-micron cartridge filtration. Two thousand gallons of G2 were produced weekly.

**Blended Lime Softened Pilot Plant**

G3 water was produced by lime softening of blended S1, RO and G1 finished waters to achieve less than 120 mg/L as CaCO₃ total hardness, or greater than 50 mg/L as CaCO₃ alkalinity. The process was the same as for G2. Softening was always required to meet the total hardness criteria of the G1, S1 and RO blends that were used in the field study. However, it is very possible that a blend of these waters would have a total hardness less than 120 mg/L as CaCO₃ and an alkalinity greater than 50 mg/L as CaCO₃.

The softened water (G3) and membrane treated water (G4) process did not require a free chlorine contact time since G1, S1 and RO had been previously disinfected. The combined chlorine residual after the softening and filtration needed to be increased to 4.0 mg/L as Cl₂. Sodium hydroxide was used for stabilization. One thousand gallons of G3 were produced every week.
**Blended Nanofiltration Pilot Plant**

G4 water was produced by an integrated membrane system (IMS) treating the same blend of G1, S1 and RO that was used to produce G3. A conventional Osmonics nanofilter was used to produce G4. Hypersperse MDC700TM was dosed at 2.7 mg/L to the NF feed stream to control scaling. The NF membrane unit was operated at 75% recovery, which produced 1.3 gpm of permeate. The G4 permeate stream was aerated using the aeration towers shown in Figure 7.

Calcium chloride and sodium bicarbonate addition were added when needed to achieve the hardness and alkalinity goals of 1 meq/L as CaCO₃. The pH of the G4 finished water was maintained at 0.1 to 0.3 units above pHₚ. A chloramine residual was maintained in the G4 feed stream at 4 mg/L as Cl₂.

**Surface Water Pilot Plant**

S1 was produced by enhanced coagulation, ozonation, biologically activated carbon (BAC) filtration, aeration and chloramination. The S1 production processes mirrored the TBW Regional Surface Water Treatment Facility's treatment process as closely as possible. The raw surface water was taken from the Hillsborough River and hauled weekly to the field facility for treatment. The raw SW was initially treated by coagulation, sedimentation and filtration (CSF) using ferric sulfate coagulation. The dose of ferric sulfate was determined based on water quality and treatment. Total organic carbon (TOC) concentration of the finished SW had to be less than 3.6 mg/L, which was the maximum allowable TOC concentration in the finished SW. The combined sulfate and chloride concentration had to be less than 3.8 meq/L, which controlled the maximum coagulant dose. Inline addition of 0.5 mg/L of Cytec N-1986 coagulant aid was
used. The flocculated water was settled for 24 hours, and filtered through a 1 micron bag filter and followed by a 0.35 micron cartridge filter in series before transfer to the CSF storage tank.

Ozone and monochloramines were used for primary disinfection and residual maintenance respectively. A 1.4 mg-min/L CT as specified for 99.9 percent reduction of Giardia Lamblia at 10 °C was maintained during ozonation. The required contact time for this CT was determined by a tracer study on the 100 gallon contact tank, which determined that 10% of the water exited the tank in 2.5 minutes (T10). A minimum ozone residual of 0.6 mg/L in the effluent stream was maintained to achieve the 1.4 mg/L-min CT. The ozonated water was filtered through 2 feet of BAC with a 5 minute EBCT and then again through a 0.35 micron bags filter. The ozone pilot plant was housed in the front trailer as shown in Figure 8.

**Integrated Membrane System Pilot Plant**

S2 pilot process was a true surface water integrated membrane system which consisted of nanofiltration (NF) of CSF surface water, which was housed in the second trailer from the front as shown in Figure 8. A conventional Osmonics nanofilter was used to produce S1. Hypersperse MDC700TM was dosed at 2.7 mg/L to the NF feed stream to control scaling. The NF membrane unit was operated at 75% recovery, which produced 1.3 gpm of permeate. The S2 permeate stream was aerated using the aeration towers as previously described.

Calcium and alkalinity were added to the S2 permeate stream to meet project specifications. Calcium chloride and sodium bicarbonate were added to reach 1 meq/L as CaCO₃ goal for Ca hardness and carbonate alkalinity. Sodium hydroxide was added to achieve a finished pH that was 0.1-0.3 pH units above pHₚ. Primary disinfection was achieved using a solution of sodium hypochlorite (free chlorine) with a minimum 4 mg/L residual and allowing a
2-minute contact time. Ammonium chloride was then added to produce a minimum 4 mg/L monochloramine concentration as secondary disinfection for distribution system protection.

**Reverse Osmosis Pilot Plant**

Reverse osmosis (Desalination) treatment pilot system produced a finished water that was described as RO. The RO pilot plant was housed in the third trailer from the front as shown in Figure 8 and utilized raw groundwater for the feed stream. The RO PWTS required the addition of TDS, calcium and alkalinity to the permeate to represent the finished water produced by the TBW Regional Desalination Facility. RO pretreatment consisted of 2.7 mg/L antiscalent addition (Hypersperse MDC700TM) followed by 5-micron cartridge filtration.

The Reverse osmosis (high rejection) membrane filtration unit was operated at 75% recovery, producing 10 gpm permeate flow, which was aerated by a 10" diameter aeration tower filled with tripack plastic packing as shown in Figure 7 previously. After aeration, 50 mg/L of sea salt was added to the aerated permeate stream to simulate the TBW desalination process. Calcium chloride and sodium bicarbonate were also added to meet the calcium and alkalinity specifications. The finished was stabilized with sodium hydroxide to 0.1 to 0.3 pH units above pH₅.

The three main finished waters (S1, G1 and RO) were blended at two different ratios and then further treated by softening (G3) and nanofiltration (G4). The percentages of finished waters used in this blend for each study period is presented in Table 6.
Table 6. Percentage of Finished Waters Blended to Make G3 and G4 Feed Source

<table>
<thead>
<tr>
<th>Study Period</th>
<th>Blend Percentage (%)</th>
<th>G1</th>
<th>S1</th>
<th>RO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase 1 and 3</td>
<td></td>
<td>23</td>
<td>45</td>
<td>32</td>
</tr>
<tr>
<td>Phase 2 and 4</td>
<td></td>
<td>60</td>
<td>30</td>
<td>10</td>
</tr>
</tbody>
</table>

A diagram showing the water flow route through the PWTS is presented in Figure 18. The finished water qualities from these pilot processes were set to match the existing or future sources of water proposed by TBW. For all the waters, a new batch was prepared at the beginning of every week. The water quality analysis schedule required in the field for monitoring finished water storage and process operation is presented in Table 7. Each of the seven finished water storage tanks was measured for water stability parameters, disinfection residual, color and UV-254 using field methods. Additionally, each of the storage tanks was measured daily for disinfection residual maintenance and pH. The feed, permeate and concentrate from the membrane process were measured prior to post treatment to evaluate membrane performance and productivity. Additional water quality monitoring was conducted to evaluate specific unit processes within a treatment system. The water quality sampling schedule for parameters measured at the UCF Laboratory is presented in Table 8. The average water quality for the different source waters are enumerated in Table 12.
Figure 18 Process Train Layout
Table 7. UCF Laboratory Analysis Schedule for Water Treatment Processes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>G1 Raw</th>
<th>S1 Raw</th>
<th>G1 Fin</th>
<th>G2 Fin</th>
<th>G3 Fin</th>
<th>G4 Fin</th>
<th>S1 Fin</th>
<th>S2 Fin</th>
<th>RO Fin</th>
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</thead>
<tbody>
<tr>
<td>Ammonia</td>
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<td>Biwk</td>
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<td>TDS</td>
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<tr>
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</tr>
<tr>
<td>Turbidity</td>
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<td>Biwk</td>
<td>Biwk</td>
<td>Biwk</td>
<td>Biwk</td>
</tr>
</tbody>
</table>

Note: Biwk = Biweekly Sample collection

Table 8. Field Analysis Schedule For Water Treatment Processes

<table>
<thead>
<tr>
<th></th>
<th>G1 Raw</th>
<th>S1 Raw</th>
<th>G1 Fin</th>
<th>G2 Fin</th>
<th>G3 Fin</th>
<th>G4 Fin</th>
<th>S1 Fin</th>
<th>S2 Fin</th>
<th>RO Fin</th>
<th>G3/G4, S1/S2 feed</th>
<th>Permeate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Cl₂</td>
<td>n/a</td>
<td>n/a</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Cl₂</td>
<td>n/a</td>
<td>n/a</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
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</tr>
<tr>
<td>Ammonia</td>
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<td>B</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
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<td>Temperature</td>
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<td>D</td>
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</tr>
</tbody>
</table>

Note: D = Daily, B = Batch = after each batch is finished.
Color and UV-254 are filtered but pH is not adjusted. Mg is determined by difference between CaH and TH. Phs is determined after all chemicals are added to adjust water quality to targeted goals. RO, S2 and G4 concentrate was measured for TDS by conductivity probe.
Process Finished Water Quality

Average raw and finished water quality parameters for the duration of the pilot study are presented in Table 10, Table 11, and Table 12.

Water quality monitoring during production was conducted to evaluate specific unit processes within a given treatment system. Raw surface water experienced the most seasonal water quality variations as shown in Table 9. The following variations in average water quality for raw surface water were observed between the dry season (December-May) and the wet season (May-November), alkalinity dropped from 143 mg/L as CaCO₃ to 87 mg/L as CaCO₃, total hardness dropped from 238 mg/L as CaCO₃ to 156 mg/L as CaCO₃, color increased from 36 CPU to 192 CPU, TDS dropped from 314 mg/L to 200 mg/L. The low alkalinity during the wet season necessitated the supplementation of raw surface water alkalinity using sodium hydroxide in order to ensure proper coagulation and achieve the target water quality goals.

Table 9. Seasonal Water Quality Fluctuations for Raw Surface Water

<table>
<thead>
<tr>
<th>Season</th>
<th>pH</th>
<th>Alk</th>
<th>TH</th>
<th>Color</th>
<th>UV-254</th>
<th>Turbidity</th>
<th>DO</th>
<th>TDS</th>
<th>SO₄²⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry</td>
<td>7.9</td>
<td>143</td>
<td>238</td>
<td>36</td>
<td>0.10</td>
<td>2</td>
<td>6.3</td>
<td>314</td>
<td>120</td>
</tr>
<tr>
<td>Wet</td>
<td>7.2</td>
<td>87</td>
<td>156</td>
<td>192</td>
<td>0.80</td>
<td>1.6</td>
<td>3.0</td>
<td>200</td>
<td>79</td>
</tr>
</tbody>
</table>

The finished waters for all processes were produced to meet target water quality objectives designed to mimic actual and expected TBW blended water quality shown in Table 10.
Table 10. Water Quality Objectives for Finished Process Waters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
<th>S1</th>
<th>S2</th>
<th>RO</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>pHs + 0.3</td>
<td>&gt;pHs</td>
<td>&gt;pHs</td>
<td>&gt;pHs</td>
<td>&gt;pHs</td>
<td>&gt;pHs</td>
<td>&gt;pHs</td>
</tr>
<tr>
<td>T. Hardness (mg/L as CaCO₃)</td>
<td>240</td>
<td>120</td>
<td>120</td>
<td>60 min</td>
<td>60 min</td>
<td>60 min</td>
<td>60 min</td>
</tr>
<tr>
<td>Alkalinity (meq/L)</td>
<td>4</td>
<td>1 min</td>
<td>1 min</td>
<td>1 min</td>
<td>1 min</td>
<td>1 min</td>
<td>1 min</td>
</tr>
<tr>
<td>Total Cl₂ (mg/L)</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Na (mg/L)</td>
<td>80 max</td>
<td>80 max</td>
<td>80 max</td>
<td>80 max</td>
<td>80 max</td>
<td>80 max</td>
<td>80 max</td>
</tr>
<tr>
<td>SO₄ &amp; Cl⁻ (meq/L)</td>
<td>3.8 max</td>
<td>3.8 max</td>
<td>3.8 max</td>
<td>3.8 max</td>
<td>3.8 max</td>
<td>3.8 max</td>
<td>3.8 max</td>
</tr>
<tr>
<td>TDS (mg/L)</td>
<td>500 max</td>
<td>500 max</td>
<td>500 max</td>
<td>500 max</td>
<td>500 max</td>
<td>500 max</td>
<td>500 max</td>
</tr>
<tr>
<td>Color (CPU)</td>
<td>15 max</td>
<td>15 max</td>
<td>15 max</td>
<td>15 max</td>
<td>15 max</td>
<td>15 max</td>
<td>15 max</td>
</tr>
<tr>
<td>TOC (mg/L)</td>
<td>3.6 max</td>
<td>3.6 max</td>
<td>3.6 max</td>
<td>3.6 max</td>
<td>3.6 max</td>
<td>3.6 max</td>
<td>3.6 max</td>
</tr>
</tbody>
</table>

Despite the reported fluctuations in surface water quality, the surface water PWTS consistently met the set finished water quality targets (Table 5 and Table 10), reducing turbidity to 0.3 NTU, color to 1 CPU and UV-254 to 0.038 cm⁻¹. The finished surface water experienced elevated sulfates (180 mg/L) due to the addition of ferric sulfate as a coagulant. Conductivity and TDS also increased to 591 µS/cm, and 394 mg/L respectively.

Raw groundwater water quality did not change appreciably for the duration of the project. As an example, conductivity presented an average of 517 µS/cm with a standard deviation of 30 µS/cm and TDS presented and average of 344 mg/L with a standard deviation of 20 mg/L. G1 finished water had similar characteristics to the raw ground water except for DO which increased from an average of 0.2 mg/L to 7.5 mg/L due to the aeration process.

Minimal variations were experienced in most finished water quality parameters measured as shown by the standard deviation values presented in Table 12. The largest fluctuations were
observed in G1, for which color had an average of 5 CPU with a standard deviation of 4 CPU, and turbidity with an average of 0.5 NTU and standard deviation of 0.5 NTU. Groundwater softening process (G2 finished water) significantly reduced calcium hardness (212 to 88 mg/L), alkalinity (211 to 93 mg/L as CaCO₃), TDS (314 to 198 mg/L). Similarly, softening of the blended S1/G1/RO water resulted in reduction in calcium hardness from 187 mg/L as CaCO₃ to 99 mg/L as CaCO₃ for G3. As expected, this calcium concentration was higher than that for RO at 64 mg/L as CaCO₃. G4, produced by nanofiltration of the blended S1/G1/RO, had significantly reduced average calcium (77 mg/L), TDS (207 mg/L) and color (0 CPU) with respect to the G3/G4 feed as shown in Table 12.

All the processes performed as expected during the project, consistently meeting water quality constraints set for the project (Table 5 and Table 10). Average finished water quality for selected parameters are provided in Table 11 and Table 12.

Table 11. UCF Laboratory Process Waters Selected Average Water Quality Characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
<th>S1</th>
<th>S2</th>
<th>RO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turbidity</td>
<td>NTU</td>
<td>0.5</td>
<td>0.2</td>
<td>0.3</td>
<td>0.1</td>
<td>0.3</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Sodium</td>
<td>mg/L</td>
<td>16</td>
<td>13</td>
<td>38</td>
<td>28</td>
<td>81</td>
<td>45</td>
<td>52</td>
</tr>
<tr>
<td>Chlorides</td>
<td>mg/L</td>
<td>28</td>
<td>22</td>
<td>48</td>
<td>41</td>
<td>32</td>
<td>67</td>
<td>79</td>
</tr>
<tr>
<td>Calcium</td>
<td>mg/L as CaCO₃</td>
<td>212</td>
<td>88</td>
<td>99</td>
<td>77</td>
<td>134</td>
<td>62</td>
<td>64</td>
</tr>
<tr>
<td>HPC</td>
<td>cfu/ml</td>
<td>96</td>
<td>9</td>
<td>62</td>
<td>109</td>
<td>29</td>
<td>19</td>
<td>66</td>
</tr>
<tr>
<td>Alkalinity</td>
<td>mg/L as CaCO₃</td>
<td>208</td>
<td>93</td>
<td>64</td>
<td>88</td>
<td>59</td>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td>SO₄</td>
<td>mg/L</td>
<td>27</td>
<td>26</td>
<td>76</td>
<td>5</td>
<td>184</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>TOC</td>
<td>mg/L</td>
<td>3.3</td>
<td>2.2</td>
<td>1.5</td>
<td>0.8</td>
<td>1.2</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Si</td>
<td>mg/L</td>
<td>14</td>
<td>14</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>HPC</td>
<td>cfu/mL</td>
<td>96</td>
<td>9</td>
<td>62</td>
<td>109</td>
<td>29</td>
<td>19</td>
<td>66</td>
</tr>
</tbody>
</table>
RO water process produced water with the lowest concentration of organics. The TOC values for RO and NF water were the lowest followed by surface water, softened water and groundwater as shown in Table 11. The complete order for the waters produced is RO<S2<G4<SW<G3<G4<GW. Membrane treated waters had the lowest turbidity, sulfates, and silica. Chlorides, sodium, calcium, magnesium and alkalinity were high for RO water due the addition sea salt to mimic desalination of sea water.

All the waters were maintained above the alkalinity limit of 1 meq/L (50 mg/L as CaCO3) as required. The total hardness target of 120 mg/L as CaCO3 for the softened waters (G2 and G3) was obtained consistently during the project. All finished waters were stabilized with respect to the Langelier index, maintaining a LSI of between +0.1 and +0.3.

The combined chlorine concentration for all finished water tanks was maintained at 4.5 mg/L, which dropped to 4.0mg/L prior to entering the pilot distribution system. HPC in all the finished water tanks were at or below 100 cfu/mL, indicating little or no microbiological activity.

Overall, all parameters were maintained within the set limits. Warning limits for the different parameters were set at two standard deviations from water quality goals, while action limits were set at three standard deviations from the target limit. No corrective action on finished water was ever required indicating consistent and good control in the production of all the finished waters.
Table 12. Water Quality Averages for the Water Pilot Treatment Processes

<table>
<thead>
<tr>
<th>Process</th>
<th>Free Cl₂ mg/L</th>
<th>T Cl₂ mg/L</th>
<th>NH₃ mg/L</th>
<th>pH</th>
<th>Alkalinity mg/L CaCO₃</th>
<th>Ca mg/L</th>
<th>H TH µg/L</th>
<th>HPC cfu/ml</th>
<th>UV 254 cm⁻¹</th>
<th>Color CPU</th>
<th>Turbidity NTU</th>
<th>D.O. mg/L</th>
<th>Cond. µS/cm</th>
<th>TDS mg/L</th>
<th>Temp °C</th>
<th>Cl⁻ mg/L</th>
<th>SO₄ mg/L</th>
<th>Fe mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>Average</td>
<td>0.1</td>
<td>4.7</td>
<td>0.2</td>
<td>7.9</td>
<td>208</td>
<td>212</td>
<td>243</td>
<td>96</td>
<td>0.070</td>
<td>5</td>
<td>0.5</td>
<td>7.5</td>
<td>515</td>
<td>346</td>
<td>23</td>
<td>25</td>
<td>28</td>
</tr>
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<td>0.1</td>
<td>0.6</td>
<td>0.1</td>
<td>0.2</td>
<td>20</td>
<td>10</td>
<td>12</td>
<td>10</td>
<td>0.005</td>
<td>4</td>
<td>0.5</td>
<td>0.6</td>
<td>31</td>
<td>39</td>
<td>3</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>G2</td>
<td>Average</td>
<td>0.1</td>
<td>4.8</td>
<td>0.2</td>
<td>8.0</td>
<td>93</td>
<td>88</td>
<td>121</td>
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<td>0.2</td>
<td>7.8</td>
<td>292</td>
<td>194</td>
<td>23</td>
<td>0.1</td>
<td>4.8</td>
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<tr>
<td></td>
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<td>0.1</td>
<td>0.2</td>
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<td>0.4</td>
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<td>Average</td>
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<td>0.3</td>
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<td>Average</td>
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<td>23</td>
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</tr>
<tr>
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<td>Std Dev</td>
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<td>0.7</td>
<td>0.1</td>
<td>0.1</td>
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<td>S1</td>
<td>Average</td>
<td>0.2</td>
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<td>0.1</td>
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<td>68</td>
<td>64</td>
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<td>8</td>
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<td>4</td>
<td>6</td>
<td>46</td>
</tr>
<tr>
<td>S1/S2</td>
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<td>0.1</td>
<td>8.3</td>
<td>68</td>
<td>62</td>
<td>69</td>
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<td>238</td>
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<tr>
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<td>Std Dev</td>
<td>0.1</td>
<td>0.7</td>
<td>0.1</td>
<td>0.2</td>
<td>6</td>
<td>5</td>
<td>6</td>
<td>3</td>
<td>0.007</td>
<td>1</td>
<td>0.1</td>
<td>0.9</td>
<td>47</td>
<td>32</td>
<td>5</td>
<td>7</td>
<td>6</td>
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<tr>
<td>G3/G4</td>
<td>Average</td>
<td>0.1</td>
<td>3.7</td>
<td>8.1</td>
<td>126</td>
<td>163</td>
<td>187</td>
<td>-</td>
<td>-</td>
<td>0.046</td>
<td>3</td>
<td>0.4</td>
<td>8.7</td>
<td>517</td>
<td>344</td>
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</tr>
<tr>
<td></td>
<td>Std Dev</td>
<td>0.1</td>
<td>0.4</td>
<td>0.1</td>
<td>25</td>
<td>26</td>
<td>30</td>
<td>-</td>
<td>-</td>
<td>0.009</td>
<td>2</td>
<td>0.3</td>
<td>1.0</td>
<td>30</td>
<td>20</td>
<td>3</td>
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</tr>
<tr>
<td>S1/S2</td>
<td>Average</td>
<td>5.4</td>
<td>16</td>
<td>150</td>
<td>179</td>
<td>-</td>
<td>-</td>
<td>0.067</td>
<td>5</td>
<td>4.3</td>
<td>6.8</td>
<td>465</td>
<td>312</td>
<td>23</td>
<td>25</td>
<td>269</td>
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<tr>
<td></td>
<td>Std Dev</td>
<td>0.8</td>
<td>13</td>
<td>52</td>
<td>58</td>
<td>-</td>
<td>-</td>
<td>0.123</td>
<td>10</td>
<td>4.6</td>
<td>1.3</td>
<td>90</td>
<td>56</td>
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<tr>
<td>G5 - GW</td>
<td>Average</td>
<td>7.5</td>
<td>211</td>
<td>212</td>
<td>241</td>
<td>-</td>
<td>-</td>
<td>0.063</td>
<td>6</td>
<td>0.3</td>
<td>0.2</td>
<td>475</td>
<td>314</td>
<td>23</td>
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<tr>
<td></td>
<td>Std Dev</td>
<td>0.1</td>
<td>7</td>
<td>10</td>
<td>12</td>
<td>-</td>
<td>-</td>
<td>0.009</td>
<td>4</td>
<td>0.1</td>
<td>0.4</td>
<td>27</td>
<td>24</td>
<td>2</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>G5 - SW</td>
<td>Average</td>
<td>7.4</td>
<td>102</td>
<td>143</td>
<td>173</td>
<td>-</td>
<td>-</td>
<td>0.6</td>
<td>135</td>
<td>1.6</td>
<td>4.7</td>
<td>346</td>
<td>228</td>
<td>22</td>
<td>19</td>
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<tr>
<td></td>
<td>Std Dev</td>
<td>0.4</td>
<td>33</td>
<td>55</td>
<td>65</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
<td>101</td>
<td>1.3</td>
<td>2.0</td>
<td>125</td>
<td>83</td>
<td>5</td>
<td>4</td>
<td>75</td>
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<td></td>
</tr>
</tbody>
</table>
**Pilot Distribution System Operation**

After construction, the PDSs were equilibrated for a period of 144 days with a conventionally treated groundwater. A requirement to provide a substantial equilibration period (approximately three months) had been reported in the literature to allow stabilization of water quality parameters after construction of pilot testing facilities. It is believed that mechanical disturbance of the pipe interior films (chemical and/or biological) could result from harvesting, transport, and reassembly of the aged pipe materials. During the first part of the equilibration period, finished water from the Tampa Bay Water Cypress Creek Facility was used. This source was characterized by a low dissolved oxygen concentration and free chlorine residual. In the latter stage of equilibration, groundwater from the pilot treatment facilities (Source G1) was employed. This water was produced by aeration of raw groundwater from the Cypress Creek Well Field, followed by stabilization and chloramination. The influent and effluent from the PDS lines were monitored during the equilibration period to verify that any transient effects associated with PDS construction had been eliminated.

In November 2001, it was determined that the PDS lines were equilibrated and a decision was made to implement the blending study. The pilot distribution system was operated at a 5 day hydraulic retention time (HRT) initially (Phase 1 to 3, 12/8/01 to 8/30/02) to simulate dead end conditions. The HRT was changed to 2 days during the final two weeks of Phase 3 through Phase 5 (8/31/02 to 4/4/03) to permit maintenance of chlorine residual. Different source waters and their blends were introduced into the PDS by dosing pumps feeding individual influent standpipes for each PDS. These blends were selected to model the anticipated operations by TBW. The source waters for the PDSs were changed during phase 2 and 4 with reassignment of individual blends to different pilot distribution lines. In most cases, the blend composition was
not altered, relative to the composition in Phase 1 and 3. In these instances, the blend was redirected to feed a different hybrid line. Preparation of G3 and G4 was accomplished with a revised ratio of the three principal sources as presented previously in Table 6. The input to the single material lines was also modified with the same ratio of G1, S1, and RO used to prepare G3 and G4. The source waters for each PDS by phase are summarized in Table 13.

Table 13. Blends used for Different Pilot Distribution Systems (PDS) over the Period of the Study.

<table>
<thead>
<tr>
<th>PDS – Material</th>
<th>Phase I (12/8/01 to 3/15/02) &amp; Phase 3 (6/15/02 to 9/13/02)</th>
<th>Phase 2 (3/16/02 to 6/14/02) &amp; Phase 4 (9/14/02 to 12/13/02)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>01 – Hybrid GW (100) G2 (100) switch with PDS02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>02 – Hybrid G2 (100) GW (100) switch with PDS01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>03 – Hybrid SW (100) S2 (100) switch with PDS13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>04 – Hybrid G4 (100) G3 (100) switch with PDS12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>05 – Hybrid RO (100) SW (100) switch with PDS03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>06 – Hybrid GW (55) SW (45) GW (68) RO (32) switch with PDS07</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>07 – Hybrid GW (68) RO (32) GW (55) SW (45) switch with PDS06</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>08 – Hybrid GW (23) SW (45) RO (32) GW (60) S2 (30) RO (10) switch with PDS09</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>09 – Hybrid GW (60) S2 (30) RO (10) GW (23) SW (45) RO (32) switch with PDS08</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 – Hybrid G2 (50) SW (50) G2 (62) SW (24) RO (14) switch with PDS11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 – Hybrid G2 (62) SW (24) RO (14) G2 (50) SW (50) switch with PDS10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 – Hybrid G3 (100) G4 (100) switch with PDS04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13 – Hybrid S2 (100) RO (100) switch with PDS05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 – Hybrid GW (62) SW (27) RO (11) GW (62) SW (27) RO (11) high frequency line</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 – Unlined Cast Iron GW (23) SW (45) RO (32) GW (60) SW (30) RO (10) Change blend pcts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 – Lined Cast Iron GW (23) SW (45) RO (32) GW (60) SW (30) RO (10) Change blend pcts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17 – PVC GW (23) SW (45) RO (32) GW (60) SW (30) RO (10) Change blend pcts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 – Galvanized Iron GW (23) SW (45) RO (32) GW (60) SW (30) RO (10) Change blend pcts</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Values in () indicate the percentage of the source water in blend.

The high frequency line (PDS14) operation includes twice-daily changes in the blend ratio. These adjustments are selected to maintain long-term average flows in line with ultimate capacities of the three major sources in the TBW service area: G1 (62%), S1 (27%), and RO (11%). Selection of actual blends was defined by Monte Carlo simulation methods. The three
scenarios represent blends resulting from removal of one principal source (G1, S1, or RO) from production. Actual alteration of the blend was performed at the beginning and end of the production day (approximately 8 AM and 5 PM, respectively).

**PDS Water Quality Schedule**

Samples were collected and analyzed in the field and at the UCF laboratory. Monitoring and analyses of many physical, chemical and biological water quality parameters was carried out on the influent and effluent to the PDS. Table 14 shows the different parameters that were monitored during the study. Weekly analyses were completed in the field laboratory and the bi-weekly samples were brought back to UCF laboratory for analysis. Quality assurance and quality control of both the laboratory and field determinations of water quality parameters was established by duplicating analyses of at least 10% of the samples. Where appropriate standards were available, 10% of the samples were spiked with known concentrations of the parameter being analyzed and the recovery measured. Blind duplicates and spikes were also used to determine the accuracy of measurements. Dynamic control charts were used to determine whether the results were acceptable.
Table 14. Pilot distribution system monitoring program phase 1 through 4

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Field Analysis</th>
<th>UCF Laboratory</th>
</tr>
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<tr>
<td></td>
<td>PDS Influent</td>
<td>PDS Effluent</td>
</tr>
<tr>
<td>pH</td>
<td>Weekly</td>
<td>Weekly</td>
</tr>
<tr>
<td>Alkalinity</td>
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<td>Turbidity</td>
<td>Weekly</td>
<td>Weekly</td>
</tr>
<tr>
<td>Free chlorine</td>
<td>Weekly</td>
<td>Weekly</td>
</tr>
<tr>
<td>Total chlorine</td>
<td>Weekly</td>
<td>Weekly</td>
</tr>
<tr>
<td>Nitrite-N</td>
<td>As needed</td>
<td>As needed</td>
</tr>
<tr>
<td>Nitrate-N</td>
<td>As needed</td>
<td>As needed</td>
</tr>
<tr>
<td>Ammonia-N</td>
<td>Weekly</td>
<td>Weekly</td>
</tr>
<tr>
<td>Dissolved oxygen</td>
<td>Weekly</td>
<td>Weekly</td>
</tr>
<tr>
<td>Temperature</td>
<td>Weekly</td>
<td>Weekly</td>
</tr>
<tr>
<td>Conductivity</td>
<td>Weekly</td>
<td>Weekly</td>
</tr>
<tr>
<td>Apparent Color</td>
<td>Weekly</td>
<td>Weekly</td>
</tr>
<tr>
<td>True color</td>
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</tr>
<tr>
<td>UV-254</td>
<td>Weekly</td>
<td>Weekly</td>
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<tr>
<td>TDS</td>
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</tr>
<tr>
<td>Aluminum</td>
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<tr>
<td>Silica</td>
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</tr>
<tr>
<td>Chloride</td>
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<tr>
<td>Sulfate</td>
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</tr>
<tr>
<td>Fluoride</td>
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</tr>
<tr>
<td>Sodium</td>
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<td></td>
</tr>
<tr>
<td>NPDOC</td>
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<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>Weekly</td>
<td>Weekly</td>
</tr>
<tr>
<td>Magnesium</td>
<td>Weekly</td>
<td>Weekly</td>
</tr>
<tr>
<td>Total hardness</td>
<td>Weekly</td>
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<tr>
<td>Total iron</td>
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</tr>
<tr>
<td>Dissolved iron</td>
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</tr>
<tr>
<td>HAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>THM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pb (corr. loops)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu (corr. loops)</td>
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<tr>
<td>Manganese</td>
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<tr>
<td>Ortho-Phosphate</td>
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<td>TC/E. Coli</td>
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</tr>
<tr>
<td>AOC</td>
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<tr>
<td>BDOC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biofilm PEPA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BFHPC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
To prevent non-representative biofilm thicknesses and sediment build-up caused by the slow velocity of flow through the PDSs the lines were flushed regularly. The objective of the flushing was to briefly attain the shear forces expected in full scale distribution systems. The PDS lines were flushed once a week during the 5-day HRT period and once every two weeks for the 2-day HRT period. The flush velocity was 1 ft/s (0.3 m/s) for at least 3 pipe volumes. An example of PDS hydraulic operation protocol is provided in Table 15 for 5-day HRT operation.

Table 15. Hydraulic Operation Protocol for the PDSs at 5-day HRT

<table>
<thead>
<tr>
<th>PDS</th>
<th>Pipe Volume (L)</th>
<th>Normal Flow rate (mL/min.)</th>
<th>Flushing Flow rate (gpm)</th>
<th>Flushing velocity (ft/s.)</th>
<th>HRT (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>01 – hybrid</td>
<td>310</td>
<td>43</td>
<td>53</td>
<td>1.0</td>
<td>5</td>
</tr>
<tr>
<td>02 – hybrid</td>
<td>310</td>
<td>43</td>
<td>53</td>
<td>1.0</td>
<td>5</td>
</tr>
<tr>
<td>03 – hybrid</td>
<td>310</td>
<td>43</td>
<td>53</td>
<td>1.0</td>
<td>5</td>
</tr>
<tr>
<td>04 – hybrid</td>
<td>310</td>
<td>43</td>
<td>53</td>
<td>1.0</td>
<td>5</td>
</tr>
<tr>
<td>05 – hybrid</td>
<td>310</td>
<td>43</td>
<td>53</td>
<td>1.0</td>
<td>5</td>
</tr>
<tr>
<td>06 – hybrid</td>
<td>310</td>
<td>43</td>
<td>53</td>
<td>1.0</td>
<td>5</td>
</tr>
<tr>
<td>07 – hybrid</td>
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<td>43</td>
<td>53</td>
<td>1.0</td>
<td>5</td>
</tr>
<tr>
<td>08 – hybrid</td>
<td>310</td>
<td>43</td>
<td>53</td>
<td>1.0</td>
<td>5</td>
</tr>
<tr>
<td>09 – hybrid</td>
<td>310</td>
<td>43</td>
<td>53</td>
<td>1.0</td>
<td>5</td>
</tr>
<tr>
<td>10 – hybrid</td>
<td>310</td>
<td>43</td>
<td>53</td>
<td>1.0</td>
<td>5</td>
</tr>
<tr>
<td>11 – hybrid</td>
<td>310</td>
<td>43</td>
<td>53</td>
<td>1.0</td>
<td>5</td>
</tr>
<tr>
<td>12 – hybrid</td>
<td>310</td>
<td>43</td>
<td>53</td>
<td>1.0</td>
<td>5</td>
</tr>
<tr>
<td>13 – hybrid</td>
<td>310</td>
<td>43</td>
<td>53</td>
<td>1.0</td>
<td>5</td>
</tr>
<tr>
<td>14 – hybrid</td>
<td>310</td>
<td>43</td>
<td>53</td>
<td>1.0</td>
<td>5</td>
</tr>
</tbody>
</table>
Table 15 (continued). Hydraulic Operation Protocol for the PDSs at 5-day HRT

<table>
<thead>
<tr>
<th>PDS</th>
<th>Pipe Volume (L)</th>
<th>Normal Flow rate (mL/min.)</th>
<th>Flushing Flow rate (gpm)</th>
<th>Flushing velocity (ft/s.)</th>
<th>HRT (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 – UCI</td>
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<tr>
<td>16 – LCI</td>
<td>457</td>
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<td>88</td>
<td>1.0</td>
<td>5</td>
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<td>17 – PVC</td>
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</tr>
<tr>
<td>18 - G</td>
<td>86</td>
<td>12</td>
<td>10</td>
<td>1.0</td>
<td>5</td>
</tr>
</tbody>
</table>

**Biological Film Characterization**

Biofilm characterization investigations were conducted using coupons made from the same MG pipes that were used to construct the PDSs. The coupons were housed in cradles following every PDS as shown in Figure 12 and Figure 13. Each cradle consisted of 4” PVC pipe housing and 3” diameter PVC pipe cut length-wise as sliding pipe coupon holders. The length of the cradles was approximately 12 feet. The pipe coupon holders were easily removed from the cradles and replaced after each experimental phase for biofilm analysis. The holders were transported in a large plastic bin with the coupons still in place. The humidity in the bin was elevated by placing a wet sponge inside. Upon arrival at the UCF labs the coupons were harvested for biofilm studies.

Pilot distribution system pipe coupons for biological sampling were approximately 3 cm in diameter with a small PVC peg made from PVC welding rod attached to what was the outer surface of the pipe. The material is drilled from aged or pristine pipe and then de-burred to give smooth edges. In each integrated pilot distribution system cradle, there were duplicate aged
coupons of each of the four materials. The sequence of the coupons in these cradles from upstream to downstream was PVC, LCI, UCI and finally G coupons.

The coupons were placed in this order to avoid transport of corrosion materials downstream to contaminate the inert materials such as PVC and LCI. Thus the two corrodbible materials (i.e. UCI and G) were placed in the downstream locations. The aged pipe coupons were obtained from used pipe segments from actual member government networks.

In each cradle for single material pilot distribution systems, there were duplicated aged and pristine coupons of the same material as the pilot distribution system material. The pristine coupons allowed separate quantification of the bioassay variability and were purely for QA/QC. They were only being used during the first year of the study. They were placed upstream of aged pipe coupons to avoid transport of corrosion products from the aged material onto the pristine coupons. Each coupon/material pair was separated by 4 inches and duplicates were within 1 inch of each other.

Since there was not enough of each member government's coupons to include origin as an experimental variable, coupon pairs were randomly distributed among the PDSs so that any bias resulting from origin would not cause a systematic bias in the data. Each coupon/material pair was separated by 4 inches and duplicates were within 1 inch of each other. The biofilm coupons were usually harvested near the end of each Phase to quantify the amount of biological growth that has occurred during the Phase.

Table 16 shows the scheduled biological sampling events for each phase of the pilot study.
Table 16. Sampling Schedule for Biological Indicators

<table>
<thead>
<tr>
<th>Phase</th>
<th>Coupon Type</th>
<th>PDS #</th>
<th>BFHPC</th>
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<td>Aged</td>
<td>15-18</td>
<td>End Phase</td>
<td>End Phase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1-14</td>
<td></td>
<td>End Phase</td>
</tr>
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<td>End Phase</td>
</tr>
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<td>11-14</td>
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<td>End Phase</td>
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<td>6</td>
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<td>End Phase</td>
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<td>9-10</td>
<td>3 weeks*</td>
<td>3 weeks*</td>
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<td></td>
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<td>11-14</td>
<td>3 weeks*</td>
<td>3 weeks*</td>
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</table>

† Sampling dates: 5/22/03, 6/12/03, 7/03/03
* Sampling dates: 4/24/03, 5/15/03, 6/05/03, and 6/26/03

Additional Studies

Confounding Effects

The operation of the pilot distribution system during phase 1 through 4 included confounding effects between factors that may be relevant to release of corrosion products. Specifically, the water source(s) with the highest alkalinity (GW and blends enriched in GW) also contain the lowest chlorides. The water source(s) with the greatest sulfate (SW and blends enriched in SW), exhibited low alkalinity. Similarly, the water source(s) with the highest chlorides (RO and blends enriched in RO), also report low alkalinity. Additional studies were needed to isolate the effect of individual parameters on effluent water quality.

Nitrification Study

A planned nitrification study was conducted to determine how to control nitrification in a system receiving blended waters of varying water quality such as desalination, enhanced surface water treatment and conventional ground water treatment. Several factors were evaluated to
determine their impact on the beginning, continuation and decline of nitrification. These factors were NH\textsubscript{3} concentration, residual, temperature, pH, Cl\textsubscript{2}/NH\textsubscript{3} ratio, AOC, HPC, AOB, and time.

The planned nitrification investigation was conducted during Phases 5 and 6. The waters utilized for the nitrification study were 100 \% G1, 100 \% S1, 100 \% RO and a blend. The blend B1 (40\% G1, 30\% S1, and 30\% RO) was used from 12/24/2002 to 04/04/2003, and the blend B2 (62\% G1, 27\% S1, and 11\% RO) from 04/05/2003 to 07/04/2003. The feed streams to PDSs 11-14 were changed on 6/13/03. The different changes in water sources for PDSs 9 through 18 are presented in Table 17.

Table 17. Feed Matrices for PDSs under Nitrification Study.

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<thead>
<tr>
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<th>01/30/03-04/04/03</th>
<th>04/05/03-06/12/03</th>
<th>06/13/03-07/04/03</th>
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<tr>
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<td>G1</td>
<td>G1</td>
<td>RO</td>
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<td>S1</td>
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<td>PDS 16</td>
<td>-</td>
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<td>Blend 2</td>
</tr>
<tr>
<td>PDS 17</td>
<td>-</td>
<td>Blend 2</td>
<td>Blend 2</td>
</tr>
<tr>
<td>PDS 18</td>
<td>-</td>
<td>Blend 2</td>
<td>Blend 2</td>
</tr>
<tr>
<td>Blend</td>
<td>G1 (40%)</td>
<td>G1 (62%)</td>
<td>G1 (62%)</td>
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<tr>
<td>Ratio</td>
<td>S1 (30%)</td>
<td>S1 (27%)</td>
<td>S1 (27%)</td>
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<td></td>
<td>RO (30%)</td>
<td>RO (11%)</td>
<td>RO (11%)</td>
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Initially, steps were taken to establish a nitrifying environment in PDS 11 to 14 from 12/24/2002 to 04/04/2003. The influent stream to these lines was fed with a 4 mg/L chloramine residual as Cl\textsubscript{2} and 1 mg/L of excess ammonia. Sodium thiosulfate was dosed to quench influent
residual and create a microbiological nitrifying environment. The pilot plant pumping and piping scheme could not accommodate separate chloramination facilities, so chloramines quenching was necessary for PDS 11 through 14.

Beginning on 04/05/2003, PDSs 9, 10, 15, 16, 17 and 18 were incubated for nitrifiers for three weeks with 1.0 mg/L excess ammonia-N and sodium thiosulfate quenching of chloramine residuals, which provided another 0.8 mg/L of ammonia N. The HRT for the first three weeks was set at 5 days to accelerate the occurrence of nitrification, thereafter a 2-day HRT was used to prevent anaerobic conditions. Table 18 shows the different operating conditions for PDS lines 9 to 18 during the nitrification study.

Different combinations of excess ammonia-N, Cl₂:N ratio, flushing frequency and flushing strength were studied in the controlled nitrification investigations as shown in Table 18. During the nitrifier incubation period an excess NH₃ concentration of 1 mg/L was added to the feed of each PDS. After incubation, three different Cl:N ratios were investigated. Excess NH₃ concentration of 0, 0.2, 0.5 mg/L were used in the feed stream of the PDSs to reach a Cl:N ratio of 5:1, 4:1, and 3:1 respectively.

Since PDSs 11 through 14 were seeded during phase 5, they were available for four cycles of study while PDSs 9,10 and 15 through 18 were available for three study cycles as noted in Table 18.

From 04/05/2003 to 07/04/2003, PDSs 9 and 10 were used for investigating the effects of flushing the system using high residual finished water. The monochloramine concentrations used for flushing PDSs 9 and 10 were 10 mg/L as Cl₂ and 7 mg/L as Cl₂, respectively. Before flushing, the finished waters were pumped to a storage water tank and the chlorine concentration was adjusted to the target concentration for flushing. The effect of flushing frequency in
nitrification was also studied, from 05/02/03 to 06/06/03, the PDSs were flushed every one and half weeks totaling three flushes, and from 06/06/03 to 07/04/03 the PDSs were flushed on a weekly basis, totaling four flushes.

The influent and effluent to each PDS were analyzed for several parameters weekly as shown in Table 19 and Table 20. Several parameters were also analyzed for the internal ports located between the different types of pipe material, the sampling dates for each PDS line are shown in Table 21.
Table 18. Nitrification Operational and Sampling Schedule

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<td>4 mg/L</td>
<td>4 mg/L</td>
<td>4 mg/L</td>
<td>4 mg/L</td>
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<td>4 mg/L</td>
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<td>0.5 mg/L</td>
<td>0.5 mg/L</td>
<td>0.5 mg/L</td>
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<td>4 mg/L</td>
<td>4 mg/L</td>
<td>4 mg/L</td>
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Phase VI Cycle 3

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Phase VI Cycle 4

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<td>0.5 mg/L</td>
<td>0.5 mg/L</td>
<td>0.5 mg/L</td>
<td>0.5 mg/L</td>
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<td>HRT days</td>
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<td>pH</td>
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<td>Free Cl2</td>
<td>DO</td>
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<td>NO2-N</td>
<td>NO3-N</td>
<td>Alkalinity</td>
<td>UV-254</td>
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Note: 0 = influent; 1 = effluent; 2 = copper loops; 3 = coupon cradle
Table 20. Analytical Plan for PDSs 9, 10, 15-18 from 04/05/2003 to 07/04/2003

<table>
<thead>
<tr>
<th>Influent, Cradles, Effluent</th>
<th>PDS 9, 10, 15-18 sampling started May 8th (Biological) 9th (WQ)</th>
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<td>Parameters</td>
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Note: 0 = influent; 1 = effluent; 2 = copper loops; 3 = coupon cradle
Table 21. Internal Ports Sampling Plan for the Period from 04/05/2003 to 07/04/2003

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<td>mg/L NO₃-N</td>
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</tr>
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<td>Nitrite</td>
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<td>x</td>
<td>X</td>
<td>x</td>
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<td>x</td>
</tr>
<tr>
<td>Alkalinity</td>
<td>mg/L as CaCO₃</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>X</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

Note: Samples dates were
05/09/2003, 05/30/2003, 06/20/2003 and 07/03/2003 for PDS 11 to 14
05/09/2003, 05/30/2003, and 06/20/2003 for PDS 15 to 18
Bioassays

As most of the bioassays used in this study are not directly derived from the Standards Methods (which is the case for most chemical water quality parameters), a short description of these techniques is provided below and detailed information can be found in Appendix 1.

Heterotrophic Plate Count (HPC)

This assay was adapted to UCF laboratory from Standard Methods (9215C, USEPA) and was used in order to quantify heterotrophic growth in the PDS bulk liquid and reported as number of colony forming unit per milliliter (cfu/mL).

Assimilable Organic Carbon (AOC)

AOC was measured using the rapid method developed by LeChevallier et al. (1993), except that plate counts were used to enumerate bacteria rather than ATP fluorescence.

Biological Dissolved Organic Carbon (BDOC)

The procedure for BDOC determination followed the technique using sand fixed bacteria (Joret and Levi, 1986; Joret et al., 1991) and relied heavily on a draft standard method procedure (Volk, personal communication).
**Total Coliform-E. Coli (TC/Ecoli)**

A rapid technique using Colilert kit and Quantitray reader was used to detect and quantify coliforms in the PDS bulk liquid.

**Biofilm Heterotrophic Plate Count (BFHPC)**

Coupons colonized by biofilm were sampled and rinsed with Phosphate Buffer Solution. Then the biofilm was manually detached from the coupon using a sterile cell scraper (sterilized by Ethanol 70%) in 4-mL of sterile PBS, and then homogenized using a tissue blender (Tissue Tearor™, Biospec products, Inc) at 3000 rpm for 2 min. Then HPC assay was used to quantify heterotrophic bacteria in the homogenized suspension. The HPC result for a given sample expressed as cfu/mL was further converted into cfu/cm² by multiplying by 4 (mL) and divided by the surface area of the scraped coupon (7.06 cm²).

**Potential of Exoproteolytic Activity (PEPA)**

The PEPA method measures the global activity of the biofilm, estimating the potential of bacteria to lysis proteins, using a proteic non-fluorescent artificial substrate (here L-Leucine β-Naphtylamide, LLβN). The enzymatic hydrolysis of this substrate leads to a fluorescent product (here β-Naphtylamine, βN), which can be detected by spectrofluorimetry. Fluorescence is plotted as a function of time and the rate of degradation gives an estimate of biological activity in the sample. This technique was derived from the work of Laurent and Servais (1995).
**Fluorescent In-Situ Hybridization (FISH)**

In order to enumerate ammonia oxidizing bacteria, fluorescent in-situ hybridization has been used. The technique implemented in the laboratory was designed by Dr. A. Randall based on personal communications from Dr. L. Blackall (University of Queensland, Australia). The method uses two molecular probes specific to the 16S rRNA of Nitrosomonas, the main genus responsible for oxidation of ammonia. The probes were developed by (Mobarry et al., 1996; largely Bruce Rittman and David Stahls group out of Northwestern University), and have been used before for drinking water biofilms successfully, specifically to look at the effects of chloramination (Regan et al., 2002). Quantification has been performed using a triple-laser Confocal Laser Scanning Microscope (CLSM) and an image analysis software package.

**Quality Control**

This portion of the final report summary is intended to provide a comprehensive overview of quality control procedures, analytical results and supplementary discussion of PDS sampling events and process sampling events associated the entirety of this project. The total number of samples, spiked samples, duplicates and blind analyses varies by parameter. The following section addresses analytical QA/QC issues and other laboratory function and information issues pertinent to those samples that were analyzed in the field as well as those that were brought back to the main ESEI lab facility for analysis.
The following describes quality assurance and quality control (QA/QC) measures taken in order to insure data of known quality throughout the entire study. QA/QC plan for all data has followed guidelines as presented in Standard Methods (APHA, 1995). Furthermore, biological QA/QC measures were taken as recommended in reports by Verner (1990) and Najm et al. (2000).

**Chemical Data- UCF Main Laboratory**

The procedures used and their analytical specifications for the duration of this project are summarized in Table 22 and Table 23. Some parameters contain multiple method listings as relevant to field applications and/or multiple concentration ranges of quantification.

Table 22. Methods and Reporting Limits for Aggregate Properties and NPDOC Measured in the Main Laboratory during the Project

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Method</th>
<th>Reporting Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color</td>
<td>SM 2120A pages 2-2 to 2-4</td>
<td>1 cpu</td>
</tr>
<tr>
<td></td>
<td>UV Absorption at 254 nm</td>
<td></td>
</tr>
<tr>
<td>Conductivity</td>
<td>SM 2510B pages 2-45 to 2-46</td>
<td>0.1 μmho/cm</td>
</tr>
<tr>
<td></td>
<td>Laboratory Method</td>
<td></td>
</tr>
<tr>
<td>NPDOC</td>
<td>SM 5310C, pages 5-19 to 5-21</td>
<td>0.1 mg C/L</td>
</tr>
<tr>
<td>Solids (TDS)</td>
<td>SM 2540 pages 2-53 to 2-58</td>
<td>1 mg/L</td>
</tr>
<tr>
<td></td>
<td>Gravimetric Method</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Estimation of TDS by major ion sum</td>
<td>1 mg/L</td>
</tr>
<tr>
<td>Turbidity</td>
<td>SM 2130B pages 2-9 to 2-11</td>
<td>0.01 NTU</td>
</tr>
<tr>
<td></td>
<td>Nephelometric Method</td>
<td></td>
</tr>
<tr>
<td>UV-254</td>
<td>SM 5910 pages 5-60 to 5-62</td>
<td>0.0001 cm⁻¹</td>
</tr>
<tr>
<td></td>
<td>UV Absorption at 254 nm</td>
<td></td>
</tr>
</tbody>
</table>

*All techniques designated with “SM” referenced in Standard Methods are from the 19th Edition (1995).*
Table 23. Methods and Reporting Limits for Inorganic Analysis in the Main Laboratory during the Project

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Method</th>
<th>Reporting Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bicarbonate</td>
<td>SM 2320B, pages 2-26 to 2-28 Titration Method</td>
<td>5 mg/L</td>
</tr>
<tr>
<td>Bromides</td>
<td>SM 4110 pages 4-2 to 4-6, Ion Chromatography with Chemical Suppression of Eluent Conductivity</td>
<td>0.1 mg/L</td>
</tr>
<tr>
<td>Calcium</td>
<td>SM 3111B pages 3-13 to 3-15, Direct Air/Acetylene Flame AAS</td>
<td>0.1 mg/L</td>
</tr>
<tr>
<td>Chloride</td>
<td>SM 4500-Cl B pages 4-49 to 4-50 Argentometric Method</td>
<td>1.0 mg/L</td>
</tr>
<tr>
<td>Copper</td>
<td>SM 4110 pages 4-2 to 4-6, Ion Chromatography with Chemical Suppression of Eluent Conductivity</td>
<td>0.1 mg/L</td>
</tr>
<tr>
<td>Iron</td>
<td>SM 3111B pages 3-13 to 3-15, Direct Air/Acetylene Flame AAS</td>
<td>0.3 mg/L</td>
</tr>
<tr>
<td>Lead</td>
<td>SM 3113 pages 3-22 to 3-27 Electrothermal Atomic Absorption</td>
<td>0.01 mg/L</td>
</tr>
<tr>
<td>Magnesium</td>
<td>SM 3113 pages 3-22 to 3-27 Electrothermal Atomic Absorption</td>
<td>0.01 mg/L</td>
</tr>
<tr>
<td>Manganese</td>
<td>SM 3113 pages 3-22 to 3-27 Electrothermal Atomic Absorption</td>
<td>1 ppb</td>
</tr>
<tr>
<td>Nitrate</td>
<td>SM 4110 pages 4-2 to 4-6, Ion Chromatography with Chemical Suppression of Eluent Conductivity</td>
<td>0.1 mg/L</td>
</tr>
<tr>
<td>Nitrite</td>
<td>SM 4110 pages 4-2 to 4-6, Ion Chromatography with Chemical Suppression of Eluent Conductivity</td>
<td>0.1 mg/L</td>
</tr>
<tr>
<td>Nitrogen (NH3,TKN)</td>
<td>SM 4500-Norg pages 4-92 to 4-94 Macro-Kjeldahl Method</td>
<td>0.1 mg/L</td>
</tr>
<tr>
<td>pH</td>
<td>SM 4500-H+ B pages 4-65 to 4-69 Electrometric Method</td>
<td>± 0.1 pH units</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>SM 4500-P C, pages 4-111 to 4-112 VM Colorimetric Method</td>
<td>0.3 mg/L</td>
</tr>
<tr>
<td></td>
<td>SM 4500-P E, pages 4-113 to 4-114 Ascorbic Acid Method</td>
<td>0.01 mg/L</td>
</tr>
</tbody>
</table>
Table 23 (continued). Methods and Reporting Limits for Inorganic Analysis in the Main Laboratory during the Project

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Method</th>
<th>Reporting Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silica</td>
<td>SM 4500-Si D, pages 4-118 to 4-120, Molybdosilicate Method</td>
<td>1 mg/L</td>
</tr>
<tr>
<td>Sodium</td>
<td>SM 3500-Na B, page 3-96 to 3-98 Flame Emission Method.</td>
<td>0.1 mg/L</td>
</tr>
<tr>
<td>Sulfate</td>
<td>SM 4500-SO42- pages 4-136 to 4-137, Turbidimetric Method</td>
<td>1.0 mg/L</td>
</tr>
<tr>
<td></td>
<td>SM 4110 pages 4-2 to 4-6, Ion Chromatography with Chemical Suppression of Eluent Conductivity</td>
<td>0.1 mg/L</td>
</tr>
</tbody>
</table>

**Chemical Data - Field Laboratory**

Maintenance of good analytical practice in the field was carried out according to the same relevant statistical principles as were used in the main laboratory. While facilities available in a field laboratory are somewhat less sophisticated than those in a full-scale laboratory, the same guidelines are applied to obtain the maximum quality possible from the data gathered in the field. The methodologies used in the field are summarized in Table 24. Many of these techniques are the same as those used in the laboratory (such as the alkalinity determination). Others are modifications of known standard laboratory methods that are commercially available for application to field usage (such as the Hach application for chlorine and for nitrogen species). In all cases involving water quality analysis during the course of this project, the commercial modifications cited and used here were widely accepted and available applications of existing standard protocols described in Standard Methods for Water and Wastewater Analysis. They are not novel or untested methods, and they are broadly used by utilities all over the US.
Statistics (accuracy, precision, etc.) were monitored in the same fashion as in the main laboratory, where applicable. Some parameters (UV254, pH, etc.) can not be monitored for accuracy under field conditions (i.e. can not be accurately spiked with a known increment of standard). In cases as these, either standard is not applicable (turbidity, color), or is not sufficiently stable (DO, sulfide). Precision was monitored for these parameters. Surrogate or secondary standards were used for alkalinity, turbidity, and pH to further assist with assessment.
Table 24. Methods and Reporting Limits for Inorganic Analysis in the Field Laboratory during the Project

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Method</th>
<th>Method Reference</th>
<th>Approx. Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkalinity</td>
<td>Titration</td>
<td>SM 2320 B</td>
<td>5 - 500 ppm</td>
</tr>
<tr>
<td>Ammonia-N</td>
<td>Membrane Probe Method</td>
<td>SM 4500-NH3 C</td>
<td>0.1 - 3 ppm</td>
</tr>
<tr>
<td>Chloride</td>
<td>Argentometric Titration</td>
<td>SM 4500-C1 B</td>
<td>1 - 100 mg/L</td>
</tr>
<tr>
<td>Chlorine, free</td>
<td>DPD colorimetric</td>
<td>SM 4500-C1 G or Hach 8021</td>
<td>0.1 - 2 ppm</td>
</tr>
<tr>
<td>Chlorine, total</td>
<td>DPD colorimetric</td>
<td>SM 4500-C1-G or Hach 8167</td>
<td>0.1 - 2 ppm</td>
</tr>
<tr>
<td>Color, apparent</td>
<td>Visual Comparison (by spectrometer)</td>
<td>SM 2120 B</td>
<td>1 - 50 cpu</td>
</tr>
<tr>
<td>Conductivity</td>
<td>Conductivity Bridge</td>
<td>SM 2510 B</td>
<td>variable</td>
</tr>
<tr>
<td>Hardness (total, calcium)</td>
<td>EDTA Titration</td>
<td>SM 2340 C</td>
<td>5 - 500 mg/L</td>
</tr>
<tr>
<td>Nitrate</td>
<td>Cadmium reduction</td>
<td>Hach 8192</td>
<td>0.1 - 0.5 mg/L</td>
</tr>
<tr>
<td>Nitrite</td>
<td>Diazotization</td>
<td>Hach 8507</td>
<td>0.1 - 0.3 mg/L</td>
</tr>
<tr>
<td>Oxygen, Dissolved (DO)</td>
<td>Membrane probe</td>
<td>SM 4500-O G</td>
<td>0.1 - 20 mg/L</td>
</tr>
<tr>
<td>pH</td>
<td>Electrometric</td>
<td>SM 4500-H⁺ B</td>
<td>2 - 13</td>
</tr>
<tr>
<td>Sulfide</td>
<td>Iodometric</td>
<td>SM 4500-S²⁻ F</td>
<td>0.1 - 5 mg/L</td>
</tr>
<tr>
<td>Temperature</td>
<td>Direct reading</td>
<td></td>
<td>0 - 100 deg C</td>
</tr>
<tr>
<td>Turbidity</td>
<td>Nephelometric</td>
<td>SM 2130 B</td>
<td>0.02 - 200 ntu</td>
</tr>
<tr>
<td>UV-254</td>
<td>UV spectrometry</td>
<td>SM 5910 A</td>
<td>0-0.5200 cm⁻¹</td>
</tr>
</tbody>
</table>
Biological Data

In order to insure quality of the biological data of this study, several measures have been taken to minimize variability and analytical errors in the bioassays. These measures can be divided in three categories: (i) sampling protocol, (ii) preparation of reagents and microorganisms, and (iii) analysis of the samples. In each category, the fate of both bulk liquid and biofilm samples is further discussed.

For all the bulk liquid assays (e.g. AOC, BDOC, HPC, TC/E.Coli., FISH), samples were taken using sterile gloves and sterile containers (either sterile disposable 100-mL Coliform test sampling containers or 1-L Pyrex bottles that had been muffled at 525°C for 5 hrs). After collection, sample bottles were capped with persulfate washed caps. Bulk liquid samples were then placed in ice for transport and brought back to the laboratory upon sampling, where they were stored in a 4°C walk-in cooler until preservation and/or analysis. Storage of bulk liquid samples did not exceed 24 hrs prior to analysis unless proper preservation measures were taken. For biofilm assays (e.g. PEPA, BFHPC, FISH AOB), coupons affixed on PVC holders were handled with sterile gloves and transported in air-tight PVC containers, in which moisture level was maintained with sponges soaked with corresponding incubation water (to prevent desiccation of the biofilms). Upon arrival at the UCF main laboratory, these containers were stored in a 4°C walk-in cooler until analysis was carried out (no later than 24hrs after sampling).

For the preparation of reagents in contact with bulk liquid samples and/or microorganisms, strict aseptic techniques were used. Sterilization of agar and reagents (such as distilled water, dilution buffer and control reagents) was performed by autoclaving at 121°C and
15 psi for 20 minutes. Preservation of AOC samples was carried out by pasteurization in a water bath at 70°C for 1 hr or with alternate pasteurization technique described by Escobar and Randall (2000) for extended storage. Aliquots of AOC samples were added to 40-mL EPA vials, previously muffled at 525°C for 5 hrs, and capped with persulfate washed caps. PVC containers used for incubation of coupons in PEPA assay were sterilized by autoclave as previously described. To prevent contamination, handling and manipulating of samples and reagents requiring aseptic conditions were carried out under a laminar flow hood, previously wiped with bactericidal detergent and irradiated with a UV lamp for 5 minutes. For biofilm samples, sterilized (with 70% ethanol solution) cell scraper and tissue homogenizer were used for cell detachment and homogenization of suspended materials.

Analysis of samples was mainly carried out under laminar flow hood (kept sterile as described above), except for PEPA, which was carried out in a fume hood. In order to check aseptic conditions and proper sterility of the reagents, control measures included the following three steps: (i) laminar flow hood atmosphere was checked using open agar plate during spreading, (ii) dilution buffer sterility was checked by spreading one plate with dilution buffer alone as a blank during the same time as the spreading of the actual samples, and (iii) sterility of the agar was verified by inclusion of one covered, non-inoculated plate in each run. These three plates were incubated along with the samples. A glass spreader (i.e. bent glass rod) was sterilized with 70% ethanol solution and flamed with a Bunsen burner. Sterile disposable pipette tips, sterile gloves and sterilized dilution vials were used to prevent contamination during experiments. For AOC, sterile needles and syringes were used to inoculate samples with
Pseudomonas fluorescens P17 and Spirillum NOX. Purity and accuracy of inocula were also monitored. Incubation of samples when required was performed in a temperature-controlled atmosphere. For biofilm samples analyzed by PEPA, North Viton® gloves were used for handling carcinogenic reagents under fume hood conditions. For bioassays involving plate spreading techniques (HPC, BFHPC and AOC), serial dilutions were always performed in duplicate and plate counts in the range of 30-300 cfu were selected whenever possible. However in the case of low (less than 30 cfu at $10^{-1}$ dilution) or high (above 300 cfu at highest dilution) density samples, plate counts out of this range have been used for interpretation. Since the sample analysis and assessment required 7 to 10 days to complete, this rendered additional duplication (i.e. re-collection of an identical duplicate of a sample in question) practically impossible.

The quality control measures on biological assays described above proved proper aseptic conditions and analysis of the samples with respect to Standard Methods guidelines for handling and analyzing biological samples throughout the entire study.

**QC Assessment**

*Chemical Data – UCF Main Laboratory*

The tables on the following pages provide a summary of the accuracy and precision of laboratory analysis during the entire project.
**Accuracy**

The mean recoveries for the inorganic parameters ranged from about 94% to 103%, as shown in Table 25. Metal recoveries ranged from 90 to 103% recovery. Similarly, anion recovery ranged from 94 to 103%. NPDOC and the other inorganic recoveries fell within 98 to 102% recovery. Individual spike measurements for all parameters were within the NIST required 80 to 120% recovery range, or the value was rejected and another spike was performed. Most warning limits were similar to or within these NIST ranges.

Spike recoveries were tracked using Shewhart control charts in accordance with Standard Methods. Table 26 summarizes the warning and control limit information gathered in the accuracy control charts for all applicable parameters analyzed during the entire project.

**Precision**

Average RPD values for all inorganic parameters analyzed in the laboratory during the entire project are shown in Table 27. The precision control chart information for inorganics and NPDOC is provided in Table 28. A similar summary is provided in Table 29 for aggregate parameters measured in the laboratory. Aluminum and phosphorus were nearly always at or below detection levels. This trend was one of a few recurring circumstances that gave rise to high RSD values and broad ranges. Values that are near/at the detection limit for a given technique and can not be accurately resolved further (for example, TKN. measurement to + 0.01 mg/L) will inevitably give rise to a high RPD. This is responsible for the bulk of the large values shown in the “High” column of Table 25.

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Table 25. Accuracy Summary for NPDOC and Inorganic Parameters in the UCF Main Laboratory during the Project.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Technique</th>
<th>Units</th>
<th># Spikes</th>
<th>Mean</th>
<th>%Rec</th>
<th>RPD</th>
<th>High</th>
<th>Low</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High Metals</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>Flame AAS</td>
<td>ppm</td>
<td>224</td>
<td>98.2</td>
<td>8.3</td>
<td>120</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Magnesium</td>
<td>Flame AAS</td>
<td>ppm</td>
<td>180</td>
<td>97.3</td>
<td>6.2</td>
<td>113</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>Sodium</td>
<td>Flame AE</td>
<td>ppm</td>
<td>195</td>
<td>99.1</td>
<td>3.8</td>
<td>104</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td><strong>Trace Metals</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aluminum</td>
<td>GFAAS</td>
<td>ppm</td>
<td>98</td>
<td>99.0</td>
<td>8.3</td>
<td>120</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Copper</td>
<td>GFAAS</td>
<td>ppb</td>
<td>117</td>
<td>100.4</td>
<td>6.3</td>
<td>116</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>Iron, diss</td>
<td>GFAAS</td>
<td>ppb</td>
<td>170</td>
<td>103.1</td>
<td>8.5</td>
<td>118</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>Iron, total</td>
<td>GFAAS</td>
<td>ppb</td>
<td>216</td>
<td>98.5</td>
<td>2.2</td>
<td>103</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>Lead</td>
<td>GFAAS</td>
<td>ppb</td>
<td>122</td>
<td>99.9</td>
<td>2.1</td>
<td>105</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>Manganese</td>
<td>GFAAS</td>
<td>ppb</td>
<td>14</td>
<td>90.3</td>
<td>4.8</td>
<td>120</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td><strong>Anions</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Bromide</td>
<td>IC</td>
<td>ppm</td>
<td>109</td>
<td>100.6</td>
<td>9.7</td>
<td>120</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Chloride</td>
<td>Titration</td>
<td>ppm</td>
<td>142</td>
<td>97.6</td>
<td>12.1</td>
<td>120</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Fluoride</td>
<td>IC</td>
<td>ppm</td>
<td>96</td>
<td>95.0</td>
<td>10.1</td>
<td>120</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Ortho-P</td>
<td>IC</td>
<td>ppm</td>
<td>90</td>
<td>101.8</td>
<td>8.7</td>
<td>120</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Sulfate</td>
<td>IC</td>
<td>ppm</td>
<td>142</td>
<td>94.1</td>
<td>10.0</td>
<td>120</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td><strong>Other Inorganics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TKN</td>
<td>Digest/Distill</td>
<td>ppm</td>
<td>64</td>
<td>102.0</td>
<td>9.6</td>
<td>120</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Alkalinity</td>
<td>Titration</td>
<td>ppm</td>
<td>140</td>
<td>97.3</td>
<td>7.1</td>
<td>117</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Total-P</td>
<td>Vis spec</td>
<td>ppm</td>
<td>40</td>
<td>103.8</td>
<td>8.8</td>
<td>118</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>Silica</td>
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<td>No. &gt; CL</td>
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<td>Total ≤ CL</td>
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<td>Pct. ≤ CL</td>
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<td>95</td>
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<td>100.0</td>
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<td>135</td>
<td>142</td>
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<td>100.0</td>
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<td>135</td>
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<td>95.1</td>
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</tr>
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Table 27. Precision Summary for Inorganic Parameters and NPDOC Analysis in the UCF Main Laboratory during the Project.

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<tr>
<th>Parameter</th>
<th>Technique</th>
<th>Units</th>
<th># Dupes</th>
<th>Mean</th>
<th>RPD</th>
<th>%RSD</th>
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<th>Low</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>Flame AAS</td>
<td>ppm</td>
<td>228</td>
<td>1.0</td>
<td>1.3</td>
<td>9.3</td>
<td>&lt; 1</td>
<td></td>
</tr>
<tr>
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<td>Flame AAS</td>
<td>ppm</td>
<td>180</td>
<td>1.0</td>
<td>1.2</td>
<td>9.2</td>
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</tr>
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<td>Flame AE</td>
<td>ppm</td>
<td>191</td>
<td>1.2</td>
<td>1.3</td>
<td>6.6</td>
<td>&lt; 1</td>
<td></td>
</tr>
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<td><strong>Trace Metals</strong></td>
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<td>ppm</td>
<td>107</td>
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<td>5.7</td>
<td>25.4</td>
<td>&lt; 1</td>
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<td>ppb</td>
<td>117</td>
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<td>3.3</td>
<td>25.5</td>
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<td>ppb</td>
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<td>4.9</td>
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<td>2.0</td>
<td>9.1</td>
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<td>ppb</td>
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<td>4.1</td>
<td>25.4</td>
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<td>14</td>
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<td>3.5</td>
<td>9.6</td>
<td>&lt; 1</td>
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<td>1.3</td>
<td>9.3</td>
<td>&lt; 1</td>
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<td>95</td>
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<td>9.2</td>
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<td>NA</td>
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<td>NA</td>
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<td>1.3</td>
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<td>4.8</td>
<td>37.3</td>
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* All phosphate and aluminum samples analyzed for QA/QC were below detection (spikes and dupes supportive)
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<th>Total &gt; WL</th>
<th>Total &gt; CL</th>
<th>Pct. &lt; WL</th>
<th>Pct. &lt; CL</th>
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<td>13</td>
<td>91.7</td>
<td>94.3</td>
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<td>5</td>
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<td>96.3</td>
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<td>4</td>
<td>92.5</td>
<td>96.3</td>
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<td>94.9</td>
<td>98.3</td>
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<td>92.3</td>
<td>95.8</td>
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<td>91.4</td>
<td>94.1</td>
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<td>91.1</td>
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<td>9</td>
<td>91.0</td>
<td>91.9</td>
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<td>8</td>
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<td>94.7</td>
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<td>96.6</td>
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<td>93.4</td>
<td>95.4</td>
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</table>

*Insufficient data to construct control chart for this parameter.
Table 29. Precision Summary for Aggregate Parameters for UCF Main Laboratory during the Project.

<table>
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<tr>
<th>Parameter</th>
<th>Technique</th>
<th>Units</th>
<th># Dupes</th>
<th>Mean RPD</th>
<th>Std dev</th>
<th>Max.</th>
<th>Min.</th>
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</thead>
<tbody>
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<td>Color, App</td>
<td>Spectrometric</td>
<td>CPU</td>
<td>31</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
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<td>Spectrometric</td>
<td>CPU</td>
<td>138</td>
<td>2.7</td>
<td>11.3</td>
<td>59.3</td>
<td>&lt; 1</td>
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<tr>
<td>TDS</td>
<td>Ion Sum</td>
<td>ppm</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Conductivity</td>
<td>Probe</td>
<td>μmho/cm</td>
<td>148</td>
<td>0.8</td>
<td>1.1</td>
<td>8.9</td>
<td>&lt; 1</td>
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<td>Abs @ 254 nm</td>
<td>/cm</td>
<td>70</td>
<td>0.8</td>
<td>0.7</td>
<td>3.1</td>
<td>&lt; 1</td>
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</table>

As an added measure of precision, blind duplicates were collected on site with every sampling event. With each work order submitted, at least two (usually more) blind duplicates were also collected. A summary of the blind precision assessment is found in Table 30. These samples were submitted along with their respective sample batch, submitted in the same work order as this batch, and analyzed accordingly. After the results for each parameter were submitted to the lab manager for the entire work order, the blind duplicate results were assessed.
Table 30. Blind Precision Summary for All Parameters for UCF Main Laboratory during the Project.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Technique</th>
<th>Units</th>
<th># Dupes</th>
<th>Mean</th>
<th>RPD</th>
<th>%RSD</th>
<th>High</th>
<th>Low</th>
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<td></td>
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</tr>
<tr>
<td>Calcium</td>
<td>Flame AAS</td>
<td>ppm</td>
<td>185</td>
<td>2.5</td>
<td>4.4</td>
<td>28.9</td>
<td>&lt; 1</td>
<td></td>
</tr>
<tr>
<td>Magnesium</td>
<td>Flame AAS</td>
<td>ppm</td>
<td>183</td>
<td>2.7</td>
<td>4.1</td>
<td>27.3</td>
<td>&lt; 1</td>
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</tr>
<tr>
<td>Sodium</td>
<td>Flame AE</td>
<td>ppm</td>
<td>158</td>
<td>2.4</td>
<td>3.6</td>
<td>26.4</td>
<td>&lt; 1</td>
<td></td>
</tr>
<tr>
<td><strong>Trace Metals</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aluminum</td>
<td>GFAAS</td>
<td>ppm</td>
<td>94</td>
<td>7.6</td>
<td>12.8</td>
<td>67.3</td>
<td>&lt; 1</td>
<td></td>
</tr>
<tr>
<td>Copper</td>
<td>GFAAS</td>
<td>ppb</td>
<td>115</td>
<td>4.2</td>
<td>6.0</td>
<td>48.3</td>
<td>&lt; 1</td>
<td></td>
</tr>
<tr>
<td>Iron, diss</td>
<td>GFAAS</td>
<td>ppb</td>
<td>125</td>
<td>16.1</td>
<td>23.0</td>
<td>100</td>
<td>&lt; 1</td>
<td></td>
</tr>
<tr>
<td>Iron, total</td>
<td>GFAAS</td>
<td>ppb</td>
<td>148</td>
<td>7.9</td>
<td>9.4</td>
<td>50.0</td>
<td>&lt; 1</td>
<td></td>
</tr>
<tr>
<td>Lead</td>
<td>GFAAS</td>
<td>ppb</td>
<td>92</td>
<td>8.7</td>
<td>15.9</td>
<td>100</td>
<td>&lt; 1</td>
<td></td>
</tr>
<tr>
<td><strong>Other Inorganics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>Titration</td>
<td>ppm</td>
<td>107</td>
<td>4.8</td>
<td>5.8</td>
<td>23.0</td>
<td>&lt; 1</td>
<td></td>
</tr>
<tr>
<td>Bromide</td>
<td>IC</td>
<td>ppm</td>
<td>78</td>
<td>7.1</td>
<td>15.7</td>
<td>66.7</td>
<td>&lt; 1</td>
<td></td>
</tr>
<tr>
<td>Chloride</td>
<td>IC</td>
<td>ppm</td>
<td>149</td>
<td>2.8</td>
<td>4.0</td>
<td>23.3</td>
<td>&lt; 1</td>
<td></td>
</tr>
<tr>
<td>Ortho-P</td>
<td>IC</td>
<td>ppm</td>
<td>104</td>
<td>0.8</td>
<td>5.6</td>
<td>48.9</td>
<td>NA</td>
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</tr>
<tr>
<td>Total-P</td>
<td>Vis spec</td>
<td>ppm</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Silica</td>
<td>Vis spec</td>
<td>ppm</td>
<td>108</td>
<td>4.7</td>
<td>6.0</td>
<td>26.7</td>
<td>&lt; 1</td>
<td></td>
</tr>
<tr>
<td>Sulfate</td>
<td>IC</td>
<td>ppm</td>
<td>149</td>
<td>3.6</td>
<td>6.1</td>
<td>40.0</td>
<td>&lt; 1</td>
<td></td>
</tr>
<tr>
<td>TKN</td>
<td>Digest/Distill ppm</td>
<td>27</td>
<td>15.0</td>
<td>13.7</td>
<td>66.7</td>
<td>&lt; 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Aggregates</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Color, True</td>
<td>Vis spec</td>
<td>cpu</td>
<td>127</td>
<td>19.5</td>
<td>31.2</td>
<td>200</td>
<td>&lt; 1</td>
<td></td>
</tr>
<tr>
<td>Conductivity</td>
<td>Probe</td>
<td>μmho/cm</td>
<td>79</td>
<td>2.2</td>
<td>9.0</td>
<td>18.9</td>
<td>&lt; 1</td>
<td></td>
</tr>
<tr>
<td>UV-254</td>
<td>UV spec</td>
<td>cm⁻¹</td>
<td>110</td>
<td>4.9</td>
<td>10.1</td>
<td>100</td>
<td>&lt; 1</td>
<td></td>
</tr>
<tr>
<td><strong>Organics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPDGOC</td>
<td>UV/persulfate ppm</td>
<td>119</td>
<td>9.8</td>
<td>10.9</td>
<td>61.8</td>
<td>&lt; 1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Comparatively few or no blind PDS samples analyzed for ortho-phosphate, lead, or aluminum during this time ever showed values above detection limit. Only one blind sample showed detectable aluminum values; both it and its blind dupe were measure at 30 ppb or below.
Table 31 and Table 32 provide a summary of the accuracy (when applicable) and precision of laboratory analysis during the entire project.

### Table 31. Accuracy Summary for Field Parameters during the Project

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Technique</th>
<th>Units</th>
<th># Spikes</th>
<th>Mean % Rec</th>
<th>RPD</th>
<th>High</th>
<th>Low</th>
</tr>
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<tbody>
<tr>
<td><strong>Metals</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca Hardness</td>
<td>Titration</td>
<td>ppm</td>
<td>73</td>
<td>102.1</td>
<td>5.8</td>
<td>119.5</td>
<td>86.4</td>
</tr>
<tr>
<td>Total Hardness</td>
<td>Titration</td>
<td>ppm</td>
<td>70</td>
<td>100.6</td>
<td>4.3</td>
<td>119.9</td>
<td>90.0</td>
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<tr>
<td>Iron, total</td>
<td>Vis spec</td>
<td>ppb</td>
<td>45</td>
<td>100.9</td>
<td>10.4</td>
<td>104.6</td>
<td>94.8</td>
</tr>
<tr>
<td><strong>Anions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloride</td>
<td>Titration</td>
<td>ppm</td>
<td>54</td>
<td>103.5</td>
<td>5.4</td>
<td>115.7</td>
<td>90.0</td>
</tr>
<tr>
<td>Nitrite-N</td>
<td>Vis spec</td>
<td>ppm</td>
<td>156</td>
<td>100.3</td>
<td>6.7</td>
<td>120.0</td>
<td>80.0</td>
</tr>
<tr>
<td>Nitrate+Nitrite-N</td>
<td>Vis spec</td>
<td>ppm</td>
<td>150</td>
<td>95.7</td>
<td>24.5</td>
<td>120.0</td>
<td>80.0</td>
</tr>
<tr>
<td><strong>Other Inorganics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkalinity</td>
<td>Titration</td>
<td>ppm</td>
<td>176</td>
<td>99.4</td>
<td>4.2</td>
<td>114.7</td>
<td>93.3</td>
</tr>
<tr>
<td>Ammonia-N</td>
<td>Probe</td>
<td>ppm</td>
<td>224</td>
<td>97.3</td>
<td>11.8</td>
<td>120.0</td>
<td>80.0</td>
</tr>
<tr>
<td>Chlorine, free</td>
<td>DPD color</td>
<td>ppm</td>
<td>533</td>
<td>97.2</td>
<td>1.7</td>
<td>101.3</td>
<td>94.5</td>
</tr>
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</table>
Table 32. Precision Summary for Field Parameters during the Project

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Technique</th>
<th>Units</th>
<th># Dupes</th>
<th>Mean</th>
<th>%RSD</th>
<th>High</th>
<th>Low</th>
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<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca Hardness</td>
<td>Titration</td>
<td>ppm</td>
<td>76</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Total Hardness</td>
<td>Titration</td>
<td>ppm</td>
<td>72</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Iron, total</td>
<td>Vis spec</td>
<td>ppm</td>
<td>44</td>
<td>0.003</td>
<td>0.004</td>
<td>0.015</td>
<td>&lt; 1</td>
</tr>
<tr>
<td><strong>Anions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloride</td>
<td>Titration</td>
<td>ppm</td>
<td>51</td>
<td>1</td>
<td>1</td>
<td>11</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Nitrite-N</td>
<td>Vis spec</td>
<td>ppm</td>
<td>177</td>
<td>0.01</td>
<td>0.03</td>
<td>0.23</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Nitrate+Nitrite-N</td>
<td>Vis spec</td>
<td>ppm</td>
<td>129</td>
<td>0.04</td>
<td>0.06</td>
<td>0.35</td>
<td>&lt; 0.01</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Inorganics</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkalinity</td>
<td>Titration</td>
<td>ppm</td>
<td>240</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Ammonia-N</td>
<td>Probe</td>
<td>ppm</td>
<td>235</td>
<td>0.01</td>
<td>0.01</td>
<td>0.11</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Chlorine, free</td>
<td>DPD color</td>
<td>ppm</td>
<td>695</td>
<td>0.02</td>
<td>0.02</td>
<td>0.03</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Chlorine, total</td>
<td>DPD color</td>
<td>ppm</td>
<td>776</td>
<td>0.06</td>
<td>0.15</td>
<td>0.54</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Oxygen, diss</td>
<td>Probe</td>
<td>ppm</td>
<td>515</td>
<td>0.03</td>
<td>0.02</td>
<td>0.44</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td><strong>Aggregates</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Color, apparent</td>
<td>Vis spec</td>
<td>cpu</td>
<td>433</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Conductivity</td>
<td>Probe</td>
<td>μmho/cm</td>
<td>211</td>
<td>2</td>
<td>2</td>
<td>34</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>ORP</td>
<td>Probe</td>
<td>mV</td>
<td>75</td>
<td>4.2</td>
<td>3.0</td>
<td>68.7</td>
<td>0.1</td>
</tr>
<tr>
<td>pH</td>
<td>Probe</td>
<td>mV</td>
<td>612</td>
<td>0.02</td>
<td>0.01</td>
<td>0.44</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Turbidity</td>
<td>Nephelometer</td>
<td>ntu</td>
<td>557</td>
<td>0.05</td>
<td>0.04</td>
<td>0.37</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>UV-254, filtered</td>
<td>UV spec</td>
<td>cm⁻¹</td>
<td>159</td>
<td>0.0008</td>
<td>0.0011</td>
<td>0.0082</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>UV-254, unfilt</td>
<td>UV spec</td>
<td>cm⁻¹</td>
<td>129</td>
<td>0.0007</td>
<td>0.0006</td>
<td>0.0027</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Biological Data

For plate counting techniques (HPC, BFHPC), all samples were run in duplicate. For each individual within each pair, the counts were assessed. The logarithm of each count was taken, and the range (difference) of the log values for each pair was calculated. The range was expected to be less than one, i.e. the counts were expected to be within one order of magnitude of each other (USEPA 1989). Out of 320 HPC analyses, the range varied from 0 to 0.64. Out of 324 BFHPC analyses throughout the project, the range varied from 0 to 0.75. In either case, the range never exceeded 1. This means that all duplicate analyses of HPC as well as BFHPC were acceptable.

Yield controls were used to assess AOC performance. Two separate solutions of carbon (100 µg/L of acetate carbon in mineral salts buffer) are inoculated with P17 and with NOX respectively. Growth yields were assessed as cfu/µg C and compared to published values (Van der Kooij 1990). The difference between the logarithm of the value obtained experimentally versus the published (theoretical) value were expected to be within 1. There were a total of 11 P17 yield controls and 11 NOX controls. All of the NOX controls complied, whereas 9 of the 11 P17 yield controls complied; 2 of these were high (about 1.3-1.5). It was found upon inspection of the logs that the P17 inocula were slightly higher for 6/20/02 and 8/01/02, resulting in a higher yield control.

For PEPA analysis as carried out in this project (i.e. on coupons), biofilm structure and pattern of colonization can not be reproduced identically on two apparently identical coupons of
the same material (Van der Kooij and Veenendaal 1992). Therefore, as long as the range of logs of the biofilm inventory of duplicate pairs were within 2, (i.e. the inventories were within two orders of magnitude), duplication was deemed acceptable. The range varied from 0 to 1.4 over the entire project (64 pairs); no range value ever exceeded 2. The majority of ranges (62 of 64) were below 1.

Bulk liquid FISH-AOB results correlated well with the sum of nitrite and nitrate concentration for a given sample location, referred to as NOx ($R^2 = 0.36$ and $R^2=0.25$, respectively). For FISH-AOB, two different probes were used to detect nitrifiers (NSO 190 and NSO 1225). Results from these two probes correlated well with each other ($R = 0.7$). It should be noted that this FISH-AOB technique was tailor-designed for this project, therefore no standard selection criteria or literature reference exist for comparison.
CHAPTER FOUR: IMPACT OF STEADY-STATE CHANGE OF WATER QUALITY ON HPC IN DISTRIBUTION SYSTEMS; TRANSITION MODEL

Introduction

Potable water distribution systems work as continuously fed bioreactors [1] in which physico-chemical (corrosion, chlorine consumption, flocculation, particles sedimentation, etc) and biological reactions (biological growth) are carried out in the bulk and at the interface bulk/surface. These reactions are often misunderstood and are always difficult to control. Once the chemical and biological deposits (biofilms) form in the system, pseudo-equilibrium takes place between the water column and the inner walls of the pipes, leading to water with a relatively stable quality and complying with regulations. However, when this equilibrium is upset by changes in the water quality (seasons, storage time, blends), these deposits can be released, which implies a new equilibrium, which can result in a failure to comply with the regulations. In most potable water distribution systems, the water/material interface is a favored site for cells and organic matter accumulation and for multiplication of bacteria [2-6]. This proliferation is followed by the detachment [7] or by their displacement from the pipe surface to the bulk liquid [8] and by their transport in the bulk liquid. According to van der Wende [6] most suspended bacteria in the bulk phase originate from the biofilms on the inner walls of the distribution systems. Regrowth can occur in the bulk phase and on the inner walls of distribution system pipes. Bacteria can attach and grow on pipes even in presence of significant disinfectant residual. Bacteria can detach and enter the bulk phase but will be inactivated if sufficient
residual is present. Regrowth becomes a concern only when it is measured in the bulk water. Disinfectant (either free chlorine or chloramines) is considered to be the most important factor for controlling bacterial proliferation in the bulk water [9]. Upon implementation of more stringent disinfection by-products regulations, chloramines have been more widely used. They are thought to produce a more stable residual than free chlorine and thus provide lasting protection against regrowth and penetrate more deeply within biofilms, which was confirmed at full-scale in Muncie (Indiana) [10]. However chloramine is a less effective disinfectant than free chlorine and therefore may not control bacterial proliferation as well if its concentration is significantly reduced. HPC measurement on low-nutrient R2A agar is considered better than other HPC assays to predict regrowth of water-based bacteria in distribution systems [11]. Reasoner [12] considers HPC as a suitable tool for (i) assessing changes in finished water quality during distribution and storage and distribution system cleanliness, (ii) measuring bacterial regrowth potential in treated drinking water, and (iii) monitoring bacterial populations changes following treatment modifications, e.g. change in type of disinfectant or finished water source. However it needs to be noted that HPC levels have yet to be correlated to adverse health effects [13-15]. The maximum HPC population of 500 cfu/mL often cited for drinking water is not a health-based standard and no EPA, FDA or WHO health-based HPC regulations exist [16]. Therefore sudden increases of HPC levels in distribution systems, while indicating greater bacterial regrowth, do not necessarily pose a threat to the consumers.

The results discussed in this paper (June 19th to July 7th 2003) originate from a tailored collaboration project between American Water Works Association Research Foundation
(AWWARF), Tampa Bay Water (TBW) and University of Central Florida (UCF) to study the effect of blending different source waters on distribution system water quality. The main objectives of the research presented in this paper were (1) to monitor HPC before and after finished water change in pilot distribution systems (PDS) under chlorination and chloramination, (2) to model HPC transition from the time of steady state water quality change, and (3) to model HPC as a function of water quality in both disinfectant scenarios.

**Materials and Methods**

**Experimental Set-Up.**

**Pilot Distribution Systems (PDS).**

The eight pilot distribution systems (PDS) used to produce the results of this paper consisted of pipe sections from actual full-scale distribution systems that were pulled out from the ground by the participating member governments and were thus aged and sometimes corroded extensively (in the case of unlined metals such as cast iron and galvanized steel). To simulate the sequence of materials likely found in full-scale distribution systems, the sequence of pipe material in each of the PDS was (upstream to downstream) polyvinyl chloride (PVC), lined cast iron (LCI), unlined cast iron (UCI) and galvanized steel (G). A diagram of one of the PDS is presented in Figure 19. The PDS were designed with a two-day Hydraulic Residence Time (HRT) and were virtually identical with respect to the condition of their pipe sections, their
hydraulic regime (HRT, flow velocities in the different pipe sections) and the environmental conditions under which they were operated (temperature, humidity). Therefore the main difference between each of these PDS was the source water used to feed the PDS. The PDS were divided into two groups. The finished waters introduced to the first group (PDS 1 to 4) were chlorinated and the finished waters introduced to the second group (PDS 5 to 8) were chloraminated.

**Water Sources.**

Each of the eight PDS under study received different source water. In each set of PDS, three PDS received a single water source, while a fourth PDS received a blend of these single water sources. The three source waters were produced from three different water treatment processes designed to simulate member government present or proposed finished waters: (i) G1 was produced from a groundwater source treated by aeration and stabilization, (ii) S1 was produced from a surface water source treated by enhanced ferric sulfate coagulation, ozonation, BAC filtration and stabilization, (iii) RO was produced from a groundwater source treated by high pressure reverse osmosis (salts were added to the permeate to simulate typical finished water from a desalination process). A blend of 63% G1, 27% S1 and 11% RO was feeding a fourth PDS. All finished waters were treated either with a chlorine or chloramines residual of 4 mg Cl₂/L total chlorine.
Heterotrophic Plate Count - HPC

Daily HPCs were performed by plate spreading on R2A agar incubated at 25°C for seven days, according to Standard Method 9215B [17]. Results were expressed in colony-forming units per milliliter (cfu/mL).

Chemical Water Quality Monitoring

Influent and effluent PDS water quality samples were collected on a daily basis. Reported information included ammonia, nitrate, nitrite, residual total chlorine, temperature, and dissolved oxygen. All analyses were performed by accepted methods according to Standard Methods [17].

Results and Discussion

An investigation on the effects of water quality change on HPC transition was conducted between June 19th and July 7th 2003. On June 27th 2003, the finished water sources were changed in PDS 1 through 8 as shown in Table 33 and the high HPC sampling frequency experiment during transition was conducted. The waters were changed by switching the feed lines among PDSs. Average water quality of the eight finished water sources under study is presented in Table 34 (calculated with 3-month dataset prior to the changes). These waters were very similar in terms of water quality, although there was noticeable alkalinity level differences. Therefore alkalinity was used as a tracer to monitor the transition of steady-state water quality.
HPC Transition over Time.

Log HPCs as a function of time through the high sampling frequency tests are shown in Figure 20 and Figure 21 for free chlorine and chloramine residuals respectively. The figures are shown side by side to facilitate comparison of HPCs in both disinfectant environments. The transition date (water quality change) is shown by a vertical dotted line in Figure 20 and Figure 21. The change in steady-state water quality was implemented on June 27, 2002 in PDS 1-8. The HRT in PDS 1-8 was two days, but as shown in Figure 20 and Figure 21 almost five days were required before steady-state water quality was achieved, which would correspond to the time when HPCs release was maximized. Alkalinity changes are reported in Figure 22. The alkalinity changes were also completed in about five days, which further supports the correlation of steady state water quality change (alkalinity) and HPC release in the PDSs. However, it is possible that the HPC transition could have been affected by the physical switching of lines among PDS 1-8. The removal of the flexible feed lines from the various peristaltic pumps controlling finished water flow rates from the selected finished water storage tanks may have resulted in disruption of biofilm that discharged a HPC spike to the PDSs. Such action could cause HPC peaks in all PDSs irrespective of water quality. However, it is difficult to rationalize identical HPC peaks in all PDSs by such actions. Identical peaks might be more likely from chemical changes in the bulk water as opposed to physical disruption.

The sharp rise in HPCs after the transition data was irrespective of PDS residual concentration and form as the same magnitude of HPC change occurred in PDS 1-8 regardless of residual concentration and form. However the HPCs in the chloraminated PDSs (5 through 8)
were one to 1.5 logs higher than the HPCs in the chlorinated PDSs (1 through 4) before the water quality change. Although the HPC population was significantly higher in the chloraminated PDS relative to the chlorinated PDS, the maximum HPC concentration achieved in the presence of either residual were approximately equal, meaning nearly the same mass of HPCs was released in the presence of either residual. Since the original HPC concentrations in the chlorinated PDSs were lower than in the chloraminated PDSs, more HPCs would have had to be released from the chlorinated PDSs to equal the HPCs in the chloraminated PDSs. However, since both the chlorine and chloraminated PDSs approach six log HPCs, this difference is practically insignificant. Finally, there was some indication of a downward HPC trend in most of the PDSs.

Clearly, a change in steady-state water quality or something occurring in the same time period of the steady-state water quality change caused a significant release of HPCs in all PDSs involved in the high frequency HPC sampling investigation. However, it can not be dismissed that the HPC change may have been caused by switching the feed lines among PDSs.

**HPC Transition Modeling**

As shown in Figure 20 and Figure 21, the HPC transition peaked about four to five days after the water quality switch. PDS effluent stream alkalinity was monitored to determine mass transport through the PDS and is shown in equation 1. This mixing model was regressed against all alkalinity observations in PDS 1 through 8 following the water quality switch. The 0.72 rate constant shown in equation 1 was significant at the 0.0001 confidence level and $R^2$ for predicted and actual alkalinity versus time was 0.95. An example of how well the model fit the PDS flow
distribution is shown in Figure 22. The significance of the PDS mixing model is that it accurately describe flow distribution through the PDS and that the reciprocal of the rate constant, \((k = 0.72 \text{ days}^{-1})\) would be the time constant (1.40 days). The time constant represents the time for 62% of the mass entering the PDS to pass through the PDS. Ninety-five percent of the mass entering the PDS passed through the PDS in approximately 4.2 days, which corresponded to the point of maximum HPC release in PDS 1 through 8.

\[
Alk_t = Alk_0 + \Delta Alk_{ss} \times \left(1 - e^{-0.72t/\text{day}}\right)
\]  

(1)

where, \(Alk_t\) = Effluent alkalinity after switch (mg/L CaCO\(_3\))

\(Alk_0\) = Steady state alkalinity before switch (mg/L CaCO\(_3\))

\(Alk\) = Steady state alkalinity after switch (mg/L CaCO\(_3\))

\(\Delta Alk_{ss}\) = Alk - Alk\(_0\) (mg/L CaCO\(_3\))

\(t\) = Elapsed time after water quality switch (days)

Equation 1 describes alkalinity transition during PDS water quality changes and it does provide a quantitative means of determining when transition was completely realized in the PDS effluent. However, it did not peak and then decrease as shown in Figure 20 and Figure 21 for HPC variation during the HPC transition experiment. The HPC transition data was regressed against time and in the second order function shown in equation 2 to develop a model that would predict HPC release and duration. Although a composite model for each PDS was statistically significant, the \(R^2\) for the composite model was low. As shown in Table 35, the \(R^2\)'s were acceptable when the model was applied to each PDS separately. The HPC transition model did predict the HPCs return to their original values in approximately ten days and peak at
approximately five days for all PDSs. Predicted and actual Log HPC for free chlorine and chloramines from a blended to a RO finished water are shown in Figure 23 and Figure 24 for PDS 4 and 8 respectively. It is unfortunate that more data was not collected for the HPC transition experiment to validate the return of HPC values to their initial condition.

\[ \text{Log}(HPC) = \text{Log}(HPC_0) + A \times \text{time} \times \exp(-k_{hpc}t) \]  

where \( \text{Log}(HPC_0) = \) Initial Log(HPC)
\( A = \) Coefficient
\( \text{time} = \) Time (days following switch)
\( k_{hpc} = \) Reaction rate (days\(^{-1}\))

Relating water chemistry to the magnitude of the peak HPC observed during the transition would have provided a useful tool in estimating the effect of blending on transient HPC release. An attempt was made to model the driving force \( (A) \) as a function of water chemistry parameters, including alkalinity, chlorides and sulfates. No significant relationship was found between the water chemistry and the driving force \( (A) \). The effect of water chemistry on transient HPC release was inconclusive.

HPC variations through PDS 1 through 4 were described as a function of temperature, time and free chlorine residual as shown in equation 3. HPC variations through PDS 5 though 8 were described in a similar manner as shown in equation 4 for chloramine residuals. Predicted and actual HPCs from both of these models were within a 95 % confidence interval. \( R^2 \) for these models were 0.52 and 0.47 respectively. Both the models indicated HPCs increased with time and temperature, and decreased with increasing residual, which is what the actual data showed.
Hence the models were descriptive of the observed HPCs during the HPC transition experiments.

\[ \log(HPC) = \frac{1.19^{(T-20)} \times 1.12^t}{1.51 \times Cl_2^{0.14}} \]  

(3)

where  
- HPC = cfu/mL  
- T = temperature (°C)  
- t = time (days)  
- Cl₂ = Free chlorine residual (mg/L as Cl₂)

\[ \log(HPC) = \frac{1.06^{(T-20)} \times 1.08^t}{0.42 \times NH₂Cl^{0.04}} \]  

(4)

where  
- NH₂Cl = chloramine residual (mg/L as Cl₂)

A three dimension graph of actual and predicted Log HPCs normalized to 20°C versus time and residual is shown in Figure 25. Log HPCs were normalized to a common temperature in order to remove the effects of temperature.

The lower plane in Figure 25 is the plane of predicted Log HPCs for free chlorine residuals for varying time and residual. The clear circles are the actual HPCs for free chlorine residuals at associated time and residuals. The higher plane in Figure 25 is the plane of predicted Log HPCs for chloramine residuals for varying time and residual. The dark circles are the actual HPCs for chloramine residuals at associated time and residuals. Both planes increase sharply at low residuals, which emphasize the importance of residual to microorganism growth in drinking water distribution systems. The chloramine plane is significantly above the free chlorine plane at
all points except where both residuals approach zero. Free chlorine more effectively retarded HPC proliferation in the PDSs.

Conclusions

The following main conclusions can be drawn from the present study:

- The high frequency HPC experiment showed a rapid increase in HPCs in all PDSs corresponding to the change in steady-state water quality.
- Modeling HPC transition from the time of steady-state water quality change with a second-order time model found that maximum HPCs were achieved approximately five days after the water quality change and that HPCs would return to the HPC concentrations prior to the water quality change in approximately ten days after the change. The time to reach maximum HPCs was related to a mixing model using alkalinity as a surrogate that confirmed alkalinity transition was complete in approximately eight days.
- HPC transition was also modeled as a function of residual, time and temperature by non-linear regression. The actual and predicted HPCs showed that HPC populations decreased as residual increased; decreased as temperature decreased and increased with time. The actual and predicted data for free chlorine and chloramine residuals demonstrated that exponential growth occurred when either residual approached zero. HPC populations associated with chloramines were significantly (1-2 logs) higher than HPC populations associated with free chlorine.
References


Figure 19. Conceptual Flow Diagram of Pilot Distribution System

“\: inches (one inch = 2.54cm); Dia: Diameter.
Figure 20 PDS Effluent Log HPC by Source Switch as a Function of Time, for Free Chlorine Residual

Figure 21 PDS Effluent Log HPC by Source Switch as a Function of Time, for Chloramine Residual
Figure 22. Alkalinity Transition during HPC Transition Investigation for PDS 1.
Figure 23. Blend to RO (Free Chlorine Residual)

Figure 24. Blend to RO (Chloramine Residual)
Figure 25. Actual and Predicted HPCs versus Residual and Time Following Steady-State Water Quality Change
Table 33. High Frequency Testing for HPC Change during Phase Transition.

<table>
<thead>
<tr>
<th>Date</th>
<th>PDS 1</th>
<th>PDS 2</th>
<th>PDS 3</th>
<th>PDS 4</th>
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<tr>
<td>Source</td>
<td>100 % G1</td>
<td>100 % S1</td>
<td>100 % RO</td>
<td>63%G1/27%S1/11%RO</td>
</tr>
<tr>
<td>Change</td>
<td>to</td>
<td>to</td>
<td>to</td>
<td>To</td>
</tr>
<tr>
<td>Disinfectant</td>
<td>Cl₂</td>
<td>Cl₂</td>
<td>Cl₂</td>
<td>Cl₂</td>
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<td>HPCs</td>
<td>3-4/wk</td>
<td>3-4/wk</td>
<td>3-4/wk</td>
<td>3-4/wk</td>
</tr>
<tr>
<td>Source</td>
<td>100 % G1</td>
<td>100 % S1</td>
<td>100 % RO</td>
<td>63%G1/27%S1/11%RO</td>
</tr>
<tr>
<td>Change</td>
<td>to</td>
<td>to</td>
<td>to</td>
<td>To</td>
</tr>
<tr>
<td>Disinfectant</td>
<td>NH₂Cl</td>
<td>NH₂Cl</td>
<td>NH₂Cl</td>
<td>NH₂Cl</td>
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<td>3-4/wk</td>
<td>3-4/wk</td>
<td>3-4/wk</td>
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Table 34. Average Water Quality of Eight Finished Waters.

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<tr>
<th>Water Source</th>
<th>Turb. NTU</th>
<th>NH₃ mg/L N</th>
<th>DOC mg/L C</th>
<th>AOC µg/L C</th>
<th>Alk. mg/L CaCO₃</th>
<th>Residual Cl₂ mg/L</th>
<th>Temp. °C</th>
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<tr>
<td>G1-free</td>
<td>1.1</td>
<td>0.02</td>
<td>2.4</td>
<td>62</td>
<td>205</td>
<td>3.97</td>
<td>27.0</td>
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<tr>
<td>S1-free</td>
<td>0.51</td>
<td>0.01</td>
<td>1.9</td>
<td>59</td>
<td>73</td>
<td>3.94</td>
<td>27.0</td>
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<tr>
<td>RO-free</td>
<td>0.3</td>
<td>0.01</td>
<td>0.5</td>
<td>23</td>
<td>79</td>
<td>6.00</td>
<td>26.8</td>
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<tr>
<td>Blend-free</td>
<td>0.9</td>
<td>0.08</td>
<td>2.1</td>
<td>58</td>
<td>150</td>
<td>4.16</td>
<td>27.1</td>
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<tr>
<td>G1-combined</td>
<td>1.28</td>
<td>0.23</td>
<td>2.2</td>
<td>19</td>
<td>203</td>
<td>3.53</td>
<td>27.1</td>
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<td>62</td>
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<td>RO-combined</td>
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<td>0.3</td>
<td>17</td>
<td>72</td>
<td>3.84</td>
<td>27.0</td>
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<td>Blend-combined</td>
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<td>0.26</td>
<td>2.0</td>
<td>45</td>
<td>162</td>
<td>3.52</td>
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Table 35. Statistical Parameters for HPC Transition Model.

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<th>Term</th>
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<th>PDS-2</th>
<th>PDS-3</th>
<th>PDS-4</th>
<th>PDS-5</th>
<th>PDS-6</th>
<th>PDS-7</th>
<th>PDS-8</th>
<th>Composite</th>
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<tr>
<td>Log(HPC&lt;sub&gt;0&lt;/sub&gt;)</td>
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<td>2.10</td>
<td>2.30</td>
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<td>3.79</td>
<td>3.62</td>
<td>3.60</td>
<td>3.09</td>
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<td>A</td>
<td>1.64</td>
<td>1.69</td>
<td>1.96</td>
<td>2.99</td>
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<td>0.68</td>
<td>1.75</td>
<td>2.49</td>
<td>1.59</td>
</tr>
<tr>
<td>K&lt;sub&gt;hpc&lt;/sub&gt;(day&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.252</td>
<td>0.201</td>
<td>0.286</td>
<td>0.584</td>
<td>0.370</td>
<td>0.182</td>
<td>0.417</td>
<td>0.518</td>
<td>0.885</td>
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<tr>
<td>p-Value Log(HPC&lt;sub&gt;0&lt;/sub&gt;)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
<td>0.00</td>
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<td>0.00</td>
</tr>
<tr>
<td>p-Value for A</td>
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<td>0.12</td>
<td>0.22</td>
<td>0.00</td>
<td>0.41</td>
<td>0.07</td>
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<td>p-Value for k</td>
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<td>0.04</td>
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<td>0.03</td>
<td>0.08</td>
<td>0.00</td>
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<tr>
<td>R&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.50</td>
<td>0.88</td>
<td>0.48</td>
<td>0.53</td>
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<td>0.51</td>
<td>0.79</td>
<td>0.62</td>
<td>0.00</td>
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<tr>
<td>Conf. Interval</td>
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<td>0.00</td>
<td>0.07</td>
<td>0.10</td>
<td>0.00</td>
<td>0.12</td>
<td>0.00</td>
<td>0.00</td>
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CHAPTER FIVE: EMPIRICAL MODEL FOR HETEROTROPHIC PROLIFERATION IN CHLORAMINATED DISTRIBUTION SYSTEMS

Introduction

Production of biologically safe drinking water in compliance with regulations at the outlet of a treatment plant is not sufficient to guarantee a safe product at the consumer’s tap due to the phenomenon of bacterial regrowth within the distribution system. Bacterial regrowth has been considered a major problem in many drinking water distribution systems [1-2]. Distribution systems act as large bioreactors [3] and unlike European utilities studied by van der Kooij, many North American utilities have high Biological Organic Matter (BOM), i.e. nutrient levels in the water entering the distribution system, with residual BOM concentration of more than 200 µg/L as C-acetate [4-5]. Bacterial regrowth can be divided into two categories: (i) attached and (ii) suspended bacteria proliferation. It has been commonly accepted that the presence and proliferation of bacteria in the bulk phase was mainly due to detachment of cells from biofilm matrices, bulk bacterial growth being considered negligible [6-8]. This concept has been challenged by Boe-Hansen et al. [9] who found that bulk phase bacterial growth can be significant and should be taken into account within bacterial growth models for drinking water distribution systems. Heterotrophic Plate Count (HPC) assay has been traditionally used to monitor the phenomenon of regrowth in drinking water distribution systems. Some authors consider that the HPC assay provides at best a gross underestimation of the number of viable bacteria present in distribution system [7, 10-12]. Nevertheless, HPC is widely used and
Reasoner [13] highlighted that HPC is a suitable tool for measuring bacterial regrowth potential in treated drinking water and monitoring bacterial population changes following treatment modification (e.g. change in disinfectant or water source). Understanding the factors governing regrowth in distribution systems is critical since bacterial regrowth can promote occurrence of pathogenic bacteria in distributed drinking water [14]. Several studies have been carried out to identify key factors that influence bacterial regrowth within distribution networks. It is considered that no regrowth occurs in the bulk phase if sufficient disinfectant residual is present but that bacteria can attach and grow on pipes even if significant residual is present. Bulk heterotrophic proliferation has been correlated to DO consumption, temperature, ammonia and oxidized nitrogen in Sydney’s chloraminated distribution system [15]. LeChevallier et al. correlated HPC with temperature and chlorine levels [16]. Disinfectant residual (either free chlorine or chloramines) was considered the most important factor in determining HPC level [17]. Gaining better understanding of factors leading to bacterial proliferation in the bulk phase of distribution systems is therefore necessary in order to find the most suitable strategies to control this phenomenon and ensure quality of drinking water delivered to consumers.

The results discussed in this paper (December 6th 2001 to December 23rd 2002) originate from a tailored collaboration project between American Water Works Association Research Foundation (AWWARF), Tampa Bay Water (TBW) and University of Central Florida (UCF) to study the effect of blending different source waters on distribution system water quality. The main objective of the research presented in this paper was to study heterotrophic proliferation measured by HPC under a wide range of conditions (e.g. water sources, PDS and seasons).
Specifically this work aimed (i) to determine the most important factors influencing bacterial proliferation in the bulk phase of distribution systems, and (ii) to build a representative and predictive empirical model for bulk HPC.

**Materials and Methods**

**Experimental set-up**

*Pilot Distribution Systems (PDS)*

The 18 pilot distribution systems (PDS) used to produce the results of this paper consisted of pipe sections from actual full-scale distribution systems that were pulled out from the ground by the participating member governments and were thus aged and sometimes corroded extensively (in the case of unlined metals such as cast iron and galvanized steel). Two sets of PDS were used. The fourteen PDS in the first set, referred to as “hybrid” PDS, consisted of a sequence of different pipe materials in order to simulate sequence of materials likely found in full-scale distribution systems. The sequence of pipe material in each of these PDS was (upstream to downstream) polyvinyl chloride (PVC), lined cast iron (LCI), unlined cast iron (UCI) and galvanized steel (G). A diagram of one of the PDS is presented in Figure 26. Each of the four PDS in the second set, referred to as “single material” PDS, consisted of a single material (either PVC, LCI, UCI or G). All PDS were designed with a five-day (December 6th 2001 to August 29th 2002) and a two-day (August 30th 2002 to December 23rd 2002) Hydraulic
Residence Time (HRT). All hybrid PDS were virtually identical with respect to the condition of their pipe sections, their hydraulic regime (HRT, flow velocities in the different pipe sections) and the environmental conditions under which they were operated (temperature, humidity). Therefore the main difference between each of these PDS was the source water used to feed the PDS. All four single material PDS were fed with the same water source, therefore isolating the effect of pipe material on water quality.

**Water Sources**

Each of the eighteen PDS under study received a different source water. A brief description of the seven water treatment processes used on this project is presented in Table 36. The finished waters produced from these processes and referred to as “single water sources” were blended in different proportions according to finished waters in use by the collaborating member governments. This one-year study was divided into four 3-months phases, i.e. phases 1 to 4, each being characterized by specific water source for each PDS. A description of these blends per phase is provided in Table 37. All finished waters were treated with a chloramine residual of 4 mg Cl₂/L total chlorine.
Heterotrophic Plate Counts - HPC

HPCs were performed by plate spreading on R2A agar incubated at 25°C for seven days, according to Standard Method 9215B [18]. Results were expressed in colony-forming units per milliliter (cfu/mL).

Assimilable Organic Carbon - AOC

AOC was measured using the rapid method developed by LeChevallier et al. (1993), except that plate counts were used to enumerate bacteria rather than ATP fluorescence [19].

Chemical Water Quality Monitoring

Influent and effluent PDS water quality samples were collected. Reported information included chloramine or total chlorine residual, temperature, dissolved oxygen, AOC, DOC or UV254, calcium, sulfates, silica, chlorides and alkalinity. All analyses were performed by accepted methods according to Standard Methods [18].

Results and Discussion

Bulk biostability was assessed by HPC population as no coliforms were ever observed in any PDS influent or effluent samples. A general assessment of biostability without regard to temperature, pipe materials or water quality is shown in Figure 27. Biostability as assessed by
HPC population is shown to be dependent on PDS effluent residual. HPC population dramatically increases when the PDS effluent residual is less than approximately 0.1 mg/L as Cl₂ and appears to enter a transition zone of visually apparent exponentially increasing growth at 0.2 to 0.3 mg/L Cl₂ total chlorine.

Transition zone and zone of biological instability can be defined by the HPC goals of 500 or 1,000 cfu/mL and 10,000 cfu/mL, respectively. The beginning of the transition zone to biological instability would be defined by a regulatory definition and the beginning of the bioinstability zone would be arbitrarily defined as an order of magnitude higher. The HPC relationship with chloramines residual shown in Figure 27 clearly shows a zone of unchecked microbiological growth corresponding to total residual, which can easily be defined as biological instability. Although the HPC relationship shown in Figure 27 does not consider temperature, water quality (e.g. AOC) or pipe material, some comments can be made concerning impact of residual on HPC. In opposition to European practice of adequately controlling biological stability without a residual in drinking water distribution systems, Figure 27 shows that this no-residual approach would not have achieved biological stability in this work.

**HPC Modeling**

Relationships of HPCs with AOC, temperature and residual were investigated by visual inspection of graphs and statistical analyses of the data in the bulk and biofilm. Bulk biostability was assessed by relating HPC proliferation to water quality parameters by non-linear regression.
Pipe material was represented by dummy variables in the regression analysis with \( H \) being assigned to the hybrid PDS. Independent water quality variables included in the initial regression were AOC, residual, temperature, DOC or UV254, calcium, sulfates, silica, chlorides and alkalinity. With the exception of AOC and the saturation constant (i.e. the constant obtained by regression that allows to consider the case of zero residual), independent variables that were not within the 95 % confidence level (CL) were eliminated from the data set and the regression was repeated until only independent variable that were within the 95 % CL remained. When more than one independent variable was outside the 95 % CL, the variable most outside the 95 % CL (highest p value) was eliminated and the regression was repeated. The final regression equation relating \( \log HPC_{AOC} \) to water quality is shown in equation 1, which is designated as \( \log HPC_{AOC} \) to clarify this model from other \( \log HPC \) models. The selected model is a non-linear model, which includes exponential water quality terms with exponents whose magnitude and sign are determined by regression. The residual term included a retardant term (saturation constant) plus the actual residual in the denominator, which maintains convergence at zero residual.
\[
\text{Log}(\text{HPC})_{AOC} = (2.6488H + 2.6477ucI + 3.3410LI + 3.0633\text{PVC} + 2.8364G) \\
\times \frac{\text{AOC}_{\text{inf}}^{0.0398}}{(0.0177 + TCI_{2-eff})^{0.1310}}
\]

(1)

where \( \text{Log HPC} = \) Log HPCs (cfu/mL) \\
\( H = \) Hybrid PDS (0,1) \\
\( UCI = \) UCI PDS (0,1) \\
\( LCI = \) LCI PDS (0,1) \\
\( G = \) G PDS (0,1) \\
\( \text{AOC}_{\text{inf}} = \) Influent AOC (µg/L as C-acetate) \\
\( TCI_{2-eff} = \) Effluent Total Chlorine Residual (mg/L as Cl\(_2\))

As shown in Table 38, all dummy coefficients for material were significant; however, AOC\(_{eff}\) (p = 0.27) and the saturation constant (p = 0.53) were not significant at the 95 % confidence level. The dummy variable coefficient for PVC is larger than the dummy variable coefficient for G, which could be incorrectly interpreted as the PVC environment was more conducive to HPC growth than the G environment, which is not the case. The relatively high PVC dummy variable coefficient was caused by the inclusion of residual in the model and the higher residual in the PVC environment relative to the G environment. If the same residuals could be maintained in the G and PVC environments, then the PVC HPCs would be predicted to exceed the G HPCs. This would not be unreasonable in the hypothesis of leaching zinc, which may be toxic to HPCs.

The predicted and actual Log HPCs versus residual and AOC are shown in Figure 28, which illustrate the impact of AOC and residual for this model. The coefficient of determination for this model is 0.51. As shown in Figure 28 and after inspection of equation 1, the predicted
HPC population is approximately one order of magnitude less at a 1 mg/L residual than at a zero
residual; whereas the predicted HPC population increases by approximately 0.2 of a log for an
AOC variation of 10 to 1000 µg/L as C. Residual impacts the predicted HPC population
significantly more than AOC. However, the highest AOC measured in this work was
approximately 300 µg/L as C. AOC did vary according to process. The highest average AOC
was 110 µg/L as C, which was in conventionally treated groundwater (G1). The lowest average
AOC was 43 µg/L as C, which was in RO treated groundwater. Using these AOCs in equation 1,
results were 500 cfu/mL higher for predicted HPCs in the G1 source than in the RO source. This
difference would be significant at low HPC populations; however the predicted HPC populations
in both sources were over 5000 cfu/mL. Consequently, the predicted difference in HPC
populations due to the low and high AOCs at the average residual is about 10% of the HPC
population, which is not significant at these levels. At zero residual, this difference increases to
approximately 9,000 cfu/mL. Both populations were in excess of 40,000 cfu/mL, but the high
and low AOC difference increases the HPC populations by approximately 20%. Finally, it must
be remembered that AOC was not statistically significant and was used to illustrate the predicted
impact on HPCs. The projections show that the impact on HPC populations increased
dramatically as residual decreased.

Another empirical model for Log HPCs was developed that only considered statistically
significant terms. The model was developed in the same manner as the model shown in equation
1 except that only statistically significant terms were retained. Independent water quality
variables included in the initial regression were AOC, residual, temperature, DOC or UV254,
calcium, sulfates, silica, chlorides and alkalinity. Dummy variables were used to represent pipe material. This Log HPC model is shown in equation 2. All pipe materials, temperature and effluent residual were statistically significant with respect to Log HPC. AOC, DOC or any other inorganic parameters were not significant.

\[
\text{LogHPC} = \left( 2.6407H + 2.5964UCI + 3.3322LCI + 2.9237PVC + 2.9111G \right) \\
\times \frac{1.029^{(\text{Temp} - 20)}}{\left( 0.0310 + T_{\text{Cl}_2-\text{eff}} \right)^{0.11476}}
\]

(2)

where \( \text{Log HPC} \) = Log HPCs (cfu/mL)
\( T \) = Temperature (°C)
\( H \) = Hybrid PDS (0,1)
\( UCI \) = UCI PDS (0,1)
\( LCI \) = LCI PDS (0,1)
\( G \) = G PDS (0,1)
\( T_{\text{Cl}_2-\text{eff}} \) = Effluent Total Chlorine Residual (mg/L as Cl\(_2\))

The coefficients and statistical parameters associated with equation 2 are shown in Table 39. All terms are statistically significant except the saturation constant, which was retained in the model to maintain convergence at zero residual and illustrate the effects of zero residual. The coefficient of determination \( (R^2) \) was 0.57 for predicted and actual Log HPC using equation 2. A three dimensional plot of predicted and actual Log HPC versus combined chlorine residual and temperature using equation 2 is shown in Figure 29. The plane or net is the grouping of predicted Log HPCs; whereas the clear circles are the actual points. The dark curves within the net are curves for constant Log HPC 3 and 4; whereas the curves on the bottom plane are the same Log HPC 3 and 4 curves projected on the combined chlorine residual and temperature
plane of Figure 29. Biostability is defined in terms of HPC populations in this work. Transition to biological instability was defined as beginning at 3 Log HPC and biological instability was defined as beginning at 4 Log HPC. Those points in Figure 29 in terms of temperature and total residual as mg/L as Cl\textsubscript{2} are for (a) 15\degree C at 0.06 mg/L for 3 Log HPC and greater than 4 mg/L for 4 Log HPC; for (b) 25\degree C at 1.1 mg/L for 3 Log HPC and 0.06 mg/L for 4 Log HPC; and for (c) 35\degree C three Log HPC is not possible from 0 to 4 mg/L and at 1.1 mg/L for 4 Log HPC. Log HPC increased from 3 to 4 when temperature was increased from 25\degree C to 35\degree C at 1.1 mg/L residual; hence a ten-degree temperature rise changes HPCs from entering the zone of transition to entering the zone of biological instability.

Given that the Log HPC relationship shown in equation 2 is non-linear, it is difficult to state whether temperature or residual has the greatest impact on HPCs. Both impacts are significant, but the impact of residual is more significant than the impact of temperature as residual approaches zero; and the impact of temperature is most significant on HPCs at residuals greater than 1 mg/L as Cl\textsubscript{2}. Given that utilities can do nothing to control temperature in a distribution system, residual is the only practically significant parameter on HPCs and requires more active management during high temperature periods. Following such reasoning conditions favoring residual maintenance are recommended to control heterotrophic proliferation in drinking water distribution systems (e.g. use of less reactive pipes).

Finally, the Log HPC model shown in equation 2 can be proactively utilized to anticipate biological instability by incorporating a chlorine dissipation model into equation 2. This combined chlorine residual model was developed by Arevalo et al. (Equation 3) utilizing the
same experimental units described in this paper and during the same time period [20]. It was found that total chlorine residual could be accurately described by a first-order kinetic model with respect to initial chlorine and time as shown in equation 3.

\[
Cl_2(t) = Cl_2(0) \times \exp \left( - \left( K_B \times UV \times 254 + \frac{K_w}{D_p} \right) \times A(T-20) \times t \right)
\]

where

- \( K_B \) = Bulk decay constant (cm/hr)
- \( K_W \) = Wall decay constant (in/hr)
- \( Cl_2(t) \) = Chlorine concentration at time \( t \) (mg/L as Cl\(_2\))
- \( Cl_2(0) \) = Initial chlorine concentration (mg/L as Cl\(_2\))
- \( A \) = Temperature correction coefficient
- \( T \) = Temperature (°C)
- \( t \) = Time (hrs)
- \( D_p \) = Pipe diameter (inches)

Figure 30 presents a plot of predicted versus actual Log HPC using the dissipation model (equation 3) merged into the HPC model (equation 2). The correlation of determination was 0.53 and the predicted data was not significantly different from the actual data as determined by a paired t test at the 95 % CL.

**Conclusions**

The following main conclusions can be drawn from the present study:

- Bulk liquid heterotrophic biostability was assessed in this work by HPC populations.
Transition to biological instability was considered as increase of HPC from three log to four logs and above four logs HPC, the system was considered biologically unstable. Selection of three and four logs HPC as the beginning of the transition and biological instability zones was correlated to a combined chlorine residual of approximately 0.1-0.2 and 0.5 mg/L as Cl₂. As these residuals were both below the regulatory minimum of 0.6 mg/L as Cl₂, biological stability in these systems was mainly controlled by chloramines residual and was maintained as long as residual regulatory requirements were met.

- An empirical model relating effluent Log HPC to pipe material, AOC and residual was developed. All terms were significant, however AOC was only significant at the 73 % CL. Residual impacted Log HPC significantly more than AOC for the anticipated ranges of residual and AOC in drinking water. Predicted Log HPC increased significantly as residual approached zero.

- A second empirical model was developed that related Log HPC to pipe material and temperature. Log HPC increased as temperature increased and residual decreased. The impact of residual is more significant than the impact of temperature on Log HPC as residual approaches zero, but the impact of temperature is most significant at residuals greater than 1 mg/L on Log HPC. Given utilities can do nothing to control temperature in a distribution system, residual is the only practically significant parameter for controlling HPCs and requires more active management during high temperature periods. Use of less reactive (i.e. with less chlorine demand) pipes is
recommended in order to prevent residual depletion and subsequent bacterial proliferation.

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Figure 26. Conceptual Flow Diagram of Pilot Distribution

"": inches (one inch = 2.54 cm); Dia: Diameter.
Figure 27. Effect of Combined Chlorine Residual (TCl₂-eff) on Effluent HPC
Figure 28. Predicted Log HPC versus Combined Chlorine Residual and Log AOC for Hybrid PDS
Figure 29. Predicted and Actual Log HPCs versus Combined Chlorine Residual and Temperature for the Hybrid PDSs
Figure 30. Actual versus Predicted Effluent Log HPC using Combined Chlorine Residual
Predicted by Residual Dissipation Model
Table 36. Single Water Source Designation and Corresponding Treatment Trains

<table>
<thead>
<tr>
<th>Designation</th>
<th>Water Source</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>GW</td>
<td>aeration and stabilization</td>
</tr>
<tr>
<td>G2</td>
<td>GW</td>
<td>lime softening</td>
</tr>
<tr>
<td>S1</td>
<td>SW</td>
<td>enhanced ferric sulfate coagulation, ozonation, BAC filtration</td>
</tr>
<tr>
<td>S2</td>
<td>SW</td>
<td>enhanced ferric sulfate coagulation, nanofiltration</td>
</tr>
<tr>
<td>RO</td>
<td>GW</td>
<td>high pressure reverse osmosis&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>G3</td>
<td>Blend</td>
<td>lime softening</td>
</tr>
<tr>
<td>G4</td>
<td>Blend</td>
<td>nanofiltration, aeration</td>
</tr>
</tbody>
</table>

<sup>a</sup> Salts were added to the permeate to simulate typical finished water from a desalination process

GW: Groundwater; SW: Surface water; Blend: Blend of G1, S1 and RO
### Table 37. Finished Water Matrix for PDS 1-14

<table>
<thead>
<tr>
<th>PDS</th>
<th>Phase 1&amp;3 Sources and (%)</th>
<th>Phase 2&amp;4 Sources and (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>G1(100)</td>
<td>G2(100)</td>
</tr>
<tr>
<td>02</td>
<td>G2(100)</td>
<td>G1(100)</td>
</tr>
<tr>
<td>03</td>
<td>S1(100)</td>
<td>S2(100)</td>
</tr>
<tr>
<td>04</td>
<td>G4(100)</td>
<td>G4(100)</td>
</tr>
<tr>
<td>05</td>
<td>RO(100)</td>
<td>S1(100)</td>
</tr>
<tr>
<td>05</td>
<td>G1(55), S1(45)</td>
<td>G1(68), RO(32)</td>
</tr>
<tr>
<td>07</td>
<td>G1(68), RO(32)</td>
<td>G1(55), S1(45)</td>
</tr>
<tr>
<td>08</td>
<td>G1(23), S1(45), RO(32)</td>
<td>G1(60), S2(30), RO(10)</td>
</tr>
<tr>
<td>09</td>
<td>G1(60), S2(30), RO(10)</td>
<td>G1(23), S1(45), RO(32)</td>
</tr>
<tr>
<td>10</td>
<td>G2(50), S1(50)</td>
<td>G2(62), S1(24), RO(14)</td>
</tr>
<tr>
<td>11</td>
<td>G2(62), S1(24), RO(14)</td>
<td>G2(50), S1(50)</td>
</tr>
<tr>
<td>12</td>
<td>G3(100)</td>
<td>G3(100)</td>
</tr>
<tr>
<td>13</td>
<td>S2(100)</td>
<td>RO(100)</td>
</tr>
<tr>
<td>14</td>
<td>G1(62), S1(27), RO(11)</td>
<td>G1(62), S1(27), RO(11)</td>
</tr>
</tbody>
</table>

### Table 38. Statistically Determined Coefficients and Parameters for Log HPC Model with AOC

<table>
<thead>
<tr>
<th></th>
<th>Coefficient</th>
<th>Std. Error</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturation Constant</td>
<td>0.0177</td>
<td>0.0283</td>
<td>0.6254</td>
<td>0.5334</td>
</tr>
<tr>
<td>AOC&lt;sub&gt;inf&lt;/sub&gt;</td>
<td>0.0398</td>
<td>0.0358</td>
<td>1.1100</td>
<td>0.2702</td>
</tr>
<tr>
<td>Residual</td>
<td>0.1310</td>
<td>0.0319</td>
<td>4.1083</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hybrid</td>
<td>2.6488</td>
<td>0.4076</td>
<td>6.4978</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>UCI</td>
<td>2.6477</td>
<td>0.4649</td>
<td>5.6947</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LCI</td>
<td>3.3410</td>
<td>0.6360</td>
<td>5.2529</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PVC</td>
<td>3.0633</td>
<td>0.5951</td>
<td>5.1474</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>G</td>
<td>2.8364</td>
<td>0.5314</td>
<td>5.3371</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Table 39. Statistical Coefficients and Parameters for Log HPC Model without AOC

<table>
<thead>
<tr>
<th></th>
<th>Coefficient</th>
<th>Std. Error</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>0.0310</td>
<td>0.0358</td>
<td>0.8654</td>
<td>0.3886</td>
</tr>
<tr>
<td>TCl$_{2\text{-eff}}$</td>
<td>0.1476</td>
<td>0.0345</td>
<td>4.2764</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>$\Theta$</td>
<td>1.0290</td>
<td>0.0087</td>
<td>117.7563</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>H</td>
<td>2.6407</td>
<td>0.1436</td>
<td>18.3883</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>UCI</td>
<td>2.5964</td>
<td>0.2460</td>
<td>10.5529</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LCI</td>
<td>3.3322</td>
<td>0.3666</td>
<td>9.0902</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PVC</td>
<td>2.9273</td>
<td>0.3401</td>
<td>8.6064</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>G</td>
<td>2.9111</td>
<td>0.2758</td>
<td>10.5535</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
CHAPTER SIX: BIOFILM PROLIFERATION IN CHLORAMINATED DISTRIBUTION SYSTEMS; STUDY AT PILOT-SCALE

Introduction

Despite the use of disinfectant, bacterial regrowth often occurs in drinking water distribution systems (DS). This bacterial proliferation can be categorized into two groups: (i) the growth occurring in the bulk phase and (ii) the biofilm-forming growth occurring on the inner surface of network pipes. Both phenomena are related to each other, as it is thought that most suspended or planktonic bacteria originate from biofilms [1]. Biofilms are commonly considered responsible for the deterioration of drinking water quality in DS, due to transport of bacteria from biofilms to the bulk phase. If sufficient disinfectant residual is present, this detachment of bacteria from pipe walls entering the bulk phase does not constitute a public concern, since the disinfectant would properly inactivate these microorganisms. However depletion of residual is often observed, which can potentially lead to bio-instability and bacterial proliferation of both suspended and attached microorganisms. Maintenance of a significant disinfectant residual is therefore required to control bacterial regrowth [2]. Due to more stringent regulations on disinfection by-products (DBP), many utilities have adopted chloramination instead of chlorination. It is generally accepted that chloramines provide a more stable residual than free chlorine, which in turn allows a better control of bacterial proliferation. Chloramines are also considered to provide more effective inactivation of attached bacteria by penetrating more deeply
within biofilms [3-5]. Norton and LeChevallier observed significant reduction of heterotrophic plate counts (HPCs) and better control of attached growth in the Muncie (Indiana) network after switching permanently to chloramines [6]. Understanding the mechanisms that lead to residual depletion, as well as the impact of disinfection on attached growth is therefore crucial for controlling bacterial proliferation in drinking water networks. Among other factors, pipe material has a strong influence on residual depletion. Hallam et al. (2002) demonstrated that plastic-based and relatively inert materials (e.g. PVC, PE, MDPE or lined cast iron) have much less wall residual demand than unlined reactive metals (e.g. cast iron)[7]. Further, several studies have shown that these reactive materials (thanks to the presence of iron corrosion products) favored colonization and biofilm growth, while inert materials host lowest biofilm inventories [8-10]. Therefore pipe material and its composition have a strong impact on bacterial regrowth in drinking water DS.

In order to control bacterial proliferation in DS, it is necessary to understand the growth mechanisms of these suspended or attached microorganisms and the relationship between these two populations. Attached bacteria in biofilms are less susceptible to changes in bulk phase water quality (temperature, pH, nutrient concentration, metabolic products and disinfectant residual) than their planktonic counterparts [11]. In turn, the cell-specific growth rate of the biofilm is lower compared to the bulk phase bacteria, due to a larger production of exopolymeric substances (EPS) for biofilm bacteria at the expense of energy not available for growth [12]. These EPS act as a shield for biofilm bacteria which explains their recalcitrance to biocides [13,14]. Gradients of respiratory activity have been demonstrated in biofilms, the activity
persisting deep in biofilms, while attached bacteria are inactivated at the bulk/biofilm interface. Since, it is not feasible to inactivate biofilms entirely, it is crucial in the US and especially in warm regions (e.g. Florida) to maintain a strong residual to avoid bacterial proliferation. Utilities in cold climates might possibly be able to control assimilable organic carbon (AOC) to less than 10 µg/L as C and successfully control distribution system HPCs and other microorganisms without a residual as that is done in European countries with cold climates. However, there is a major utility (WMO) in the Netherlands that maintains chlorine residuals in the drinking water distribution system because the quality of the raw water is not adequate to do otherwise. As with WMO, a residual must be maintained in Florida and the majority of the US to maintain biological water quality.

The results discussed in this paper (December 6th 2001 to December 23rd 2002) originate from a tailored collaboration project between American Water Works Association Research Foundation (AWWARF), Tampa Bay Water (TBW) and University of Central Florida (UCF) to study the effect of blending different source waters on distribution system water quality. The main objectives of the research presented in this paper were (i) to model biofilm growth and assess significant parameters involved, (ii) to assess the impact of pipe material and process on biofilm inventories, and finally (iii) to compare results between the potential of exoproteolytic activity (PEPA) assay and the traditional culture-based HPC assay for quantification of biofilm inventories.
Materials and Methods

Experimental Set-Up

Pilot Distribution Systems (PDS)

The 14 pilot distribution systems (PDS) used to produce the results for this paper consisted of pipe sections from actual full-scale distribution system pipes that were pulled out from the ground by the participating member governments and were thus aged and sometimes corroded extensively (in the case of unlined metals such as cast iron and galvanized steel). To simulate the sequence of materials likely found in full-scale distribution systems the sequence of pipe material in each of the PDS was (upstream to downstream) polyvinyl chloride (PVC), lined cast iron (LCI), unlined cast iron (UCI) and galvanized steel (G). A diagram of one of the PDS is present in Figure 31. All PDS were designed with a five-day (December 6th 2001 to August 29th 2002) and a two-day (August 30th 2002 to December 23rd 2002) Hydraulic Residence Time (HRT) and were virtually identical with respect to the condition of their pipe sections, their hydraulic regime (HRT, flow velocities in the different pipe sections) and the environmental conditions under which they were operated (temperature, humidity). Therefore the main difference between each of these PDS was the source water used to feed the PDS.
**Biofilm sampling**

Coupons cut from each of the material pipe sections were sampled prior to each phase change after three months incubation and replaced with new un-colonized coupons. Duplicate coupons were 3 cm in diameter and affixed to a coupon holder that was placed in a PVC coupon cradle. Each PDS was provided with a coupon cradle operated in parallel and fed by the same source water as the PDS, under similar hydraulic conditions. Intact biofilms were analyzed (using biofilm HPC or BFHPC and PEPA assay) upon sampling, after scraping and resuspending of the cells from the entire surface of the coupons.

**Water Sources**

Each of the fourteen PDS under study received a different source water. A brief description of the seven water treatment processes used on this project is presented in Table 40. The finished waters produced from these processes and referred to as “single water sources” were blended in different proportions according to finished waters in use by the collaborating member governments. A description of these blends is provided in Table 41. All finished waters were treated with a chloramine residual of 4 mg Cl₂/L total chlorine.
**Heterotrophic Plate Counts - HPC**

Daily HPCs were performed by plate spreading on R2A agar incubated at 25°C for 7 days, according to Standard Method 9215B [17]. Results were expressed in colony-forming units per milliliter (cfu/mL).

**Assimilable Organic Carbon**

AOC was measured using the rapid method developed by LeChevallier et al. (1993), except that plate counts were used to enumerate bacteria rather than ATP fluorescence [15].

**Potential of ExoProteolytic Activity Assay - PEPA**

The PEPA method measures the global activity of the biofilm, estimating the potential of bacteria to lysis proteins, using a proteic non-fluorescent artificial substrate (here L-Leucine β-Naphtylamide, LLβN). The enzymatic hydrolysis of this substrate leads to a fluorescent product (here β-Naphtylamine, βN), which can be detected by spectrofluorimetry. Fluorescence is plotted as a function of time and the rate of degradation gives an estimate of biological activity in the sample, which can be translated into biofilm density (cfu/cm²). This non-destructive technique was adapted from the work of Laurent and Servais [16].
Chemical Water Quality Monitoring

Influent and effluent PDS water quality samples were collected on a daily basis. Reported information included ammonia, nitrate, nitrite, residual total chlorine, temperature, and dissolved oxygen. All analyses were performed by accepted methods according to Standard Methods [17].

Results and Discussion

Biofilm Activity Modeling

An empirical model for Log PEPA was developed in which Log (bulk HPCs), AOC, residual, temperature, DOC or UV254, calcium, sulfates, silica, chlorides and alkalinity were the independent variables that were regressed against Log PEPA; and dummy variables were used to represent pipe material. The empirical Log PEPA model is shown in equation 1 and shows that all material terms, temperature, residual, Log HPC and HRT were statistically significant at the 95 % CL. The statistically determined coefficients, exponents and associated parameters are shown in Table 42. The coefficient of determination, $R^2$, for the Log PEPA model was 0.87.
\[
\log \text{PEPA} = (8.6966UCl + 8.3791LCI + 7.7651PVC + 8.3993G) \\
\times \frac{1.0232^{(\text{Temp} - 20)}}{T \\text{Cl}_{2-\text{eff}}^{0.0112}(\log \text{HPC})^{0.0292} \text{HRT}^{0.0271}}
\]

where \( \log \text{PEPA} = \log \text{PEPAs (cells/cm}^2) \)
\( UCl = \text{UCI PDS (0,1)} \)
\( LCI = \text{LCI PDS (0,1)} \)
\( G = \text{G PDS (0,1)} \)
\( \text{Temp} = \text{Temperature (°C)} \)
\( \text{HRT} = \text{Hydraulic Residence Time (days)} \)
\( \text{TCI}_{2-\text{eff}} = \text{Effluent Total Chlorine Residual (mg/L as Cl}_2) \)
\( \log \text{HPC} = \log \text{HPCs (cfu/mL)} \)

Further review of equation 1 reveals the impact of material, HRT, HPC and residual on PEPA. PEPA increases as temperature increases and decreases as residual increases, which is intuitively rational. However, PEPA increases as HPCs and HRT decrease, which may not be intuitively understood at first glance. Indirect relationships between bulk and biofilm terms were frequently observed in this work and, as previously noted, can be explained using a thermodynamic equilibrium relationship between biofilms and bulk HPCs and other bacteria in this work. Given that pseudo-equilibrium exists between the bacteria in the biofilm and the bulk, bacteria would seek the path of least resistance for proliferation and vary according to the equilibrium relationship. The biofilm also acts as a reservoir of bacteria, which allows bacteria to enter the bulk by diffusion during periods of low bacterial growth in the bulk. During periods of high bacterial activity in the bulk, bacteria could enter the biofilm from the bulk. Bacteria could also reproduce and die in the biofilm and the bulk. If the bulk environment allows bacteria to easily proliferate, bacteria in the biofilm could enter the bulk from the biofilm as that environment is the path of least thermodynamic resistance to growth. HPCs in the bulk would
increase with time because of growth, which will increase as time and DO consumption increase; and decrease with HPCs in the biofilm. Consequently, the hypothesis of an indirect relationship between biofilm HPCs and bulk HPCs as seen in equation 1 is supported by a thermodynamic equilibrium hypothesis. Predicted versus normalized actual log PEPA is shown in Figure 32 and Figure 33 using equation 1. Since there are four independent variables in equation 1, two different figures were required to represent the effects of the independent variables. Log PEPA was normalized by holding Log HPC and HRT constant at 4 and 2 days respectively, and then using equation 1 to calculate Log PEPA for variable temperature and total residual as shown in Figure 32. Log PEPA was normalized by holding total residual and temperature constant at 2 mg/L as Cl₂ and 25°C, and then using equation 1 to calculate Log PEPA for varying Log HPC and HRT as shown in Figure 33. As shown in Figure 32, chloramine residual has little impact on the magnitude of estimated Log PEPA; however, temperature has a significant impact on Log PEPA. The biofilm density hardly changes from 7 logs beginning at near zero residual to a total residual of 4 mg/L as Cl₂; whereas, the biofilm density increases from approximately 7 logs to more than 9 logs from 15°C to 30°C. The impact of HRT and Log HPC_eff is also slight relative to temperature as shown in Figure 33. Predicted PEPA varies from about 8.4 to 8.8 logs from 2 days to 7 days HRT; whereas, varying Log HPC in the effluent from 7 to 1 increases PEPA by approximately one-half of a log.
Differences in Biofilm Activities by Treatment Train

The average Log PEPAs are shown by process in Figure 34. The highest average Log PEPAs were produced on coupons receiving G1 (conventionally treated groundwater) and RO (simulated desalinated seawater) finished waters, which is surprising relative to RO finished water. The lowest average Log PEPAs were produced on coupons receiving nanofiltered surface and blended waters, which makes the average Log PEPA from the RO environment seem out of sequence. The sequence of ground water average Log PEPAs is rational assuming that increasing degrees of treatment would reduce the Log PEPA potential accordingly as G1 Log PEPA > G2 Log PEPA > G3 Log PEPA > G4 Log PEPA. The sequence of SW average Log PEPAs is also rational based on degree of treatment as S1 Log PEPA > S2 Log PEPA. There are over 200 PEPA observations considered in Figure 34, which eliminates the possibility of an anomaly for the RO average Log PEPA observation. The p-values for the t tests of average Log PEPAs by process is shown in Table 43. Average G1 Log PEPA is statistically higher than the average Log PEPA at the bracketed % confidence level (CL) for G4 (80%), S1 (80%) and S2 (90%). Average G2 Log PEPA is statistically higher than average S2 Log PEPA at the 90 % CL. Average G4 and S2 Log PEPA are statistically higher than the average RO Log PEPA at the 80 % CL.
**Differences in Biofilm Activities by Pipe Material**

The average Log PEPAs by coupon materials are shown in Figure 35. The most inert materials with regards to corrosion potential (PVC and LCI) produced the lowest average Log PEPAs. The unlined metal materials (UCI and G) produced the highest average Log PEPAs. Although there is less than an order of magnitude between the average Log PEPAs for any of the materials, a considerable number of cells are gained when drinking water distribution system biofilms increase from $10^8$ to $10^9$ cell/cm². The magnitude of difference in the average Log PEPA by material can be seen in the paired t test for difference shown in Table 44. The average Log PEPA for every material is statistically significant at either the 95, 90 or 80 % CL except the G average Log PEPA that is statistically higher than the UCI average Log PEPA at the 79.17 % CL.

**Relationship between Biofilm Activity and Biofilm HPC (BFHPC)**

The correlation between Log PEPA and Log BFHPC is shown in Figure 36. As expected there is a linearly positive correlation between Log PEPA and Log BFHPC. Both Log PEPA and Log BFHPC are higher in the biofilms associated with the unlined metal coupons (UCI and G) than in the coupons associated with the non-metal surfaces (PVC and LCI). PEPA results were two to five orders of magnitude greater than BFHPC because PEPA is an account of global activity of the biofilm, while BFHPC only enumerates viable, cultivable heterotrophic bacteria. Plate count techniques are often considered to underestimate total counts while non-destructive enzymatic
techniques are considered to provide better estimation of bacterial populations.

**Conclusions**

The following main conclusions can be drawn from the present study:

- A non-linear model was developed for biofilm density (PEPA) as a function of pipe material, temperature, total residual, Log HPC and HRT. More than 200 observations of PEPA were included in the data set that was regressed to develop the model. PEPA increased with temperature and decreased with total residual, Log HPC and HRT. The variation of PEPA with Log HPC indicated that cells in the bulk and biofilm were in pseudo-equilibrium and sought the environment that was most suitable for their growth. Log HPCs varied directly with HRT, which then resulted in the negative relationship with biofilm cell density (PEPA). Temperature impacted PEPA significantly more than HRT, Log HPC_{eff} and residual, all of which had relatively little impact on PEPA. The model predicts biofilms will always be active for environments common to drinking water distribution systems, which is consistent with the hypothesis that biofilms act as reservoirs for microorganisms and are a constant source of microorganisms to the bulk water under normal conditions. As American utilities do not control biofilms with extensive and costly AOC reduction, American utilities must maintain a strong residual to maintain biological integrity and stability in drinking water distribution systems.
• Average biofilm density (PEPA) of PDS fed by treated ground waters with the exception of RO was ordered as G1>G2>G3>G4. The average process biofilm density of PDS fed by treated SWs was ordered as S1>S2. The order of biofilm densities of treated surface and ground waters excepting RO indicated that biostability increased as the degree of treatment increased with nanofiltered finished waters being the most biostable. Average biofilm density (PEPA) was the highest in RO finished water, which was seemingly out of sequence.

• Average biofilm densities on different pipe materials were ordered as UCI>G>LCI>PVC.

• Biofilm density results obtained with PEPA were positively correlated with results from traditional culture-based HPC assay (BFHPC). PEPA results were several orders of magnitude greater than BFHPC results.

References


Figure 31. Conceptual Flow Diagram of Pilot Distribution System

*: inches (one inch = 2.54cm); Dia: Diameter.
Figure 32. Predicted and Normalized Log PEPA to 2 days HRT and 4 Log HPC versus Temperature and $T_{Cl_2-eff}$.
Figure 33. Predicted versus Actual Normalized PEPA to 2 mg/L Chloramine Residual and 25°C for PVC Pipe Material at Varying HRT and Log HPC_{eff}.
Figure 34. Average and Standard Error of Finished Water Log PEPA versus Water Source
Figure 35. Average and Standard Error of Log PEPA versus Material
Log PEPA = 0.39Log BFHPC + 6.74

$R^2 = 0.22$

Figure 36. Log PEPA versus Log BFHPC by Material
### Table 40. Single Water Source Designation and Corresponding Treatment Trains

<table>
<thead>
<tr>
<th>Designation</th>
<th>Water Source</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>GW</td>
<td>aeration and stabilization</td>
</tr>
<tr>
<td>G2</td>
<td>GW</td>
<td>lime softening</td>
</tr>
<tr>
<td>S1</td>
<td>SW</td>
<td>enhanced ferric sulfate coagulation, ozonation, BAC filtration</td>
</tr>
<tr>
<td>S2</td>
<td>SW</td>
<td>enhanced ferric sulfate coagulation, nanofiltration</td>
</tr>
<tr>
<td>RO</td>
<td>GW</td>
<td>high pressure reverse osmosis $^a$</td>
</tr>
<tr>
<td>G3</td>
<td>Blend</td>
<td>lime softening</td>
</tr>
<tr>
<td>G4</td>
<td>Blend</td>
<td>nanofiltration, aeration</td>
</tr>
</tbody>
</table>

$^a$ Salts were added to the permeate to simulate typical finished water from a desalination process.

GW: Groundwater; SW: Surface water; Blend: Blend of G1, S1 and RO.
### Table 41. Finished Water Matrix for PDS 1-14

<table>
<thead>
<tr>
<th>PDS</th>
<th>Phase 1&amp;3 Sources and (%)</th>
<th>Phase 2&amp;4 Sources and (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>G1 (100)</td>
<td>G2 (100)</td>
</tr>
<tr>
<td>02</td>
<td>G2 (100)</td>
<td>G1 (100)</td>
</tr>
<tr>
<td>03</td>
<td>S1 (100)</td>
<td>S2 (100)</td>
</tr>
<tr>
<td>04</td>
<td>G4 (100)</td>
<td>G4 (100)</td>
</tr>
<tr>
<td>05</td>
<td>RO (100)</td>
<td>S1 (100)</td>
</tr>
<tr>
<td>06</td>
<td>G1 (55), S1 (45)</td>
<td>G1 (68), RO (32)</td>
</tr>
<tr>
<td>07</td>
<td>G1 (68), RO (32)</td>
<td>G1 (55), S1 (45)</td>
</tr>
<tr>
<td>08</td>
<td>G1 (23), S1 (45), RO (32)</td>
<td>G1 (60), S2 (30), RO (10)</td>
</tr>
<tr>
<td>09</td>
<td>G1 (60), S2 (30), RO (10)</td>
<td>G1 (23), S1 (45), RO (32)</td>
</tr>
<tr>
<td>10</td>
<td>G2 (50), S1 (50)</td>
<td>G2 (62), S1 (24), RO (14)</td>
</tr>
<tr>
<td>11</td>
<td>G2 (62), S1 (24), RO (14)</td>
<td>G2 (50), S1 (50)</td>
</tr>
<tr>
<td>12</td>
<td>G3 (100)</td>
<td>G3 (100)</td>
</tr>
<tr>
<td>13</td>
<td>S2 (100)</td>
<td>RO (100)</td>
</tr>
<tr>
<td>14</td>
<td>G1 (62), S1 (27), RO (11)</td>
<td>G1 (62), S1 (27), RO (11)</td>
</tr>
</tbody>
</table>

### Table 42. Statistical coefficients and exponents for Log PEPA model

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>Std. Error</th>
<th>T</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>θ</td>
<td>1.0232</td>
<td>0.0013</td>
<td>796.8906</td>
</tr>
<tr>
<td>T Cl&lt;sub&gt;2-eff&lt;/sub&gt;</td>
<td>0.0112</td>
<td>0.0024</td>
<td>-4.6295</td>
</tr>
<tr>
<td>Log HPC</td>
<td>0.0292</td>
<td>0.0103</td>
<td>-2.8377</td>
</tr>
<tr>
<td>HRT</td>
<td>0.0271</td>
<td>0.0069</td>
<td>-3.9388</td>
</tr>
<tr>
<td>UCI</td>
<td>8.6966</td>
<td>0.1329</td>
<td>65.4293</td>
</tr>
<tr>
<td>LCI</td>
<td>8.3791</td>
<td>0.1302</td>
<td>64.3680</td>
</tr>
<tr>
<td>PVC</td>
<td>7.7651</td>
<td>0.1204</td>
<td>64.4815</td>
</tr>
<tr>
<td>G</td>
<td>8.3993</td>
<td>0.1279</td>
<td>65.6646</td>
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</table>
Table 43. Paired t-test results (p-values) for PEPA by treatment train

<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
<th>S1</th>
<th>S2</th>
<th>RO</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>-</td>
<td>0.6328</td>
<td>0.2723</td>
<td>0.1189</td>
<td>0.1751</td>
<td>0.0783</td>
<td>0.9034</td>
</tr>
<tr>
<td>G2</td>
<td>0.6328</td>
<td>-</td>
<td>0.5337</td>
<td>0.2769</td>
<td>0.3776</td>
<td>0.1967</td>
<td>0.7214</td>
</tr>
<tr>
<td>G3</td>
<td>0.2723</td>
<td>0.5337</td>
<td>-</td>
<td>0.6404</td>
<td>0.7941</td>
<td>0.5014</td>
<td>0.3284</td>
</tr>
<tr>
<td>G4</td>
<td>0.1189</td>
<td>0.2769</td>
<td>0.6404</td>
<td>-</td>
<td>0.8366</td>
<td>0.8370</td>
<td>0.1499</td>
</tr>
<tr>
<td>S1</td>
<td>0.1751</td>
<td>0.3776</td>
<td>0.7941</td>
<td>0.8366</td>
<td>-</td>
<td>0.6804</td>
<td>0.2164</td>
</tr>
<tr>
<td>S2</td>
<td>0.0783</td>
<td>0.1967</td>
<td>0.5014</td>
<td>0.8370</td>
<td>0.6804</td>
<td>-</td>
<td>0.1006</td>
</tr>
<tr>
<td>RO</td>
<td>0.9034</td>
<td>0.7214</td>
<td>0.3284</td>
<td>0.1499</td>
<td>0.2164</td>
<td>0.1006</td>
<td>-</td>
</tr>
</tbody>
</table>

Italicized, underlined and bold alpha values are less than 0.05
Underlined alpha values are greater than 0.05 and less than 0.10
Italicized alpha values are greater than 0.10 and less than 0.20

Table 44. Paired t-test results (p-values) for PEPA by pipe material

<table>
<thead>
<tr>
<th></th>
<th>PVC</th>
<th>LCI</th>
<th>UCI</th>
<th>G</th>
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</thead>
<tbody>
<tr>
<td>PVC</td>
<td>-</td>
<td>0.0905</td>
<td>&lt;0.0001</td>
<td>0.0037</td>
</tr>
<tr>
<td>LCI</td>
<td>0.0905</td>
<td>-</td>
<td>0.0046</td>
<td>0.2083</td>
</tr>
<tr>
<td>UCI</td>
<td>&lt;0.0001</td>
<td>0.0046</td>
<td>-</td>
<td>0.1065</td>
</tr>
<tr>
<td>G</td>
<td>0.0037</td>
<td>0.2083</td>
<td>0.1065</td>
<td>-</td>
</tr>
</tbody>
</table>

Italicized, underlined and bold alpha values are less than 0.05
Underlined alpha values are greater than 0.05 and less than 0.10
Italicized alpha values are greater than 0.10 and less than 0.20

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CHAPTER SEVEN: ESTABLISHMENT OF NITRIFICATION IN PILOT DISTRIBUTION SYSTEMS UNDER OPTIMIZED CONDITIONS

Introduction

Odell et al. [1] found that two thirds of US systems using chloramination experienced problems with nitrification in the distribution system. Nitrification is caused by the growth of ammonia-oxidizing bacteria (AOB), and nitrite oxidizing bacteria (NOB), in the distribution system. AOB use ammonia as an energy source, converting ammonia to nitrite. Nitrite is known to accelerate monochloramine decay through an abiotic oxidation-reduction reaction that liberates ammonium, chloride, and nitrate as end products. The subsequent disappearance of the disinfectant residual along with production of ammonium can lead to the proliferation of AOBs, producing more nitrite and consuming more chloramines in a residual “death spiral”. In addition the disappearance of residual can lead to an increase in heterotrophic plate count (HPC) in the distribution system and may result in violation of the disinfectant residual requirements in the Surface Water Treatment Rule.

Studies showed that reduced residual was conducive to nitrification whether ammonia levels were low or high and was an important prerequisite for the acceleration of nitrification events [1-2]. Odell et al. noted that nitrification was accompanied by a decrease in ammonia concentrations below 0.2 mg/L as N, an increase in nitrite levels from 0.01 to > 0.1 mg/L as N, and no noticeable change in nitrate at about 1.5 mg/L as N [1]. Relationship between HPC and nitrification has not been clearly defined. Increased HPCs may or may not be observed during a
nitrification episode [2]. A decrease in dissolved oxygen often accompanied nitrification episodes. Odell et al. found from their survey of full-scale utilities that utilities with lower DO concentrations in their distribution systems may have less potential for nitrification to occur [1]. The impact of ammonia concentration in finished waters was not clearly elucidated. Higher ammonia concentrations do not necessarily mean more nitrification will occur [1]. High temperatures facilitated nitrification.

Wilczak et al. noted that no single water quality parameter was a good indicator of nitrification [2]. Nitrite or alternately nitrate were sometimes the predominant form of oxidized nitrogen observed. Typically it was nitrite, probably because nitrite reacts so rapidly with chlorine/chloramine residual. Thus the kinetics of nitrite consumption are probably more rapid than the kinetics for nitrite oxidation to nitrate in many systems. However in a few systems nitrate was observed, and thus must be monitored as well. Wilczak et al. noted from a full scale survey that measurement of both nitrite and nitrate was absolutely necessary since either or both could be an important indicator of a nitrification event [2].

Other parameters that are useful in assessing nitrification are chloramine dosage and residual, ammonia, pH, HPCs, and dissolved oxygen [2]. HPC and AOB numbers have been observed to be correlated with each other above HPC counts of 350 cfu/mL in a Metropolitan Water District study [2]. Ammonia, alkalinity, pH, and TOC were not found to be very sensitive or accurate indicators of nitrification in the same full scale survey by Wilczak et al. [2].

The results discussed in this paper, over the period of January 16th to April 3rd 2003, originate from a tailored collaboration project between American Water Works Association
Research Foundation (AWWARF), Tampa Bay Water (TBW) and University of Central Florida (UCF). The main objective of this 3-year project (initiated in December 2001) was to study the effect of blending different source waters on distribution system water quality. The subset of data presented thereafter describes the establishment of nitrification under controlled optimum conditions. The objective of the investigation was to determine the most suitable water quality parameters for detection of the onset of nitrification in distribution systems.

**Materials and Methods**

**Experimental set-up**

*Pilot distribution systems (PDS)*

The four pilot distribution systems (PDS) used to produce the results of this paper consisted of pipe sections from actual full-scale distribution system pipes that were pulled out from the ground by the participating member governments and were thus aged and sometimes corroded extensively (in the case of unlined metals such as cast iron and galvanized steel). To simulate sequence of materials likely found in full-scale distribution systems the sequence of pipe material in each of the PDS was (upstream to downstream) polyvinyl chloride (PVC), lined cast iron (LCI), unlined cast iron (UCI) and galvanized steel (G). A diagram of one of the PDS is present in Figure 37. Standpipes at the beginning and end of each system were used to maintain a surcharge to prevent the units from ever emptying. The PDS were designed with a two-day Hydraulic Residence Time (HRT) and were virtually identical with respect to the
condition of their pipe sections, their hydraulic regime (HRT, flow velocities in the different pipe sections) and the environmental conditions under which they were operated (temperature, humidity). Therefore the main difference between each of these PDS was the source water used to feed the PDS.

**Water Sources**

Each of the four PDS under study received a different source water. Three PDS received a single water source, while a fourth PDS received a blend of these single water sources. The three source waters were produced from three different water treatment processes designed to simulate member government present or proposed finished waters. A brief description of these water treatment processes used for this study is presented with an abbreviated identification:

- **GW**: *Ground water source*. Treatment by aeration, disinfection by free chlorine with a residual of 4 mg/L after a 2 minute contact time, disinfection residual maintenance of 4 mg/L combined chlorine.

- **SW**: *Surface water source*. Treatment by ferric sulfate coagulation, flocculation, settling, filtration, disinfection by ozonation, biologically activated carbon filtration, disinfection residual maintenance of 4 mg/L combined chlorine.

- **RO**: *Ground water source*. Treatment by high pressure reverse osmosis, aeration, disinfection by free chlorine with a residual of 4 mg/L after a 2 minute contact time, disinfection residual maintenance of 4 mg/L combined chlorine. Salts were added in
RO permeate to simulate typical finished water from a desalination process.

- **Blend.** A blend of 40% GW, 30% SW and 30% RO was feeding a fourth PDS.

Below several of the measures used to promote onset of nitrification in the PDS are summarized:

- Ammonium chloride (NH$_4$Cl) was added to a final bulk concentration of 3.8 mg N/L in the feed waters.
- Addition of sodium thiosulfate (Na$_2$S$_2$O$_3$) to the feed waters was implemented on January 31$^{st}$, 2003 to quench disinfectant residual.
- Flushing operations were discontinued on February 8$^{th}$, 2003.

**Heterotrophic Plate Counts - HPC**

HPCs were performed by plate spreading on R2A agar incubated at 25°C for 7 days, according to Standard Method 9215B (1995). Results were expressed in colony-forming units per milliliter (cfu/mL).

**Assimilable Organic Carbon - AOC**

AOC was measured using the rapid method of LeChevallier et al. [3], except that plate counts were used to enumerate bacteria rather that ATP fluorescence, in conjunction with
Standard Methods 9217 [4] and the method of van der Kooij [5]. The procedure used a temperature of 25°C for sample incubation and is outlined in great detail in an article by Escobar and Randall [6]. Quality control for the AOC bioassay was performed using blank controls and 100 µg/l sodium acetate standards. The 100 µg/l sodium acetate standards inoculated with P17 produced an average AOC of 93.80 ± 20.00 µg/l as acetate-C, while for NOX, they produced an average AOC of 77.20 ± 12.53 µg/L as acetate-C. Experimental yield values from acetate standards for P17 (4.08 ± 0.81 × 10^6 cfu/µg of acetate-C) and NOX (9.26 ± 1.50 × 10^6 cfu/µg of acetate-C) compared reasonably well with the literature values as specified in Standard Methods [4] (4.1 × 10^6 and 1.2 × 10^7 cfu/µg of acetate-C for P17 and NOX, respectively). Literature yield values were used for the AOC calculations to conform to the Standard Method [4].

**Chemical Water Quality Monitoring**

Influent and effluent PDS water quality samples were collected on a weekly basis. Reported information included ammonia, nitrate, nitrite, residual total chlorine, temperature, and dissolved oxygen. All analyses were performed by accepted methods according to the Standard Methods [4].
Results and Discussion

Heterotrophic Plate Count - HPC

Figure 38 shows levels of HPC at 102 cfu/mL at the beginning of this study. A sudden increase of HPC in the PDS fed by RO and SW between January 16th, 2003 and January 30th, 2003 is suspected to have been caused by the flushing operation on January 17th, 2003. All PDS resumed similar levels by January 30th, 2003. This was followed by a significant increase in HPC (over 3 orders of magnitude) in all PDS between January 30th, 2003 and February 6th, 2003. This corresponded to the implementation of residual quenching with sodium thiosulfate in feed waters on January 31st. No significant variations were observed after this date (less than one order of magnitude). HPC reach a plateau at about 10^5 cfu/mL after February 8th, 2003. It can be noted that HPC in the PDS fed by RO remained the lowest until the end of the phase, while HPC in the PDS fed by GW showed the most variability.

Assimilable Organic Carbon - AOC

Two sampling events for AOC were carried out at the beginning and the end of the phase (Figure 39). On January 23rd, 2003 AOC levels for feed waters SW and blend (PDS 12 and 14) were higher than normal (274 and 150 µg C/L, respectively) and also significant AOC consumption was observed in these PDS (155 and 41 µg C/L, respectively). No significant changes between influent and effluent AOC values (i.e. delta AOC) were observed on the March, 23rd, 2003, sampling event. Thus the 2 to 3 order of magnitude increase in HPC following
removal of the residual (Figure 40) was not reflected by the AOC data.

**Total Chlorine Residual**

Effluent total chlorine levels ranged from 1.5 to 3.0 mg Cl₂ between January 16th, 2003 and January 30th, 2003. After February 8th, 2003, feed waters had no residual entering the PDS due to quenching by sodium thiosulfate (Figure 40).

**Nitrates**

Delta Nitrate results, i.e. formation of nitrates, are shown in Figure 41. Influent levels were close or equal to zero, therefore the observed increases were probably due to biological activity (Figure 41). A linear increase of nitrate concentrations was observed in the four PDS after depletion of residual. Formation of nitrates in PDS fed by SW occurred one month later than in the other PDS.

**Nitrites**

Production of nitrite in the PDS followed the same pattern as described above for nitrates. Figure 42 shows the evolution of nitrite production in the PDS during the same period.
Dissolved Oxygen - DO

Between January 16th, 2003 and February 5th, 2003 consumption of DO (i.e. delta DO) ranged from 0.76 to 1.98 mg O₂/L (Figure 43). A significant increase of DO consumption (4.67 mg O₂/L for GW and up to 6.87 mg O₂/L increase for RO) was observed between February 5th, 2003 and February 19th, 2003. The increase coincided with the residual quenching and the subsequent increase of heterotrophic growth and nitrification in the PDS. Within 2 weeks of quenching there was a rapid increase in DO consumption in all the PDS, and though there were some oscillations in GW and SW essentially all four PDS stayed at a much higher level of DO consumption than they experienced prior to quenching.

Sodium thiosulfate was used to quench the combined residual in the storage tanks, with an excess of 2mg/L entering the PDS. It is possible that DO could have also been consumed by this reducing agent. Equation 1 shows the stochiometry of the redox reaction between dissolved oxygen and thiosulfate ion S₂O₃²⁻, which shows that one mole of S₂O₃²⁻ neutralizes 0.25 mole of O₂.

\[
4S_2O_3^{2-} + O_2 + 4H^+ \rightarrow 2S_4O_6^{2-} + 2H_2O \quad (1)
\]

Therefore an excess of 2 mg/L of sodium thiosulfate neutralizes a maximum of 0.1 mg/L O₂. The observed consumption of DO in the PDS thus cannot be explained by the presence of an excess of sodium thiosulfate.

In order to understand variations in DO consumption, comparison with changes in turbidity has been carried out. Figure 44 to Figure 47 show these comparisons for PDS fed by
G1, S1, RO and the blend respectively. The increase of turbidity between influent and effluent, i.e. delta turbidity correlated well with the increase in DO consumption throughout the length of the study for PDS fed by G1 and S1. This was only true between February 5th, 2003 and February 19th, 2003 for the PDS fed by RO and the blend. This period corresponds to the dramatic increase of DO consumption observed in the four PDS. Data from these PDS during this period were pooled and a strong correlation was found between DO consumption, i.e. delta DO and turbidity increase, i.e. delta turbidity (Figure 48, $r^2 = 0.90$). Therefore the sudden increase in DO consumption during this period can be, in part, interpreted as a consequence of particulate or bacterial release in the system from the pipe surfaces (and subsequent turbidity increase) leading to higher dissolved oxygen demand in the PDS.

**Ammonia**

Delta ammonia (the difference between influent and effluent values) was calculated and is presented in Figure 49. A significant increase in ammonia consumption started by March 6th, 2003 for GW, RO and blend water sources, while that increase started on March 20th, 2003, for SW.

High consumption of ammonia also corresponded to increased DO consumption, production of nitrate and nitrite, and elevated HPC. Ammonia concentrations in the feed waters were about 2 mg N/L due to feed of ammonium chloride into the PDS and the release of that ammonia due to the destruction of monochloramines by sodium thiosulfate in the feed water.
storage tanks. Equation 2 shows that one mole of monochloramines immediately releases one mole of ammonia after reaction with sodium thiosulfate.

\[ \text{NH}_2\text{Cl} + 2\text{S}_2\text{O}_3^{2-} + \text{H}^+ \rightarrow \text{NH}_3 + \text{Cl}^{-} + \text{S}_4\text{O}_6^{2-} \]  

(2)

Ammonia was almost totally consumed in PDS fed by SW, RO and the blend towards the end of the phase, while about 1 mg N/L of ammonia was still present in the effluent of the PDS fed by GW.

**Statistical Analysis**

In order to understand nitrate and nitrite formation in the PDS during the last month of the phase and to confirm observed trends, a simple linear regression was carried out. Delta NOx (the sum of delta nitrate and delta nitrite) was the dependent variable, while delta DO and delta ammonia were the independent variables. The data from all four PDS were used. Table 45 presents the regression analysis in which both independent variables were considered. It can be seen that there was a significant correlation of NOX production with both variables.

**Conclusions**

The following main conclusions can be drawn from the present study:

- Measurement of nitrite and nitrate production was considered suitable for accurately assessing onset of nitrification episode in distribution system and to allow earliest
detection of a nitrification episode.

- No noticeable difference was observed in time of nitrification onset and magnitude of nitrification episode with respect to the different water treatment trains under study. No correlation between treatment processes and nitrification was found.

- Dramatic increase in DO consumption correlated with increase in turbidity and heterotrophic proliferation upon total residual depletion. This may be explained by bacterial and inorganic release from the biofilms due to sudden change in water quality caused by residual quenching, thus increasing oxygen demand in the bulk phase. Onset of nitrification, i.e. production of nitrite and nitrate did not lead to HPC increase in the distribution systems.

- Production of nitrite and nitrate was correlated to consumption of ammonia and DO in the distribution systems. Therefore DO and ammonia consumptions were symptomatic of nitrification. However these parameters may not be suitable as indicators of nitrification since they are not specifically related to nitrification occurrences.

References


Figure 37. Conceptual Flow Diagram of Pilot Distribution System

**Notes:**
- "": inches (one inch = 2.54 cm); Dia: Diameter.
- "": inches (one inch = 2.54 cm); Dia: Diameter.
- "": inches (one inch = 2.54 cm); Dia: Diameter.
- "": inches (one inch = 2.54 cm); Dia: Diameter.
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Figure 38. HPC Effluent as a Function of Time, Sorted by Water Source.
Figure 39. AOC Consumption (Delta AOC) Sorted by Water Source and Residual.

Figure 40. Effluent Total Chlorine Residual as a Function of Time, Sorted by Water Source.
Figure 41. Nitrate Production (Delta Nitrates) as a Function of Time, Sorted by Water Source.

Figure 42. Nitrite Production (Delta Nitrites) as a Function of Time, Sorted by Water Source.
Figure 43. DO Consumption (Delta DO) as a Function of Time, Sorted by Water Source.

Figure 44. Comparison of DO Consumption (Delta DO) and Turbidity Formation (Delta Turbidity) for PDS fed by GW
Figure 45. Comparison of DO Consumption (Delta DO) and Turbidity Formation (Delta Turbidity) for PDS fed by SW
Figure 46. Comparison of DO Consumption (Delta DO) and Turbidity Formation (Delta Turbidity) for PDS fed by RO

Figure 47. Comparison of DO Consumption (Delta DO) and Turbidity Formation (Delta Turbidity) for PDS fed by Blend
Figure 48. Correlation between DO Consumption (Delta DO) and Turbidity Formation (Delta Turbidity), 2/05/03-2/19/03
Figure 49 Ammonia Consumption (Delta Ammonia) as a Function of Time, Sorted by Water Source.
Table 45. Statistical analysis for NOx Production (Delta NOx) as a function of Consumption of NH3 and DO (Delta NH3 and Delta DO)

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*Independent Variable - P-value*

<table>
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<tbody>
<tr>
<td>Delta NH3</td>
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<tr>
<td>Delta DO</td>
<td>$7.30 \times 10^{-2}$</td>
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</tbody>
</table>

*Equation*

$$\text{Delta NOX} = -1.44 + 0.76 \times \text{Delta NH3} + 0.39 \times \text{Delta DO}$$
CHAPTER EIGHT: CHARACTERIZATION OF ON-GOING NITRIFICATION IN CHLORAMINATED DISTRIBUTION SYSTEMS USING FLUORESCENT IN-SITU HYBRIDIZATION

Introduction

Nitrification is caused by the growth of ammonia-oxidizing bacteria (AOB), and nitrite oxidizing bacteria (NOB), in the distribution system. AOB use ammonia as an energy source, converting ammonia to nitrite. Nitrite is known to accelerate monochloramine decay through an abiotic oxidation-reduction reaction which liberates ammonium, chloride, and nitrate as end products. The subsequent disappearance of the disinfectant residual along with production of ammonium can lead to the proliferation of AOBs, producing more nitrite and consuming more chloramines in a residual “death spiral”. In addition the disappearance of residual can lead to an increase of heterotrophic plate count (HPC) bacteria in the distribution system and may result in violation of the disinfectant residual requirements in the Surface Water Treatment Rule. Therefore to maintain good water quality both AOB inactivation and AOB regrowth prevention strategies must be considered. The presence of AOB in chloraminated systems depends on temperature, chloramine residual levels, and chlorine to ammonia ratio [1]. Odell et al. showed a marked difference in AOB regrowth potential for different chloramines dosage in Metropolitan Water District of Southern California (MWDSC)[2]. At 1.7 mg/L AOB regrowth occurred in 77% of the samples tested; and at 2.5 mg/L AOB regrowth appeared in 26% of test conditions. Chlorine to ammonia ratio of 4:1 and 5:1 are also favored to reduce free ammonia levels and thus
AOB nutrient supply.

Over the years more stringent Disinfection By-Products (DBP) regulation has driven many utilities to switch from free chlorine to chloramines as secondary and primary disinfectant. The D/DBP rule states levels of THMs not to exceed 80 µg/L and levels of HAAs not to exceed 60 µg/L [3]. Chloramines reduce the potential for DBP formation with respect to free chlorine [4-6]. In 1990 22% of the 438 surveyed US utilities were using chloramines as a secondary disinfectant [7]. However utilities using chloramination, while reducing THM and HAA formation, face potential onset of nitrification in their distribution systems. It was shown in a survey by Wilczak et al. that nitrification occurred in 63% of US utilities using chloramines [7]. The issue of potential nitrification in chloraminated distribution systems is therefore critical for drinking water quality management. The fate of AOB within the distribution system (in bulk phase and biofilms) needs to be especially investigated in order to determine prevention and control measures for nitrification.

The results discussed in this paper (April 4th to June 26th 2003) originate from a tailored collaboration project between American Water Works Association Research Foundation (AWWARF), Tampa Bay Water (TBW) and University of Central Florida (UCF). The main objective of this 3-year project initiated in December 2001 was to study the effect of blending different source waters on distribution system water quality. The subset of data presented thereafter characterizes AOB inventories in the bulk phase and biofilm under different water quality scenarios using fluorescent in-situ hybridization and the impact of water quality changes on AOB proliferation.
Materials and Methods

Experimental Set-Up

Pilot distribution systems (PDS)

The 4 pilot distribution systems (PDS) used to produce the results of this paper consisted of pipe sections from actual full-scale distribution system pipes that were pulled out from the ground by the participating member governments and were thus aged and sometimes corroded extensively (in the case of unlined metals such as cast iron and galvanized steel). To simulate sequence of materials likely found in full-scale distribution systems the sequence of pipe material in each of the PDS was (upstream to downstream) polyvinyl chloride (PVC), lined cast iron (LCI), unlined cast iron (UCI) and galvanized steel (G). A diagram of one of the PDS is present in Figure 50.

The PDS were designed with a two-day Hydraulic Residence Time (HRT) and were virtually identical with respect to the condition of their pipe sections, their hydraulic regime (HRT, flow velocities in the different pipe sections) and the environmental conditions under which they were operated (temperature, humidity). Therefore the main difference between each of these PDS was the source water used to feed the PDS.
**Biofilm sampling**

Coupons cut from each of the material pipe sections were sampled prior to each chlorine to ammonia ratio change after 3 weeks incubation and replaced with new un-colonized coupons. Duplicate coupons were 3 cm in diameter and affixed to a coupon holder that was placed in a PVC coupon cradle. Each PDS was provided with a coupon cradle operated in parallel and fed by the same source water as the PDS, under similar hydraulic conditions. Intact biofilms were analyzed for heterotrophic and nitrifying bacteria (using HPC and FISH assays respectively), after scraping and resuspending of the cells from the entire surface of the coupons.

**Water Sources**

Each of the four PDS under study received a different source water. Three PDS received a single water source, while a fourth PDS received a blend of these single water sources. The three source waters were produced from three different water treatment processes designed to simulate member government present or proposed finished waters: (i) GW was produced from a groundwater source treated by aeration and stabilization, (ii) SW was produced from a surface water source treated by enhanced ferric sulfate coagulation, ozonation, BAC filtration and stabilization, (iii) RO was produced from a groundwater source treated by high pressure reverse osmosis (salts were added to the permeate to simulate typical finished water from a desalination process). A blend of 40% GW, 30% SW and 30% RO was feeding a fourth PDS. All finished waters were treated with a chloramines residual of 4 mg Cl₂/L combined chlorine.
**Heterotrophic Plate Counts - HPC**

HPCs were performed by plate spreading on R2A agar incubated at 25°C for 7 days, according to Standard Method 9215B [8]. Results were expressed in colony-forming units per milliliter (cfu/mL).

**Fluorescent In-Situ Hybridization – FISH**

Based on works by Mobarry et al. [9], Baudart et al. [10], Regan et al. [11], and Blackall [12], 200mL of bulk liquid samples were filtered through 0.45µm black polyethylene filter and cells removed by filtration were fixed by placing filter on an absorbent pad soaked with 4% paraformaldehyde and incubating at 4°C overnight. Cells were dehydrated with 50%, 80%, and 96% ethanol solutions [13]. Sequential whole cell hybridization was performed using the 16S ribosomal RNA-targeted oligonucleotide probes listed in Table 46 at a concentration of 1µg/µL of sample. Cells were first hybridized by NSO190 and NSO 1225 for detection of all sequenced ammonia-oxidizing bacteria of the β subclass of Proteobacteria [9] and then with EUB338 for detection of all bacteria [14]. The probes were labeled with fluorescent dyes (Table 46, Sygma-Genosys, The Woodlands, TX, USA). Optimal hybridization was achieved by adjusting formamide concentration in hybridization buffer and sodium chloride concentration in washing buffer as described in Table 46. Hybridization and washing step were carried out at 46°C for 2h and 48°C for 30min, respectively. Biofilm suspensions underwent the same procedure. 2-mL of the original 4-mL bacterial suspension was filtered through 0.45µm black polyethylene filter and
similar procedure as described above for bulk liquid samples was carried out [10]. Slides were mounted using CITIFLUOR AF-2. A Zeiss LSM 510 confocal laser scanning microscope equipped with a Zeiss Plan-Apo 40x (NA 1.3) objective and set for three-color fluorescence of FITC, Rhodamine and Cy5 was used for microscopic analysis of stained slides, with 3 lasers set at 488, 543 and 633 nm respectively. Five randomly chosen areas of each slide were serially scanned resulting in 50 - 75 optical sections (0.5µm apart) per slide. The section series were analysed using Carl Zeiss and Photoshop image analysis software to calculate the relative volume occupied by each oligonucleotide probe (expressed as percent of total volume scanned). Results were expressed in cfu/mL and cfu/cm² for bulk liquid and biofilm samples respectively after conversion using HPC results.

Chemical Water Quality Monitoring

Influent and effluent PDS water quality samples were collected on a weekly basis. Reported information included ammonia, nitrate, nitrite, residual total chlorine, temperature, and dissolved oxygen. All analyses were performed by accepted methods according to Standard Methods [8].
Results and Discussion

AOB Proliferation in Bulk Phase

Table 47 presents average water quality data for the four PDS under study. On-going nitrification was confirmed by significant nitrite and nitrate production, DO consumption and residual depletion. Figure 51 shows the linear correlation between AOB inventories and the sum of nitrite and nitrate (NOx) production, which proves that NOx can be used as a monitoring parameter for on-going nitrification in distribution systems. Maintaining significant chloramine residual helps controlling bioinstability (Figure 52). Unless residual levels were maintained above 0.60 mg Cl₂/L at all times within the distribution system, significant autotrophic and heterotrophic proliferations were observed. The data also show that greater residual levels reduced the magnitude of nitrification without completely stopping on-going nitrification. Consumption of DO corresponded with increased bacterial growth (Figure 53). While DO can be consumed through several pathways (oxidation of organic matter, reaction with network metallic pipe material, biological reactions), these linear correlations show that DO is a valuable parameter for assessing bioinstability, in conjunction with other parameters (e.g. residual, nitrite, nitrate and temperature measurements). The above observations show that heterotrophic and especially autotrophic proliferations are related to residual and DO levels.
AOB Proliferation in Biofilms

Figure 54 shows a decreasing trend between AOB inventories in the biofilm and the production of NOx in the bulk phase, for all pipe materials. The same trend was observed with DO consumption (Figure 55). Similar relationship was observed between biofilm HPC and DO consumption (data not shown). Since production of NOx and DO consumption have been presented as indicative of nitrification in the bulk phase (Figure 51 and Figure 53), these trends suggest that AOB proliferation in the bulk led to smaller AOB inventories in the biofilm. This hypothesis was confirmed by Figure 56. In the biofilms, conditions leading to greater HPC inventories also led to greater AOB inventories since the two are linearly correlated (Figure 57). Residual did not have significant effect on either biofilm HPC or AOB inventories (data not shown).

Comparison of Bulk and Biofilm AOB Inventories

In order to determine which AOB populations (i.e. bulk or biofilm) was responsible for the ongoing nitrification, bulk and biofilm AOB total inventories were compared for each PDS. The total AOB density in the bulk corresponds to the estimated total number of AOB present in the PDS bulk liquid volume, while the total AOB density in the biofilm corresponds to the estimated total number of AOB attached to the inner surface of the PDS. Total AOB density in bulk and biofilm were calculated assuming that the AOB colonization of the pipe walls was uniform, and that the concentration of bulk AOB in the effluent of the PDS was representative of the average
bulk AOB concentration within the PDS. Table 48 shows that there was no significant difference between bulk and biofilm AOB density for each PDS, which suggests similar potential for nitrification in the bulk phase and in the biofilms. However the results presented above showed that only bulk AOB results were correlated to signs of nitrification (e.g. nitrite, nitrate production and DO consumption). Furthermore, the opposite trends observed with biofilm AOB suggest that AOB activity was predominantly in the bulk phase. Such dichotomy could be explained by the fact that bulk AOB and HPC activity, by consuming DO, may have caused DO limitations in the biofilms. Formation of a layer of heterotrophs on top of the nitrifying biofilm may also have limited AOB oxygen supply, by diffusion limitation (Nogueira et al., 2002). Therefore while AOB were present in both the bulk phase and biofilms, only bulk AOB inventories correlated with nitrification in the bulk, i.e. nitrite and nitrate production. Since chloramine residual depletion is accelerated by the presence of nitrite (produced by AOB), leading to further bioinstability within the distribution system (i.e. heterotrophic proliferation), bulk AOB inventory is the parameter of interest with respect to on-going nitrification.

Conclusions

The following main conclusions can be drawn from the present study:

- Monitoring nitrite and nitrate levels in the bulk phase of a distribution system is a suitable way of assessing the magnitude of an on-going nitrification event.
- Residual maintenance is an option for reducing nitrification and controlling
heterotrophic growth in the bulk phase but has little impact on biofilm inventories.

- Monitoring DO in conjunction with residual and nitrite and nitrate is an inexpensive option for assessing bioinstability in distribution systems (i.e. bulk and biofilm proliferations).

- Heterotrophic and autotrophic growths have been correlated with each other in bulk and biofilms.

- Bulk AOB caused nitrification in the bulk phase. The contribution of biofilm AOB activity to nitrification was shadowed by bacterial activity in the bulk phase, which may have resulted in DO limitation within the biofilms.

- While FISH is not yet a cost-effective option for utilities for monitoring nitrification and AOB populations in distribution systems, it is a valuable research tool to investigate nitrification and it proved to correlate well with nitrification indicators.

References


Figure 50. Conceptual Flow Diagram of Pilot Distribution System
Figure 51. Log effluent AOB as a Function of Production of Nitrite and Nitrate
Figure 52. Log Effluent AOB and HPC as a Function of Effluent Residual
Figure 53. Log Effluent AOB and HPC as a Function of DO Consumption
Figure 54. Log Biofilm AOB as a Function of Production of Nitrite and Nitrate

Figure 55. Log Biofilm AOB as a Function of DO Consumption, by Pipe Material
Figure 56. Log Biofilm AOB as a Function of Log Effluent AOB, by Pipe Material

Biofilm AOB = 0.84(Biofilm HPC) - 0.33  
$R^2 = 0.67$

Figure 57. Log Biofilm AOB as a Function of Log biofilm HPC, by Pipe Material

Biofilm AOB = 0.84(Biofilm HPC) - 0.33  
$R^2 = 0.67$
Table 46. Probe Description and Corresponding Hybridization Conditions.

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Table 47. Average Water Quality.

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Table 48. PDS Average AOB Inventories.

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CHAPTER NINE: CONCLUSIONS AND RECOMMENDATIONS

Water quality changes can occur in distribution systems due to several reasons (e.g. water source change, disinfectant change, infiltration of groundwater through leaking pipes…). These changes can have adverse effects on distribution system water quality, which in turn can affect the consumers. This study specifically investigated impact of finished water change on HPC levels in chlorinated and chloraminated distribution systems. Chlorination was more effective than chloramination in reducing HPC levels (1-2 log difference). A rapid increase in HPC corresponding to the change in steady state water quality was observed in all PDS. Modeling effort demonstrated that HPC levels reached a maximum within five days after water quality change and return to initial level ten days after change. As alkalinity was used to monitor completion of steady-state water quality change, time to reach maximum HPC was related to a mixing model using alkalinity as a surrogate that confirmed alkalinity transition was complete in approximately eight days.

Since no coliform was ever detected in the PDS, biostability assessment was based on heterotrophic proliferation measured by HPC. It was observed that HPC levels would be above four logs if residual drops below 0.1-0.2 mg/L as Cl₂, which is below the regulatory minimum of 0.6 mg/L as Cl₂. Therefore bacterial proliferation is controlled in distribution systems as long as residual regulatory requirements are met. An empirical modeling effort showed that residual, pipe material and temperature were the most important parameters in controlling HPC levels in distribution systems, residual being the only parameter that can be practically used by utilities to
control biological stability in their distribution systems. Use of less reactive (i.e. with less chlorine demand) pipes is recommended in order to prevent residual depletion and subsequent bacterial proliferation.

Attached growth on the inner surface of distribution system pipes is an important factor in biostability assessment. This study is unprecedent as it investigated biofilm growth simultaneously with suspended growth under a wide range of water quality scenarios and pipe materials. It was found that increasing degree of treatment led to reduction of biofilm density, except for reverse osmosis treated groundwater, which exerted the highest biofilm density of all waters. Pipe material is a significant factor for biofilm growth. Biofilm densities on corrodible, highly reactive materials (e.g. unlined cast iron and galvanized steel) were significantly greater than on PVC and lined cast iron. Biofilm modeling showed that attached bacteria were most affected by temperature and much less by HRT, bulk HPC and residual. The model predicts biofilms will always be active for environments common to drinking water distribution systems, which is consistent with the hypothesis that biofilms act as reservoirs for microorganisms and are a constant source of microorganisms to the bulk water under normal conditions. As American utilities do not control biofilms with extensive and costly AOC reduction, American utilities must maintain a strong residual to maintain biological integrity and stability in drinking water distribution systems.

As nitrification is a recurrent issue in chloraminated distribution systems, especially in warm seasons, a controlled experiment was designed to study this phenomenon. Nitrite and nitrate were considered the most suitable indicators for utilities to predict onset of a nitrification
episode in the bulk phase. DO and ammonia were correlated to production of nitrite and nitrate but are not directly related to nitrification.

AOB densities in the bulk phase correlated well with nitrite and nitrate production, reinforcing the fact that nitrite and nitrate are good monitoring tools to predict nitrification. Chloramine residual proved to be helpful in reducing nitrification in the bulk phase but has little effect on biofilm densities. As DO has been related to bacterial proliferation and nitrification, it can be a useful and inexpensive option for utilities in predicting biological instability, if monitored in conjunction with residual, nitrite and nitrate. Autotrophic (i.e. AOB) and heterotrophic (i.e. HPC) were correlated in the bulk phase and biofilms.
APPENDIX A

BIOASSAYS – STANDARD OPERATING PROCEDURES
Appendix A-1: Heterotrophic Plate Count – HPC

EQUIPMENT AND CHEMICALS

100mL disposable sample vials
Dilution Buffer
Isopropyl alcohol
Test tubes
Test tube rack
Labels
Bunsen burner
Glass spreader
Viable agar plates
Mechanical pipet aid
Turntable
100-1000 µL pipet
Redi Tip disposable pipet tips
Reagent Bottle
Latex gloves
Spatula
Weighing dish
Graduated cylinder
Volumetric flask
Parafilm
Top load balance
1:1 HCl
Muffler Oven
Aluminum Foil
Detergent
DI water
500 mL Beaker
250 mL Beaker
Magnetic Stirrer
pH Meter
10-100 µL pipet
Electric vortex shaker
PROCEDURE

A. Preparation of Glassware

1. Wash all glassware with detergent and water.
2. Rinse at least 2x with 1:1 HCl.
3. Rinse at least 3x with DI.
4. Dry and cap with aluminum foil.
5. Heat all glassware at 525°C for 6 hours

B. Preparation of Reagents

Wear latex gloves when handling reagents and samples to minimize contamination.

1. Phosphate Buffer
   a. Weigh out 17.0 g KH₂PO₄ into a weighing dish and transfer to 500 ml beaker.
   b. Add 250 mL DI water and use magnetic stirring rod to dissolve.
   c. Adjust pH to 7.2 +/- .05 using 1 N NaOH (20g/500 mL DI).
   d. Transfer solution to 500 mL volumetric flask and dilute to mark using DI water.
   e. Cover flask with parafilm and invert several times to mix the solution.
   f. Transfer to a reagent bottle for storage. Store at 4°C and use within 4 weeks.

2. Magnesium Chloride Hydrate
   a. Weigh out 8.11 g MgCl₂•6 H₂O into a weighing dish and transfer to 250 ml flask.
   b. Add 100 mL DI water and stir using magnetic stirrer.

3. Dilution Buffer
   a. Using 100-1000 µL pipet, pipet 1.25 mL of stock Phosphate buffer into a 1 L volumetric flask.
   b. Using a graduated cylinder, measure 5 mL of MgCl₂•6 H₂O and add to flask.
   c. Dilute to 1L mark using DI water.
   d. Autoclave solution for 15 min.
C. Collection of Water Samples

Wear latex gloves when handling samples to minimize contamination.

1. Collect samples in carbon free bottles (refer to section A – Preparation of Glassware.)
2. Transfer samples to sterile sample container.
3. Store at 4°C and analyze within 24 hours.

D. Water Sample Preparation

Wear latex gloves when handling samples and reagents to minimize contamination.

1. Determine amount of dilutions needed.
2. Label test tubes with sample number and location.
3. Flame lip of Dilution Buffer bottle prior to use.
4. Place 9 mL of Dilution Buffer in each test tube using a 10mL disposable pipet and mechanical pipet aid.
5. Using 100-1000µL pipet, pipet 1000µL of sample into 102 test tube.
6. Dispose of pipet tip.
7. Shake tube on vortex for five seconds.
8. Using a new pipet tip, place 1000µL of 102 solution into 103 test tube.
9. Dispose of pipet tip and shake on vortex.
10. Repeat steps 4-8 to obtain as many samples as needed. (the initial sample is the 101 dilution.)

E. Plating Samples

Wear latex gloves when handling samples to minimize contamination.

1. Label all plates with sample number and location.
2. Using a 10-100µL pipet, place 100µL of sample in the center of the correct plate and place plate on the turntable.
3. Begin with the highest dilution of each sample.
4. Dip the glass spreader into isopropyl alcohol and flame.
5. Cool spreader under hood then place in the middle of the sample and gently move back and forth until the sample is spread across the entire glass rod.
6. Spin the turntable and evenly spread the sample over the entire plate.
7. Repeat steps 2-6 until all samples have been plated.
8. After completion of final plate, wait 15 minutes then invert and place in a 35
degree Celsius incubator for 48 hours.

F. Sample Analysis

*Wear latex gloves when handling samples to minimize contamination.*

1. Recover sample from the incubator within 48 hours of experiment.
2. Count and record all colonies for all dilutions.
3. Optimal dilution contains between 30-300 colonies.
4. After completion of analysis, spray 10% bleach solution on each plate and dispose of in separate trash bag.

G. Calculation

1. Select the counts that are between 30-300 colonies
2. Multiply by the dilution factor
3. Express as cfu/mL.
Appendix A-2: Assimilable Organic Carbon – AOC

EQUIPMENT AND CHEMICALS

A. Equipment and Chemicals for Preparing Incoming Samples

1. Sample Containers
   a. Teflon-coated Silicone Septae for 40ml EPA vials
   b. Kimble Borosilicate Glass 40ml EPA Vials
   c. Screw Caps GPI thread 24-400
   d. Reagent Grade Sodium Persulfate

2. Cultures
   a. P17 and NOX Working Stock Cultures
   b. 16 x 5 mm Petri Dishes
   c. R2A Plates
   d. Sterile bent Pasteur Pipettes

B. Equipment and Chemicals for Inoculation Set-up

1. Wheaton Step-pettor
2. 6 x 8 Array Vial Racks (12½ x 9½ x 4 Rack for 25-30 mm tubes)
3. 3 x 8 Array Vial Racks
4. (3) BD 9585 3 cc Syringes with 21 G 1½ “ Needles
5. Destruclip
   16 x 5 Sterile Petri Dishes
   P17 Working Stock Culture
   NOX Working Stock Culture
   10% Sterile Sodium Thiosulfate Solution
   0.1-0.2 ml pipette tips
   1.0 ml pipette tips
   0.1 and 1.0 ml Pipetters
   Class A TD (serological) Pipettes (5ml and 10ml)

C. Equipment and Chemicals for Analysis Set-up

1. Turner Designs 20e Luminometer
2. Hoefer Scientific manifold with Stainless Steel Weights
3. Turner Designs Luciferin/Luciferase (20-2101)
4. Turner Designs Hepes Buffer (20-2011)
5. Turner Designs Releasing Reagents (20-2130)
6. Turner Designs 8 x 50 Polypropylene Test Tubes
7. 0.1-0.2 ml pipette tips
8. 1.0 ml pipette tips
9. 0.1 and 1.0ml Pipettors
10. Timer
11. Wheaton Step-pettor
12. Wheaton Disposable 37.5ml Syringe
13. Working Stock ATP Standard
14. Sartorius Cellulose Acetate Filters, 0.22µm 25mm
15. 12 x 75 Sterile Disposable Polystyrene Culture Tubes without a cap
16. Test Tube rack for 12 x 75ml tubes
17. Micro Sample Tube rack for 8 x 50test tubes
18. (2) 2L Side Arm Filtering Flasks
19. Plastic Tubing and corks for filtering flasks

PROCEDURE

A. Freezer Stock Cultures

1. Obtain P17 of NOX seeded slants.
2. From slants, streak for purity several R2A plates for each stain.
3. Grow plated cultures at 25± 1°C for 3 to 5 days.
4. From an isolated colony, seed an R2A plate by streaking the entire plate in a close zigzag pattern.
5. Make several seeded plates for each stain and incubate at 25± 1°C for 3 to 5 days.
6. Prepare a 2% peptone / 20% glycerol solution by adding 2g peptone to a mixture of 80 ml ultra-pure water and 20ml glycerol/ Sterilize by autoclaving.
7. Loosen colonies from a seeded plate with 2ml 2%peptone/ 20% glycerol solution.
8. Using sterile Pasteur pipettes, transfer mixture to sterile 2 ml Nalgene cryovials and freeze at -70°C.
9. Repeat steps 7-8 for all seeded plates made.
B. Reagent Preparation

Wear latex gloves when handling reagents and samples to minimize contamination

1. Mineral Salts Buffer Stock Solution

Dissolve following constituents in prescribed volume of water using pre-cleaned glassware:
   A. 7.0g K$_2$HPO$_4$
   B. 3.0g KH$_2$PO$_4$
   C. 0.1g MgSO$_4$·7H$_2$O
   D. 1.0g (NH$_4$)$_2$SO$_4$
   E. 0.1g NaCl
   F. 1.0mg FeSO$_4$
   G. 1.0L Ultra-pure H$_2$O

2. Mineral Salts buffer Working Solution

Using a pre-cleaned volumetric pipette or calibrated pipettor with sterile tip, add 1 ml Mineral Salts Buffer Stock Solution to 999 ml ATP-free water (ultra-pure) in a pre-cleaned volumetric flask

3. Stock Sodium Acetate Solution (200mg/L)

In a pre-cleaned volumetric flask, add ATP-free water (ultra-pure) to 1.134g of Sodium Acetate until volume is 1L.

4mg/L Sodium Acetate
Using a pre-cleaned volumetric flask and pipette, mix the following constituents:
   a. 20ml Stock Sodium Acetate
   b. 980ml Mineral Salts Buffer Working Stock

2mg/L Sodium Acetate
Using a pre-cleaned volumetric flask and pipette, mix the following constituents:
   a. 10ml Stock Sodium Acetate
   b. 990ml Mineral Salts Buffer Working Stock
200µg/L Sodium Acetate
Using a pre-cleaned volumetric flask and pipette, mix the following constituents:

a. 50ml 4mg/L Sodium Acetate  
b. 950 ml Mineral Salts Buffer Working Stock

100µg/L Sodium Acetate
Using a pre-cleaned volumetric flask and pipette, mix the following constituents:

a. 25ml 4mg/L Sodium Acetate  
b. 975 ml Mineral Salts Buffer Working Stock

50µg/L Sodium Acetate
Using a pre-cleaned volumetric flask and pipette, mix the following constituents:

a. 12.5ml 4mg/L Sodium Acetate  
b. 987.5 ml Mineral Salts Buffer Working Stock

C. Sample Analysis

1. Prepare working stock by inoculating 100ml of 2 mg/L Sodium Acetate with a loopful (10µl) of the freezer stock culture. Grow working stock culture at 25±1°C for 7 days. At the end of 4 days growth, plate each working stock culture at 10^{-4} / 10^{-5} final dilution.

2. Inoculation (Friday)
Do colony counts on plates that were prepared on Tuesday and dilute using the following procedure in order to obtain the Working Stock Cultures. Given that the inoculation volume is 50µL for both P17 and NOX, the working stock culture concentrations have to be adjusted to 8x10^6 cfu/mL by proper dilution with dilution buffer and 50mL of Working Stock Culture have to be produced for both P17 and NOX.

3. Pour 40ml of samples into vials for P17 (6 vials: 3-day; 2 vials, 4-day; 2 vials, 5-day; 2 vials) and NOX (6 vials). Pour Mineral Salts buffer
Working Solution into vials of 2 (P17 and NOX) x 3 (3-day, 4-day, 5-day) vials for Blank, and pour Sodium Acetate Solutions (200µg/L, 100µg/L, 50µg/L) into vials of 3 x 6 for Yield control (200µg/L, 100µg/L, 50µg/L). To make starting count plates, prepare additionally Two Blank vials (P17/NOX).

Pre-filtration
Raw and settled water samples and other waters that contain large amounts of particles require pre-filtration using glass fiber filters before pasteurization is performed

Sample Containers
a. Vials
   All 40ml vials used in AOC sampling are prepared for use by cleaning in Miele dishwasher Model 67733 (with acid rinse). The vials are then muffled at 525°C for 6 hours.

b. Septae
   Septae for 40ml vials are pre-cleaned at approximately 70°C in a 10% Sodium Persulfate Solution for 1 hour. Rinse septae 4 times in ultra-pure water after cooling.

4. With forceps, place septae with Teflon side up into open-top screw thread caps. Carefully place septae and caps onto vials and screw down.

5. Pasteurization

   AOC samples containing a chlorine residual must be heat-treated at 70°C for 30 minutes within 24 hours of sample collection. Until pasteurization is initiated, all AOC samples, regardless of disinfectant residual, must be refrigerated at 4°C ± 1°C or kept cold using blue ice. AOC samples without a chlorine residual must be pasteurized immediately after collection and subsequently refrigerated at 4°C ± 1°C or kept cold on blue ice.

6. Aseptically pour a small amount of 10% Sterile Sodium Thiosulfate into a sterile 16 x 5 mm Petri Dish.

7. Affix a sterile 3 ml BD syringe with 1½ “ 21 G needle to the Wheaton Step-pipettor. Make sure the Step-pipettor has the blue dial set to 0.05ml.
8. Fill the syringe and inject each sample vial with 0.05ml of sterile 10% Sodium Thiosulfate for a final concentration of 0.0125% into sample vials containing chlorine.

9. Make dilutions of the Working Stock Cultures using sterile TD serological pipettes, sterile 16 x 5 mm petri dishes, Mineral Salts buffer Working Solution as diluent, and the appropriate Working stock Cultures.

10. Adjust the blue dial on the Wheaton Step-pettor to the 0.1 ml mark.

11. Using a new sterile 3ml BD syringe with 1 ½ “ 21 G needle, fill the syringe with P17 Working Stock Culture Dilution and inject 0.1 ml into the sample vials labels with a ‘P17’ as well as the Blank an Yield Experiment vials.

12. Repeat step 10 for the NOX Working Stock Culture Dilution and inject 0.1ml into the sample vials labeled with ‘NOX’ as well as the Blank and Yield Experiment vials.

13. Make starting count plates from Blank vials (P17/NOX).

14. Incubate plates and all inoculated vials at 25°C for 3 days.

15. At the end of growth (3-day, 4-day, 5-day), all vials are plated on R2A plates. Different dilutions of each inoculated vial are prepared and plated in duplicate. All plates are incubated at 25°C for 3 days. The cell density of all plates is determined after 3 days. Plate each vial: P17 Blank at $10^2$ / $10^3$ final dilution, P17 Yield and samples at $10^3$ / $10^4$ final dilution, NOX Blank at $10^3$ final dilution, NOX Yield and samples at $10^4$ / $10^5$ final dilution.

**METHOD PERFORMANCE**

Since the Mineral Salts Buffer Working Solutions and the Sodium Acetate Solution are used in a separate experiment on a ‘per run’ basis, these solutions are monitored for method performance as a result. These solutions constitute the Blank and Yield Controls; which are designed to monitor vital contamination (Blank Control-MSB Working Solution) and growth yield (Yield Control -200µg/L, 100µg/L, 50µg/L Acetate) of the indicator organisms P17 and NOX. The Blank Control further constitutes a low level control, whereas the Yield Control is a mid-range control.

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CALCULATION

\[
AOC(\mu g/L) = \left[ \frac{\text{average} P17 \text{ cfu/mL}}{4100000} + \frac{\text{average NOX cfu/mL}}{1200000} \right] \times 1000mL/L
\]

\[
[Yield = \frac{[P17\text{ cfu/mL : yield control} - (P17\text{ cfu/mL : blank control})]}{100\mu g \text{ acetate} - C/L} \times (1000mL/L)]
\]

QUALITY CONTROL

Blank control = 1 x 10⁴ cfu/mL

0.003-0.035 ppm AOC

Yield controls = 1 x 10⁵ cfu/mL for P17

0.1±0.01 ppm AOC

2 x 10⁵ cfu/mL for NOX

0.1±0.01 ppm AOC

All solutions are tested for TOC and must adhere to the following standards for quality assurance:

A. Mineral Salts Buffer Stock Solution- 1mg/L ± 0.3 mg/L
B. Mineral Salts Buffer Stock Solution- 0.1-0.3 mg/L
C. Ultra-Pure Water used in preparing all Solutions 0.1-0.3 mg/L
D. Stock Sodium Acetate Solution- 200mg/L ± 20mg/L
E. 4mg/L Sodium Acetate Solution- 4mg/L ± 0.4mg/L
F. 2mg/L Sodium Acetate Solution- 2mg/L ± 0.2 mg/L
G. 100μ/L Sodium Acetate Solution- 0.1- 0.3 mg/L

TOC values for the Mineral Salts buffer (MSB) Working Solution should be no higher than an additional 10- 12% of the TOC value obtained for the Ultra-pure Water on the same day that the MSB working solution was made. The TOC value obtained for the 100μg/L Sodium Acetate Solution should be 0.1 ± 0.03mg/L higher than the TOC value obtained on the MSB Working Solution, each solution is monitored for TOC level on the same day since all are prepared on the same day.
Appendix A-3: Biodegradable Dissolved Organic Carbon – BDOC

EQUIPMENT AND CHEMICALS

- Biological sand (quartz sand, 1-2 mm from sand filter of water treatment plant without pre-chlorination or from freshwater fish tank)
  - 10.5 x 20 x 11.5 inches Fish tank
- Reusable polypropylene scoop
- Aquarium air pump
- ¼ in. ID ⅜ in. OD Rubber tubing
- 5-Outlet gang valve
- Air stones
- Rubber stopper
- Glass tubing
- 1.2 or 2 L Stainless steel beakers
- 13 gal Carboy with spigot
- 500 mL gas wash bottle
- 500 mL Erlenmeyer flask
- 5 mL Sterile serological pipet
- 35-45 mm Plastic foam stoppers
- 30 mL Luerlock glass syringe
- Gelman syringe type holders for 25 mm diameter filter
- 0.45 micron 25 mm Polycarbonate membrane filter
- 3-way Stopcock (female luer to male luerlock, side arm female luer)
- 40 mL Borosilicate glass EPA vials
- 47 mm diameter Whatman 934-AH glass fiber filter
- Glass filtration set-up
- Test tube rack
- Latex gloves
- Spatula
- Weighing dish
- Graduated cylinder
- Volumetric flask
- Volumetric pipet
- Eppendorf pipet
- Reagent bottle
- Parafilm
- Aluminum foil
- Evian water
- Sodium acetate
Sodium thiosulfate
Dechlorinated tap water
1:1 HCl
Bleach
Muffler Oven
UV-VIS Spectrophotometer
Top load balance
TOC Analyzer

PROCEDURE

A. Sand Preparation

Wear latex gloves when handling reagents and samples to minimize DOC contamination.

I. Sand from water treatment plant

1. Procure biological sand from a sand filter of a water treatment plant without pre-chlorination stage.
2. Wash sand with dechlorinated tap water until washing is clear and colorless. Sand washing can be conducted using a backwashing system in a column. Dechlorinated tap water is continuously pumped at the bottom of the column and the washing goes into an overflow. A recycle system can be used but the dechlorinated tap water should be regularly changed once it becomes turbid or colored. If anthracite is present, sieve using 1 mm mesh size. Any remaining anthracite (~1%) will not affect the BDOC analysis.
3. Distribute sand into stainless steel beaker. Fill beakers with about \( \frac{1}{3} \) sand.
4. Fill beakers with \( \frac{3}{4} \) DI and stir sand with a polypropylene scoop to insure complete washing. Decant to discard washing.
5. Continue washing with DI water until the UV-254* of the washing is about 0.0050 cm\(^{-1}\). This will be approximately 0.2 mg DOC/L. For UV-254 measurement, filter samples prior to analysis. Refer to section G - Sample Analysis #9.
6. The sand is ready for use if DOC\(_{\text{wash}}\) ≤ DOC\(_{\text{DI}}\).
7. For storage: store sand in dechlorinated tap water. Fill beakers with \( \frac{1}{4} \) dechlorinated tap water and aerate using aquarium air pump.
8. Cover with aluminum foil and keep in the dark.

*Establish UV-254 and DOC correlation first if this analysis is being conducted for the first time.
II. Sand from fish tank

1. Purchase 1 to 2 mm quartz sand.
2. Soak the sand in bleach for 2 h.
3. Rinse the sand several times with tap water until washing is clear and then at least 3x with DI.
4. Store sand overnight in DI. Check that the sand is not releasing DOC by measuring DOC of the washing. For DOC measurement, filter samples prior to analysis. Refer to section G - Sample Analysis #9.
5. Set-up an aquarium tank with an air pump.
6. Pour about 20 lbs of the washed sand into the tank.
7. Fill the tank to about 3 inches from the top of the sand with dechlorinated tap water.
8. Aerate using an aquarium air pump. Cover the tank with aluminum foil.
9. Incubate in the dark for a month without changing the water.
10. Mix the sand 2x a day (early morning and late afternoon) using a polypropylene scoop to avoid anaerobic sites.
11. Proceed as in section A - Sand Preparation, I. Sand from water treatment plant #3.
12. To test bacterial growth, run BDOC experiment using sodium acetate standards.

B. Preparation of Glassware

1. Wash all glassware with detergent and water.
2. Rinse at least 2x with 1:1 HCl.
3. Rinse at least 3x with DI.
4. Dry and cap with aluminum foil.
5. Heat all glassware at 525 °C for 6h (except analytical glassware) or soak in 100 g/L sodium persulfate solution for 1h at 70 °C or soak in sulfochromic solution (concentrated sulfuric acid saturated with potassium dichromate) and rinse 3x with DI.
6. For pretreatment of glass fiber filter: wrap glass fiber filters in foil and heat at 525°C for 6 h together with the glassware.

C. Preparation of BDOC Set-up

1. Prepare BDOC set-up as illustrated in the following diagram (Fig.1). Use rubber tubings for connections. Air from the pump is scrubbed through an Erlenmeyer flask and a gas wash bottle filled with DI.
2. Assemble an air scrubber using 500 mL Erlenmeyer flask capped with a rubber stopper. Insert 2 glass tubings through the rubber stopper and into the flask with one of the tubing longer than the other. One of the tubing should be long enough to reach about 1 ½ inches from the bottom of the flask and the other about 1 inch long from the stopper. The longer tubing will be submerged in DI. Both tubings should have portions of about 1 inch on top of the stopper for connections from the pump and to the gas wash bottle.

3. Fill the Erlenmeyer flask and the gas wash bottle halfway with DI and replace daily during the experiment.

4. Conduct the experiment at 20 ± 2°C in the dark.

Figure A1. BDOC Set-up
D. Preparation of Reagents

*Wear latex gloves when handling reagents and samples to minimize DOC contamination.*

1. DI – DI with DOC < 0.2 mg/L or HPLC grade water
2. Blank water – Evian mineral spring water
3. Sodium acetate stock solution, 200 mg DOC/L
   - Weigh out 1.133 g CH₃COONa.3H₂O into a weighing dish and transfer into a 1L volumetric flask.
   - Add blank water and swirl to dissolve the reagent.
   - Fill the flask to the mark with blank water.
   - Cover flask with parafilm and invert several times to mix the solution.
   - Transfer to a reagent bottle for storage. Store at 4°C and use within 4 weeks.
4. Sodium acetate standard solution, 2 mg DOC/L
   - Using a volumetric pipet, transfer 10 mL of the sodium acetate stock solution into 1L volumetric flask.
   - Fill the flask to the mark with blank water.
   - Cover flask with parafilm and invert several times to mix the solution.
5. Sodium thiosulfate solution, 10%
   - Weigh out 100 g Na₂S₂O₃ into a weighing dish and transfer into 1L volumetric flask.
   - Add DI and swirl to dissolve the reagent.
   - Fill the flask to the mark with DI.
   - Cover flask with parafilm and invert several times to mix the solution.
   - Transfer to a reagent bottle for storage. Store at 4°C and use within 4 weeks.
6. Nonchlorinated/dechlorinated drinking water
   - Using an Eppendorf pipet, add about 0.2 mL 10% Na₂S₂O₃ solution to 1L of tap water.
   - Stir to mix.
E. Water Sample Collection

*Wear latex gloves when handling samples to minimize DOC contamination.*

1. Collect samples in carbon free bottles (refer to section B – Preparation of Glassware).
2. Store at 4°C and analyze within 24 h.

F. Water Sample Preparation

*Wear latex gloves when handling reagents and samples to minimize DOC contamination.*

1. Filter samples only if it contains very high concentration of suspended solids. Use pretreated glass fiber filter in a glass filtration set-up.
2. If present, neutralize chlorine residual by adding 0.2 mL 10% Na$_2$S$_2$O$_3$ per 1L sample using an Eppendorf pipet.
3. Dilute high DOC samples with blank water. The DOC concentration should be less than 10 mg C/L.

G. Sample Analysis

*Wear latex gloves when handling reagents and samples to minimize DOC contamination.*

1. Weigh out 100 ± 10 g sand into the 500 mL Erlenmeyer flask using a top load balance.
2. Add approximately 100 mL sample or sodium acetate standard (activity control flask) using a graduated cylinder. Set aside two 40 mL of the sample for DOC analysis (DOC$_a$). For DOC measurement, filter samples prior to analysis. Refer to section G - Sample Analysis #9.
3. Gently swirl sample in the sand.
4. Stand for 10 min and drain the sample or the sodium acetate standard by decanting.
5. Using a graduated cylinder, fill the flask with 300 mL sample or sodium acetate standard. Prepare sample dupe (SF#2), activity control (ACF) and inhibitor control (ICF) for QA/QC. Refer to table 1 for the sample set-up. For each sample type, there should be 1 sample, 1 sample dupe, and 1 inhibitor control. There will be a total of 3 flasks for each sample type. Only 1 activity control will be necessary for a set of BDOC experiment.

<table>
<thead>
<tr>
<th>Flask ID</th>
<th>Water Sample</th>
<th>CH$_3$COONa Stock</th>
<th>CH$_3$COONa Standard</th>
<th>Sand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>300 mL</td>
<td>x</td>
<td>x</td>
<td>100 g</td>
</tr>
<tr>
<td>Sample Dupe</td>
<td>300 mL</td>
<td>x</td>
<td>x</td>
<td>100 g</td>
</tr>
<tr>
<td>Inhibitor Control</td>
<td>300 mL</td>
<td>3 mL</td>
<td>x</td>
<td>100 g</td>
</tr>
<tr>
<td>Activity Control</td>
<td>x</td>
<td>x</td>
<td>300 mL</td>
<td>100 g</td>
</tr>
</tbody>
</table>

6. Connect sample flasks to the BDOC set-up.
7. Aerate sample with a continuous steady supply of air bubbles (~1 bubble/sec) coming out through the pipet.
8. Collect 40 mL samples for DOC analysis after 3 to 5 min contact with the sand (DOC$_1$)
   - Assemble the syringe filtration set-up for sample collection.
   - Attached in sequence to a 30 mL glass luerlock syringe: a 3 way stopcock with a rubber tubing for the sample inlet and a syringe type filter holder.
   - Place a 0.45 µm polycarbonate membrane filter into the filter holder.
   - Set the valve position in the stopcock to be able to draw sample from the flask.
   - Slowly pull the syringe plunger to draw the sample into the syringe.
   - Change the valve position in the stopcock to be able to inject the sample through the filter and into a 40 mL EPA vial.
   - Slowly push the syringe plunger to allow the sample to be filtered into the vial.
   - Collect 40 mL sample for the DOC analysis. Make sure that there is no residual sample in the tubing when collecting the next sample.
   - The filter can be used repeatedly during one sample collection period until a pressure increase limits the filtration process.
   - Store DOC samples at 4°C and analyze within 24 h of collection.
9. Allow the experiment to run for 6 days and collect samples daily from Day 3 to Day 6 for DOC analysis. If conducting the BDOC test for the first time, collect samples for Day 1 to Day 4/5 to determine the kinetics of the test.
10. Refer to #9 for the sample collection procedure.
11. Sand used for analysis should be allowed to regenerate for about a month before re-use. After the experiment, rinse sand at least 3x with dechlorinated tap water and store. Refer to section A – Sand Preparation, I. Sand from water treatment plant #7 and #8.

H. Results and QA/QC Assessment

\[
BDOC = DOCi - DOC_m \\
\text{Final}BDOC = \text{average}BDOC_{\text{each flask}}
\]

where:
DOC\text{_i} = mean initial DOC (DOC_1 and DOC_o) in each flask, mg C/L
DOC\text{_m} = minimum mean DOC, mg C/L
DOC_1 = DOC of the sample after 5 min contact with the sand, mg C/L
DOC_o = DOC of the sample, mg C/L

\[
BDOC_{\text{avg}} = 2mgC/L \pm 0.4mgC/L \\
BDOC_{\text{avg}} = BDOC_{\text{avg}} + BDOC_{\text{avg}} \pm 20\%mgC/L \\
BDOC_{\text{avg}} - BDOC_{\text{avg}} < 30\%
\]
Appendix A-4: Total Coliform/E. Coli – Fecal Coliform

TOTAL COLIFORM – E.COLI

EQUIPMENT AND CHEMICALS

EZ-CFU Microorganisms:
- *Eschericia coli* (3.3 x 10³ CFU/ml)
- *K. pneumoniae* (3.3 x 10³ CFU/ml)
- *Pseudomonas aeruginosa*

Hydrating Fluid

100 mL Sterile sample containers
2000 mL Beaker
Colliert Presence/Absence Powder (for 100 mL samples)
Quanti Tray/2000 (for 100 mL samples)
Quanti-Tray sealing machine
Rubber Quanti-Tray holder
100-1000 µL Pipet
Redi Tip disposable pipet tips
Reagent Bottle
Latex gloves
Spatula
Weighing dish
Graduated cylinder
Volumetric flask
Parafilm
Top load balance
1:1 HCl
Muffler Oven
Aluminum Foil
Detergent
DI water
500 mL Beaker
250 mL Beaker
Magnetic Stirrer
pH Meter
10-100 µL Pipet
1 µL Plastic, disposable inoculating loop
10 µL Plastic, disposable inoculating loop
Electric shaker
PROCEDURE

A. Preparation of Glassware

1. Wash all glassware with detergent and water.
2. Rinse at least 2x with 1:1 HCl.
3. Rinse at least 3x with DI.
   a. Dry and cap with aluminum foil.
   b. Heat all glassware at 525°C for 6 hours

B. Preparation of Reagents

*Wear latex gloves when handling reagents and samples to minimize contamination.*

1. Phosphate Buffer
   a. Weigh out 17.0 g KH₂PO₄ into a weighing dish and transfer to 500 ml beaker.
   b. Add 250 mL DI water and use magnetic stirring rod to dissolve.
   c. Adjust pH to 7.2 +/- .05 using 1 N NaOH (20g/500 mL DI).
   d. Transfer solution to 500 mL volumetric flask and dilute to mark using DI water.
   e. Cover flask with parafilm and invert several times to mix the solution.
   f. Transfer to a reagent bottle for storage. Store at 4°C and use within 4 weeks.

2. Magnesium Chloride Hydrate
   a. Weigh out 8.11 g MgCl₂●6 H₂O into a weighing dish and transfer to 250 ml flask.
   b. Add 100 mL DI water and stir using magnetic stirrer.

3. Dilution Buffer
   a. Using 100-1000 µL pipet, pipet 1.25 mL of stock Phosphate buffer into a 1 L volumetric flask.
   b. Using a graduated cylinder, measure 5 mL of MgCl₂●6 H₂O and add to flask.
   c. Dilute to 1L mark using DI water.
   d. Autoclave solution for 15 min.
4. Nutrient Broth
   a. Measure .800 g Nutrient broth powder into weighing dish and transfer to beaker.
   b. Add 100 mL of DI water.
   c. Mix and heat on magnetic stirrer.
   d. Autoclave for 15 min.

C. Preparation of Standards

Wear latex gloves when handling reagents and samples and flame all reagent caps and necks prior to use to minimize contamination.

1. Pseudomonas aeruginosa
   a. Add 500 µL Nutrient Broth, using 100-1000 µL pipet, to sample of Pseudomonas aeruginosa.
   b. Shake well to mix.
   c. Remove blue chlorine pellet from sterile sample container and fill to 100 mL line with dilution buffer.
   d. Add 10 µL of Pseudomonas aeruginosa sample to container using larger end of disposable, plastic inoculating loop, stir with loop to mix.
   e. Add Colliert powder to sterile sample container and shake well to mix.
   f. Label Quanti-Tray with bacteria name.
   g. Pour mixture into Quanti-Tray and place tray on rubber grid.
   h. Pass the grid, with the tray, through machine to seal.
   i. Place all used pipet tips and contaminated containers in 2000 ml beaker.
   j. Incubate at 37°C for 24 hours.

2. K. pneumoniae
   a. Remove 1 pellet of bacteria from container and add to vial of hydrating fluid.
   b. Place on electric shaker to dissolve and mix.
   c. Remove blue chlorine pellet from sterile sample container and fill to 100 mL line with dilution buffer.
   d. Using smaller end of plastic, disposable inoculating loop, transfer 1 µL of microorganism sample to container.
   e. Label K.P. 1 µL.
   f. Remove blue chlorine pellet from another sterile sample container and fill to 100 mL line with dilution buffer.
   g. Using 100-1000 µL pipet, transfer 1000 µL of “K.P. 1 µL” to new sample container, using loop to stir.
h. Label the new container: $K.P. \ 10^3 \ \mu L$
i. Add Colliert powder to “$K.P. \ 10^3 \ \mu L$” sterile sample container and shake well to mix.
  j. Label Quanti-Tray with bacteria name.
k. Pour mixture into Quanti-Tray and place tray on rubber grid.
l. Pass the grid, with the tray, through machine to seal.
m. Place all used pipet tips and contaminated containers in 2000 ml beaker.
n. Incubate at 37°C for 24 hours.

3. **E. coli**
   a. Remove 1 pellet of bacteria from container and add to vial of hydrating fluid.
   b. Place on electric shaker to dissolve and mix.
   c. Remove blue chlorine pellet from sterile sample container and fill to 100 mL line with dilution buffer.
   d. Using 10-100 µL pipet, add 75 µL of microorganism sample to container.
   e. Add Colliert powder to sterile sample container and shake well to mix.
   f. Label Quanti-Tray with bacteria name.
   g. Pour mixture into Quanti-Tray and place tray on rubber grid.
   h. Pass the grid, with the tray, through machine to seal.
   i. Place all used pipet tips and contaminated containers in 2000 ml beaker, autoclave all 2000 mL beakers for 15 min to avoid contamination, discard materials.
   j. Incubate at 37°C for 24 hours.

D. **Collection of Water Samples**

*Wear latex gloves when handling samples to minimize contamination.*

1. Collect samples in carbon free bottles (refer to section A – Preparation of Glassware)
2. Transfer samples to sterile sample container.
3. Store at 4°C and analyze within 24 hours.
E. Water Sample Preparation

Wear latex gloves when handling samples and reagents to minimize contamination.

1. Add Colliert powder to sterile sample container and shake well to mix.
2. Label Quanti-Tray with sample name.
3. Pour mixture into Quanti-Tray and place tray on rubber grid.
4. Pass the grid, with the tray, through machine to seal.
5. Incubate at 37°C for 24 hours.

F. Sample Analysis

Wear latex gloves when handling samples to minimize contamination.

1. Recover samples and standards from incubator within 24 hours of experiment.
2. Analyze each Quanti Tray:
   ● yellow = coliform positive
   ● yellow & glows under UV light = E. coli positive.
3. Record sample names and coliform and E. coli positive/negative for each sample.
4. Autoclave all coliform and E. coli positive Quanti Trays for 15 min upon analysis to avoid contamination.

Fecal Coliform – Coliscan EasyGel

"Coliscan" is a type of commercially available bacterial growth media that contains a combination of color producing chemicals and nutrients that result in the growth of colonies of general coliform and fecal coliform bacteria in different colors. A test sample of water is added to the Coliscan media and general coliform bacteria will grow as pink-magenta colonies while E. coli (fecal coliform) will grow as purple colonies, and other bacterial types will grow as non-colored colonies. Many coliforms are normally found in soil and water and do not necessarily indicate the presence of fecal contamination, but E. coli is the primary bacterium in the mammalian (including humans) intestinal tract and its presence in food or water indicates fecal contamination. Therefore, E. coli is the coliform that is used as an indicator for fecal contamination.
The Coliscan media contains two color-producing substrates that are acted upon by the presence of the enzymes galactosidase and glucuronidase to produce pigments of different colors. General coliforms will produce the enzyme galactosidase (by fermenting lactose), and the colonies that grow in the medium will be pink in color. Fecal coliforms (*E. coli*) produce both galactosidase and glucuronidase and will grow as purple (or purple-blue) colonies in the medium. A count of the number of purple colonies will indicate the number of fecal coliforms per sample. The pink colonies indicate the total number of general coliforms per sample. The combined general coliform and fecal coliform number equals the total coliform number. Any non-colored colonies that grow in the medium are not coliforms, but may be other members of the family *Enterobacteriaceae*.

**Materials Needed:**

Sterile collection container (sterile bottle or test tube) with water sample  
Sterile pipets or sterile transfer pipets (dropper pipets)  
Sterile petri dish  
Coliscan Easygel (Micrology Laboratories)  
Incubator set at 37° C

**Procedure:**

1. Label a petri dish with your name and the location of your water sample.  
2. Wash your hands with antibacterial soap, then open a bottle of Coliscan Easygel.  
3. Use a sterile pipet (or a sterile transfer pipet) to add between 1 and 5 ml of your water sample (the amount of water you add depends on the extent of fecal contamination you think is in the water).  
4. Swirl the bottle to mix the water with the Coliscan Easygel.  
5. Pour the mixture into a sterile petri dish. Gently swirl the dish to cover the bottom evenly.  
6. Allow the petri dish to solidify for about 40 minutes.  
7. Incubate the plate upside down (to minimize condensation on the agar surface) at 37° C.  
8. Count colonies 24-48 hours later. See "Interpreting Coliscan pour plates" guide to assist you in determining which colonies are fecal coliforms.  
9. Colonies should be analyzed further by bacterial staining and microscopy. Do simple stains and gram staining.

**NOTE:** You may want to include a negative control and a positive control in your experiment. Sterile water may be used for the negative control. Water from a toilet can be used as a positive control. Be sure to use reasonable precaution in collecting this sample!

To look at the total number of bacteria in your water sample, you may repeat this method using "Total Count" Easygel (instead of Coliscan Easygel). Decrease the amount of sample water that you add to the Total Count Easygel (0.1-1.0 ml should be sufficient). The resulting colonies that
grow indicate the total number of colony-forming bacteria that are in your sample. These include both coliforms and non-coliforms. These colonies may be tested further, using microscopic staining.

*Time required: 30-45 minutes (day 1); 30-45 minutes (day 2)*
Appendix A-5: Potential of ExoProteolytic Activity – PEPA

GENERAL

The PEPA method measures the global activity of the biofilm, estimating the potential of bacteria to lysis proteins, using a proteic non-fluorescent artificial substrate (here L-Leucine β-Naphtylamide, LLβN). The enzymatic hydrolysis of this substrate leads to a fluorescent product (here β-Naphtylamine, βN), which can be detected by spectrofluorimetry. Fluorescence is plotted as a function of time and the rate of degradation gives an estimate of biological activity in the sample.

REFERENCE


EQUIPMENT AND CHEMICALS

1. PVC incubator made with a 1 ¼ in. cap (SCH 40) and a 1 ¼ in. tube section (L = 3 in.) described in Appendix 2.
2. 1.3 in. diameter coupons; surface area = 7.06 cm²
3. Incubator set up at 25°C
4. Spectrofluorimeter RF-1501, Shimadzu, Columbia, Maryland, USA
5. Fume hood
6. Dryer set up at 105°C
7. Fluorescence-Suprasil quartz cells (Lightpath = 10 mm, Vol. = 3 mL), Fisherbrand
8. Disposable 15 mL sterile plastic centrifuge tubes
9. Disposable sterile transfer pipets (2 mL)
10. 1.5 mL glass tubes with rubber septae
11. Sterile latex gloves
12. North Viton gloves
13. Micro syringe 0-250 μL
14. Kimwipes EXL, Kimberly-Clark
15. Aluminum foil
16. Teflon tape
17. Beaker (Liquid trash)
18. Safety container for hazardous wastes
19. Stop Watch
20. β-Naphtylamine (βN.), Sigma, St Louis, MO ; (MW=143.2 g/mol⁻¹)
21. L-Leucine β-Naphtylamide (LLβN.), Sigma, St Louis, MO ; (MW=292.8 g/mol⁻¹)
PREPARATION OF βN. STANDARDS

1. Weight 114.6mg of βN.
2. Dissolve in 20 mL of pure ethanol
3. Mix thoroughly to homogenize the concentration
4. Transfer to a bottle capped with a septum
5. Label with date, initials and concentration [βN.] = 40 mM
   This is the βN. Stock solution = [I]
6. Prepare Working Stock solution [II] at 40 µM by diluting 50 µL of Stock solution [I] into 50mL of sterile distilled water.
7. Dilute [II] to 1mM, 500 µM, 250 µM and 100 µM, using 15 mL sterile plastic centrifuge tubes
   • 1 mM = 250 µL from [II] in 10 mL of sterile distilled water
   • 500 µM = 125 µL from [II] in 10 mL of sterile distilled water
   • 250 µM = 62.5 µL from [II] in 10 mL of sterile distilled water
   • 100 µM = 25 µL from [II] in 10 mL of sterile distilled water
8. Protect tubes from light by covering with aluminum foil
9. Do not store standards, use immediately to build the standard curve
10. Process measuring as described in Section G. Measurements – Standard Curve

Note: Steps 2 to 7 have to be carried out in the fume hood, wearing North Viton™ gloves.

PREPARATION OF SAMPLES

1. Weight 117.2 mg of LLβN.
2. Dilute in 10 mL of pure ethanol in a sterile centrifuge tube
3. Mix thoroughly by reversing several times the tube until LLβN. is dissolved
4. Transfer to 1.5 mL glass tubes and cap with rubber septum
5. Cover tubes with aluminum foil
6. Label with date, initials and concentration [LLβN.] = 40 mM
   This is the LLβN. stock solution [II]
7. Store in the refrigerator at 4°C
8. Dilute 200 µL of [II] in 8 mL of sterile distilled water to reach [LLβN.] = 1mM, in a 15mL sterile centrifuge tube
9. Mix thoroughly to homogenize the concentration
10. Cover the tube with aluminum foil
11. Do not store, use immediately for measurements
12. Process experiment as described in Section F. Experiment Protocol
Note: Steps 8 to 10 have to be followed for each coupon: 1 tube / coupon; no more than 30 minutes before the experiment starts.

EXPERIMENT PROTOCOL

A. Preliminary check-up

1. Ensure that the PVC devices have been cleaned properly, i.e. rinsed thoroughly with distilled water and dried at 105°C.
2. Place them in the fume hood, capped with aluminum foil.
3. The spectrofluorimeter has to be set up: Emission = 410 nm and Excitation = 340 nm.
4. Ensure that two quartz cells (if duplicates) are clean (rinse it with DI 3x and wipe it out with Kimwipes) and ready to use.
5. Place a Kimwipes box in the fume hood.
6. Place a beaker for the liquids trash, in the fume hood.
7. Place a squeeze bottle of distilled water in the fume hood.
8. Have a stop watch ready to start.

B. Preparation of the coupons

1. Remove carefully the coupons (see limiting quantity below) from the incubator, wearing sterile latex gloves.
2. Remove the Teflon tape from the back of the coupon and replace it by a new one (only the inner surface of the coupon will be exposed to the substrate).
3. Place one coupon in each PVC device, in the fume hood.
4. Cap each PVC device with aluminum foil.
5. Discard the gloves in a regular trash box.
6. Proceed immediately with the measurement.

Note: The biofilm is very fragile, be careful for these steps not to touch the upper surface of the coupon. Plus, only the coupons needed for the next experiment has to be taken. The others remain in the incubator until a new experiment is set up.

C. Experiment sequence

1. Do not place more than 6 PVC-devices/Coupons in the fume hood for a given experiment.
2. Wear North Viton gloves.
3. Take 2 LLβN tubes, mix by reversing twice.
4. Simultaneously empty each in a PVC device (1 LLβN tube / PVC device) and start the time.
5. Proceed with measurement (Section D.- Samples), respecting the sequence.
D. Measurements

Standard Curve

1. Transfer approximately 2 mL of standard with a sterile transfer pipet from the 15 mL centrifuge tube to the cell
2. Clean cell walls with Kimwipes
3. Place the cell in the compartment
4. Measure and record the fluorescence intensity (FI) with the concentration of the standard.
5. Discard the standard in the Trash beaker
6. Rinse the cell 3 X with distilled water in the trash beaker
7. Stand the cell on Kimwipes to allow it to dry out for a bit.
8. Proceed with another standard, i.e. start over to step 1.
9. Build the corresponding standard curve \([\beta N]\) versus FI.
10. Record the linear regression equation with the date in the notebook:

\[
[\beta N] (nM/L) = a \cdot FI + b
\]

where \(a\) = slope of the trendline

\(b\) = intercept

Sample Analysis

Note: Steps 2 to 7 have to be carried out in the fume hood, wearing North Viton™ gloves.

1. Remove the aluminum foil protection
2. Transfer approximately 2 mL of sample with a sterile transfer pipet from the PVC device to the cell
3. Place the cell in the compartment
4. Measure and record the fluorescence intensity (FI) with the time, the matrix used (ex: SW, GW, or blend) and the material of the coupon (ex: PVC, Galvanized or Ductile Iron)
5. Turn the sample back into the PVC device
6. Put the aluminum foil back onto the device
7. Rinse the cell 3X with distilled water in the trash beaker
8. Stand the cell on Kimwipes to allow it to dry out for a bit.
9. Proceed with the other sample according to the sequence (Section F).
E. Calculations – Results interpretation

1. The FI values have to be converted to concentration of $\beta$N in **nanomoles** per liter with the Standard curve (**Section D.**)
2. Plot $[\beta N](\text{nM/L})$ versus Time (min.)
3. Get the slope of the linear part ($r^2 \geq 0.90$)
4. Divide this by the Surface area of the coupon in square centimeter (here: 7.06 cm$^2$)
   
   We obtain exoproteolytic activity expressed in nanomoles of $\beta$-naphtylamine produced per liter and square centimeter in each minute.
5. Convert this to bacterial biomass using the correlation PEPA/Biomass (Billen, 1991)

   Thus multiply the PEPA by $6.7 \left( \frac{\mu\text{g C} \times \text{min}}{\text{nM} \beta N} \right)$ to yield the biomass in micrograms of carbon per liter and square centimeter. The calculation procedure is summarized on the scheme below.
## Experiment Sequence Template

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This sequence has been set up for 6 coupons = 3 materials * 2 duplicates and can be adjusted to another scenario. The clear cells correspond to the samples that have to be analyzed for a given time. The **time in bold** represent the corrected time for each sampling time and each material. For example: at time = 36 min. only the duplicates from the material 3 have to be analyzed. The results for this will be labeled later under the corrected sampling time = 30 min.

**G. PVC Device and Coupon**

![Diagram of PVC device and coupon]

- 1/4 in. PVC Cap
- 1/4 in. PVC tube section
- 11 RN solution

**Coupon**

\[(D = 1.3 \text{ in. (3 cm)}, \text{ Surface area} = 7.07)\]
Appendix A-6: Biofilm Heterotrophic Plate Count – BFHPC

Coupons colonized by biofilm are sampled and rinsed very carefully with Phosphate Buffer Solution (PBS) twice. Then the biofilm is manually detached from the coupon using a sterile cell scraper (sterilized by Ethanol 70%) in 4-mL of sterile PBS, and then homogenized using a tissue blender (Tissue Tearor™, Biospec products, Inc) at 3000 rpm for 2 min. Then HPC assay (Appendix 1-1) was used to quantify heterotrophic bacteria in the suspension. The HPC result for a given sample expressed as cfu/mL was further converted into cfu/cm² by multiplying by 4 (mL) and divided by the surface area of the scraped coupon (7.06 cm²).
Appendix A-7: Fluorescent In-Situ Hybridization – FISH

EQUIPMENT AND CHEMICALS

- Probes EUB338, NSO190 and NSO1225 labeled respectively with Texas-Red-X/Cy-5, Oregon Green and Rhodamine Red (Sygma-Genosys, The Woodlands, TX 77380)
- Sodium Chloride, NaCl
- Na$_2$HPO$_4$
- NaH$_2$PO$_4$
- Paraformaldehyde
- Tris
- Ethylenediamine tetraacetate, disodium salt, EDTA, certified
- 1N HCl
- 2M NaOH
- Sodium Laural Sulfate, certified
- Formamide, certified
- Tween 20
- Nitrile gloves
- 100mL dilution bottles
- 100µL Cryovials
- 0.2µm filters
- Distilled water, DI
- Black Polyethylene filters : 0.45µm, 47mm
- 4% Paraformaldehyde
- Filter manifold
- Absorbent pads, 47mm
- Sterile transfer pipets
- Hybridization Buffer
- Sterile Petri dishes
- Non-denatured ethanol
- 50mL sterile disposable conical centrifuge tubes
- Surgical scissors
- 0-25µL pipettor
- 1-100µL pipettor
- 10-1000µL pipettor
PROCEDURE

Note: Two incubators must be respectively set at 46°C and 48°C, about an hour before starting the experiment. Hybridization buffers (HB) must be placed in 46°C incubator and washing buffers must be placed in 48°C incubator for prewarming.

A. Preparation of reagents

1. Phosphate Buffered Saline - PBS

   - Combine the following in 500mL of DI water

     \[
     \begin{align*}
     38.7 \text{ g } & \text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O} \\
     6.6 \text{ g } & \text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O} \\
     113.1 \text{ g } & \text{NaCl}
     \end{align*}
     \]

   - Dilute 1:30 to obtain 1X PBS
   - Autoclave at 120°C, 15psi for 20 minutes

2. 4% Paraformaldehyde

   Note: Nitrile gloves must be worn and work must be carried out in a fume hood, when handling paraformaldehyde (PFA), in accordance with Material Safety Data Sheets (MSDS) for PFA.

   - Set up a balance and a heated stirrer in a fume hood
   - Warm 65mL of DI water to 60°C
   - Weight out 4g of PFA powder (Do not inhale)
Add PFA to the DI water. It should be cloudy.
Add 2 drops of 2M NaOH and the PFA should dissolve in 1-2min.
Cool to room temperature and add 33mL of 3X PBS (safe to remove from the fume hood at this point)
Adjust pH to 7.2 with 1M HCl
Filter through 0.2µm filter to remove any undissolved crystals
Aliquot and freeze

3. Ethanol solutions

Dilute 200 proof non-denatured ethanol to 50%, 80% and 96% ethanol solutions with DI water
Aliquot in 50mL sterile conical tubes and store at -20°C in freezer

4. Tris-EDTA Buffer

Add 0.121g Tris to 75mL sterile DI water
Add 0.0372g EDTA
Adjust to pH 7.6 with 1N HCl
Dilute to 100mL with sterile DI water
Pass through a 0.2µm filter to sterilize
Pour into a 100mL sterile dilution bottle

5. Tris-HCl Buffer 1 (20mM, pH 7.6)

Add 2.42g Tris to 900mL sterile DI water
Adjust pH to 7.6 with 1N HCl
Dilute to 1000mL with sterile DI water
Aliquot and store at 4°C

6. Tris-HCl Buffer 2 (0.1mM, pH 7.5)

Add 12mg Tris to 900mL sterile DI water
Adjust pH to 7.5 with 1N HCl
Dilute to 1000mL with sterile DI water
Aliquot and store at 4°C
7. Hybridization Buffers (HB)

**NSO HB (30% Formamide)**
- Add 5.2596g to 60mL of sterile 20mM Tris-HCl
- Add 0.01g Sodium Laural Sulfate, Certified
- Add 30g Formamide, Certified
- Dilute to 100mL with 20mM Tris-HCl
- Pass through a 0.2µm filter to sterilize
- Pour into a sterile 100mL bottle
- Store at 4°C

**EUB HB (20% Formamide)**
- Add 5.2596g to 60mL of sterile 20mM Tris-HCl
- Add 0.01g Sodium Laural Sulfate, Certified
- Add 20g Formamide, Certified
- Dilute to 100mL with 20mM Tris-HCl
- Pass through a 0.2µm filter to sterilize
- Pour into a sterile 100mL bottle
- Store at 4°C

8. Washing Buffers

**NSO WB (112mM NaCl)**
- Add 0.05g Sodium Laural Sulfate, Certified to 480mL 20mM Tris-HCl
- Add 3.27g NaCl, Certified
- Dilute to 500mL with 200mL Tris-HCl
- Pass through a 0.2µm filter to sterilize
- Pour into a sterile 100mL bottle
- Store at 4°C

**EUB WB (250mM NaCl)**
- Add 0.05g Sodium Laural Sulfate, Certified to 480mL 20mM Tris-HCl
- Add 7.31g NaCl, Certified
- Dilute to 500mL with 200mL Tris-HCl
- Pass through a 0.2µm filter to sterilize
- Pour into a sterile 100mL bottle
➤ Store at 4°C

9. TNT Buffer

➤ Add 8.77g NaCl to 500mL 0.1mM Tris-HCl
➤ Add 0.5g Tween 20
➤ Dilute to 1000mL with 0.1mM Tris-HCl
➤ Aliquot and store at 4°C

B. Preparation of probes

For each probe, depending upon the specifications provided by Sygma-Genosys:

1. Storage of stock

➤ Make 10µL aliquots of at 1µg/µL with in cryovials
➤ Store at -80°C in cryofreezer

2. Preparation of working stocks

   Note: To be prepared less than 30 min. before dehydration step.

➤ Take one 10µL aliquot of each probe
➤ Dilute each to 25ng/µL with 190µL of Tris-EDTA
➤ Vortex thoroughly to homogenize concentration
➤ Store at 4°C in the dark until use for hybridization steps

C. Fixation of bulk liquid samples

➤ Filter 200mL of sample onto 0.45µm, 47mm black polyethylene filter
➤ Use sterile forceps to handle filters and autoclaved filter manifold to filter
➤ Rinse manifold with sterile distilled water between two filtrations of different samples
➤ Set absorbent pad in sterile Petri dish
➤ Pipet 2mL of 4% Paraformaldehyde onto absorbent pad with sterile transfer pipet
➤ Lay filter onto soaked pad
➤ Close Petri dish
➤ Incubate at 4°C in refrigerator for 4-16hrs.
D. Fixation of biofilm samples

- Manually detach biofilm from coupon (surface area: 7.07 cm²) with sterile cell scraper (sterilized in 70% ethanol)
- Resuspend in 4mL PBS in sterile 15mL conical centrifuge tube
- Homogenize with Tissumizer (Tissue Tearor™, Biospec products, Inc) at 3000rpm for 2 min.
- Filter 2mL of homogenized cell suspension onto 0.45µm, 47mm black polyethylene filter
- Use sterile forceps to handle filters and autoclaved filter manifold to filter
- Rinse manifold with sterile distilled water between two filtrations of different samples
- Set absorbent pad in sterile Petri dish
- Pipet 2mL of 4% Paraformaldehyde onto absorbent pad with sterile transfer pipet
- Lay filter onto soaked pad
- Close Petri dish
- Incubate at 4°C in refrigerator for 4-16hrs.
- Remount filter onto autoclaved manifold
- Wash twice with 10mL PBS
- Place filter into sterile Petri dish
- Label Petri dish accordingly
- Store in freezer at -20°C until hybridization

E. Dehydration

- Set absorbent pad in sterile Petri dish, presoaked with 2mL of 50% non-denatured ethanol, referred to as “50% absorbent pad”
- Take out filter/Petri dish from freezer
- Transfer filter from Petri dish onto 50% absorbent pad
- Close Petri dish
- Stand for 4 min. at room temperature
- Set absorbent pad in sterile Petri dish, presoaked with 2mL of 80% non-denatured ethanol, referred to as “80% absorbent pad”
- Transfer filter from 50% absorbent pad onto 80% absorbent pad
F. Hybridization

1. Preparation of filters

- Remove filter from 96% absorbent pad
- Cut 1 to 4 sections from center of the filter with surgical scissors
- Set one microscope slide (wiped with 70% ethanol)
- Pipet 15µL of prewarmed NSO Hybridization Buffer onto the slide
- Mount filter section onto the slide

At this point, two types of hybridization can be chosen. Sequential Hybridization is considered by certain teams to give finer staining than combined hybridization.

2. Combined Hybridization

- Pipet 4µL of each probe onto the center of the filter section
- Pipet 10µL of prewarmed NSO HB
- Mix with sterile pipet tip
- Place glass coverslip over filter section
- Put microscope slide in sterile Petri dish
- Close and seal Petri dish with Parafilm
- Incubate at 46°C for 2hrs
- Open dish and remove/discard coverslip
- Move filter section off the slide onto absorbent pad presoaked with 2mL of NSO WB, prewarmed at 48°C in sterile Petri dish
- Seal with Parafilm
- Incubate at 48°C for 30 min.
- Transfer filter section to absorbent pad presoaked with 2mL of TNT buffer in sterile Petri dish
- Stand for 15 min. in the dark at room temperature

At this point filter sections can be stored from 15 min. to overnight in this
conditions (Dr. M. Prevost, personal communication).

3. Sequential Hybridization

This hybridization technique includes the steps from combined hybridization described above, except that only NSO probes are pipetted onto the center of the filter section. Here hybridization of NSO probes (described in combined hybridization) is performed separately from the hybridization of EUB338 probe. The following describes the steps for this specific hybridization

- Pipet 4µL of EUB338 probe onto the center of the filter section
- Pipet 10µL of prewarmed EUB HB
- Mix with sterile pipet tip
- Place glass coverslip over filter section
- Put microscope slide in sterile Petri dish
- Close and seal Petri dish with Parafilm
- Incubate at 46°C for 2hrs
- Open dish and remove/discard coverslip
- Move filter section off the slide onto absorbent pad presoaked with 2mL of EUB WB, prewarmed at 48°C in sterile Petri dish
- Seal with Parafilm
- Incubate at 48°C for 30 min.
- Transfer filter section to absorbent pad presoaked with 2mL of TNT buffer in sterile Petri dish
- Stand for 15 min. in the dark at room temperature

At this point filter sections can be stored from 15 min. to overnight in these conditions (Dr. M. Prevost, personal communication). The following step is the common final step for both hybridization techniques.

- Remove and mount filter section onto clean microscope slide using 4 points of nail polish and CITIFLUOR AF-2
- Store in microscope slide tray at 4°C in the dark until microscopic examination
G. Confocal Laser Scanning Microscopy

Stained slides were initially examined on the fluorescence microscope. Having determined that successful staining was present, the slides were scanned using a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, Thornwood, NY), equipped with a Zeiss Plan-Apo 40x (NA 1.3) objective. Initially, slides were checked for specific antigen staining by comparing them to their positive and negative controls. The filters on the confocal microscope were set for three-color fluorescence of FITC, Rhodamine and Cy5 stained slides. To achieve this, the primary beam-splitter (dichroic filter) was chosen for excitation wavelengths of 488, 543 and 633 nm. FITC staining was detected in channel 1 using an emission band-pass filter of 530 +/- 15 nm. Rhodamine was detected in channel 2 using an emission bandpass filter of 600 +/- 15 nm, and Cy5 was detected in channel 3 using a 650 nm long-pass filter. The photomultiplier tube (PMT) of each channel of the CLSM was set to just include light from the appropriate negative control. Using the same PMT settings, five areas of each slide was serially scanned for fluorescence (areas were chosen using the halogen lamp alone to ensure that areas of high or low fluorescence were not inadvertently “selected”). For each field of view, a series of ten to fifteen (depending on specimen thickness) serial optical sections, 0.5 µm apart, were be scanned (i.e. 50 - 75 optical sections per slide). Data was stored on an IBM compatible computer and on CD for image analysis. The section series were analysed using Carl Zeiss and Photoshop image analysis software to calculate the relative volume in the sample occupied by each oligonucleotide probe (expressed as percent of total volume scanned).
APPENDIX B

BIOLOGICAL DATA
## Appendix B-1: HPC Data

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