State of the Science and Research Needs for Opportunistic Pathogens in Premise Plumbing
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State of the Science and Research Needs for Opportunistic Pathogens in Premise Plumbing

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Sponsored by:
Water Research Foundation
6666 West Quincy Avenue, Denver, CO 80235

Published by:

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CONTENTS

LIST OF TABLES ..................................................................................................................... XIII

LIST OF FIGURES ..................................................................................................................... XV

FOREWORD ............................................................................................................................ XVII

ACKNOWLEDGMENTS ......................................................................................................... XIX

EXECUTIVE SUMMARY ....................................................................................................... XXI

CHAPTER 1: OVERVIEW OF OPPORTUNISTIC PREMISE PLUMBING PATHOGENS (OPPPS) ........................................................................................................................................ 1
  Opportunistic premise plumbing pathogens ....................................................................... 1
  Development of this Document and Role of the Expert Workshop ................................... 2
  Document Scope and Organization .................................................................................... 2

CHAPTER 2: EPIDEMIOLOGY OF OPPPS ................................................................................ 3
  OPPPSs: Definitions and surveillance ................................................................................ 3
  Habitats, exposure routes, and implications for OPPP control .......................................... 4
  epidemiology of l. pneumophila .......................................................................................... 5
    Legionella Strains and Serotypes .................................................................................. 6
    Risk Factors for Legionnaires’ Disease .......................................................................... 7
    Linkage of Premise Plumbing and Legionella Disease .................................................. 8
    Transmission of Legionella ........................................................................................... 8
    Legionella Risk Assessment and Management ............................................................ 9
  Epidemiology of M. avium and Other Nontuberculous Mycobacteria (NTM) .................. 11
    NTM History, Disease, and Prevalence ......................................................................... 11
    Role of Various NTM Species in Disease ...................................................................... 11
    Risk Factors for M. avium Complex Disease ............................................................... 12
    Linking NTM and M. avium in Premise Plumbing to Disease ....................................... 13
    NTM Risk Assessment and Management ................................................................... 14
  Epidemiology of Pseudomonas aeruginosa .................................................................... 14
    Pseudomonas aeruginosa Disease and Prevalence ....................................................... 14
    Risk Factors for Pseudomonas aeruginosa Infection .................................................... 16
    Linking Pseudomonas in Premise Plumbing to Disease ............................................... 16
    Transmission of Pseudomonas ...................................................................................... 17
    Pseudomonas Risk Assessment and Management ....................................................... 17
  Epidemiology of Acanthamoeba ....................................................................................... 18
    Acanthamoeba Disease and Prevalence ....................................................................... 18
    Risk Factors for Acanthamoeba Infection .................................................................... 18
    Linking Acanthamoeba in Premise Plumbing to Disease .............................................. 18
    Transmission of Acanthamoeba .................................................................................... 19
    Acanthamoeba Risk Assessment and Management ..................................................... 19

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### CHAPTER 3: MICROBIAL PHYSIOLOGY AND ECOLOGY

<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidemiology of <em>N. fowleri</em></td>
<td>20</td>
</tr>
<tr>
<td><em>Naegleria</em> Disease and Prevalence</td>
<td>20</td>
</tr>
<tr>
<td>Transmission of <em>Naegleria</em></td>
<td>20</td>
</tr>
<tr>
<td>Linking <em>Naegleria</em> in Premise Plumbing to Disease</td>
<td>21</td>
</tr>
<tr>
<td><em>Naegleria</em> Risk Assessment and Management</td>
<td>22</td>
</tr>
<tr>
<td>Physiology and Microbial Ecology of <em>L. pneumophila</em></td>
<td>24</td>
</tr>
<tr>
<td><em>Legionella</em> Physiology</td>
<td>24</td>
</tr>
<tr>
<td><em>Legionella</em> Ecology</td>
<td>25</td>
</tr>
<tr>
<td>Physiology and Microbial Ecology of NTM and <em>M. avium</em></td>
<td>28</td>
</tr>
<tr>
<td>Physiology of NTM and <em>M. avium</em></td>
<td>28</td>
</tr>
<tr>
<td>Ecology of NTM and <em>M. avium</em></td>
<td>31</td>
</tr>
<tr>
<td>Physiology and Ecology of <em>P. aeruginosa</em></td>
<td>33</td>
</tr>
<tr>
<td>Physiology of <em>P. aeruginosa</em></td>
<td>33</td>
</tr>
<tr>
<td>Microbial Ecology of <em>P. aeruginosa</em></td>
<td>34</td>
</tr>
<tr>
<td>Physiology and Ecology of <em>Acanthamoeba</em></td>
<td>35</td>
</tr>
<tr>
<td>Physiology of <em>Acanthamoeba</em></td>
<td>35</td>
</tr>
<tr>
<td>Ecology of <em>Acanthamoeba</em></td>
<td>36</td>
</tr>
<tr>
<td>Physiology and Ecology of <em>N. fowleri</em></td>
<td>37</td>
</tr>
<tr>
<td>Physiology of <em>N. fowleri</em></td>
<td>37</td>
</tr>
<tr>
<td>Ecology of <em>N. fowleri</em></td>
<td>38</td>
</tr>
</tbody>
</table>

### CHAPTER 4: DETECTION METHODOLOGY

<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. pneumophila</em></td>
<td>41</td>
</tr>
<tr>
<td>Overview</td>
<td>41</td>
</tr>
<tr>
<td>Sampling and Monitoring Considerations</td>
<td>43</td>
</tr>
<tr>
<td>Sample Concentration Procedures</td>
<td>43</td>
</tr>
<tr>
<td>Culture-Based Techniques</td>
<td>44</td>
</tr>
<tr>
<td>Molecular and Serological Methods</td>
<td>46</td>
</tr>
<tr>
<td>Phenotypic Assays</td>
<td>47</td>
</tr>
<tr>
<td>Methodology Gaps for <em>L. pneumophila</em></td>
<td>48</td>
</tr>
<tr>
<td><strong>NTM and <em>M. avium</em></strong></td>
<td>49</td>
</tr>
<tr>
<td>Field Sampling Techniques</td>
<td>49</td>
</tr>
<tr>
<td>Culture-Based Techniques</td>
<td>51</td>
</tr>
<tr>
<td>Molecular Methods</td>
<td>52</td>
</tr>
<tr>
<td>Methodology Gaps for NTM</td>
<td>53</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>54</td>
</tr>
<tr>
<td>Sampling and Monitoring Considerations</td>
<td>54</td>
</tr>
<tr>
<td>Culture-Based Techniques</td>
<td>54</td>
</tr>
<tr>
<td>Most Probable Number (MPN) and Phenotypic Assays</td>
<td>55</td>
</tr>
<tr>
<td>Molecular Methods</td>
<td>55</td>
</tr>
<tr>
<td>Methodology Gaps for <em>P. aeruginosa</em></td>
<td>55</td>
</tr>
<tr>
<td><em>Acanthamoeba</em> spp.</td>
<td>56</td>
</tr>
<tr>
<td>Sampling and Monitoring</td>
<td>56</td>
</tr>
<tr>
<td>Microscopic examination</td>
<td>56</td>
</tr>
</tbody>
</table>
Contents

Culture-Based techniques .................................................................................. 57
Molecular Methods .......................................................................................... 58
Methodology Gaps for *Acanthamoeba* spp...................................................... 60
*N. fowleri* ...................................................................................................... 61
Sampling and Monitoring Considerations ...................................................... 61
Culture-Based Techniques .............................................................................. 62
Molecular Methods .......................................................................................... 62
Methodology Gaps for *N. fowleri* ................................................................. 64

CHAPTER 5: METHODOLOGY FOR SIMULATING DRINKING WATER SYSTEM
ENVIRONMENT ......................................................................................................................... 65
General Laboratory Assay Challenges for Opportunistic Pathogens .................. 65
overview of systems for simulating drinking water environment .................. 65
Batch Reactors ................................................................................................... 67
Continuous flow stirred tank reactors (CFSTRs) .............................................. 67
Centers for Disease Control and Prevention (CDC) Reactor ......................... 68
Annular Reactors ............................................................................................. 68
Plug Flow Reactors (PFRs) ............................................................................ 69
Flow Cells ......................................................................................................... 70
Robbins Device and Modified Robbins Device ............................................. 70
Biofilm Monitor ............................................................................................... 71
Tubing Reactors ............................................................................................... 71
Pipe Reactors ................................................................................................... 72
Different Modes of Operating CFSTRs and PFRs ........................................... 73
Sidestream Devices ......................................................................................... 73
Considerations on Reactor Selection ............................................................. 73
Considerations on System Operation ............................................................. 74
Pure culture versus mixed populations ......................................................... 74
Choice of the base biofilm ........................................................................... 75
Influent water – choice and composition ....................................................... 75
Choice of pathogen ....................................................................................... 75
Pathogen inoculation ............................................................................... 76
Continuous versus intermittent flow ............................................................. 76
Biofilm Sampling Techniques ........................................................................ 77
Methodological Gaps for Simulating Drinking Water Environment for Laboratory Study ........................................................................................................................................... 78

CHAPTER 6: POTENTIAL AND LIMITATIONS OF ENGINEERED CONTROLS FOR
OPPORTUNISTIC PREMISE PLUMBING PATHOGENS ....................................................... 79
Engineering and Regulatory Realities ............................................................ 79
Shared Responsibility Approach .................................................................... 82
Engineered Approaches that Influence OPPPs .............................................. 84
Source Water Treatment and Distribution System Maintenance ................ 85
In-Building Temperature Control ................................................................. 86
Disinfection with Chlorine/Chlorine Dioxide/Chloramine ............................ 88
Other In-Building Disinfectants ................................................................. 88
Nutrient Control and Limitation ................................................................. 89

©2013 Water Research Foundation. ALL RIGHTS RESERVED.
CHAPTER 7: REQUESTS FOR PROPOSALS (RFPS) ................................................................. 97
#1–EPI 1: Prevalence, incidence and trends of OPPP disease ........................................ 98
  Background: .................................................................................................................. 98
  Problem: ......................................................................................................................... 98
  Approach: ....................................................................................................................... 98
  Risk of Approaches and Anticipated Rewards: .............................................................. 98
  Stakeholders: .................................................................................................................. 98
  Estimated Budget: ......................................................................................................... 98
#2 RFP –Meth 1: Appropriate sampling and monitoring strategies for OPPPs ............... 99
  Background: .................................................................................................................. 99
  Problem: ......................................................................................................................... 99
  Approach: ....................................................................................................................... 99
  Risk of Approaches and Anticipated Rewards: .............................................................. 100
  Stakeholders: ................................................................................................................ 100
  Estimated Budget: ........................................................................................................ 100
#4 RFP –Meth 2: Molecular-based spatial and temporal survey of OPPPs ....................... 102
  Background: .................................................................................................................. 102
  Problem: ......................................................................................................................... 102
  Approach: ....................................................................................................................... 102
  Risk of Approaches and Anticipated Rewards: .............................................................. 102
  Stakeholders: ................................................................................................................ 103
  Estimated Budget: ........................................................................................................ 103
#5 RFP-Ecol 1: Relationship between distribution system and microbiota of premise plumbing ........................................................................................................................... 104
  Background: .................................................................................................................. 104
  Problem: ......................................................................................................................... 104
  Approach: ....................................................................................................................... 104
  Risk of Approaches and Anticipated Rewards: .............................................................. 104
  Stakeholders: ................................................................................................................ 105
  Estimated Budget: ........................................................................................................ 105
#6 RFP-Eng 2: Fundamental factors/mechanisms controlling detachment and release of OPPPs from biofilms in premise plumbing ......................................................... 106
  Background: .................................................................................................................. 106
Problem: .................................................................................................................... 106
Approach: .................................................................................................................. 106
Risk of Approach and Anticipated Rewards: ............................................................. 106
Stakeholders: ............................................................................................................. 106
Estimated Budget: ..................................................................................................... 106

#7 RFP-Eng 1: Impact of water treatment on control of OPPPs in premise plumbing. 107
Background: .............................................................................................................. 107
Problem: .................................................................................................................... 107
Approach: .................................................................................................................. 107
Risk of Approach and Anticipated Rewards: ............................................................. 107
Stakeholders: ............................................................................................................. 107
Estimated Budget: ..................................................................................................... 107

#8 RFP –Ecol 3: Mechanisms of chloramine and chlorine impact on OPPPs ............... 108
Background: .............................................................................................................. 108
Problem: .................................................................................................................... 108
Approach: .................................................................................................................. 108
Risk of Approaches and Anticipated Rewards: ........................................................ 108
Stakeholders: ............................................................................................................. 108
Estimated Budget: ..................................................................................................... 108

#9 RFP –EPI- 3: Communication of OPPP prevalence, incidence and trends. .............. 109
Background: .............................................................................................................. 109
Problem: .................................................................................................................... 109
Approach: .................................................................................................................. 109
Risk of Approaches and Anticipated Rewards: ........................................................ 109
Stakeholders: ............................................................................................................. 110
Estimated Budget: ..................................................................................................... 110

#10 RFP –Meth 3: Comparison between culture-based and molecular-based techniques for OPPPs .................................................................................................................... 111
Background: .............................................................................................................. 111
Problem: .................................................................................................................... 111
Approach: .................................................................................................................. 111
Risk of Approaches and Anticipated Rewards: ........................................................ 112
Stakeholders: ............................................................................................................. 112
Estimated Budget: ..................................................................................................... 112

#11 RFP –Eng 5: Optimizing the design of hot water systems to achieve temperature control of OPPPs and prevent amplification ....................................................... 113
Background: .............................................................................................................. 113
Problem: .................................................................................................................... 113
Approach: .................................................................................................................. 113
Risk of Approaches and Anticipated Rewards: ........................................................ 113
Stakeholders: ............................................................................................................. 113
Estimated Budget: ..................................................................................................... 113

#12 RFP –Ecol 2: Role of free-living amoebae (FLA) in enhancing persistence, survival, and growth of OPPPs ......................................................................................... 114
Background: .............................................................................................................. 114
Problem: .................................................................................................................... 114
Approach: .................................................................................................................... 114  
Risk of Approaches and Anticipated Rewards: ........................................................ 114  
Stakeholders: ............................................................................................................. 114  
Estimated Budget: ..................................................................................................... 115  

#13 RFP - Eng 3: Understanding chlorine, chlorine dioxide and chloramine demand in hot water premise plumbing ................................................................. 116  
Background: ............................................................................................................. 116  
Problem: .................................................................................................................... 116  
Approach: .................................................................................................................. 116  
Risk of Approaches and Anticipated Rewards: ........................................................ 116  
Estimated Budget: ..................................................................................................... 116  

#14 RFP - Ecol 4: Potential for microbial ecological controls on OPPPs. ................. 117  
Background: ............................................................................................................. 117  
Problem: .................................................................................................................... 117  
Approach: .................................................................................................................. 117  
Risk of Approaches and Anticipated Rewards: ........................................................ 117  
Estimated Budget: ..................................................................................................... 117  

#15 RFP - EPI 4: Is Hartmanella spp. or Acanthamoeba spp. of more significance for bacterial OPPPs? ................................................................. 118  
Background: ............................................................................................................. 118  
Problem: .................................................................................................................... 118  
Approach: .................................................................................................................. 118  
Risk of Approaches and Anticipated Rewards: ........................................................ 118  
Estimated Budget: ..................................................................................................... 119  

#16 RFP - Eng 4: Determining why electronic faucets sometimes harbor high levels of OPPPs. ................................................................................................. 120  
Background: ............................................................................................................. 120  
Problem: .................................................................................................................... 120  
Approach: .................................................................................................................. 120  
Risk of Approaches and Rewards: ........................................................................... 120  
Stakeholders: ............................................................................................................. 120  

#17 RFP - EPI 5: Contributions of bacterial, viral, and eukaryotic microbiota to OPPP disease ............................................................................................................. 121  
Background: ............................................................................................................. 121  
Problem: .................................................................................................................... 121  
Approach: .................................................................................................................. 121  
Risk of Approaches and Anticipated Rewards: ........................................................ 121  
Stakeholders: ............................................................................................................. 121  
Estimated Budget: ..................................................................................................... 121  

#18 RFP - Ecol 5: Potential contribution of disinfection and other management practices on emergence of multi-antibiotic resistant pathogens? ........................................ 122  
Background: ............................................................................................................. 122  
Problem: .................................................................................................................... 122  
Approach: .................................................................................................................. 122  
Risk of Approaches and Anticipated Rewards: ........................................................ 122  

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LIST OF TABLES

2-1 Premise Plumbing Pathogens of Concern: Diseases, Cases, Modes of Exposure, and Regulations (CCL = Contaminant Candidate List) ................................................................. 4

3-1 Characteristics of Opportunistic Premise Plumbing Pathogens .......................................................... 23

3-2 Frequently isolated nontuberculous mycobacteria (NTM) .................................................................. 29

4-1 Summary of Available Methodology for L. pneumophila ................................................................. 50

4-2 Summary of Available Methodology for Detection of NTM ............................................................ 53

4-3 Summary of Available Methodology for P. aeruginosa .................................................................. 56

4-4 Summary of Available Methodology for Acanthamoeba spp ........................................................ 60

4-5 Summary of Available Methods for N. fowleri .............................................................................. 64

5-1 Removal techniques found in the published literature and their associated advantages and limitations .................................................................................................................. 77

6-1 Comparing and contrasting problems of traditional pathogens to opportunistic premise plumbing pathogens ........................................................................................................ 82

6-2 Analogies between shared responsibility models for general bacteria regrowth, inorganics, taste and odor, premise plumbing pathogens, and materials failure ........................................... 84

6-3 Engineering approaches to control opportunistic premise plumbing pathogens ....................... 87

6-4 Summary of Prior Research Reports on OPPP in electronic faucets ............................................. 94

6-5 Hypothetical Shared Responsibility Model for Management of Opportunistic Pathogens in Drinking Water ............................................................................................................ 95
LIST OF FIGURES

1-1 Illustration of the current delineation of public versus private responsibility with respect to the water distribution system and premise plumbing. ................................................................. 1

2-1 Pathogen exposure in premise plumbing systems. ................................................................. 5

3-1 Role of amoeba hosts in enhancing replication of Legionella and other amoeba-resisting microbes (ARMs)........................................................... ....................................................... 27

6-1 Overview of major factors under control of utilities and consumers ..................................... 81
FOREWORD

The Water Research Foundation (Foundation) is a nonprofit corporation dedicated to the development and implementation of scientifically sound research designed to help drinking water utilities respond to regulatory requirements and address high-priority concerns. The Foundation’s research agenda is developed through a process of consultation with Foundation subscribers and other drinking water professionals. The Foundation’s Board of Trustees and other professional volunteers help prioritize and select research projects for funding based upon current and future industry needs, applicability, and past work. The Foundation sponsors research projects through the Focus Area, Emerging Opportunities, and Tailored Collaboration programs, as well as various joint research efforts with organizations such as the U.S. Environmental Protection Agency and the U.S. Bureau of Reclamation.

This publication is a result of a research project fully funded or funded in part by Foundation subscribers. The Foundation’s subscription program provides a cost-effective and collaborative method for funding research in the public interest. The research investment that underpins this report will intrinsically increase in value as the findings are applied in communities throughout the world. Foundation research projects are managed closely from their inception to the final report by the staff and a large cadre of volunteers who willingly contribute their time and expertise. The Foundation provides planning, management, and technical oversight and awards contracts to other institutions such as water utilities, universities, and engineering firms to conduct the research.

A broad spectrum of water supply issues is addressed by the Foundation's research agenda, including resources, treatment and operations, distribution and storage, water quality and analysis, toxicology, economics, and management. The ultimate purpose of the coordinated effort is to assist water suppliers to provide a reliable supply of safe and affordable drinking water to consumers. The true benefits of the Foundation’s research are realized when the results are implemented at the utility level. The Foundation's staff and Board of Trustees are pleased to offer this publication as a contribution toward that end.

Denise Kruger
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ACKNOWLEDGMENTS

We thank our Water Research Foundation Research Manager, Mary Smith, for her helpful support and guidance in this research project. We also sincerely thank the Project Advisory Committee: Marilyn M. Marshall (University of Arizona); Marsha Pryor (Pinellas County Florida); Maggie Rogers (Cleveland Division of Water); and Andrew J. Whelton (University of South Alabama) for their insight and enthusiasm that they offered to this project. Finally, we thank the students: Jordan Bird, Randi Brazeau, Lindsay Hamilton, Amanda Martin, Sheldon Masters, William Rhoads, and Hong Wang for their impeccable attention to detail in the organization of the workshop and for their support in preparing the document. A special thanks to Randi Brazeau for her special touch in managing the project and organizing the workshop. Finally, we thank participants from the U.S. Environmental Protection Agency (U.S. EPA) and the U.S. Centers for Disease Control (CDC). The views expressed in this report are those of the individual authors and do not necessarily reflect the views and policies of the U.S. Environmental Protection Agency, CDC, or otherwise the employers of the authors.

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EXECUTIVE SUMMARY

OBJECTIVES

The objectives of this project were to: 1.) Host an expert workshop to identify research needs for opportunistic premise (i.e., building) plumbing pathogens (OPPPs); 2.) With the assistance of the workshop participants, prepare this research report that critically reviews the state of the knowledge of OPPPs and identifies key research gaps; and 3.) Develop and rank a list of requests for proposals (RFPs) that address these key research gaps.

BACKGROUND

The successful mitigation of microbiological hazards in drinking water represents one of the 10 greatest engineering achievements of the 20th century. Widespread implementation of water treatment and disinfection have virtually eliminated incidence of diseases such as typhoid in the United States. In 2008, however, the U.S. Centers for Disease Control and Prevention acknowledged that a greater incidence of waterborne disease outbreaks are attributable to microbes that persist and grow in premise plumbing (especially Legionella pneumophila) versus traditional fecal-borne pathogens leaving the treatment plant. “Premise plumbing” refers to the portion of potable water distribution systems beyond the property line and in buildings (e.g., businesses, schools, private homes, apartments). Addressing these OPPPs poses a logistical challenge because community water systems are designed and regulated to control pathogens leaving the water treatment facility, whereas OPPPs reside and multiply in building water systems. The five model OPPPs that were the focus of this study were: Legionella pneumophila, Mycobacterium avium Complex (MAC), Pseudomonas aeruginosa, Acanthamoeba spp., and Naegleria fowleri. In particular, addressing these OPPPs is challenged by research gaps in four key areas: 1.) Epidemiology. Increasing incidence of infection and outbreak and broad, non-traditional susceptibility groups suggest that OPPPs are more than “opportunistic” in that a significant and increasing portion of the population is at risk. However, because L. pneumophila is the only reportable OPPP, and only on a voluntary basis, there is very little information on the actual incidence of diseases caused by OPPPs. Further, while several studies have demonstrated premise plumbing to be a source of opportunistic pathogen infection, very little is known about dose-response and to what extent potable water contributes to disease relative to other sources. 2.) Methodology. There are no detailed accepted standardized methodologies or protocols for detecting and quantifying viable and infectious OPPPs in potable water, especially when the objective requires simultaneous monitoring of multiple pathogens. Specifically, knowledge gaps exist with respect to optimal selection of monitoring points, sampling methods, and sample processing. Further, culture-based methods can be influenced by reduced recovery of viable cells, can provide poor quantitation, are time-consuming, and are subject to contamination. While molecular-based methods are gaining ground, improvements are still generally needed in terms of their target specificity and detection limits for live and infectious pathogens. 3.) Microbial Ecology. Premise plumbing is a unique ecological niche and home to a robust microbial ecology of which OPPPs are integral members. In particular, the bacterial OPPPs have a tendency to infect and grow in pathogenic and non-pathogenic amoeba, a process that not only enhances bacterial OPPP reproduction, but can also increase their virulence. Many OPPPs are also relatively resistant to disinfectants (especially M. avium Complex) and thrive under the
extreme oligotrophic condition of drinking water. Understanding the range of environmental conditions and key interactions with other microbes is critical to development of strategies to limit OPPP proliferation in building plumbing. **4.) Engineering Controls.** There are currently no known engineering controls that reliably limit proliferation of all OPPPs under all circumstances. Further, some approaches may reduce numbers of one pathogen, but may stimulate another, or have unintended consequences on the integrity of the plumbing system as a whole. In spite of the complexity of the situation and depending on the circumstances, it appears that combined efforts of various stakeholders, ranging from water utilities to building proprietors, may be effective in reducing risks to consumers.

**APPROACH**

The workshop participants gathered and consulted to guide the development of this critical review document. Expertise included representatives from academia, utilities, government agencies, and industry. For the preparation of this document the participants were assigned to teams and challenged to summarize the state of the knowledge and identify knowledge gaps in the four key areas of Epidemiology, Methodology, Microbial Ecology, and Engineering Controls. Nine conference calls were conducted from September to December, 2011, for discussion and development of this literature review. Contributing authors developed written text, which were integrated into this final document and further reviewed by the workshop participants and the PAC. On March 25 and 26, 2012, an expert workshop was conducted in Falls Church, Virginia, where key outcomes of the review, critical knowledge gaps, and emergent research needs were presented and discussed in great depth. The purpose was to recommend a path forward to generate the fundamental and practical knowledge necessary to addressing the importing public health challenge that OPPPs pose. A total of 20 RFPs were developed and ranked by the workshop participants. These RFPs may be considered by research sponsors that share in their understanding of the importance of OPPPs.

**RESULTS/CONCLUSIONS**

The ultimate products of this report are the 20 ranked RFPs, described in the final chapter. These summarize and highlight the most pressing research needs for addressing OPPPs. Two of the top three RFPs dealt with Epidemiology research needs. Specifically, determining the prevalence, incidence and trends of OPPP disease was identified as the top research need. Expanding this to identify sources and weights of various sources of OPPP disease ranked third. The second highest ranked RFP highlighted the need for improved Methodology in terms of appropriate sampling and monitoring strategies that provide meaningful data for determining risk of OPPP infection or outbreaks. The fourth highest ranked RFP called for a molecular-based spatial and temporal survey of OPPPs in premise plumbing systems. The fifth highest ranked RFP called for a study to determine the level of control that water treatment plants and the distribution system microbiome have on premise plumbing Microbial Ecology. The top ranked RFP with respect to Engineering Controls is research calling for identification of the fundamental factors/mechanisms that control detachment and release of OPPPs from biofilms in premise plumbing, which ranked 6th overall. Specifically, the 20 RFP titles, in ranked order, were:
1. **RFP – EPI 1**: Prevalence, incidence and trends of OPPP disease.
2. **RFP – Meth 1**: Appropriate sampling and monitoring strategies for OPPPs.
3. **RFP – EPI 2**: Sources and weights involved in OPPP disease and relationship between OPPP numbers and disease.
4. **RFP – Meth 2**: Molecular-based spatial and temporal survey of OPPPs.
5. **RFP – Ecol 1**: Relationship between distribution system and microbiota of premise plumbing.
6. **RFP – Eng 2**: Fundamental factors/mechanisms controlling detachment and release of OPPPs from biofilms in premise plumbing.
7. **RFP – Eng 1**: Impact of water treatment on control of OPPPs in premise plumbing.
10. **RFP – Meth 3**: Comparison between culture-based and molecular-based techniques for OPPPs.
11. **RFP – Eng 5**: Optimizing the design of hot water systems to achieve temperature control of OPPPs and prevent amplification.
12. **RFP – Ecol 2**: Role of free-living amoebae (FLA) in enhancing persistence, survival, and growth of OPPPs.
13. **RFP – Eng 3**: Understanding chlorine, chlorine dioxide and chloramine demand in hot water premise plumbing.
15. **RFP – EPI 4**: Is Hartmanella spp. or Acanthamoeba spp. of more significance for bacterial OPPPs?
16. **RFP – Eng 4**: Determining why electronic faucets sometimes harbor high levels of OPPPs.
17. **RFP – EPI 5**: Contributions of bacterial, viral, and eukaryotic microbiota to OPPP disease.
18. **RFP – Ecol 5**: Potential contribution of disinfection and other management practices on emergence of multi-antibiotic resistant pathogens?
20. **RFP – Meth 4**: Characterization of OPPP pangenomes for advancing molecular targets for OPPPs.

**APPLICATIONS/RECOMMENDATIONS**

Although OPPPs are now the primary source of waterborne disease outbreaks in the U.S. and other developed countries, they pose a complex challenge that cannot be addressed solely by water utilities using traditional pathogen control approaches. This document provides vital information to stakeholders regarding what is known and not known with respect to OPPPs. This document is intended to provide a state of the science assessment with respect to Epidemiology, Methodology, Microbial Ecology, and Engineering Controls of OPPPs. Further, based on existing knowledge, a multi-stakeholder approach for managing OPPPs is considered necessary. The ultimate product of this report is a list of twenty ranked RFPs, which summarize and highlight the most pressing research needs for addressing OPPPs.
PARTICIPANTS

We thank the Virginia Tech Institute for Critical Technology and Applied Science (ICTAS) for providing $25,000 cash match to support the expert workshop. We also offer a special thanks to our utility participants: American Water, Pinellas County Florida, Long Beach Water, and the City of Philadelphia.
CHAPTER 1: OVERVIEW OF OPPORTUNISTIC PREMISE PLUMBING PATHOGENS (OPPPS)

OPPORTUNISTIC PREMISE PLUMBING PATHOGENS

The successful mitigation of microbiological hazards in drinking water represents one of the 10 greatest engineering achievements of the 20th century (NRC, 2006). Widespread implementation of water treatment and disinfection have virtually eliminated incidence of diseases such as typhoid in the United States (U.S.). In 2008, however, due in part to past success in controlling traditional pathogens and in part to increased monitoring, the U.S. Centers for Disease Control and Prevention (CDC) acknowledged that a greater incidence of waterborne disease outbreaks are attributable to microbes that grow in premise plumbing (especially L. pneumophila) versus more traditional pathogens leaving the treatment plant (CDC, 2008). “Premise plumbing” refers to the portion of potable water distribution systems beyond the property line and in buildings (e.g., businesses, schools, private homes, apartments). Addressing opportunistic premise plumbing pathogens (OPPPs) poses a logistical challenge because water systems are designed and regulated to control pathogens leaving the treatment facility, whereas OPPPs reside and multiply beyond the property line (Figure 1-1). However, it is increasingly being recognized that water treatment and chemistry factors may play a role in downstream proliferation of opportunistic pathogens, and utilities therefore play some role in controlling outbreaks. Clearly, premise plumbing systems themselves, including water heaters, showers, filters, pipe and fixture materials, and HVAC systems, demand serious attention, yet there is a lack of sound scientific understanding that could be used to formulate advice for property owners, property managers, and manufacturers. Hence, factors influencing the growth of opportunistic pathogens in premise plumbing were identified as a “high priority” for research by the National Research Council (NRC) and remain a high priority through the present day (NRC, 2006).

Figure 1-1. Illustration of the current delineation of public versus private responsibility with respect to the water distribution system and premise plumbing.
DEVELOPMENT OF THIS DOCUMENT AND ROLE OF THE EXPERT WORKSHOP

To assess the state of the knowledge of OPPPs and identify critical knowledge gaps, a team of experts gathered and provided consultation to guide the development of this synthesis document. Expertise included representatives from academia, utilities, government agencies, and industry, each of whom was invited to contribute written material for this document. In the preparation of this document the participants were assigned to one of four teams to address the following major themes:

1. Epidemiology
2. Methodology
3. Microbial Ecology
4. Engineered Controls

Nine conference calls were conducted from September to December, 2011, three for each team, for discussion and development of the literature review. Contributing authors developed written text, which were integrated into this final document and further reviewed by participants. On March 25 and 26, 2012, an expert workshop was conducted in Falls Church, Virginia, where key outcomes of the review, critical knowledge gaps, and emergent research needs were discussed. The purpose was to recommend a path forward for generating the fundamental and practical knowledge necessary to address the importing public health challenge that OPPPs pose. As summarized in Chapter 7, several requests for proposals (RFPs) were developed that may be considered by research sponsors that are also important stakeholders in addressing various challenges associated with OPPPs.

DOCUMENT SCOPE AND ORGANIZATION

Although knowledge regarding OPPPs of concern is emerging and there are numerous pathogens drawing attention, this report focuses on five representative pathogens of concern: Legionella pneumophila, Mycobacterium avium, Acanthamoeba spp., Naegleria fowleri and Pseudomonas aeruginosa. The review begins with the topic of epidemiology (Chapter 2), which highlights what is known about the incidence, nature, and evidence of disease caused by OPPPs. Also noted are risk factors and the status of risk assessment for quantifying the nature of this challenge and prioritizing response efforts by water and public health professionals. Subsequently, a basic introduction to the ecology and physiology of opportunistic pathogens is provided (Chapter 3). This lays a critical foundation for subsequent sections on methodology (Chapters 4 and 5) and the potential for engineering controls to take advantage of OPPP vulnerabilities (Chapter 6). In the final section, critical knowledge gaps are summarized and a recommended path forward is provided in the form of 20 ranked RFPs developed at the Workshop (Chapter 7).
CHAPTER 2: EPIDEMIOLOGY OF OPPPS

OPPPS: DEFINITIONS AND SURVEILLANCE

The representative OPPPs of concern that are the focus of this report are summarized in Table 2-1. The term “opportunistic” is commonly used to describe pathogens that tend to pose a higher risk to sensitive populations possessing specific risk factors, such as compromised immune system, HIV infection, age, or other physical or genetic factors. However, as detailed in this chapter, a greater proportion of the population may be at risk to OPPP infection than implied by the term “opportunistic”. Many of the known OPPP risk factors are not intuitive [e.g., slender, older females are susceptible to nontuberculous mycobacterial (NTM) infections (Chan and Iseman, 2010)], and surely many more remain to be identified. Also, OPPP infections may sometimes occur in healthy individuals with no known risk factors. Even when considering only groups with known risk factors, it becomes apparent that a significant portion of the population in the U.S. is “at risk.” For example, in the case of M. avium, simply being over age 60 is a risk factor, and infections of individuals in this group outnumber such infections relative to people with other risk factors. As the population of elderly and immunocompromised individuals in the U.S. increases, and our ability to properly detect and diagnose causes of illness improves, the overall impact of OPPPs is likely to increase and receive greater attention. Thus, it is important to recognize that “opportunistic” pathogens likely affect a significant portion of the population and have important health and economic implications. We have chosen to use the term “opportunistic” in this document as it is widely used, while acknowledging that it is an imperfect descriptor of the true importance of this group of pathogens.

The Safe Drinking Water Act (SDWA) Amendments of 1996 directed the EPA to consider emerging waterborne contaminants for potential regulation in drinking water (EPA, 1996). This periodically updated list of chemical and microbial contaminants became known as the Contaminant Candidate List (CCL). To be included on the list (EPA, 1996):

1. “the contaminant may have an adverse effect on the health of persons;”
2. “the contaminant is known to occur or there is a substantial likelihood that the contaminant will occur in public water systems with a frequency and at levels of public health concern;” and
3. “…regulation of such contaminant presents a meaningful opportunity for health risk reduction for persons served by public water systems”.

During the formulation of the third CCL, a systematic approach was used to narrow microbial contaminants from a “universe” of all human pathogens to a list of contaminants that may be of particular concern in water. In the interest of parsimony, a representative pathogen from related groups of pathogens was selected to represent the larger group (EPA 2007). Thus, M. avium is included on the CCL as the representative organism for waterborne NTM. L. pneumophila and N. fowleri have also been included on the latest version of the list, CCL3.

Since 1971, the CDC, the U.S. Environmental Protection Agency (EPA) and others have maintained a collaborative Waterborne Disease and Outbreak Surveillance System (WBDOSS) for collecting and reporting data related to occurrences and causes of waterborne-disease outbreaks and cases of waterborne disease. This passive surveillance system is the primary
source of data concerning waterborne disease outbreaks in the U.S. In 2001, *Legionella* was added to the WBDOSS (Blackburn et al., 2004) and is the primary OPPP tracked by the system.

Table 2-1. Premise Plumbing Pathogens of Concern: Diseases, Cases, Modes of Exposure, and Regulations (CCL = Contaminant Candidate List)

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Disease(s)</th>
<th>Cases/Deaths (Year(s))</th>
<th>Mode of Exposure</th>
<th>Reportable</th>
<th>Regulations</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. pneumophila</em></td>
<td>Legionnaires' disease (pneumonia) or Pontiac fever</td>
<td>4,107 U.S. cases (2011; CDC, 2013)</td>
<td>Inhalation or aspiration</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><em>M. avium</em></td>
<td>Pulmonary disease, cervical lymphadenitis (children)</td>
<td>19,600 U.S. cases (2010; Prevots, 2010)</td>
<td>Inhalation or aspiration</td>
<td>No</td>
<td>No, but listed on CCL3</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>Urinary tract infections, respiratory infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, GI infections</td>
<td>1,400 U.S. pneumonia deaths (Anaissie, 2002) 2.4 million U.S. ear cases (2003-2007; CDC 2011c)</td>
<td>Wound infection; inhalation</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><em>Acanthamoeba</em></td>
<td><em>Acanthamoeba</em> keratitis (AK) <em>Granulomatous amoebic encephalitis (GAE)</em></td>
<td>&gt;3,000 global cases (2004; Schuster and Visvesvara, 2004)</td>
<td>Wound infection; contact lens solution</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><em>N. fowleri</em></td>
<td>Primary amoebic meningoencephalitis</td>
<td>111 cases (1962-2008; Yoder et al. 2010)</td>
<td>Nasal aspiration</td>
<td>No</td>
<td>No, CCL3</td>
</tr>
</tbody>
</table>

HABITATS, EXPOSURE ROUTES, AND IMPLICATIONS FOR OPPP CONTROL

OPPPs flourish in biofilms and other microenvironments within hot and cold potable water distribution systems, which can include shower heads, faucets, in pipe scale/rust, sediments, water heaters, and water treatment devices. Pathogens of historic concern to water utilities generally originate in the source water from fecal contamination and do not amplify (i.e., multiply or reproduce) in potable water itself. Thus, in contrast to traditional pathogens, humans and OPPPs find themselves cohabitating within the same household and work environments. Also, the primary modes of OPPP transmission tend to be via inhalation, aspiration, or wound infection, which differ from the traditional route of ingestion (Table 2-1). Major routes of OPPP exposure are summarized in Figure 2-1. Although we may be surrounded by OPPPs in the household environment, specifically linking outbreaks to a premise plumbing source poses several challenges, which are noted in sections devoted to describing the epidemiology of...
individual OPPPs that follow. Thus, there are some stark contrasts between OPPPs and traditional pathogens, which will require a paradigm shift in terms of responsibility, guidance, and approaches that will be necessary to control waterborne disease.

An emerging theme is that it may not be practical to control all OPPPs, as will be explored later in this document in Chapter 6. As a result, it is recognized that approaches that attempt to protect all individuals all the time may not be realistic. Public education and other risk management strategies may be necessary to reduce exposure risk for sensitive populations. Ideally, quantitative assessment of risk should drive management strategies. Thus, where applicable in this section we draw from quantitative microbial risk assessment (QMRA) to estimate the number of individuals exposed to OPPPs and at risk of infection under various scenarios. QMRA is a formalized approach based on quantitative risk assessment methodology developed by the National Academy of Sciences, adjusted to account for the peculiarities of microorganisms (Haas et al. 1999; NRC, 1983). QMRA can also serve as a guide for effective environmental sampling and assay practices and has been useful in identifying gaps in essential data (Haas, 2002).

**Figure 2-1. Pathogen exposure in premise plumbing systems.** acanthamoebae and other protists occur in cyst (A) and trophozoite (B) forms. Vesicles within trophozoites can harbor up to 20-1500 pathogenic bacteria such as *L. pneumophila* (C), which can eventually burst and lead to pathogen occurrence in tap water and shower water (D). Contact lens wearers are vulnerable to keratitis infection from acanthamoebae (E-F). Inhaling mists containing *L. pneumophila* and nontuberculosis mycobacteria (NTM) can cause lung infections (G). Exposure to acanthamoebae, *P. aeruginosa* and NTM through skin lesions can cause infection (H). (Brazeau & Edwards, 2011).

**EPIDEMIOLOGY OF L. PNEUMOPHILA**

*Legionella* History, Disease, and Prevalence

*Legionella* is a bacterial genus containing several pathogenic members that was unrecognized prior to 1977. It was first identified in connection with a mysterious 1976 outbreak of severe respiratory infections at an American Legion convention in Philadelphia, PA. Twenty-nine of the 182 cases were fatal (Fraser et al., 1977). The illness quickly became known as
Legionnaires’ disease. The causative agent, identified in January 1977, was isolated and assigned to the family *Legionellaceae*, the genus *Legionella*, and the species *pneumophila* (McDade et al., 1977). The bacterium was presumed to have originated in the hotel’s air-conditioning system and cooling tower. Although Legionnaires’ disease historically has been associated with cooling towers, premise plumbing is now recognized as an equally important source (Addiss et al., 1989).

The term “legionellosis” includes two distinct clinical entities. Legionnaires’ disease is characterized by severe pneumonia and high mortality rates. Pontiac Fever is a milder, self-limiting, non-pneumonic febrile illness. Legionnaires’ disease develops in a small percentage (e.g., 5%) of exposed persons and therefore is considered an opportunistic infection. Pontiac fever is believed to affect up to 95% of exposed individuals (Glick et al., 1978), and thus is not truly an “opportunistic” infection. Because it is relatively mild and self-limiting, cases of Pontiac Fever are rarely identified or reported. For the purposes of this review, legionellosis refers to Legionnaires’ disease, unless otherwise noted.

*Legionella* has been included in the passive WBDOSS surveillance system since 2001 (Blackburn et al., 2004). Outbreaks are believed to be substantially underreported, in part because pneumonia caused by *Legionella* is indistinguishable on the basis of clinical symptoms or chest x-rays, along with a general lack of clinical awareness. Half of the disease outbreaks related to drinking water that have been reported were attributed to *Legionella* and all occurred as a result of colonization of plumbing and pipes (Yoder et al., 2008). *Legionella* was added to EPA’s Contaminant Candidate List in 2009.

CDC has estimated that between 8,000-18,000 people in the U.S. are hospitalized each year with Legionnaires’ disease (CDC, 2008a). Others have estimated that Legionnaires’ disease accounts for 2-5% (i.e., 12,000-30,000) of approximately 600,000 annual adult cases of community-acquired pneumonia requiring hospitalization (Marston et al., 1994). According to the CDC, reported cases of Legionnaires’ disease have nearly tripled from 1990-2005 (Neil and Berkelman, 2008).

A relatively large number of reported outbreaks have occurred in hospitals, healthcare facilities and nursing homes (Craun et al., 2010). The British Communicable Disease Surveillance Centre reported that 19 of 20 hospital outbreaks of Legionnaires' disease reported in the United Kingdom from 1980 to 1992 were attributed to contaminated potable water systems (Joseph et al., 1994). Some researchers have estimated that 25% of the estimated 18,000 or more cases of Legionnaires’ disease in the U.S. were acquired in healthcare facilities (Marston et al., 1994; Neil and Berkelman, 2008). In case reports submitted to the Legionnaires’ Disease Supplemental Surveillance System (LDSSS) from 2005–2007, acute care hospitals accounted for 88% of healthcare-acquired Legionnaires’ disease cases, with long-term care and rehabilitation facilities accounting for the remaining 12%. Based on CDC analysis of the 2005–2007 LDSSS, the estimated case-fatality rate for healthcare-acquired Legionnaires’ disease is approximately 34% (Tucker, 2009). In addition to healthcare-associated cases, outbreaks of Legionnaires’ disease also have been linked to engineered water systems in hotels, cruise ships, industrial facilities, public buildings, and residences.

**Legionella Strains and Serotypes**

To date, more than 50 *Legionella* species and more than 70 serogroups have been identified. *L. pneumophila* is the most prominent, comprehensively studied *Legionella* species
with subgroups that have been identified as the primary causative agents of legionellosis. More than 70% of Legionnaires’ disease cases identified in Europe and the U.S. have been attributed \textit{L. pneumophila} serogroups-1, -3, -4 and -6, with an additional 20% attributed to other \textit{L. pneumophila} serogroups. Non-\textit{pneumophila} \textit{Legionella} species that also have been linked to clinical illness include \textit{L. micdadei}, \textit{L. bozemanii} and \textit{L. dumoffii} (Fang et al., 1989). The predominant clinical diagnostic test for Legionnaires’ disease is a urinary antigen test specific for \textit{L. pneumophila} serogroup 1. Infections by species or serogroups other than \textit{L. pneumophila} serogroup 1 generally go undetected and are probably underreported (Fields et al., 2002).

\textbf{Risk Factors for Legionnaires’ Disease}

The list of groups with higher susceptibility to Legionnaires’ disease is wide ranging and growing. Risk factors for Legionnaires’ disease include those associated with general immune deficiency, e.g., advanced age, HIV infection, and immunosuppressive therapy. Additional risk is imposed by diminished lung defenses related to smoking, alcohol abuse, and chronic pulmonary disease. Such conditions can affect the proper function of cilia in the lung epithelial lining. Properly functioning cilia and other clearance mechanisms in healthy lungs normally expel particles larger than about 2 microns before they can reach the pulmonary alveoli in the deep lungs. However, larger particles can penetrate and lodge into the deep alveolar regions of compromised lungs. Difficulty swallowing may also enhance risk, as it can result in aspiration into the lungs.

Attributable mortality for Legionnaires’ disease is approximately 20% (CDC, 1997). However, case fatality rates are greater in patients who are younger than one year, older than 60, male, or with predisposing underlying conditions such as malignancy (Stout et al., 2007). Case fatality also increases with delayed administration of appropriate antimicrobial therapy (Stout et al., 2007). Corticosteroid use is an independent risk factor. Neoplastic disease, diabetes, and renal failure are also frequently cited risk factors. Mortality is reportedly as high as 40% for healthcare-associated cases (Stout et al., 2007).

Increasing use of diagnostic tests for \textit{Legionella} has led to recognition of new risk groups, including elderly residents of long term care facilities and immune-compromised children in pediatric hospitals. In a CDC survey of reported pediatric legionellosis cases, 72% were healthcare-associated, linked to \textit{Legionella} from tap water (Stout et al., 2007). Further, increasing use of diagnostic tests has revealed that many patients with Legionnaires’ disease do not fall into identified risk groups. In one study, 22% of the reported cases did not have any of the usual risk factors (Squier et al., 2005). Similar findings were reported in a large study of a German town (von Baum et al., 2008). Factors thought to contribute to wide variations in reported cases of Legionnaires’ disease include differences in the extent of colonization of building water systems, environmental transmission, susceptibility of exposed persons, and recognition of the clinical entity by physicians (Stout et al., 2007).

As highlighted in Chapter 3, \textit{Legionella} are subject to a unique ecology that further affects their ultimate risk of causing infections in humans. Specifically, in the drinking water environment, \textit{Legionella} replication is thought to take place exclusively within a protozoan host. Once phagocytized by the host, \textit{Legionella} survive, replicate, and are eventually dispersed to infect new hosts. This process has been observed to result in the upregulation of virulence genes, and thus directly affects their capability to infect humans and cause disease. The role of protozoa in enhancing the risk of infection is explored in detail by Thomas and Ashbolt (2011).
Linkage of Premise Plumbing and Legionella Disease

The association of Legionnaires’ disease with the presence of *L. pneumophila* in premise plumbing is well-established (Alary and Joly, 1992; Straus et al., 1996). Most early epidemiological observations of Legionnaires’ disease were of hospital-acquired cases. In a number of surveys, up to 70% of institutional water systems tested positive for *Legionella* (Alary and Joly, 1992; Britain, 1987). However, the sporadic nature of the disease, the variety of environmental sources, and intrinsic limitations of environmental sampling methods have challenged the linkage of Legionnaires’ disease with environmental isolates of *Legionella* (Stout et al., 1988). Nonetheless, the important connection between *Legionella* in the hospital drinking water systems and cases of hospital-acquired Legionnaires' disease was made definitively and confirmed independently by a number of researchers (Stout et al. 1982; Best et al. 1983; Tobin-D’Angelo et al., 2004). In subsequent years, the development of genomics and advent of molecular methods proved invaluable in confirming the link between environmental sources of *Legionella* and clinical disease. Variations in the macrophage infectivity potentiator (*mip*) gene, in particular, have proven useful to distinguish different strains and track sources of *Legionella* exposure (Ratcliff et al., 1998). Monoclonal antibody subtyping and pulsed-field gel electrophoresis (PFGE) has also been useful for linking sources to infections, and in one study identified decorative fountains as a source of infection in several attendees of a convention in Florida (Hlady et al. 1993).

Transmission of Legionella

The presence of *Legionellae* in a building’s plumbing is not, in-and-of itself, sufficient to cause disease. *Legionella* must reach the deep alveolar region of the lungs of a susceptible host to cause Legionnaires’ disease. *Legionellae* from colonized plumbing can enter the environment when pieces of biofilm break off, exit the system through a tap and are broadcast in bioaerosols. The water in the microscopic droplets can evaporate quickly, leaving small infectious particles that can readily travel more than hundred yards, with complex dispersion patterns that are a function of a number of variables. Under extreme circumstances, particles have reportedly traveled more than 2 miles (Addiss et al., 1989; Hoque et al., 2011). When the airborne, virulent *Legionella* are inhaled deep into the alveolar region of the respiratory system of a susceptible host, they infect and replicate within alveolar macrophages, causing Legionnaires’ disease. *Legionella*-parasitized protozoa and *Legionella*-containing protozoan vacuoles may be similarly released and disseminated.

Aerosols are of thought to be the primary mode of transmission of *Legionella* to humans. Water pressure changes have been shown to disturb the biofilm, with a dramatic increase in the concentration of *Legionella* in the bulk water and, presumably, in dispersed aerosols (Mermel et al., 1995). Shower aerosols are widely thought to represent an important means of environmental transmission. While shower heads are known to generate aerosols in the respirable size range containing *Legionella* (Bollin et al., 1985; Dennis et al., 1984), one early study employing simulations using computational models suggested that showerheads were a relatively insignificant source of *Legionella* bioaerosol transmission compared to humidifiers and rising ventilation bags in hospitals (Woo, 1986). More recently, a shower exposure model was developed by Schoen and Asbolt (2011). Sensitivity analysis of their model identified the target
deposited dose associated with infections, the pathogen air water partitioning coefficient, and the quantity of detached biofilm from in-premise plumbing surfaces as important parameters needing attention in future research. Aspiration of contaminated water or oropharyngeal secretions also has been proposed as an important mode of transmission in the hospital setting (Blatt et al., 1993). There also is as some evidence suggesting that instillation into the lung during respiratory tract manipulation may provide an additional route of infection, which is logical given reports that tap water is often used to rinse respiratory apparatus and tubing used for ventilators. Three cases of \textit{L. pneumophila} pneumonia were reportedly acquired from contaminated transesophageal echocardiography probes that were rinsed with contaminated tap water (Levy 2003). Nasogastric tube placement has been identified as a significant risk factor for healthcare-associated legionellosis in intubated patients, with micro-aspiration of \textit{Legionella}-contaminated water the presumed mode of entry (Blatt et al., 1993; Marrie et al., 1994). The risk of Legionnaires’ disease is reported to be significantly greater for patients who have been intubated more frequently or for longer duration. As a result, use of sterile water is recommended for all nasogastric suspensions and for flushing tubes. A recent case of Legionnaires’ disease was linked to contaminated water in a dental unit, with definitive confirmation by monoclonal antibody typing, sequence-based typing, and amplified fragment length polymorphism (AFLP) typing (Ricci et al. 2012). Contaminated ice and water from ice machines also have been implicated as a source of healthcare-associated infections, and one study in particular noted the same relatively rare serotype 8 to be found both in a nearby ice machine and in several infected patients (Schuetz et al. 2009).

\textbf{Legionella Risk Assessment and Management}

The dose-response relationship between \textit{Legionella} and Legionnaires’ disease is unknown. Notwithstanding 40 years of scientific research, a safe concentration of \textit{Legionella} has not been established, and there is no consensus on endpoints for remediation. The maximum contaminant limit goal (MCLG) for \textit{Legionella} in finished water produced by U.S. public water systems is zero (EPA, 2002). However, the MCLG is not an enforceable standard, and does not carry any expectation that the finished water received by customers will be free of \textit{Legionella}. There are no enforceable regulatory limits, nor any monitoring requirements, for \textit{Legionella}.

Recently published work has employed QMRA methodology specifically in connection with issues of legionellosis associated with engineered systems (Armstrong and Haas, 2007; Armstrong and Haas, 2008; Buse et al., 2011). There are substantive gaps in information necessary to calculate risk across all major QMRA components, i.e., hazard identification, dose-response and exposure assessment. For example, there is scientific consensus that contamination of premise plumbing by \textit{Legionella} is primarily a function of natural biofilms (Declerck, 2010; Murga et al., 2001). Yet, methods used for sampling building water systems (discussed in greater detail in the methods Chapter 4) are not standardized, and bear little relationship to the way biofilm colonizes, distributes and releases from premise plumbing. Further, parasitized protozoa play a well-documented, defining role in \textit{Legionella} virulence, proliferation and transmission. However, culture on enriched media, the reference standard for detection of \textit{Legionella} in environmental samples, does not readily detect \textit{Legionella} present inside of protozoan hosts, is blind to viable but non-culturable (VBNC) organisms, and overlooks many \textit{Legionella} species.

Nonetheless, environmental sampling and culture has provided qualitative information to institutions that house highly vulnerable patients, such as chronic care and transplant facilities,
especially for outbreak investigations (Butler and Guthertz, 2001; Moore et al., 2006; Yu, 1998). However, routine surveillance based on Legionella culture, especially the notion that it provides meaningful quantitative information, is the subject of ongoing controversy (Yu, 1998). The CDC has no recommendations for routine surveillance testing of building water systems except in special circumstances, such as hospital units that house severely immunosuppressed patients, e.g., stem cell transplant recipients presumed to be at high risk from Legionnaires’ disease. The Allegheny County Health Department issued guidelines that require annual surveillance sampling and culture of water in hospitals, including hot water heaters and a small percentage of distal sites (faucets or showers), with the recommendation that sites on multiple floors and wings should be selected, and that high-risk areas (e.g., hematology oncology, transplant units) be given priority (ACHD, 1997). Several other organizations, including the Veterans Administration, have followed Allegheny County’s lead (VHA, 2008). These guidelines share two underlying assumptions: 1) that L. pneumophila cultures of hot water samples can yield valid quantitative estimates of the extent of colonization, and 2) that a threshold level of 30% positive outlets provides a meaningful action level. The 30% threshold figure is frequently cited in some guidelines (VHA, 2008; ACHD, 1997) and is based on a study of ten to twenty sites at a single hospital using culture-based techniques (Best et al., 1983). A more recent investigation of the relationship between positive sites and infections involved twenty U.S. hospitals in 13 States. The results were highly variable, e.g., one hospital with 25% culture-positive sites had a confirmed case of Legionnaires’ Disease, another with 83% positivity had no cases (Stout et al., 2007). The results support the qualitative link between site positivity and infection, but also demonstrate the insufficiency of current environmental sampling and assay methods for establishing a meaningful, quantitative action level. Thus, development of appropriate monitoring protocols and an effective action level is a critical need.

The CDC, the American Society of Heating, Refrigerating, and Air Conditioning (ASHRAE), the World Health Organization (WHO) and others publish similar guidelines for reducing the risk of legionellosis in building water systems. Just prior to the publication of this document, ASHRAE released a new public review draft of Standard 188P, Prevention of Legionellosis Associated with Building Water Systems. Standard 188P, a minimum practices consensus standard developed in accordance with the rules of American National Standards Institute (ANSI), is structured on the principles of Hazard Analysis and Critical Control Points (HACCP), a formalized methodology widely used in connection with food safety. In this document we reference ASHRAE, 2011 as the most recently published version. However, at the time of the preparation of the present document, the ASHRAE guidelines are currently under revision for a third release and are pending public comment. The two versions were reviewed by the project team and only minor changes were noted pertaining to drinking water recommendations. These include the regrouping of risk factors associated with consumption of drinking water versus exposure through cooling towers, evaporative condensers and aerosols. Key provisions of Standard 188P include maintaining hot and cold water within prescribed temperature ranges and maintaining adequate disinfectant residuals throughout the building water system. Notably, Standard 188P does not recommend routine environmental sampling or culture (ASHRAE, 2011).
EPIDEMIOLOGY OF M. AVIUM AND OTHER NONTUBERCULOUS MYCOBACTERIA (NTM)

NTM History, Disease, and Prevalence

NTM infections first emerged as important health events occurring among HIV-infected and immunosuppressed persons during the 1980s (Horsburgh and Selik, 1989). Subsequently, NTM infections have been increasingly recognized among apparently immunocompetent adults and children (Marras et al., 2007; Winthrop et al., 2010). Pulmonary infection is most frequently reported, but extrapulmonary infections are commonly described (Bodle et al., 2008; Winthrop et al., 2002).

NTM lung disease tied to drinking water is a growing concern (CDC, 2008b; Falkingham III et al., 2008; Marras et al., 2007). Estimates of NTM disease incidence range from 15-30/100,000 population with some 30,000 NTM infected patients in the U.S. (Winthrop et al., 2010). Because susceptibility to NTM increases with age and diagnosis is improving, incidence of documented waterborne disease from premise plumbing pathogens will likely increase (Falguera et al., 2001; Prince et al., 1989). In particular, epidemiological studies are needed to identify host (e.g., age), microbial (e.g., numbers of opportunistic pathogens), and human behavioral (e.g., showering) factors influencing the acquisition of disease due to household exposures.

Population-based studies are uncommon and the public health burden of NTM-associated disease is difficult to quantify. NTM infections are not reportable diseases in the U.S. (EPA, 2011). Two recent population-based studies in the U.S. described rates of NTM isolation and infection as higher among older persons (Prevots et al., 2010; Winthrop et al., 2010). Among studies that evaluated numbers of isolations among multiple years, NTM isolation rates appear to be increasing. These include reports from the U.S. (du Moulin et al., 1985), Canada (Al Houqani et al., 2011), Taiwan (Chen et al., 2012), South Korea (Ryoo et al., 2008), and China (Wang et al., 2010). Among studies where age is reported, generally older age is associated with a higher prevalence of respiratory disease. However, factors other than age alone may be associated with increased rates reported year over year in many studies even in countries with aging populations. Although the median age of the population is increasing in Canada, age and other known risk factors do not appear to fully account for the increased prevalence of disease and infection in Ontario (Al Houqani et al., 2011).

Role of Various NTM Species in Disease

Members of the M. avium complex (MAC) appear to be the most common NTM group identified among human NTM isolations, particularly among pulmonary specimens in Australia (Haverkort, 2003), the Netherlands (van Ingen et al., 2009), and east Asia (Simons et al., 2011). Among reports where NTM are speciated, an analysis of isolates among residents of Naples, Italy, identified M. intracellulare, M. gordonae, and M. kansasii as the most common species isolated. However, disease was reported to be most commonly associated with M. intracellulare, M. kansasii, and M. fortuitum (Del Giudice et al 2011), but strains identified in association with disease vary widely. Koh et al. (2006) reported that during the 2 year study period at a large medical center in Seoul, South Korea, M. intracellulare and M. abscessus were more frequently associated with pulmonary disease than M. avium. The North Moravia region of the Czech
Republic is known for heavy industrial activity and coal mining. *M. kansasii* is frequently isolated from drinking water, industrial fluids, and people living in this area (Chobot et al., 1997; Kaustová et al., 1981). A useful review of NTM occurrence by species among different countries highlights some of the geographic differences in the prevalence of specific NTM pathogenic species isolated from people (Marras and Daley, 2002).

**Risk Factors for *M. avium* Complex Disease**

Susceptibility to NTM disease is poorly characterized. In addition to older age being a risk factor, as noted above, gender and race may also be factors. Two recent reports from the U.S. reinforced previous observations that the prevalence of NTM pulmonary disease appeared to be higher among women (Prevots et al., 2010; Winthrop et al., 2010). However, studies do not always agree about the relative prevalence of disease with respect to gender. Older persons, but not necessarily women, were more commonly infected in a Japanese case control study using bronchiectatic controls (Maekawa et al., 2011). Males predominated among a small cohort in Naples, Italy (Del Giudice et al., 2011) and in a multiyear survey of isolations and NTM disease among medical centers in the Netherlands (van Ingen et al., 2009). In a U.S. survey of persons from whom NTM had been isolated, males were more likely to have associated disease (O'Brien et al., 1987). NTM sensitization prevalence appears to be increasing in the U.S.; risk factors associated with skin sensitization include male gender and African American race (Khan et al., 2007).

Besides these general sensitive groups, multiple specific host risk factors have been associated with NTM infection. Competent cell-mediated (innate) immunity is an essential first line of defense against mycobacterial infections. Defects in these pathways or in T-cell function (either acquired or induced with therapeutic agents) may be a risk factor for infection (Collins, 1989; Holland, 2007). Chronic lung disease such as chronic obstructive pulmonary disease, bronchiectasis (Fowler et al., 2006; Maugéin et al., 2005), silicosis (Bailey et al., 1974; Morrow and Armen, 1956), cystic fibrosis (Olivier et al., 2003), alveolar proteinosis (Witty et al., 1994), pneumoconiosis (Fujita et al., 2004), and previous infection with *M. tuberculosis* (Sonnenberg et al., 2000) have all been identified as risk factors for NTM-associated pulmonary disease. Other risk factors for NTM pulmonary disease include connective tissue disorders and abnormal cystic fibrosis genotypes (Iseman et al., 1991; Kim et al., 2008). Exposure risks of NTM-associated hypersensitivity pneumonitis, sensitization, and disease include: fish tanks and other aquaria, water aerosols, respiratory silica exposure, mining, metal working, and soil (Bailey et al., 1974; Corbett et al., 1999; De Groote et al., 2006; Gray et al., 1990; Kahana et al., 1997; Khan et al., 2007; Nel et al., 1977; Shelton et al., 1999).

In short, exposure to NTM is inescapable. It is useful, then, to consider ways to limit exposure of humans to conditions that might deliver bacteria in ways that enhance infectivity. Particularly notable conditions that enhance infectivity by inhalation are those that create aerosols, suspended microscopic droplets and particles that can carry mycobacteria. Such particles are sufficiently small to enter the deep airways and can serve as foci for infection or immunological irritation. Pulmonary disease and other health risks, such as asthma and hypersensitivity pneumonitis (e.g. “hot tub lung” (Embil et al., 1997) and “lifeguard lung” (Angenent et al., 2005), seen in indoor pool settings), are associated with inhalation of bacteria and microbial degradation products. Indoor settings such as swimming and therapeutic pools, hot tubs, spas and other facilities are known as settings for exposure to NTM.
Linking NTM and *M. avium* in Premise Plumbing to Disease

Drinking water contains both rapid- and slow-growing mycobacterial species, some of which have been identified as human pathogens (Bullin et al., 1970; Covert et al., 1999; Fox et al., 1992). Reports of NTM isolation from drinking water at point-of-use within buildings have documented the occurrence of NTM in drinking water in both public and private buildings (Falkinham et al., 2008; Fernandez-Rendon et al., 2012; Perkins et al., 2009). NTM isolation from hospital water supplies is of particular concern due to the potential for exposure of immunosuppressed patients to pathogenic NTM (Baird et al., 2011; du Moulin et al., 1988; Fernandez-Rendon et al., 2012; Hussein et al., 2009; Lockwood et al., 1989).

Multiple reports document MAC in drinking water and in people exposed to the water. Specific associations between human infection/colonization and water have been characterized using genetic and epidemiologic methods. Genotyping techniques have greatly improved the specificity of evidence for human infection/colonization associated with NTM isolated from drinking water. Multiple species of NTM have been isolated from drinking water and have been genetically related to strains producing human infection or colonization: *M. avium* (von Reyn et al., 1994), *M. xenopi* (Bennett et al., 1994; Costrini et al., 1981); *M. fortuitum* (Burns et al., 1991); *M. kansasii* (Picardeau et al., 1997); multiple NTM (Falkinham III, 2011), *M. simiae* (Conger et al., 2004; Sahly et al., 2002); *M. lentiflavum* (Marshall, 2011); and *M. porcinum* (Brown-Elliott et al., 2011). *M. avium* pulmonary infection in an individual was linked with the isolation of *M. avium* from water that shared the same DNA fingerprint as that of the patient’s infecting strain (Falkinham III et al., 2008). Further, in a study of the premise plumbing of individuals with nontuberculous mycobacterial (NTM) disease across the U.S. and Canada, it was shown that 17 of 37 households (46 %) were populated with NTM isolates of the same NTM species as that infecting the patient and, further, in 7 of those 17 households (41 %) the DNA fingerprint of isolates from the premise plumbing were indistinguishable to that recovered from the patient (Falkinham III, 2011). Additionally, NTM disease (predominantly caused by *M. avium*), has been linked to exposure to aerosols generated in hot tubs and spas (Mangione et al., 2001).

Related strains of *M. avium* as defined by PFGE pattern analysis were isolated from hospital, residential, and community water and from patients exposed to the water (Aronson et al., 1999; Falkinham III, 2010; Falkinham III et al., 2008; Hilborn et al., 2008; von Reyn et al., 1994). Hypersensitivity pneumonitis has been reported in association with exposure to tap water during showering, and PFGE pattern analysis has revealed related MAC strains isolated from household water samples and the resident’s sputum (Marras et al., 2005). Hypersensitivity pneumonitis is sometimes associated with exposure to water aerosols such as indoor hot tubs. However, because people bathe in hot tubs and introduce soil organisms, the microbiology of hot tubs is not necessarily representative of the microbiology of household water. Reports of NTM isolation and patient isolation associated with modified drinking water such as ice, whirlpool tubs, pools, footbaths, prepared cleaning, and irrigation solutions are common (Gubler et al., 1992; Holmes et al., 2002; Kahana et al., 1997; Laussuq et al., 1988). However, other sources of environmental contamination (such as soil, De Groote et al., 2006) cannot be excluded.
NTM Risk Assessment and Management

The prevalence of NTM isolations and infections appears to be increasing among many developed and some developing countries. Although *M. avium* is most commonly identified as the cause of disease, it is not the only pathogenic NTM of concern to public health. Source water, water treatment and distribution system characteristics along with premise plumbing conditions appear to have an impact on the occurrence of *M. avium* and other NTM in drinking water at point of use. Host factors and multiple NTM exposure sources and characteristics appear to significantly modify the risk of NTM infection given the presumed common occurrence of NTM exposure via drinking water. NTM enumeration and occurrence in environmental samples by species and site is inconsistent among studies due to methodological differences and limitations associated with sampling and analysis.

Effective control of these emerging bacterial pathogens is poorly characterized and may vary greatly among drinking water systems. Targeted control such as filtration devices in systems serving drinking water consumers at the highest risk for infection and subsequent disease progression may be the most cost-effective initial approach to decreasing the burden of NTM disease among these persons. However, unless changed frequently, filters may actually degrade water quality as they become colonized by NTM (Falkinham III, 2010; Williams et al., 2011). Given the occurrence of NTM in other sources, it is unknown how effective control measures on drinking water systems will be at reducing the total public health burden of NTM-associated disease.

Many homes and public facilities contain devices or machinery that produce aerosols, for instance humidifiers, some dehumidifiers, HVAC systems, fountain sprays and other devices. Activities that generate aerosols range from taking showers to flushing toilets to working with potting soils. Some investigators believe that the increased incidence of NTM pulmonary disease in recent decades has paralleled increased usage of showers instead of baths for personal hygiene and perceived energy savings in western societies (O’Brien et al., 2000). Indeed, pathogens recovered from some patients have been shown to be genetically indistinguishable to NTM in patient household waters (Falkinham III et al., 2008; Shin et al., 2007). Thus, aerosol-generation is a public health concern, particularly for individuals with compromised immune systems (Falkinham III, 2003). Use of dust masks could significantly reduce exposure to NTM in some activities, for instance working with potting soils or other activities that generate dust. This is an educational issue that has received too little attention.

**Epidemiology of Pseudomonas aeruginosa**

*Pseudomonas aeruginosa* Disease and Prevalence

*P. aeruginosa* is a ubiquitous Gram-negative rod-shaped bacterium commonly associated with soil and water with minimal nutritional requirements, which enables it to survive in nearly any environment. *P. aeruginosa* has been isolated from water (Favero et al., 1971; Mena and Gerba, 2009), antimicrobial soaps (Bertrand et al., 2000; Fanci et al., 2009; Lanini et al., 2011), and disinfectants (Russell, 1999). *P. aeruginosa* is rarely carried by healthy individuals (2-10% of individuals) but can be recovered from 50-60% of hospitalized patients (Cholley et al., 2008). *P. aeruginosa* is considered to be an opportunist pathogen and is responsible for causing infections in individuals with cystic fibrosis (Hata and Fick, 1988; Høiby et al., 2010) and
individuals receiving medical care. Many of these infections occur in healthcare facilities (Aumeran et al., 2007; Ferroni et al., 1998; Floret et al., 2009; Hardalo & Edberg, 1997; Mena and Gerba, 2009; Squier et al., 2000; Venier et al., 2012). Community-acquired infections among normal healthy individuals are primarily limited to infections of eyes, ears, and skin. These latter infections are most often associated with recreational water, a normal habitat of *P. aeruginosa*.

Very little data are available about the occurrence of *P. aeruginosa* disease outside of the healthcare setting. Much of what is known about infections in the community setting is from reports in the published literature from case reports and outbreak investigations. Infections produced by *P. aeruginosa* are also non-reportable, so burden of disease is difficult to assess. The primary infections (ear and skin) acquired in the community involve the use of contaminated swimming pools, hot tubs, and whirlpools where there has been a failure to maintain sufficient residual disinfectant (CDC, 2000; Greene et al., 1984; Highsmith et al., 1985; Jacobson, 1985; Mena and Gerba, 2009; Ratnam et al., 1986; Thomas et al., 1985; Yu et al., 2007).

In a meta-analysis of 43 waterborne-associated outbreaks in hospitals covering 35 years (1966-2001), it was estimated that there were approximately 1,400 nosocomial pneumonia deaths per year in the U.S. caused by waterborne *P. aeruginosa* (Anaissie et al., 2002). Among Gram-negative healthcare-associated pathogens, *P. aeruginosa* is the second most frequent pathogen causing ventilator-associated pneumonia (VAP) and the third or fourth most frequent pathogen causing septicemia, urinary tract infections, and surgical wound infections (Trautmann et al., 2009).

Folliculitis is considered to be the most commonly recognizable *P. aeruginosa* infection among bathers using whirlpools and hot tubs (Feder et al., 1983; Ratnam et al., 1986; Silverman and Nieland, 1983; Solomon, 1985). Folliculitis occurs in settings where there is a failure of pool maintenance and disinfection that permits survival and growth of *P. aeruginosa* (CDC, 2000; Hopkins et al., 1981; Tate et al., 2003). There are a few reports of cases reported in the literature of *P. aeruginosa* folliculitis not associated with either bathing in pool, hot tub or whirlpool, but rather with bathing/showering (Hogan, 1997; Zichichi et al., 2000). There has also been one report linked to occupational exposure to water at a cardboard factory (Hewitt et al., 2006). Some of these authors believe that these types of infections may be under reported (Mena and Gerba, 2009; Zichichi et al., 2000).

*P. aeruginosa* skin infections are typically self-limited but occasionally require treatment, and rarely some may be severe (Khabbaz et al., 1983). *P. aeruginosa* dermatitis has been described as either folliculitis or pruritic skin rash and may be accompanied with low-grade fever, malaise, and other systemic symptoms (Chandrasekar et al., 1984; Feder et al., 1983). *P. aeruginosa* has also been implicated in lesions on hands and feet called hot hands or hot foot syndrome (Fiorillo et al., 2001; Michl et al., 2011; Yu et al., 2007).

Swimmer’s ear (*otitis externa*) caused by *P. aeruginosa* is associated with swimming or bathing in either fresh or other natural water bodies, swimming pools, whirlpools, or hot tubs (CDC, 2000; Greene et al., 1984). These infections primarily affect individuals less than 18 years of age who spend greater than average time in the water (Jacobson, 1985). In individuals with diabetes, *P. aeruginosa* ear infections can become a severe and potentially life-threatening if untreated (Weinroth et al., 1994).
Risk Factors for \textit{Pseudomonas aeruginosa} Infection

The major risk factor for \textit{P. aeruginosa} pulmonary disease (i.e., pneumonia) is cystic fibrosis (Hata and Fick, 1988) and hospitalization (Aumeran et al., 2007; Ferroni et al., 1998; Floret et al., 2009; Hardalo and Edberg, 1997; Mena and Gerba, 2009; Squier et al., 2000). In hospitals, exposure to \textit{P. aeruginosa}-contaminated fluids used with patients, including disinfection solutions and water in inhalation devices has been linked to \textit{P. aeruginosa} pneumonia. As \textit{P. aeruginosa} is a normal inhabitant of natural waters, abrasions, cuts or burns can be infected with \textit{P. aeruginosa}. Such dermal infections reflect the loss of the normal barrier to infection provided by normal skin.

It is well known that hospitalized patients are at increased risk for developing infections. Those patients at risk include intensive care unit (ICU) patients (including neonatal ICU), transplant patients, neutropenic patients, burn patients, hydrotherapy patients, patients with malignancies, cystic fibrosis patients, and those with underlying medical conditions. \textit{P. aeruginosa} infections occurring in the ICU was associated with mechanical ventilation (Thuong et al., 2003).

Linking \textit{Pseudomonas} in Premise Plumbing to Disease

The role of tap water as a source of \textit{P. aeruginosa} disease has been established in several published studies. The modes of transmission have included direct contact with water and aerosols, aspiration, indirect transfer from moist environmental surfaces, and via healthcare worker hands (Bert et al., 1998; Hollyoak et al., 1995; Widmer et al., 1993). A number of such studies used molecular markers to demonstrate relatedness of tap water and patient isolates.

Tap water samples from sinks within ICUs have been found to contain \textit{P. aeruginosa} strains that were indistinguishable by molecular typing to those obtained from infected and colonized patients. Tap water faucets were colonized with the same \textit{P. aeruginosa} strain for more than 2 years, even though \textit{P. aeruginosa} was not recovered from the mains supplying the sinks (Reuter et al., 2002). Tap water and outlets appear to be the reservoir for \textit{P. aeruginosa} within healthcare facilities (Trautmann et al., 2006; Trautmann et al., 2008; Trautmann et al., 2009; Trautmann et al., 2005; Trautmann et al., 2001). However, in a surveillance study of hospitalized patients, most were colonized before admission (Cholley et al., 2008). \textit{P. aeruginosa} in tap water was shown to be the source of infection in 1 or 14 patients based on the identity of water and patient isolates (Cholley et al., 2008).

A 10 year molecular epidemiological study attempted to determine the respective roles of exogenous and endogenous flora and time on infection and the effect of \textit{P. aeruginosa} infection in ICU patients (Cuttelod et al., 2011). Isolates fell into three types: 1.) indistinguishable patient and faucet isolates, 2.) indistinguishable patient isolates, but none in faucets, and 3.) unrelated patient and faucet isolates. Higher levels of faucet contamination with \textit{P. aeruginosa} were correlated with higher numbers of cases in group 1; namely 34 per 1,000 patient admissions (Cuttelod et al., 2011). The number of the third type or “endogenous” cases was considerably lower and stable over time (Cuttelod et al., 2011).
Transmission of *Pseudomonas*

Most of what is known of the role of tap water and premise plumbing as a source for infections caused by *P. aeruginosa* comes from outbreaks in the healthcare setting. Exposure to water in these cases may be through direct contact with the patient (hydrotherapy, pulsatile lavage, wound debridement, patient bathing, etc.), its use to prepare solutions, and its use in the reprocessing of certain medical devices, oral hygiene, and drinking. Modes of transmission for waterborne infections include: 1.) direct contact, such as bathing; 2.) ingestion of water; 3.) indirect-contact transmission, through contamination of a device or fomite; 4.) inhalation of aerosols dispersed from water sources; and 5.) aspiration of contaminated water.

*Pseudomonas* Risk Assessment and Management

Community acquired infections attributed to water are primarily limited to recreational water exposures. Even among severely neutropenic patients with long term use of catheters, the risk of *P. aeruginosa* infection from household tap water appears to be low (von Baum et al., 2010). Therefore, *P. aeruginosa* risk assessment and management is primarily focused on hospitals.

Healthcare acquired infections of *P. aeruginosa* occur both endemically and in outbreaks. Infections can arise indirectly or directly from exposure to water or patient-to-patient. Outbreaks have frequently been reported among the different types of intensive care units, hematology-oncology units, burn units, and bone marrow transplant units among the most severely ill patients in the hospital. In particular, *P. aeruginosa* outbreaks appear to arise from colonization of plumbing fixtures, rather than the source water. Plumbing fixtures, including “no-touch” faucets, can become problematic and become contaminated even in what appears to be the absence of water feeding these fixtures (Aumeran et al., 2007; Blanc et al., 2004; Bukholm et al., 2002; Cholley et al., 2008; Ferroni et al., 1998; Halabi et al., 2001; Trautmann et al., 2008; van der Mee-Marquet et al., 2005; Wang et al., 2009).

*P. aeruginosa* outbreaks have been overcome by a variety of interventions. A number of the outbreaks reported in the literature have been resolved with the installation of point-of-use filters (Trautmann et al., 2008; Vianelli et al., 2006) with or without disinfection of faucets, sinks, and traps, and removal and replacement of sinks and fixtures. Successful outbreak control strategies include: installation of point-of-use (POU) faucets and increased disinfectant concentrations (Aumeran et al., 2007; Fanci et al., 2009; Ferroni et al., 1998), replacement of inactivated disinfectant solutions (Bert et al., 1998), cleaning and chlorination of sinks (Berthelot et al., 2001; Hollyoak et al., 1995), weekly “pasteurization” of faucets and use of sterile water for preparation of all fluids coming into patient contact (Bukholm et al., 2002), installation of a bedpan washer (Falkiner et al., 1977), hyperchlorination (Fanci et al., 2009), faucet aerators replaced on a weekly basis with autoclaved aerators (Grundmann et al., 1993), disinfection of showers and piping (Kolmos et al., 1993), and re-education of staff for proper hand-washing technique (Orrett, 2000). These successful interventions identify the sources (e.g., faucets) and highlight the importance of regular, thorough, and effective cleaning and disinfecting of faucets and sinks.
**EPIDEMIOLOGY OF ACANTHAMOEBA**

*ACANTHAMOEBA Disease and Prevalence*

Various *Acanthamoeba* spp. can cause serious infections in humans, including *Acanthamoeba* keratitis (AK), skin ulcerations, and granulomatous amoebic encephalitis (GAE). AK is a severe form of eye infection, while GAE is an infection of the central nervous system. *Acanthamoeba castellani* and *Acanthamoeba polyphaga*, in particular, are commonly cited as causative agents of AK (Omaña-Molina et al., 2004). Several strains have been implicated in GAE (Akpek et al., 2011). AK is primarily associated with poor contact lens hygiene, but has also been associated with corneal trauma and exposure to water containing *Acanthamoeba*. The increase in incidence of AK is likely due to the increase in the use of contact lenses and the omnipresence of *Acanthamoeba* in all types of environments (Joslin et al. 2006; De Jonckheere, 1991; Verani, 2009; CDC, 2011a; Dorsch et al., 1983). In 2003, it was estimated that the incidence of AK was 1 case per 30,000 contact lens wearers per year (Seal, 2003). In the Chicago area alone, 40 cases of AK were identified from 2003-2005, and the incidence rate appeared to be increasing (Joslin et al., 2006). Though GAE had traditionally been thought of as a threat to immunocompromised individuals (Martinez and Janitschke, 1985), GAE can also impact healthy people (Lackner et al., 2010). As of 1996, 103 cases of GAE induced by *Acanthamoeba* spp. had been identified world-wide, 72 in the U.S. and ≥50 with AIDS (Martinez and Visvesvara, 1997). It is anticipated that, with the increase of immunocompromised individuals, the incidence of *Acanthamoeba*-associated skin ulcerations and encephalitis will continue to increase. An important factor in *Acanthamoeba* epidemiology is the ability to form desiccation- and disinfection-resistant cysts (De Jonckheere, 1991).

**Risk Factors for Acanthamoeba Infection**

The primary risk factor of AK is contact lens use. In particular, poor hygiene, wearing lenses overnight, smoking, infrequent lens case replacement, and cleaning lenses with tap water increase risk (Stapleton et al. 2012; Kilvington et al., 2004). In addition, any corneal trauma can pose a risk to keratitis, even in non-contact lens wearers (Wanachiwanawin et al. 2012). People at risk for GAE include individuals who are debilitated or malnourised, patients undergoing immunosuppressive therapy for organ transplants, and patients with AIDS (Martinez and Visvesvara 1997). Like *P. aeruginosa* skin infections, *Acanthamoeba*-associated skin infections (ulcerations) are a consequence of loss of the normal barrier protection provided by undamaged skin and exposure to the pathogen (Martinez and Visvesvara, 1997). Skin and lung infections have been cited as pre-cursors to the development of GAE (Martinez and Visvesvara, 1997).

**Linking Acanthamoeba in Premise Plumbing to Disease**

*Acanthamoeba* species have a cosmopolitan distribution and have been isolated from soils, dusts, drinking water, bottled water, shower water, swimming pools, therapy pools, dental units, cooling waters, and contact lens cases (Mergeryan, 1991; Thomas and Ashbolt, 2011). Over the period of 1990-1992, 2,454 water samples from 467 households were examined and *Acanthamoeba* were found in 238 (51%) of the households (Stockman et al., 2011). Showerheads (52%) and kitchen sprayers (50%) were the sites most likely to yield amoebae.

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That is most likely due to the fact that amoebae detection was higher in biofilms than water samples (Stockman et al., 2011).

Kilvington and colleagues (2004) conducted an investigation of the homes of 27 AK patients in the U.K. and compared mitochondrial (mt) DNA of Acanthamoeba isolated from the patients and their plumbing. Acanthamoeba were successfully isolated from 24 of the homes and in six cases the mt DNA fingerprint presented an identical match between the patient and their tap water. In the analysis of incidence of Acanthamoeba infections in the Chicago-area, Joslin and colleagues (2006) noted a skewed pattern that coincided with location in the water distribution system. They hypothesized that changes in chlorination practice in response to the 1998 U.S. EPA Stage 1 Disinfectants and Disinfection Byproducts Rule may have compromised distal reaches of the distribution system, increased proliferation of Acanthamoeba and thus incidence of AK. However, a follow-up study by Methvin (2009) indicated that many of the implicated sites were less than 2 days from the treatment plant, and no change in disinfection practice occurred in Chicago during the relevant timeframe (2000-2006).

Mitochondrial DNA RFLP was shown useful in identifying 4 pathogenic types associated with AK amongst 13 different types (Seal, 2003). The four pathogenic mt-RFLP types were heterogeneous as each could be subdivided based on 18S rDNA sequence (Seal, 2003). Amplification of portions of the small subunit ribosomal RNA gene (ssrDNA) was shown to distinguish between pathogenic and nonpathogenic strains. Specifically, a 195-bp product was only found amongst pathogenic strains using one primer pair and a 485-bp PCR product was only found for non-pathogenic isolates (Howe et al., 1997). In a prior study, RFLP analysis of (mt) DNA of patient and environmental Acanthamoeba spp. isolates yielded common patterns, but was reported to lack the discrimination required for source tracking (Gautom et al., 1994).

**Transmission of Acanthamoeba**

The primary route of transmission of Acanthamoeba is through exposure to water, lens solution, or aerosols containing Acanthamoeba (Schuster and Visvesvara, 2004). Acanthamoeba keratitis has been linked to wearing contact lenses while swimming and the presence of Acanthamoeba, presumably cysts, in contact lens cases and cleaning fluids (Schuster and Visvesvara, 2004; Verani et al., 2009). The presence of Acanthamoeba in the respiratory tract (Łanocha et al., 2009) suggests that GAE is due to inhalation and migration of Acanthamoeba through the nasopharynx. Both infected skin and lung tissue have been cited as ports of entry for Acanthamoeba-induced GAE (Martinez and Visvesvara, 1997).

**Acanthamoeba Risk Assessment and Management**

In the face of a ubiquitous presence of Acanthamoeba in the human environment and the resistance of Acanthamoeba cysts to disinfection, it is unlikely that there exists a single, simple way to reduce human exposure. Clearly, contact lens wearers can engage in several effective behaviors to reduce acquisition of AK; namely ensuring that contact lens cases and contact lens cleaning fluids are sterile and that appropriate hygienic practices are implemented. Further, contact lens wearers should not wear contact lens while swimming or showering. As is the case for NTM bacteremia, immunocompromised individuals should be aware that exposures to Acanthamoeba-containing droplets or aerosols (e.g., showering) could place them at risk for disease; in this case GAE (Mayer et al., 2011).
As emphasized in the microbial ecology and physiology sections of Chapter 3, *Acanthamoeba* spp. are of much broader concern to public health than the direct role that they play as pathogens. *Acanthamoeba*, and other free living amoeba (FLA), play a critical role in the life cycle of amoeba-resisting microorganisms (ARM) by offering them protection from disinfection, enhancing their reproduction, and even upregulating virulence genes. ARM include all three of the bacterial pathogens that are the topic of this report, especially *L. pneumophila*, which is thought to be dependent on amoeba uptake for replication in water systems (Lau and Ashbolt 2009; Guerrieri et al. 2005; Wadowsky et al. 1988). For this reason, it has been argued that *Acanthamoeba* are likely a greater health concern in terms of the role they play as hosts to bacterial pathogens, which can enhance amplification, disinfection resistance, and virulence and aid in their overall dissemination in the water environments (Thomas and Ashbolt, 2011).

**EPIDEMIOLOGY OF N. FOWLERI**

**Naegleria Disease and Prevalence**

*N. fowleri* is the causal agent for primary amoebic meningoencephalitis (PAM). PAM is a disease with a high fatality rate of 98%, with death normally occurring within 1-12 days post-infection (CDC, 2012b). PAM occurs primarily in children and young adults who have had contact with fresh water a few days preceding the infection. The protozoan enters through the mucous membranes of the nasal cavity and can migrate to the central nervous system through the cribriform plate to the brain via the olfactory nerve. Symptoms of PAM include headache, fever, nausea, vomiting and stiff neck that eventually lead to confusion, lack of attention to the surroundings, loss of balance, seizures, hallucinations and death. Amphotericin B is the drug of choice in treating victims, but early diagnosis is essential. PAM is rare, with 0 to 8 cases reported per year in the US during 1962-2008, but almost always leads to death within 3-7 days after onset of symptoms (Yoder et al., 2010).

Most victims of PAM are children or young adults in good health with a history of swimming in naturally warm or thermally polluted waters, where growth of the amoebae is favored. Proper chlorination practice is protective in swimming pools and spas. Free chlorine or chloramines at 0.5 mg/L or higher is sufficient to control *N. fowleri*, provided that the disinfectant persists through the water supply system (Chen et al., 1985). Amoebae have been isolated from sun-warmed domestic water supplies in both the U.S. and Australia (Dorsch et al., 1983), but risk of infection from drinking water is minimal and is mostly associated with rinsing nasal passages with contaminated water (Yoder et al. 2012).

**Transmission of Naegleria**

Although infection of *N. fowleri* is rare, *N. fowleri* has been frequently isolated from both natural and human-influenced environments. They have especially been found in soil and fresh recreational water in southern tier states of the U.S., and thermally polluted waters such as cooling towers, hospital hydrothermal pools and sewage sludge (Visvesvara and Stehr-green, 1990). *N. fowleri* causes infection by entering through the nasal pathway of its victim. Water related activities that allow water to enter the nasal passages in contaminated natural or artificially warm recreational waters and recently with the use of contaminated water for flushing or rinsing sinuses are activities that could lead to a *N. fowleri* infection (CDC, 2012b). *N. fowleri*
is not transmitted by ingestion of contaminated waters (CDC, 2012b). No animal reservoir of \textit{N. fowleri} has been reported.

**Linking \textit{Naegleria} in Premise Plumbing to Disease**

Meningoencephalitis in a child caused by \textit{N. fowleri} was linked to the presence of \textit{N. fowleri} in a well water source (Shenoy et al., 2002). A reported case of PAM is typically linked to its source by investigation of the victim's recent water activities. Initial diagnosis of a clinical case is made by extracting a clinical specimen from the patient through lumbar puncture and microscopic identification of trophic amoebae. \textit{Naegleria} amoebae are recognized by their characteristic limacine movement and an anterior eruptive pseudopod (Visvesvara, 1999). Due to the acute nature of the disease, there is normally little or no antibody response for serological confirmation. Diagnosis is also performed postmortem by hematoxylin and eosin (H & E) or immunofluorescence (IIF) staining of brain tissue.

Traditionally, rapid identification techniques such as cytometry and the API ZYM system (detection of enzyme activity) have been used for distinguishing \textit{N. fowleri} from other \textit{Naegleria} species (Kilvington and White, 1985; Pougnard et al., 2002). Recently, rapid molecular techniques such as qPCR and melt curve analysis have been used for differentiating pathogenic \textit{N. fowleri} from non-pathogenic \textit{Naegleria} (Robinson et al., 2006; Qvarnstrom et al., 2006; Puzon et al., 2009). For presumptive \textit{N. fowleri} culture, pathogenicity can be confirmed by flagella formation in distilled water at 37°C and exposure to 42°C; \textit{N. fowleri} is able to grow at this temperature while most non-pathogenic \textit{Naegleria} spp. may not (De Jonckheere 2002).

Environmental isolation of \textit{N. fowleri} was traditionally performed based on enzyme activity such isozyme profiling or ELISA (Reveiller et al., 2003). Recently, real-time PCR and melt curve analysis have been used for rapid identification of pathogenic \textit{Naegleria} (Behets et al., 2006; Madarová et al., 2010; Robinson et al., 2006). In addition, a triplex real-time PCR reaction has been developed for diagnostic identification of \textit{N. fowleri}, \textit{Acanthamoeba} spp., and \textit{Balamuthia mandrillaris} (Qvarnstrom et al., 2006). Cultivation of \textit{N. fowleri} can be easily performed on non-nutrient agar plates coated with bacteria, such as \textit{E. coli} or in liquid axenic media but may take up to 20 days (De Jonckheere, 1977; Teixeira et al., 2009). Current fingerprinting is based on targeting species-specific DNA sequence of \textit{N. fowleri}; for example, the MpC15 sequence has been used in real-time PCR detection of \textit{N. fowleri} (Madarová et al., 2010).

In the U.S., \textit{N. fowleri} infections are sporadic and mostly associated with swimming in warm recreational waters (CDC, 2008b). However, the two most recent cases in the U.S. were associated with nasal irrigation using treated and disinfected drinking water in Louisiana (LA DHH, 2011). \textit{N. fowleri} infections are very rare and, until recently, premise plumbing did not seem to be a source for \textit{N. fowleri} infections in the U.S. Two \textit{N. fowleri}-related deaths associated with two different chloramine-treated public water systems (one surface water and one groundwater) were reported in Louisiana in 2011 (LA DHH 2011). Between 2001 and 2010, only 32 infections were reported in the U.S. Of those cases, 93.75% (30 cases) were attributable to contaminated recreational water and 6.25% (2 cases) were attributed to water from a geothermal (naturally hot) drinking water supply (CDC, 2012b). Common sources of \textit{N. fowleri} are warm recreational waters (i.e. lakes and rivers in Southern-tier U.S. States), geothermal recreational and drinking water sources, thermally-polluted effluents from industrial plants, poorly-maintained swimming pools and water heaters with temperatures less than 47°C (CDC,
As of date, there were four reported premise plumbing-related cases in the U.S. In 2002, two five-year-old boys from Peoria, Arizona, died of PAM after being exposed to un-disinfected drinking water supplied from a groundwater source (Maricopa County Department of Public, 2004).

In June 2011, a young Louisiana man died after flushing his sinuses with tap water and amoebae were detected in the home's water system in Saint Bernard Parish. A 51-year-old DeSoto Parish woman died in October 2011 after using tap water in a neti pot to irrigate her sinuses and becoming infected with the deadly amoeba. The two fatal cases of PAM in Louisiana in 2011 occurred in two separate drinking water systems in different parishes, De Soto in the northwest on the Texas border and in Saint Bernard Parish in the southeast near New Orleans. Both victims used neti pots to irrigate which push water into and irrigate the sinuses. In both cases, water in the premise plumbing was positive for \textit{N. fowleri}. Active investigation of the public water systems continues (Yoder et al., 2012). Note that neti pots are typically labeled for use with sterile water.

\textit{Naegleria} Risk Assessment and Management

Maintenance of cooler temperatures in distribution system (i.e., avoiding above-ground transmission lines) has been identified as one effective management strategy for \textit{N. fowleri} (Dorsch et al., 1983). The following are a number of knowledge gaps whose answers will inform \textit{Naegleria} risk assessment and management:

1. Ecology of \textit{Naegleria} in response to climate change has not been elucidated (prediction: increased occurrence due to increase in distribution of warm bodies of water);
2. Limited studies on seasonal and geographical trends of \textit{Naegleria} diseases;
3. Prevalence of \textit{Naegleria} in biofilm and associated health risks has not been extensively investigated; and
4. Lack of studies that look at the occurrence of \textit{N. fowleri} in drinking water distribution systems and in premise plumbing.
CHAPTER 3: MICROBIAL PHYSIOLOGY AND ECOLOGY

COMMON PHYSIOLOGICAL AND ECOLOGICAL FEATURES AMONG OPPPS

A basic overview of the microbial physiology and ecology of opportunistic pathogens is provided in this chapter. A discussion of basic physiology is necessary in order to define the pathogens of interest, identify the dominant physiologic features that are determinants of the microorganism’s ecology, and provide context for detection methodology, as discussed in Chapter 4. Microbial ecology is particularly relevant given that opportunistic pathogens are a part of the resident flora of premise plumbing systems. Highlighted in this chapter is the complex nature of the interaction of OPPPs with other members of the microbial community as well as their response to abiotic factors, such as plumbing material, water chemistry, and disinfectants. Similarities and differences are noted among the pathogens in terms of how they interact with the surrounding environment. Establishing a fundamental understanding of the complex ecology of OPPPs will be vital for developing appropriate research systems that simulate the drinking water environment, as discussed in Chapter 5, and for identifying appropriate engineered control strategies, as discussed in Chapter 6. Table 3-1 provides an overview of some of the key physiological and ecological considerations of OPPPs.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Structure</th>
<th>Growth</th>
<th>Amoeba-Resisting</th>
<th>Physical</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. pneumophila</td>
<td>Outer membrane</td>
<td>Fastidious, obligate, intracellular?</td>
<td>Obligate Growth, Amoebae-killing</td>
<td>Hydrophilic, Cl5</td>
</tr>
<tr>
<td>M. avium</td>
<td>Impermeable hydrophobic outer membrane</td>
<td>Slow, omnivorous</td>
<td>Growth</td>
<td>Surface attached Hydrophobic ClR</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>Outer membrane</td>
<td>Rapid, omnivorous</td>
<td>Growth</td>
<td>Biofilm former Hydrophilic, Cl5</td>
</tr>
<tr>
<td>Acanthamoeba</td>
<td>Single membrane-Cyst form</td>
<td>Organotroph, phagocytic</td>
<td>Not applicable</td>
<td>Surface attached Cyst ClR</td>
</tr>
<tr>
<td>N. fowleri</td>
<td>Single membrane-Cyst form</td>
<td>Organotroph, phagocytic</td>
<td>Not applicable</td>
<td>Surface attached Cyst ClR</td>
</tr>
</tbody>
</table>

A major challenge to the control of OPPPs is that they are native to the household environment. *Legionella* and *Mycobacteria* are readily aerosolized from water (Parker et al., 1983) and both are found in biofilms with FLA (Thomas and McDonnell, 2007; Torvinen et al., 2006). As *Mycobacteria* in biofilms are more disinfectant-resistant (Steed and Falkinham, 2006), and both *Legionella* and *Mycobacteria* grow in FLA (Cirillo et al., 1997; Thomas and McDonnell, 2007), premise plumbing provides an ideal habitat for these opportunistic pathogens to establish and grow. As FLA, such as *Naegleria* and *Acanthamoeba*, are hosts for intracellular growth of both *Legionella* and *Mycobacterium* and are also themselves pathogens (Brown and
Barker, 1999), a comprehensive understanding of premise plumbing ecology would be beneficial to appropriately inform engineered control strategies.

Biofilms are central to the microbial ecology of opportunistic pathogens and their role cannot be overstated. Biofilms provide a direct source of carbon and nutrients to amoeba and other protozoa. Artificial biofilms added to batch reactors in one study appeared to directly favor *L. pneumophila* growth (Valster et al., 2009; Kuiper et al., 2004). However, the function of biofilms in stimulating *L. pneumophila* has not clearly been defined. Biofilms also present unique methodological challenges in laboratory assays in terms of their complexity, non-uniformity, and sampling limitations. Of particular value to advancing laboratory assays incorporating biofilms will be information on how to best establish a representative biofilm, which strains or other sources to use, ideal time of acclimation, appropriate support media, flow conditions, and residence times. Efficient and representative biofilm sampling also poses a non-trivial challenge.

Complex networks of relatively small diameter pipes provide high surface-to-volume ratios, where water can stagnate for long periods and potentially promote biofilms. Indoor plumbing materials (e.g. galvanized iron, polymers) provide nutrients and scavenge chemical disinfectants (NRC, 2006). Nutrients are also available from common constituents of tap water including other resident microbes. Premise plumbing, especially in large buildings, comprises many local environments that differ from each other in ways that may bear significantly on the microbial ecology. These differences include hydrodynamics, use patterns, temperature gradients and water chemistry. Conditions widely thought to affect *Legionella* proliferation include stagnant or slow flowing water and system maintenance. However, some reports indicate that the importance of stagnation in the promotion of *Legionella* replication may be overestimated (Liu et al., 2006). Certain maintenance procedures once thought to be important, e.g., removal and disinfection of showerheads and aerators, have been shown to be ineffective (ASHRAE, 2000; Stout, 2007).

**PHYSIOLOGY AND MICROBIAL ECOLOGY OF L. PNEUMOPHILA**

*Legionella* Physiology

*Legionella* is a genus of Gram-negative, aerobic, non-spore forming, fastidious, usually-rod-shaped bacteria within the class Gamma Proteobacteria. *Legionellae* is comprised of at least 50 species and 70 serogroups. The number of species, serogroups and subgroups of *Legionella* continues to increase, owing to continuing discovery and the evolution of new strains, e.g., by horizontal gene transfer (Coscollá et al., 2011). Within the genus *Legionella*, the DNA relatedness between strains of a given species typically exceeds 90%, but between one species and another is less than 70% (Brenner, 1987). Of about 0.3-0.9 microns in width and 2-20 microns in length, *Legionellae* are facultative intracellular parasites. Some *Legionellae* are able to survive outside of hosts in appropriate ecological niches that support their fastidious and restrictive growth requirements. In particular, *Legionella* are known to be capable only of utilizing certain amino acids as a growth substrate (Tesh et al., 1983); they are not known to use other organic carbon sources.

VBNC is a well-documented protective state entered in response to environmental stressors by some microorganisms, including *Legionella*. Typically, the VBNC organism loses its ability to grow on culture media, at least temporarily, but retains its ability to infect a
susceptible host. Stressors that can induce Legionella to enter VBNC state include chemical disinfectants, such as monochloramines (Alleron et al., 2008). Some legionellae have been identified that cannot be cultivated on media but are able to multiply in hosts, and can be grown in species of FLA. These have been termed Legionella-like amoebal pathogens (LLAP). At least some LLAP are human pathogens. Several strains have been formally classified as Legionella species (Adeleke et al., 2001; Scola et al., 2004). Some legionellae have been determined to be human pathogens, with significant differences in virulence among subspecies (Helbig, et al., 1995). It is thought that most Legionella that can grow at body temperatures are also able to cause human infections under some conditions (Fields, 1996).

Legionella survive well over a wide range of temperatures, and poorly maintained or tepid hot water systems are particularly vulnerable to colonization. Legionellae are acid tolerant to pH 2.0 (Anand et al., 1983) and able to survive temperatures of up to 70°C (Sheehan et al., 2005). Subject to the availability of necessary nutrients (e.g., iron, L-cysteine), Legionella can grow in water at 20-50°C (Konishi et al., 2006). They proliferate vigorously in water at 32-42°C with low levels of available nutrients, e.g., unsterilized tap water (Yee and Wadowsky, 1982), especially in slow-flowing or stagnant water. As an engineered control, it is generally accepted that temperatures exceeding 55°C are protective of hot water systems of hospitals and other buildings (Darelid et al., 2002).

Chemical disinfectant residual can be effective for controlling Legionella in building water systems (Lin et al., 2011). In particular, chloramine appears to be more effective against Legionella than chlorine (Flannery et al., 2006). Legionella are comparatively less susceptible to chlorination than E. coli, and reportedly can survive chlorine doses of up to 50 ppm when contained inside protozoan hosts (Lin et al., 1998; Lin et al., 2011).

Legionella probably are not completely eliminated by standard water treatment practices. Even if substantially removed from source water in the treatment plant, Legionella is a normal part of the ecology of water distribution systems and is frequently present in high quality, regulation compliant drinking water distributed to customers. Legionella colonization of premise plumbing is not necessarily an indication poor operating or maintenance practices by community water systems. Rather, it is a function of the conditions characteristic of premise plumbing that support proliferation. For example, one of the most recent outbreaks of Legionella occurred at the new five star Aria Resort in Las Vegas, NV, about a year after its grand opening (James and Conley, 2011).

**Legionella Ecology**

**Legionella Strain and Serotype Distinctions**

There are numerous Legionella strains, exhibiting varying degrees of pathogenicity and preferred ecological niches. In most cases, studies that observed successful growth of L. pneumophila in an oligotrophic environment (e.g., tap water) incorporated naturally occurring L. pneumophila strains (Wadowsky et al., 1988; Kuiper et al., 2004). One study specifically demonstrated that L. pneumophila serogroup 6 exhibits a higher prevalence and stronger host infection efficiency (Messi et al., 2011). A later study also revealed that different Legionella strains vary in their abilities to multiply in tap water co-cultures (Wadowsky et al., 1991). For example, L. pneumophila-Philadelphia 1 strain could not multiply in replicate co-cultures containing H. vermiformis. Differences among Legionella strains stand as a methodological
challenge, raising important research questions with potentially significant implications for sampling procedures, assay methods and engineering controls that have been developed based on information about a sub-set of pathogenic strains. For example, the standard culture method and predominant clinical assay are biased towards detection of *L. pneumophila* serotype 1. As a result, the relative environmental and clinical importance of other strains may be grossly under-reported.

**Legionella Distribution and Habitat**

Legionellae have been found in a wide range of natural and artificial environments, including fresh water, saltwater and soil (Fliermans et al., 1981). The natural habitat for *Legionella* appears to be aquatic bodies including rivers, streams and thermally polluted waters, where the bacteria typically are found in small numbers. *Legionella* bacteria have been detected in all segments of water distribution, from the source water to the tap (Buse et al. 2012).

*Legionella* species have been recovered from a wide variety of hot and cold domestic water systems, in numbers that fluctuate widely. *Legionella* have reportedly been found in approximately 50% of large building water systems and 10-30% of home water systems in the U.S. In a series of surveys, 12-70% of hospitals had water systems that tested positive for *Legionella* (HMSO, 1987; Alary and Joly, 1992). Water sources that have been associated with cases of Legionnaires’ disease include warm water systems of buildings and private residences, air-cooling systems, a decorative fountain, a grocery store mist machine, hot water tanks, whirlpool spas and hot springs. Buildings with hot water distribution systems with lower water temperatures (less than 60°C) were significantly more likely to be colonized with *L. pneumophila* (Lin et al. 1998). Cold-water sources, such as ice machines and drinking fountains, have also been implicated as a source of infection. Cooling towers and evaporative condensers were suspected in investigations of early outbreaks. However, cooling towers are easier to control with biocides and good practice than building system water, and are now considered a less important reservoir than potable water systems in the dissemination of *Legionella* (Muder et al., 2002). The number of investigations citing cooling towers as the source of legionellosis has declined markedly, relative to those in which premise plumbing systems have been implicated.

*Legionella* can be introduced into a building’s potable water systems in several ways. In addition to the relatively small numbers characteristic of high quality, finished water, *Legionella* also can be introduced in relatively larger numbers from non-potable building water systems (e.g., cross-connections with fire protection piping, backflow from plumbing fixtures) and from disruptions in the supply water distributions system (e.g., water main breaks, service line failures, hydraulic transients). Once legionellae have been introduced, premise plumbing differs from the main water distribution system in ways that promote colonization. Noteworthy characteristics of plumbing include high surface-to-volume ratios, excessive water age, and water temperatures within ranges optimal for *Legionella* growth (32-42°C) (Yee and Wadowsky, 1982). Other distinguishing characteristics of premise plumbing systems include lack of disinfectant residual due to reaction with pipes and sediment, extensive residence time, and heating.

Colonization of premise plumbing by *Legionella* is primarily a function of natural biofilms, the complex heterogeneous microbial ecosystems that develop on virtually all surfaces in contact with non-sterile water (Murga et al., 2001). The biofilm can facilitate replication of legionellae within FLA present within the biofilm (Guerreri et al., 2008; Buse and Ashbolt,
In addition to providing nutrients, FLA sequester legionellae from chemical disinfectants and may provide an additional route for transmission (Barbaree et al., 1986). The extracellular polymeric substances (EPS) that make up much of the biofilm structure, typically a hydrated polyanionic polysaccharide matrix, also affords Legionella protection from environmental stressors, including many chemical disinfectants. Legionella resident in biofilms have been shown to be able to survive without benefit of a host, and to be far more resistant to disinfection than their free-floating, planktonic counterparts (Cargill et al., 1992; LeChevallier et al., 1988; Surman-Lee et al., 2002).

**Host Relationship with Free-Living Amoebae (FLA)**

Largely, the widespread distribution of Legionella overlaps that of free-living, phagocytic protozoa that serve as hosts of Legionella (Thomas and Ashbolt, 2011). The association of Legionella with FLA was first reported in 1980 (Rowbotham, 1980). The set of varied, complex relationships are a defining aspect of the Legionella life cycle (Buse & Ashbolt, 2011; Rowbotham, 1984, 1986) (Figure 3-1). At temperatures below 22°C, Acanthamoeba can graze on and digest legionellae without adverse consequences (Nagington and Smith, 1980), but at higher temperatures the legionellae may bypass the predator’s normal processes, multiply rapidly, increase in virulence, and eventually kill the host. L. pneumophila has been shown able to parasitize and multiply in more than twenty different protozoan species, including Acanthamoeba, Naegleria, and Hartmanella (Donlan et al., 2005; Hwang et al., 2011; Steinert et al., 1995; Kuiper et al., 2004). These protozoa are much larger than the legionellae on which they graze. For example Acanthamoeba spp. are about 15 to 35 microns long and ovoid to triangular. Notwithstanding their size, they reportedly can be broadcast in bioaerosols. Protozoan hosts, including those not known to be human pathogens in their own right, afford protection from chemical biocides (Barker et al., 1992) and insulation from high temperatures (Storey et al., 2004). Clearly, FLA play a key role in the survival, proliferation and environmental transmission of Legionella and must also be accounted for in any consideration of Legionella and legionellosis (Lau and Ashbolt, 2009; Thomas and Ashbolt, 2011).

Figure 3-1. Role of amoeba hosts in enhancing replication of *Legionella* and other amoeba-resisting microbes (ARMs)
Differences in the relationships between *Legionella* and different FLA hosts are not well defined. *L. pneumophila* has been demonstrated to employ distinct mechanisms in the attachment to and evasion of these two hosts (Harb et al., 1998). Interestingly, a co-incubation experiment in Page’s amoeba saline solution showed that *L. pneumophila* released from *H. vermiformis* exhibited significantly lower degrees in cultivability reduction and membrane deterioration when compared with those from *A. castellanii*; while fewer *L. pneumophila* cells replicated from *H. vermiformis* than *A. castellanii* (Chang et al., 2010).

**Other Microbial Ecological Relationships of L. pneumophila**

*Legionella* also exhibit complex relationships with other bacteria. These relationships vary considerably in studies using laboratory culture versus those in the natural environment. *Flavobacterium breve*, *Aeromonas*, *Pseudomonas vesicularis*, *P. paucimobilis*, *P. maltophilia*, and *Vibrio fluvialis* are known to stimulate growth of *Legionella* on media lacking cysteine, apparently providing sufficient amounts of cysteine necessary to support *Legionella* growth (Yee and Wadowsky, 1982; Toze et al., 1990). In other studies, 32% of heterotrophic bacteria from chlorinated drinking water inhibited *Legionella* (Toze et al., 1990). *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Flavobacterium meningosepticum*, *Pseudomonas aeruginosa*, and some *Staphylococcus* and *Bacillus* species are known to inhibit *Legionella* growth on culture media (Rowbotham, 1984; Paszko-Kolva et al., 1993). For example, *B. subtilis* strain BS104 has been reported to induce cell lysis of *L. pneumophila*, possibly due to inhibitory metabolic byproducts produced by BS 104 or to a decline in *L. pneumophila* growth due to nutrient or habitat competition (Temmerman et al., 2006). In other work, *L. pneumophila* was reportedly inhibited by bacterocin-like substances (BLS), proteinaceous, and bacteriocidal substances produced from heterotrophic species (Guerrieri et al., 2008).

**PHYSIOLOGY AND MICROBIAL ECOLOGY OF NTM AND M. AVIUM**

**Physiology of NTM and *M. avium***

**Physiology Overview**

NTM constitute a large group of opportunistic pathogens that are ubiquitous in water and soil, including drinking water distribution systems (Falkingham et al., 2001) and households (Falkingham III, 2011). Because of the difficulties presented by these bacteria in terms of detection and diagnosis (Griffith et al., 2007), a considerable literature has accrued and the subjects have been widely reviewed. Particularly valuable reviews are those of Marras and Daley (2002), Griffith et al. (2007) and Kim et al. (2005) for clinical presentation and diagnosis; Turenne et al. (2008) for molecular diagnostics; Tortoli (2003) for taxonomy and emergence of new *Mycobacterium* species; and Primm et al. (2004) and Falkingham III (2009) for environmental mycobacteria.
NTM Species Distribution

The most frequently isolated of approximately 20 named NTM (Griffith et al., 2007) include about 10 species, listed in Table 3-2 (Marras and Daley, 2002; Wallace et al., 1998). To date there are approximately 150 named *Mycobacterium* species, many only described in the past 10 years as isolates from clinical samples of HIV-infected patients (Tortoli, 2003). During 1993-1996, 75 % of NTM cases were pulmonary, 5 % blood, 2 % soft tissue, and 0.4 % lymphatic (Marras and Daley, 2002). The most frequently reported *Mycobacterium* species in that study were *M. avium* and *M. intracellulare* (the *Mycobacterium avium* complex, MAC) (~ 80 %), *M. fortuitum* (~ 15 %), and *M. kansasii* (~ 5 %). Although *M. avium* is usually cited as the major NTM pathogen, it is not a singular bacterium. Rather, it is a genetic complex of subspecies, as many as 7 currently and named in Table 3-2. Indeed, each of the named species undoubtedly is a collection of close relatives, with genetic contents that overlap with other species. This renders identifications difficult. For instance, no cultural, biochemical, or enzymatic test can distinguish between *M. avium* and *M. intracellulare*; many clinical laboratories still report isolates as *M. avium* complex or *M. avium-intracellulare*. Isolates similar to previously characterized NTM are readily recovered from many environments (Falkinham, 2009; Primm et al., 2004; Wallace et al., 1998), but experience has taught that at least 30% of environmental isolates are not identifiable with named species (Tortoli et al., 2001). In the face of all this variation, however, it is clear that certain strains of *M. avium* are recovered from multiple different cases of disease over the course of years (Horan et al., 2006).

Table 3-2
Frequently isolated nontuberculous mycobacteria (NTM).

<table>
<thead>
<tr>
<th>Slowing Growing Species</th>
<th>Rapidly Growing Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mycobacterium avium</em></td>
<td><em>Mycobacterium abscessus</em></td>
</tr>
<tr>
<td>subspecies <em>avium</em></td>
<td>subspecies <em>hominisuis</em></td>
</tr>
<tr>
<td>subspecies <em>silvaticum</em></td>
<td>subspecies <em>paratuberculosis</em></td>
</tr>
<tr>
<td>subspecies <em>marseillense</em></td>
<td>subspecies <em>intracellulare</em></td>
</tr>
<tr>
<td>subspecies <em>kansasii</em></td>
<td><em>Mycobacterium fortuitum</em></td>
</tr>
<tr>
<td><em>Mycobacterium malmoense</em></td>
<td><em>Mycobacterium marinum</em></td>
</tr>
</tbody>
</table>

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Mycobacteria have received considerable attention from molecular phylogenetic studies using ribosomal RNA (rRNA) and other gene sequences (Devulder et al., 2005; Stahl and Urbance, 1990). They are representative of the bacterial phylum Actinobacteria, which is one of about 100 large bacterial phyla and includes other extensively studied bacteria such as corynebacteria, actinomycetes, propionibacteria, and other groups (Ventura et al., 2007). Mycobacteria fall into a coherent phylogenetic group and most NTM cluster very closely in phylogenetic trees, distinct from members of the non-environmental, obligately pathogenic Mycobacterium tuberculosis complex (e.g., M. tuberculosis and M. bovis) (Devulder et al., 2005; Stahl and Urbance, 1990). The slowly growing NTM differ from the rapidly growing NTM by the occurrence of a 100 bp deletion in the rRNA gene (Stahl and Urbance, 1990). Additionally, slowly growing NTM possess a single rRNA cistron, whereas the rapidly growing NTM have two rRNA cistrons (Bercovier et al., 1986). In general, the NTM listed in Table 3-2 are extremely closely related, with rRNA sequences that are identical or nearly so. The close relationship of these bacteria confounds classical and molecular identification schemes (below). But the close relationships also indicate that members of the genus Mycobacterium share many properties and much biochemistry.

Key Physiological Properties of NTM

The general properties of NTM make them well-suited for life in aqueous, flowing environments. This predisposes them to enrichment in habitats that mycobacteria share with humans; drinking water distribution systems, building and household plumbing, and moist building environments (Falkinham III, 2009). Although slow growing heterotrophs in the laboratory (1 generation or less per day), they are adapted to many habitats and seem to be selected by some, for instance acidic swamp and peat waters (Iivanainen et al., 1999; Kirschner et al., 1999) and drinking water distribution systems (Falkinham III et al., 2001). NTM studied are similar in their high content of a particularly waxy, hydrophobic cell surface. This is a consequence of the presence of long chain (C40 - C80) mycolic acids (Brennan and Nikaido, 1995; Daffé and Draper, 1998). These waxy, hydrophobic, and impermeable cell envelopes often are invoked as the basis for antibiotic- (Rastogi et al., 1981) and disinfectant-resistance (Falkinham III, 2003; Taylor et al., 2000), a common property of all NTM. Resistance to chlorine and other disinfectants used in water treatment likely is responsible for the apparent enrichment of NTM in drinking water distribution systems (Falkinham III et al., 2001; Norton et al., 2004), one proven source of NTM infection (Falkinham III, 2011; Falkinham III et al., 2008; von Reyn et al., 1994). It is not known whether or not different water sources and treatment regimens influence the types of mycobacteria that occur in water distribution systems.

The waxy, hydrophobic surface of NTM cells also results in reduced transport of hydrophilic nutrients (Jarlier and Nikaido, 1990), resistance to hydrophobic antimicrobials (Rastogi et al., 1981), and surface adherence and biofilm formation (Steed and Falkinham, 2006; Torvinen et al., 2006; van Loosdrecht et al., 1987). One example of NTM enrichment in biofilm formation is seen in showerheads (Feazel et al., 2009). Biofilm formation is probably an important aspect of the enrichment of the slowly growing NTM in drinking water distribution systems and household plumbing. Hydrophobicity is also a determinant of NTM dispersion as aerosols (Angenent, 2005; Parker et al., 1983) and phagocytosis by amoeba (Cirillo et al., 1997) and protozoa (Strahl et al., 2001). In general, NTM are not viewed as “professional pathogens” such as M. tuberculosis. Rather, the properties that result in their successful proliferation and...
survival in the environment, contribute, as well, to their survival and propagation in human or other hosts. In fact, inclusion of NTM in the group of “amoeba-resistant bacteria” has been cited as a virulence factor (Loret et al., 2008).

**NTM Evolution and Genomic Variation**

Studies of bacterial genome composition and structure over the past decade powerfully influence the way that we need to consider the diversity of NTM and the relationships of environmental NTM to pathogenic varieties. Specifically, it has become clear that bacterial “species” do not constitute singular entities, rather, are comprised of closely related organisms that nonetheless can contain substantial genomic variation. For instance, the typical strain of *Escherichia coli* contains only about 4,500 genes, but the 20 sequenced *E. coli* genomes from different strains collectively contain about 18,000 genes; that is to say, 20-30% of the genes in any representative of that group of genomes are unique to that representative (Medini et al., 2005; Tenaillon et al., 2010). Such collective genome populations have been termed the “pangenome”, as it is likely that genes are readily shared among closely related bacteria in the environment, mediated by bacteriophage transduction, conjugation, or other mechanisms. Although environmental gene-exchange has not been demonstrated for NTM, the extensive differences seen among NTM in gene contents, presence or absence of specific insertion sequences, plasmids, or allelic markers in even limited genomic studies make the occurrence likely (Horan et al., 2006; Turenne et al., 2008; Wynne et al., 2011). Such would certainly be true for the NTM that are preferentially found in biofilms along with other bacteria. Thus, bacterial “species,” probably including *Mycobacterium* spp., have a far larger repertoire of genetic potential than present in any instance of the particular species (Vieira et al., 2011).

Genetic variation and complexity is a challenge to NTM classification and to understanding the pathogenesis of environmental NTM; particularly for the opportunistic NTM pathogens such as those listed in Table 3-2. Moreover, it is not clear that all environmental NTM are potentially pathogenic although some, for instance the many *M. avium* strains, seem to have a particular propensity for pathogenesis (Horan et al., 2006). Uncertainties regarding pathogenesis of different NTMs pose a significant barrier for developing a framework for risk assessment of NTM exposure. For example, which of the many genetic types of *M. avium* identified by PFGE (von Reyn et al., 1994), insertion sequence RFLP (Falkinham III et al., 2008) or repeated sequence polymorphism assays (Falkinham III, 2011) are of importance for risk assessment?

**Ecology of NTM and *M. avium***

**NTM Habitat and Ecological Niches**

NTM commonly occur in natural and engineered waters, biofilms and materials associated with household and work plumbing. Although indoor settings such as swimming and therapeutic pools, hot tubs, spas and other facilities are known as settings for exposure to NTM, the distributions of specific NTM and other mycobacteria are little studied. Typically >10% of municipal drinking water microbes are mycobacteria (Feazel et al., 2009); NTM are readily cultured from potting soils (De Groote et al., 2006). Because of their hydrophobic qualities, mycobacteria are particularly susceptible to water-air transport as aerosols (Angenent et al.,
2005; Blanchard and Syzdek, 1982). For instance, one molecular study of mycobacteria associated with aerosols in a therapeutic swimming pool found that 30-70 percent of aerosolized bacteria were mycobacteria, including NTMs (Angenent et al., 2005). Enhanced air exchange rates probably could combat risk of NTM exposure in such settings.

NTM have been shown to be amenable to studies of their behavior in pilot systems simulating drinking water distribution systems. For example, *M. avium* was introduced into a closed pilot drinking non-sterile water system and its ability to form biofilms in the presence and absence of different concentrations of organic matter (as humic acid) and disinfectant measured (Norton et al., 2004). Several valuable insights came from those measurements: 1.) a clinical isolate of *M. avium* was able to colonize, grow, and persist, 2.) the *M. avium* strain was capable of growing at an assimilable organic concentration (AOC) of ≥ 50 μg/L, and 3.) introduction of chlorine into the system led to an increase in *M. avium* colony counts as a proportion of total microbial counts. The understanding that disinfection selects for NTM in drinking water distribution and household plumbing is based not only on the observations made in that pilot system, but also is documented in other studies (Falkinham III et al., 2001).

Strains of *M. avium* isolated from humans have been shown to be competent biofilm formers (Carter et al., 2003; Williams et al., 2009). An in vitro study suggests that a *M. avium* strain’s ability to form biofilm is associated with pathogenicity and invasion of bronchial epithelial cells (Yamazaki et al., 2006). These reports suggest that *M. avium* strains able to successfully colonize drinking water distribution systems may present a chronic source of pathogen exposure.

**Predictors of NTM Occurrence**

Many factors appear to interact in complex and poorly characterized ways to support or inhibit *M. avium* growth in water pipes. Factors include: temperature, water flow, nutrients, pipe material and condition, and residual disinfectant. Reports exist for some of these factors, but none alone predict *M. avium* concentration at point of use. Concentrations of *M. avium* in water were significantly correlated with organic carbon concentrations (Falkinham III et al., 2001); *M. avium* with hot water (du Moulin et al., 1988); NTM with hot water at ≤ 50°C (Falkinham III, 2011); and NTM with plastic pipe material (Schulze-Röbbecke et al., 1992), although Norton et al. (2004) reports significant *M. avium* concentrations in water independent of pipe material.

**Resistance of NTM to Disinfection**

*M. avium* is more resistant to chlorine than indicator bacteria and survives in distribution systems despite ambient chlorine residual concentrations. *M. avium* grown in water is more chlorine resistant than the same strains grown in culture medium, and most strains appear to be more resistant to chloramine compared to free chlorine disinfection (Taylor et al., 2000).

However, NTM species vary in almost an order of magnitude in their ability to survive chlorine disinfection, with *M. fortuitum* exhibiting the most chlorine resistance among NTM evaluated (Le Dantec et al., 2002; Lee et al., 2010). It should be understood that all mycobacteria are at least 100-fold more resistant to chlorine and other disinfectants compared to *Escherichia coli* (Taylor et al., 2000). *M. avium* and *M. intracellularare* are less chlorine resistant than other NTM, but more so than *M. scrofulaceum* (Falkinham III, 2003). Pelletier (1988) reports no bacteriocidal effect at free chlorine concentrations at ≤ 0.15 ppm. Colonies of *M. chelonae* and
M. abcessus on PVC pipe surfaces have been reported to survive 7 days of exposure to 10 – 15 ppm free chlorine (Vess et al., 1993). Yet chlorine concentrations of 4 mg /L (4 ppm) represent the maximum residual disinfectant level allowed in distributed drinking water (EPA, 1998). Bohrerova and Linden (2006) report that M. terrae are resistant to disinfection when in clumps or aggregates in wastewater, but if disaggregated, are readily inactivated by free chlorine and UV treatment. This suggests that turbidity may be an important factor during inactivation of planktonic NTM in drinking water.

PHYSIOLOGY AND ECOLOGY OF P. AERUGINOSA

Physiology of P. aeruginosa

P. aeruginosa is a Gram-negative, non-spore forming, aerobic, rod shaped, motile (via a single polar flagellum), and free living prokaryote of the phylum Proteobacteria of which there are five classes (Alpha, Beta, Gamma, Delta and Epsilon proteobacteria) (Madigan et al., 2008). P. aeruginosa falls into the Gamma-Proteobacteria class, order Pseudomonales, family Pseudomonadaceae, genus Pseudomonas (based on 16S rRNA) (Bacteriology, 2005).

P. aeruginosa is an environmentally ubiquitous organism predominant in soil and water with the ability to survive in poor nutrient waters, even in distilled or deionized water (Penna et al., 2002). P. aeruginosa are extremely versatile in their metabolic capabilities and thus are the subject of interest in a variety of arenas, including bioremediation of xenobiotic compounds. Pseudomonas also serve as model biofilm-forming organisms for research purposes. Recent genome sequencing has revealed a remarkably large genome, which is congruent with their broad range of metabolic capabilities (Mathee et al., 2008).

Physiologic determinants of P. aeruginosa ecology include its ability to utilize over 75 organic compounds. It prefers organic acids and fatty acids as its source of carbon, but can use a wide range of carbon sources at very low concentrations (<100 ug/L) (van der Kooij et al., 1982) and can survive in deionized or distilled water (Warburton et al., 1994). Adaptation to low substrate concentration will vary by specific serotypes. It will not use polymers or lactose as a source of carbon, but can use multiple sources of nitrogen, preferring amino acids, organic acids and DNA as nitrogen sources. P. aeruginosa can be found in low nutrient (oligotrophic) environments, including aquatic environments of moderate salinity and in high nutrient (copiotrophic) environments.

Temperature, pH, and oxygen all influence P. aeruginosa growth. Optimum growth temperature in rich medium and suspended form is between 30 - 37°C, but P. aeruginosa can grow at temperature between 10-42 °C (Brown, 1957) and can grow in cold tap water (15°C) (van der Kooij et al., 1982). P. aeruginosa grows relatively well under microaerobic conditions (i.e., O₂ < 6 %). It is a facultative aerobe using oxygen as a terminal electron acceptor, but can utilize nitrate as a terminal electron acceptor under forming nitrogen gas (Bacteriology, 2005). P. aeruginosa fails to grow under acidic conditions (< pH 4.5) (Bacteriology, 2005).
Microbial Ecology of *P. aeruginosa*

**P. aeruginosa Habitat, Ecological Niches, and Importance of Biofilms**

Given its extreme versatility, *P. aeruginosa* is found in a variety of environments. Critchley and colleagues (2001) observed a significant relationship between water chemistry parameters and the quantity of biofilm detected. In filtered systems, inverse relationships exist between both water conductivity ($P < 0.05$) and alkalinity; positive correlation between water total organic carbon (TOC) and biofilm total biomass. Turbulence was inversely related to viable biofilm bacteria. Also, aqueous soluble phosphate concentrations may have an influence on biofilm formation and virulence in *Pseudomonas* spp. (Haddad et al. 2009).

Biofilms are largely viewed to be the major habitat of *P. aeruginosa* in engineered water distribution systems and premise plumbing. *P. aeruginosa* are renowned for their ability to colonize biofilm in plumbing fixtures (i.e., faucets, showerheads, etc.) and can be found within large buildings distribution system (Chaidez & Gerba, 2004; Rogues et al., 2007; Trautmann et al., 2008; Trautmann et al., 2005). *P. aeruginosa* grows in a highly structured biofilm with distinct architectural/chemical properties (Costerton et al., 1995).

Often used as model organism to study biofilm, the flagellar and twitching motility of *P. aeruginosa* are essentials to its ability to develop into a biofilm (O'Toole and Kolter, 1998). They also appear to play a key role in the second phase of the biofilm formation. One key characteristic is the abundant production of EPS by *P. aeruginosa*, helping it colonize surfaces and organize within a biofilm. It has also been observed to play a role in the increased attachment of other bacteria (e.g., *E. coli*) in nutrient rich broth, depending on the strain of *P. aeruginosa* (Liu, 2008).

The production of an EPS layer adds to the viability and impermeability of *P. aeruginosa* in the biofilm community. The EPS mechanism is largely responsible for its ability to colonize biofilm and the low nutrient requirement increases its viability. A recent study showed there are at least three polysaccharides (Psl, Pel and alginate) involved in the formation of biofilm by this organism and each plays a critical role (Ghafoor et al., 2011). The production of Psl is largely active during the planktonic state and mediates initial attachment to human tissue or a surface moving the organism to a sessile state. Psl is a “cellulose-like polymer” essential for the early stages of structural formation of a biofilm. The production of alginate contributes to decreased susceptibility of a biofilm to antibiotic therapies and host defense mechanisms (Ghafoor et al., 2011).

**Relationship Between P. aeruginosa and FLA**

In as much as many of the habitats occupied by *P. aeruginosa* are shared with FLA, it is not surprising that *P. aeruginosa* is an ARM as are *Legionella* and *M. avium* complex. Amoebae predation of *P. aeruginosa*, depends on the stage of biofilm formation as there is a succession of amoebae species as the biofilm matures, with early grazers feeding on suspended bacteria (Wang and Ahearn, 1997). Resistance of *P. aeruginosa* to grazing depends on the *P. aeruginosa* strain, with environmental strains being more resistant than mucoid laboratory strains, and the type of amoeba present (Weitere et al., 2005). *P. aeruginosa* grow within amoeba (Greub and Raoult, 2004). *P. aeruginosa* numbers in *Acanthamoeba polyphaga* in a synthetic drinking water were estimated at $4 \times 10^4$ CFU/amoeba (Hwang et al., 2011). Further, Matz et al. (2008) showed that
97% of *A. castellanii* were colonized by *P. aeruginosa* readily within 24 hours. The same was also observed for *Acanthamoeba* and *Echinamoeba* isolated in a hospital drinking water system, showing intracellular multiplication of *P. aeruginosa* (Michel et al., 1995).

*P. aeruginosa* can also be inhibitory to amoeba (Wang and Ahearn, 1997). *A. castellanii* growth and numbers were suppressed in an enriched suspended culture initiated with a ratio of >100 *P. aeruginosa* per amoeba cell. No toxicity was observed for ratios of amoeba/*P. aeruginosa* at ratio < 1, attributed to the toxicity of the excessive density of food source and cell association (Wang and Ahearn, 1997). In a flowcell coculture of a young biofilm of environmental and mutant *P. aeruginosa* inoculated with *A. castellani*, *P. aeruginosa* caused rapid (48 hours) lysis (63%), suggesting a Type III secretion system (T3SS), a quorum sensing independent inhibitory mechanism (Matz et al., 2008). Finally, Pickup et al. (2007) showed that *P. aeruginosa* was not toxic to *H. vermiformis* or *A. castellanii*, but it slowed the movements of the amoebas and their ingestion process.

**Other Microbial Ecological Relationships of P. aeruginosa**

*P. aeruginosa* is capable of antagonistic interactions with other microbes, thus widening its ecologic impact. Demonstrated examples of antagonism include production of 1.) siderophores (pyoverdine) to compete against other bacteria for iron present in the environment (Harrison 2008); 2.) antimicrobial compounds (Kerr, 1999); 3.)HQNO (Krausse et al., 2005); 4.) rhamnolipids (Haba et al., 2003); 5.) penazine (Kerr et al., 1999); and 6.) cyanide (Gallagher and Manoil, 2001). Although the antagonistic effects of *P. aeruginosa* against other microorganisms have been shown in medical and lab environments using suspended bacteria grown in nutrient rich conditions, there is only scarce data on the occurrence and relative importance of these effects in nutrient poor biofilm structures.

**PHYSIOLOGY AND ECOLOGY OF ACANTHAMOEBA**

**Physiology of Acanthamoeba**

**Acanthamoeba Life Cycle and Morphology**

*Acanthamoeba* spp. are often viewed as model host organisms in characterizing the life-cycle of ARMs. *Acanthamoeba* exists in two primary life stages, the trophozoite and the cyst (Figure 3-1). *Acanthamoeba* are named because of the spiny appearance of the trophozoites. During the trophozoite stage, *Acanthamoeba* actively phagocytize bacterial prey and reproduce via mitosis. *Acanthamoeba* enter the cyst stage in response to stressors, such as low nutrients, temperature shock, or presence of a disinfectant (Martinez and Visvesvara, 1997). Cysts range in length from 15 to 28 mm, whereas trophozoites are 15-45 mm with spine-like projections called acanthapodia (EPA, 2003), from which *Acanthamoeba* bear their name.

The genus *Acanthamoeba* consists of as many as 20 species classified into three groups based on cyst morphology (EPA, 2003). Group I *Acanthamoeba* are characterized by large cysts with rounded outer walls (ectocysts) that are clearly separated from the inner walls (endocysts). Group II *Acanthamoeba* cysts are smaller, with variable endocyst shapes. Group III *Acanthamoeba* cysts are even smaller than Group II cysts, with poorly separated walls. The endocyst consists of cellulose, while the exocyst consists of a wrinkly proteinaceous material.
The absence of cellulose in the trophozoite form can be useful for differentiating the two forms. Differentiating the two forms is important because the trophozoite is largely considered to be the pathogenic form, whereas the cyst plays a role in protecting bacterial pathogens from disinfectant and delivering the pathogens to the consumer.

**Acanthamoeba Identification and Classification**

*Acanthamoeba* belong to the Amoebozoa supergroup of Eukaryotes, which is phylogenetically defined based on both small subunit rRNA and actin protein sequences (Baldauf et al., 2000; Fahrni et al., 2003). Several species of *Acanthamoeba* are known to cause infections in humans, including: *A. astronyxis*, *A. castellanii*, *A. culbertsonii*, *A. divionensis*, *A. griffini*, *A. healyi*, *A. rhysodes*, *A. hatchetti*, *A. palestinensis* and *A. polyphaga*. Most are classified as Group II, except *A. culbertsonii* is Class III.

Identification of *Acanthamoeba* is relatively straightforward after they are grown in culture, but assignment of species is challenging (Stothard et al., 1998). Even if an *Acanthamoeba* isolate can be assigned to a species using rDNA sequence (Łanocha et al., 2009), that provides no indication of whether the isolate is pathogenic or not. *Acanthamoeba* isolates differ in the maximum temperature for growth (i.e., 30°-37° C), yet those isolates able to grow at 37° C were no more likely to be recovered from cases of AK, than those isolates with lower temperature maxima (Dagget et al., 1982). The pattern of proteins recovered from *A. castellani* and *A. polyphaga* by sonication differed as shown by gel electrophoresis, gel diffusion, or immunoelectrophoresis, but the differences are not linked to ecology or pathogenicity (Visvesvara and Healy, 1975).

**Ecology of Acanthamoeba**

*Acanthamoeba Habitat and Ecological Niches*

*Acanthamoeba* have been detected in air and soil (CDC, 2011a) and in a wide variety of natural waters (lakes, ponds, rivers, and streams) and engineered water systems, including premise plumbing (Thomas and Ashbolt, 2011). For example, the amoebae pathogens *Naegleria fowleri* (Marciano-Cabral et al., 2003) and *Acanthamoeba* spp. (Stockman et al., 2011) have been detected in well water (Blair et al., 2008), household waters and drinking water treatment plants (Hoffman et al., 2006; Thoma et al., 2008).

In addition to natural and engineered water systems, *Acanthamoeba* species have a cosmopolitan distribution and have been isolated from soils, dusts, drinking water, bottled water, shower water, swimming pools, therapy pools, dental units, cooling waters, and contact lens cases (Thomas and Ashbolt, 2011; Mergeryan, 1991). Over the period of 1990-1992, 2,454 water samples from 467 households were examined and *Acanthamoeba* were found in 238 (51%) of the households (Stockman et al., 2011). Showerheads (52%) and kitchen sprayers (50%) were the sites most likely to yield amoebae. That is most likely due to the fact that amoebae detection was higher in biofilms than water samples (Stockman et al., 2011). The absence of a flagellated stage for *Acanthamoeba*, is likely responsible for the fact that *Acanthamoeba* are more likely found on surfaces or attached to particulates, unlike *Naegleria* that has a flagellated stage and found in surface water (De Jonckheere, 1991). Such knowledge is useful to direct the
collection of field samples. *Acanthamoeba* are also known to sometimes colonize healthy individuals, without inducing disease (Chappell et al., 2001).

**Carbon Sources and Environmental Tolerance**

*Acanthamoeba* can grow on dissolved organic compounds or can grow on bacterial prey by phagocytosis. The fact that amoebae numbers in soils are correlated with bacterial numbers indicates that in soils, predation is the dominant mode of growth. A number of environmental constraints influence the ecology and behavior of *Acanthamoeba*. These include temperature, pH, salinity, oxygen concentration (Rodríguez-Zaragoza, 1994). For example, a higher fraction of *Acanthamoeba* isolates in water of high temperature are pathogenic compared to isolates from cold water (De Jonckheere, 1991). However, not all isolates with higher temperature-maxima for growth were pathogenic (Daggett et al., 1982). Seasonal abundances of FLA are related to water temperature. For example, high numbers of *Acanthamoeba* and *Naegleria* are found at the end of summer, suggesting that these microorganisms can persist under high temperatures and anoxic conditions (Rodríguez-Zaragoza, 1994).

**Resistance of Acanthamoeba Cysts to Disinfection**

The lack of influence of the presence or absence of chlorine on *Acanthamoeba* numbers or frequency of detection is probably due to cyst formation as the cysts are very resistant to disinfectants and other microbial inhibitors (De Jonckheere, 1991). The cyst’s disinfection resistance is likely responsible for the persistence of *Acanthamoeba* in contact lens cleaning solutions and cases and hence keratitis. Various strains of *Acanthamoeba* have been observed to survive the disinfecting agents in contact lens solution (Boost et al., 2012). It should be noted that enumeration or detection of *Acanthamoeba* from environmental samples, may reflect cysts, not growing cells (e.g., trophozoites) as trophozoites are susceptible to disinfection and many of the methods used to concentrate amoebae (e.g., filtration or centrifugation).

**PHYSIOLOGY AND ECOLOGY OF N. FOWLERI**

**Physiology of N. fowleri**

*N. fowleri* and other *Naegleria* spp.

*N. fowleri* is the only human pathogenic species in the genus *Naegleria*, which has been reported to contain at least 47 species (De Jonckheere, 2011). *Naegleria fowleri* is a free-living trophic amoebae that occurs naturally in soil and water. It grows well at temperatures between 25°C and 42°C. Although more than 30 species of *Naegleria* have been identified through DNA sequencing, *Naegleria fowleri* is the only species of *Naegleria* that causes diseases in humans (De Jonckheere, 2002). Thermotolerant species of *Naegleria* are known (e.g. *N. lovaniensis*), but are not pathogenic to humans. *N. australiensis* and *N. italica* cause infections in lab animals only.
**N. fowleri Life Cycle and Morphology**

*N. fowleri* has three stages in its life cycle: trophozoite, flagellate, and cyst. *N. fowleri* trophozoites are known to feed on *E. coli* and other Gram-negative bacteria and it is thought that *N. fowleri* trophozoites may be more likely to be found in a water body within the sediment layer, or possibly within biofilm formed at the air:water surface (e.g., an algal bloom) (Hsu et al., 2011). *N. fowleri* trophozoites measure 10-25 µm and have a single, round nucleus containing a large, centrally located nucleolus. *N. fowleri* is referred to as an ameboflagellate because trophozoites can transform into the flagellate stage in response to sudden changes in the environment (e.g., using flagella to move to areas with more ideal water conditions or to find food). *N. fowleri* trophozoites can transform into an environmentally stable cyst form during adverse conditions when the food supply becomes scarce or the water environment becomes stressful (e.g., due to low temperature). The *N. fowleri* cyst is usually spherical, measuring 7-14 µm.

Like other amoebae, *N. fowleri* can also act as a host to pathogenic amoeba-resisting bacteria. In biofilms of water distribution pipes, it has been observed that *Naegleria fowleri* acts as a reservoir and enhances the survival of *L. pneumophila* and other pathogenic microorganisms (Buse and Ashbolt, 2011; Marciano-Cabral et al., 2010).

**Ecology of N. fowleri**

*N. fowleri Habitat and Ecological Niches*

*Naegleria fowleri* is an environmental amoeba that occurs worldwide and is most often associated with warm fresh water. Cases of PAM usually occur during summer months when the ambient temperature is high and water temperatures are warm, but has also been found to be associated with geothermally heated water (e.g., hot springs) (Sheehan et al., 2003) and thermally enriched water bodies (e.g., thermal heating from power plants) (Jamerson et al., 2009). *N. fowleri* are often found in fresh water lakes and slow-moving rivers, either in the water column or in sediment, where it is thought that *N. fowleri* survives during cold weather conditions (Kyle and Noblet, 1985; Wellings et al., 1977). *N. fowleri* is sensitive to elevated levels of osmolarity and has not been recovered from seawater (Visvesvara et al., 2007).

Relatively little is known about the presence and ecology of *N. fowleri* in surface water bodies, and even less is known about the dynamics of these amoebas in ground water and drinking water systems. Beyond the association of PAM with exposure to warm fresh water, little is known about the environmental factors associated with the presence and ecological dynamics of *N. fowleri*. Part of this is due to the inherent challenges of understanding the ecology of an autochthonous opportunistic pathogen such as *N. fowleri*, but the knowledge gaps are also due to a relative lack of funding to support research on new techniques for sampling, detection, quantitation, and genomic analysis of *N. fowleri*.

**N. fowleri in Potable Water**

Few studies have investigated the prevalence and dynamics of FLA, including *N. fowleri*, in drinking water distribution systems or associated premise plumbing. However, from the
studies available, it appears that FLA are common in drinking water systems, including systems performing primary and secondary disinfection (Stockman et al., 2011; Thomas and Ashbolt, 2011). One study reported detecting various FLA, including Acanthamoeba spp. and Naegleria spp., in tap water samples collected from a chlorinated drinking water system in the U.S. upper-midwest, but N. fowleri was not specifically detected (Marciano-Cabral et al., 2010). These researchers suggested that the lack of N. fowleri detections may have been due to low water temperatures associated with the geographic location and seasonal time frame of the study. Other research also suggests that seasonal water temperatures may affect FLA prevalence and concentrations in drinking water systems (Thomas and Ashbolt, 2011). It is not clear how FLA, including N. fowleri, colonize piped water systems, but it appears that biofilm likely plays a role (Storey et al., 2004). Biofilm may present a favorable niche for N. fowleri by providing a ready source of food (e.g., bacteria) and protection from disinfectants in the drinking water.

There have been reports of PAM associated with drinking water systems and N. fowleri has been detected in drinking water, including tap water and well water (Blair et al., 2008; Bright et al., 2009; Yoder et al., 2012). In Australia, numerous PAM patients became infected by N. fowleri in the 1970s and early 1980s after swimming in backyard pools, dunking their heads in bathwater, or washing their noses in water from public water supplies that were drawn from surface water and distributed in overland pipelines for long distances. While in the overland pipelines, the water was heated by the sun, which created ideal temperature conditions for N. fowleri growth and also lead to the decay of residual chlorine in the water. Implementation of improved drinking water distribution system chlorination was reported to effectively control N. fowleri (Dorsch et al., 1983).

In Arizona in 2002, N. fowleri was detected in multiple household drinking water samples associated with a cluster of two PAM cases in which the boys were thought to have been exposed while playing in bath water (Marciano-Cabral et al., 2003). The public water supply associated with the Arizona PAM cases was from a ground water source and was not routinely chlorinated (Bright et al., 2009).

In Louisiana in 2011, two separate cases of PAM in adults were associated with nasal rinsing using tap water from public water supplies that used chloramination for secondary disinfection (Yoder et al., 2012; LA DHH 2011). N. fowleri was detected by real-time PCR in household water samples from the residences of both individuals. N. fowleri trophozoites and cysts have been reported to be sensitive to chlorine disinfection (Dorsch et al., 1983), but little information is available to evaluate the effectiveness of monochloramine for inactivating N. fowleri. One study, in which N. lovaniensis was studied as a surrogate for N. fowleri, reported that an 8x higher dose of monochloramine was needed to achieve the same level of N. lovaniensis inactivation compared to sodium hypochlorite (Ercken et al., 2003).
CHAPTER 4: DETECTION METHODOLOGY

OVERVIEW

The ability to provide rapid, reliable, sensitive, and quantitative detection of live and infectious pathogens remains as a grand challenge for OPPPs and traditional pathogens alike. Thus, the purpose of this section is to assess and compare existing laboratory techniques, and to identify a viable path forward for addressing OPPP analytical challenges.

At the heart of the challenge for pathogen detection methodology is the yet unresolved dichotomy of culture-based and molecular-based methods. This is a topic explored in depth by the recent Water Research Foundation publication, *Synthesis Document on Molecular Techniques for the Drinking Water Industry* (Nocker et al. 2009). Current regulatory and action limits are typically derived from culture-based methods, as they have been in place much longer and most existing standardized techniques are culture-based. A primary advantage of culture-based techniques is their ability to confirm the presence of live pathogens. Unfortunately, there are no universal standards for culturing opportunistic pathogens, and the standards that exist are specific to different countries or organizations, such as the International Organization for Standardization (ISO). Culture-based detection also tends to be costly, labor intensive, time-consuming and requires specialized expertise in practice. Furthermore, culture-based techniques tend to be geared towards single strains or serotypes, and, as noted in above sections, awareness of the significance of the pathogenicity of a broader range of related strains is growing for many OPPPs. Indeed, as we enter the metagenomic era, the microbial “species” concept is altogether eroding. In its place, the “pan-genome” concept-- the notion that pathogenicity is defined by the presence of a key sub-set of genes among closely related organisms-- is rapidly gaining support (Medini et al., 2005). Thus, the urgency for the development of appropriate, and ideally standardized, molecular tools is stronger than ever.

Molecular tools currently present their own strengths and limitations. The term “molecular” generally refers to methods that target DNA, RNA (i.e., nucleic acids), or proteins, which enables the ability to hone in on precise sequences specific to the pathogen of interest. This is a major advantage over the ambiguity presented by culture-based methods. Furthermore, by circumventing the need for culturing, molecular methods provide a means to overcome the fact that the vast majority of microbes are not amenable to growth on a Petri dish. Thus, molecular techniques can detect cells in the VBNC status, which remains a critical challenge for the characterization of OPPPs. Unfortunately, this capability of molecular techniques remains as a double-edged sword because they generally target all nucleic acids, including that of dead cells, living cells, and even extracellular DNA that has been released to the environment. For this reason, RNA is considered to be a more ideal target than DNA, as it has a much shorter half-life upon cell death. However, this same characteristic often makes RNA an unrealistic lab target, especially for drinking water samples that are already challenged by extremely low microbial numbers. Recently, the addition of chemicals, such as ethidium monoazide (EMA) and propidium monoazide (PMA), which enters cells with damaged membranes and binds to nucleic acids, has offered a promising means to restrict certain downstream analysis only to living cells with intact membranes (Delgado-Viscogliosi et al., 2009). Methods targeting proteins (proteomics) are also gaining ground and are promising given that expressed proteins are the ultimate manifestation of cell function.
Molecular techniques are also generally employed as multi-step procedures, each associated with its own limitations and overall recovery efficiencies. First and foremost is nucleic acid extraction, in which cells are broken open and the DNA or RNA is isolated and purified for further analysis. Nucleic acid extraction is not 100% efficient and can be biased towards certain cell types and nucleic acid sequences (Feinstein et al., 2009). Nucleic acid extraction is especially challenging for drinking water, which must be concentrated significantly to yield sufficient cells for extraction. These concentration steps, such as filtering or freeze-drying, are subject to biases of their own and also can concentrate other constituents present in the water that may interfere with downstream analysis (Schober and Kurmayer, 2006; Hill et al. 2010). The application of polymerase chain reaction (PCR) for drinking water pathogen detection has grown exponentially since it was first invented in the late 1980s. PCR is a means of selectively amplifying target DNA sequences of interest (e.g., signature sequences specific to the pathogen of interest) to enable downstream detection. PCR is particularly attractive for drinking water analysis because it has the lowest detection limit of any known method. For example, under ideal conditions, it is possibly to detect one DNA molecule originating from a pathogen of interest. PCR, however, can be inhibited by other water constituents, particularly those subject to co-concentration during nucleic acid extraction, leading to false negatives. A variety of common drinking water constituents, including divalent metals, humic acids, and disinfectant residuals are known to interfere with PCR. (Hill et al., 2010) Some such interferences may be dealt with if known. For example, higher disinfectant residuals (i.e. >1.0 mg/L) do interfere and should be neutralized before proceeding (Lee et al., 2011).

Also important to note is that PCR in and of itself is not generally quantitative, although numerous PCR adaptations have evolved to address its shortcomings, including quantitative polymerase chain reaction (qPCR). As noted in the following sections, qPCR is gaining popularity for the detection of many pathogens due to its extremely sensitive and quantitative nature. Not all molecular methods require DNA extraction and PCR. For example, fluorescence in situ hybridization (FISH) and flow cytometry employ fluorescently-labeled nucleic acid probes capable of entering intact cells and binding to targets. Cells may then be detected and enumerated by epifluorescence microscopy or using a flow cytometry. These techniques circumvent the disadvantages of DNA extraction and PCR, but the trade-off is higher detection limits.

A third class of techniques also noted as relevant are more general “assay” type techniques, which target unique aspects of the cell’s phenotype. Such assays consider characteristics such as staining, morphology of cell cultures, biochemical composition, serology, infectivity and antibiotic susceptibility. Phenotypic assays, such as immunoassays, are typically aimed at rapid screening of at risk samples in addition to providing a second line of evidence to confirm detection of live, virulent pathogens isolated by culture-based techniques. As a sub-class of phenotypic techniques, characteristic morphological features may be identified by microscopic examination of pathogens, which is particularly common for the protozoan pathogens. In general these techniques have limited specificity, but still have utility as screening tools. Other emerging techniques include biosensors and protein characterization.

The microbial physiology and ecology sections above highlight distinct features of OPPPs, which may be exploited in terms of methodology, but may also need to be taken into special consideration for their detection. For example, the amoebic OPPPs emphasized in this document, *Acanthamoeba* and *N. fowleri*, possess distinct characteristics impacting their sample processing, cell culture, and DNA extraction relative to the bacterial OPPPs. In general, there is
a need for development and application of methods for broader surveys of FLA so that their true role in enhancing the risk of amoeba resisting pathogens may be estimated (Thomas and Ashbolt, 2011). Fundamental to all detection methods, culture-based and molecular alike, is the sampling and monitoring technique, which can profoundly influence the ultimate conclusions of a study.

For each pathogen discussed in the following sections we highlight specific aspects unique to their detection methodology, in particular highlighting the following:

• Sample collection and handling
• Sample concentration and pre-treatment
• Nucleic acid extraction
• Detection limits
• Interferences, including PCR inhibitors
• Quantitative capabilities
• Status of standardized methods

**L. PNEUMOPHILA**

**Sampling and Monitoring Considerations**

There are no consensus protocols for the selection of *Legionella* sampling sites or sampling frequency (Lucas et al., 2011). Considerable variability in recovery of legionellae from repeated sampling of sites, even seeded tap water, has been reported.

A CDC publication, *Procedures for the Recovery of Legionella from the Environment* (CDC, 2005), includes limited guidelines based on a protocol developed during the investigation of a Legionnaires’ disease outbreak in a healthcare facility (Barbaree et al., 1987). The protocol advises that specific sample locations should be determined based on “epidemiologic data proving an association between patients and possible exposure to aerosols containing the agent,” and lists eight general categories of locations from which specimens should be collected:

• potable water outside or on boundary of the property;
• general potable water systems;
• pharmacy;
• air compressor systems;
• potable water final distribution outlets (such as showers and ice machines);
• air-conditioning systems;
• whirlpools; and
• “other,” such as decorative fountains.

To the extent *Legionella* sampling of building water systems is practiced, it is carried out almost exclusively by two complementary methods:

• swab samples (Rioux et al., 2003)
• water samples (CDC, 2005)

Swab samples are collected prior to water samples, in order to avoid loss of biofilm during collection of water samples. First, the aerator, showerhead, etc. are removed. Sterile
polyester swabs with wooden shafts are used to swab the surfaces of the aerator and the interior of the faucet. For water samples, the tap is opened and a 1-liter water sample is collected immediately in a sterile wide-mouth screw cap polypropylene plastic bottle. If the water source has been recently treated with an oxidizing biocide (e.g., chlorine, chlorine dioxide), a reducing agent such as 0.1N sodium thiosulfate is added to each sample to neutralize the disinfectant. Though 1-liter is the norm for potable water samples, up to 10 liters may be collected for subsequent concentration, especially if microbial concentrations are believed to be very low. Samples as small as 50 ml are frequently taken from cooling towers and other non-potable sources that are not expected to be concentrated. All swab and water samples are transported to a testing laboratory in insulated, opaque coolers to avoid inactivation of Legionella by extreme temperature and light. Samples that will not reach the laboratory within 24-72 hours are supposed to be refrigerated during shipment, but never frozen. Samples that reach the laboratory but cannot be processed within 72 hours of collection are refrigerated. Swabs are submerged in 3-5 ml of water or saline taken at the same time to prevent drying during transport. Recent studies suggest that sample holding time may have profound effects on the results of subsequent microbial assays (McCoy et al., 2011).

Sample Concentration Procedures

Environmental samples, especially in potable water, typically are dilute and contain a relatively small number of legionellae compared to competing microbial flora. Techniques used to concentrate samples for Legionella spp. detection and quantification include membrane filtration, centrifugation and selective collection of legionellae on antibody-coated magnetic media (Allegra et al., 2011). In addition, because Legionella have greater acid tolerance and thermal resistance than many freshwater bacteria, environmental water samples are often acidified and heated prior to evaluation (Leoni and Legnani, 2001). Recently, researchers have reported use of a 2-step procedure employing immuno-magnetic separation followed by density gradient centrifugation to isolate and concentrate Legionella-containing vacuoles (Urwyler et al., 2010). For downstream recovery of nucleic acids, freeze-dry concentrating is commonly employed instead of or as an additional step after membrane filtration (Schober and Kurnmayer, 2006).

Culture-Based Techniques

Legionellae are fastidious, slow-growing bacteria that require iron and an amino acid, L-cysteine, for growth. The standard culture techniques employed for the detection of Legionellae are complex, require specialized expertise and are difficult to replicate. Primary isolation of Legionella is usually carried out by spread plating samples on a defined agar medium containing buffered charcoal yeast extract (BCYE) and supplemented with L-cysteine (ISO Method 11731-2, 2004). The BCYE aids in scavenging toxins and radicals, as Legionellae are extremely sensitive to toxins and oxidative stress. Controls are plated on matched media that is lacking the supplementary nutrient. Antibiotics and protein biosynthesis inhibitors typically are added to the BYCE to reduce the numbers of competing bacterial flora and yeasts. All cultures, including controls, are incubated at 35°C in a humidified atmosphere of 2.5% CO₂ in air. After 72 to 96 hours, the cultures are examined macroscopically with a dissecting microscope; oblique lighting is used to detect bacterial colonies resembling Legionella, which are typically convex and round

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with entire edges. The center of a *Legionella* colony is usually bright white with a textured “cut-glass like” or speckled appearance. The white center of the colony is often bordered with blue, purple, green, or red auto-fluorescence. Each suspect colony is aseptically picked onto plate that has not been supplemented with nutrient. The inoculated portions of each plate are manipulated with a sterile loop to provide areas of heavy growth, and incubated for 24 hours. If no growth is detected after 24 hours, incubation is continued for an additional 24 hours. Colony picks that grow only on nutrient-supplemented BCYE agar are presumptive *Legionella* species.

A major challenge of *Legionella* isolation is the co-culture of non-*Legionella* bacteria, which may interfere with identification and enumeration. Selective pre-treatment steps (see Section 4.2.2) are used to try to eliminate background contamination. However, pre-treatment is not 100% efficient and can also reduce the recovery of *Legionella* (Leoni and Legnani, 2001). Molecular methods for speciation (e.g., *mip* or 16S sequence) are increasingly used. Because *Legionella* are relatively slow-growing bacteria, negative plates should be incubated for an additional period and re-examined.

The media and methods used to cultivate *Legionella* were designed to be supportive for growth of *L. pneumophila* serogroup 1 and may not be optimal for other *Legionella* species or serogroups. Approaches for broader cultivation of the genus have been reported. Culture of some non-*pneumophila Legionella* species requires supplementation of the agar with bovine serum albumin (Morrill et al., 1990). *L. micdadei* and several strains of *L. bozemanii* show a preference for BCYE with 1.0% albumin (ABCYE) over standard BCYE. Significant variation in the effectiveness of culturing methods has been observed across sample types and *Legionella* concentration (Leoni and Legnani, 2001; De Luca et al., 1999).

The Environmental *Legionella* Isolation Techniques Evaluation (ELITE) Program has been invaluable in assessing the culture-based techniques for *Legionella* detection and enumeration. ELITE is a proficiency-testing scheme for U.S. laboratories that culture *Legionella* from environmental samples. CDC recently reported results of accuracy and precision of *Legionella* isolation by U.S. laboratories in the ELITE program. The results indicated that U.S. laboratories are generally capable of a qualitative assessment of environmental samples for the presence of legionellae but that quantitation displays significant inter- and intra-laboratory variability (Lucas et al., 2011). Damage to *Legionella* during sample transport and concentration, the prevalence of the VBNC organisms, and the limited recovery *Legionella* present within protozoan hosts (Thomas and Ashbolt, 2011) are thought to play a role in inconsistencies obtained using culture based methods and their tendency to underestimate total *Legionella*.

Amoebal co-culture is an alternative to standard *Legionella* culture methods, in which *Legionella*, VBN and LLAP in environmental samples reportedly can be successfully cultivated in protozoan hosts, such as *Acanthamoeba* (La Scola et al., 2001; Rowbotham, 1983; Scola et al., 2004; Seno et al., 2006). Similar results have been reported for co-culture of other fastidious waterborne pathogens that parasitize protozoan hosts and pose similar challenges to cell culture methods (Lamrabet et al., 2012). Amoebal co-culture shares with standard *Legionella* culture certain inherent drawbacks: both are complicated, time consuming, laboratory-based procedures that rely on the skill of the practitioner and take up to two weeks to produce definitive results.
Molecular and Serological Methods

Culture-independent methods used to evaluate environmental samples are substantially limited to immuno-hybridization techniques and variations of PCR using Legionella-specific DNA templates. However, methods used for characterizing isolates and clinical samples (but not applied directly to environmental samples) merit mention. Molecular methods used for typing Legionella isolates include serotyping, monoclonal antibody (MAb) subtyping, isoenzyme analysis, protein and carbohydrate profiling, plasmid analysis, restriction endonuclease analysis, restriction fragment length polymorphism (RFLP) analysis of rRNA (ribotyping) or chromosomal DNA, amplified fragment length polymorphism analysis, restriction endonuclease analysis of whole-cell DNA with or without pulsed-field gel electrophoresis (PFGE), arbitrarily primed (AP) polymerase chain reaction (PCR), repetitive element (REP) PCR, and infrequent-restriction-site PCR (Manual of Clinical Microbiology, 2003). Many of these techniques, applied alone or in combination, have shown to be useful for characterization of Legionella spp.

Several comprehensive reviews of molecular methods for the detection and enumeration of Legionella have recently been published (Aw and Rose, 2011; Tronel and Hartemann, 2009). Molecular methods promise to overcome limitations of culture-based methods by offering rapid, sensitive, unambiguous quantitative detection of Legionella (Merault et al., 2010). PCR assays have been evaluated for environmental sources and are commercially available. qPCR assays have also been applied for detection of Legionella in environmental water samples (Merault et al., 2010; Nazarian et al., 2008). Clinical diagnostic tests for Legionnaires’ disease using molecular methods, such as PCR also have been developed but are not yet been approved by the U.S. Food and Drug Administration (FDA).

Legionella culture and PCR results typically do not correlate. Generally, PCR shows a consistently higher negative predictive value (80–100%) than culture (Tronel and Hartemann, 2009). PCR and qPCR methods generally have a higher detection rate and indicate higher densities of Legionella than culture-based techniques. This owes to the amplification by PCR of nucleic material from VBNC Legionella and of DNA extracted from Legionella within amoeba hosts, with additional contribution from amplification of DNA from nonviable sources (e.g., cell fragments). Discrepancies also have been attributed, in part, to constraint of nutrients or stress from disinfectants (Türetgen and Cotuk, 2007; Mogoa et al., 2010; Turetgen, 2008). In general, the species probe (for L. pneumophila only) performs better than the genus probe (for all species of Legionella), especially in its specificity (Nazarian et al., 2008).

Quantitative PCR methods targeting Legionella (Maurin et al. 2010) employ fluorescent dyes (e.g., SYBR Green) or other specialized reagents and offer extremely high sensitivity and results within 1-2 hours. PCR assays of Legionella have centered principally on three main gene targets, the macrophage infectivity potentiator (mip) gene (Ratcliff et al., 1998), the 16S ribosomal RNA gene (16S rRNA)(Stolhaug and Bergh, 2006), and the 23S-5S rRNA gene spacer region (Yang et al., 2010; Herpers et al., 2003). The mip gene is thought to be a unique biomarker to Legionella and enables its pathogenicity to both amoeba and human macrophages. The 16S rRNA gene and the 23S-5S gene spacer region represent phylogenetic markers. The publication of complete Legionella genome sequences is enabling the use of new target genes and considerable enhancement of molecular diagnostic tools (Yang et al., 2010). PCR holds the potential to detect reliably all Legionella species, including LLAP and VBNC organisms, with a high degree of sensitivity and selectivity. The primary drawback of molecular techniques has been to inability to limit results to viable organisms, i.e., those presumably able to cause
infection. Other challenges include identifying and correcting for PCR inhibitors that may be present in environmental samples.

Recent groundbreaking work has employed selective nucleic acid intercalating dyes, ethidium monoazide (EMA) (Delgado-Viscoglioso et al., 2009) and propidium monoazide (PMA) (Yáñez et al., 2011). PMA selectively penetrates membrane-compromised cells and irreversibly binds to DNA, rendering it unsuitable for PCR; this restricts PCR detection to microorganisms with intact cell membranes. In combination with qPCR, these methods show significant promise for the detection and enumeration of viable legionellae with resolution at the sub-species level (Chen and Chang, 2010). However, PCR technology is evolving rapidly and still requires the development of standardized consensus protocols.

Molecular techniques may be combined with culture-based techniques to confirm specificity. For example, sequence based typing (SBT) (Gaia et al., 2011; Gaia et al., 2003) is a convention for sub-typing Legionella. DNA from L. pneumophila colonies is extracted, amplified, and sequenced by PCR using both forward and reverse primers. Sequences, comprising a series of seven allele numbers, are assigned in accordance with a standardized protocol. Having the exact sequence in a standardized format facilitates complete inter-laboratory comparisons. The primary aim of SBT is to provide a portable consensus method for the epidemiological typing of Legionella isolates in order to facilitate outbreak investigations.

Phenotypic Assays

A number of immunoassay methods have been developed for detection and identification of Legionella. These methods are often used for early, presumptive identification of bacteria grown in cell cultures, ahead of microscopic identification. Direct immunofluorescence assays (DFAs) for L. pneumophila, which use antibody conjugated with a fluorochrome stain, are widely employed. Results are dependent on the skill and experience of the technician. One validation study indicated that DFA provided good sensitivity for Legionella and correlated well with a PCR detection assay (Lye et al., 1997). Lateral flow immunoassay, the basis for urinary antigen tests for L. pneumophila (Helbig et al., 2006) has been adapted for evaluation of environmental water samples. In practice, the sample flows along a solid substrate, generally by capillary action. The sample reagent mixes with the sample and transits the substrate encountering lines (zones) that have been pretreated with an antibody or antigen. If the target analyte is present, the colored reagent will bind at the test line or zone, and is visible. Evaluations have shown that performance of this technique corresponds well with that of latex agglutination (Helbig et al., 2009). Products incorporating lateral flow immunoassay are being promoted commercially for immediate analysis of environmental water samples in the field. These are not intended to be used for screening, not as a substitute for culture, and should not be the sole basis for reporting final results.

Latex agglutination is another method used to confirm Legionella cultivated from environmental samples (Reyrolle et al., 2004; Wilkinson et al., 1990). Each of several reagents is sensitized with Legionella-specific antibodies. Suspect colonies are emulsified and mixed with each latex reagent separately. In the presence of homologous antigens, the latex particles agglutinate to give a clearly visible positive reaction in a few minutes. However, interpretation of the results can be highly subjective, and different serogroups of L. pneumophila can cross-react, confounding results and making interpretation difficult (Wilkinson et al., 1990).

One
study indicated that the latex agglutination test provided relatively good sensitivity (85.7%), compared to PCR targeting 16S rRNA or rpo genes (Yong et al., 2010).

Although Gram negative, legionellae stain poorly in standard Gram procedures, likely owing to the high lipopolysaccharide content of its outer cell membrane. Alternatives (e.g., silver and Gimenez stains) (Roy, 1983; Greer et al., 1980) and modified staining procedures have yielded somewhat better results.

Microscopy is used for identification of Legionella, often in conjunction with staining. Optical reflectance and fluorescence microscopy have long been used to magnify two-dimensional images of colonies grown in cell culture. Recent advances in confocal laser scanning microscopy (CLSM) have enabled high-resolution, three-dimensional imaging of Legionella, with elucidation of individual structures, proteins and other molecules of interest, and their spatial relationships. CLSM capabilities include dynamic imaging, allowing observation of cellular activity (e.g., protein expression) in real time (Dailey, M. et al. 2006).

A number of spectroscopy methods have been used to characterize Legionella, based on detection of structural and functionally expressed surface proteins. Variations of Raman spectroscopy are used to measure detailed aspects of microbial proteins (Benevides et al., 2004) and essential lipid-protein interactions, (Ashton et al., 2011) Optical waveguide lightmode spectroscopy (OWLS) is reportedly capable of rapid, real-time detection of L. pneumophila in water samples (Cooper et al., 2009). Recent work has demonstrated matrix-assisted laser desorption/ionization-time of flight mass spectroscopy (MALDI-TOF) to identify “Legionella at the species level, reliably and with a high-degree of accuracy” (Fujinami et al., 2011; Gaia et al., 2011).

Magnetic immunoassay (MIA) is a relatively new method that utilizes paramagnetic particles conjugated to specific antigens or antibodies. The conjugated particles attach to the analyte and are detected by an instrument that measures changes in magnetic field. The signal is reportedly proportional to the analyte in the sample. The method is compatible with immunomagnetic separation techniques. The relatively limited data published on the use of MIA for Legionella is promising (Nikitin et al., 2007).

Methodology Gaps for L. pneumophila

A summary of available methodology for L. pneumophila is provided in Table 4-1. Methodology gaps are described in the following paragraphs.

Relating culture-based and molecular-based results

Several investigators have compared traditional culture methods and qPCR in the quantification of Legionella in varying kinds of water samples, including hot water samples (Bonetta et al., 2010; Morio et al., 2008), cooling tower water samples (Lee et al., 2011; Yaradou et al., 2007), distribution drinking water samples (Wullings et al., 2011), and spa water (Guillemet et al., 2010). Although a common trend has been identified—i.e., that qPCR yields a consistently higher estimate of Legionella than culture—there is otherwise little or no correlation between the methods. There are some studies that reported less method-to-method difference for cold/hot water samples compared to cooling tower water samples (Yaradou et al., 2007; Lee et al., 2011), leaving open the possibility that there may be some future value in continuing culture-based assays, to be used as a complement to emerging molecular methods.
**Sampling methods**

Criteria for selecting sampling sites, especially in building water systems, have been substantially neglected. Sampling, as currently practiced, does not take fully into account the microbial ecology of building water systems or the way in which *Legionella* are released from biofilm. While current sampling methods can help produce important qualitative information, the value of the information for quantitative use is minimal. Research needs include:

- Development of criteria for determining the number and location of sample points.
- Development of criteria for determining the frequency and duration of sampling.
- Determination of the relationship between colonization of premise plumbing and the release of *Legionella* in bioaerosols.
- Development of methods that account for *Legionella* contained in parasitized protozoa and protozoan vacuoles.
- Determination of the effects of handling, transport, and holding times on *Legionella* environmental samples.

**Assay methods**

Methods are critically needed that consider the essential role that protozoa play in *Legionella* virulence, persistence, and transmission. Metagenomic approaches may also be of value for better identifying pathogenic strains and characterizing their ecological roles.

**NTM AND *M. AVIUM***

Rates of NTM isolation may be affected by each clinical care provider’s diagnostic index of suspicion and each laboratory’s capacity to detect NTM. Multiple European countries were surveyed about NTM isolations during the 1970s to 1996; isolation numbers and laboratories reporting NTM isolation increased dramatically over time (Martín-Casabona et al., 2004). As laboratory capabilities to detect multiple NTM species increases, and as health care practitioners are increasingly likely to suspect NTM infections, numbers of isolations increase; this is very difficult to differentiate from an increase in the prevalence of occurrence in the population.

Surveys of NTM occurrence in water are typically performed as research activities and may be free of some the biases associated with the clinical activities detailed above. However, pretreatment of water samples and laboratory culture methods and conditions are potential sources of biases and may determine which *Mycobacterium* spp. are detected and subsequently reported (Thomson et al., 2008).

**Field Sampling Techniques**

NTM are hydrophobic, biofilm-loving organisms. These properties emphasize the importance of appropriate biofilm sampling for their recovery. Because the occurrence of NTM in distribution systems and household plumbing is not uniform, it is important to collect several samples; at least 10 are recommended to ensure isolation or detection of NTM (Falkinham III, 2011). Biofilm (swab) samples yield a higher frequency of NTM than water samples, as NTM prefer surface attachment rather than suspension in water (Falkinham III, 2011). Relatively large
volumes of water (500 mL concentrated to 2 mL in the laboratory) are necessary to ensure isolation of NTM, as culturable numbers in water are low (Falkinham III et al., 2001). A surface (e.g. inside a showerhead, (Feazel et al., 2009) can be swabbed and the swab frozen or held moist with sterile water for transfer to a laboratory. Filters, for example granular activated carbon (GAC) in point of use water filters, are also good sources of NTM as the GAC traps NTM and nutrients where they can grow. NTM grow and die slowly and are resistant to a wide range of environmental temperatures (5-50° C), so little change in NTM numbers will occur during shipping. Samples for DNA or other molecular analyses should be transported frozen or in the cold to prevent overgrowth by non-NTM microbes.

Table 4-1
Summary of Available Methodology for *L. pneumophila*.

<table>
<thead>
<tr>
<th>Method</th>
<th>Target</th>
<th>Assay Performance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td><em>Legionella</em> spp.</td>
<td>LOD: 10 CFU mL⁻¹</td>
<td>Lucas et al., 2011</td>
</tr>
<tr>
<td>Culture French</td>
<td><em>Legionella</em> spp.</td>
<td>LOD: 50 CFU L⁻¹</td>
<td>Parthuisot et al., 2011</td>
</tr>
<tr>
<td>Standard AFNOR</td>
<td></td>
<td>LOQ: 250 CFU L⁻¹</td>
<td></td>
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<tr>
<td>T90-431</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amoebal co-</td>
<td><em>Acanthamoeba</em> and</td>
<td>LOD: &lt;1 CFU mL⁻¹ after 72 hr</td>
<td>Seno et al., 2006</td>
</tr>
<tr>
<td>culture</td>
<td><em>L. pneumophila</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>qPCR</td>
<td><em>Legionella</em> spp., <em>L. pneumophila</em></td>
<td>LOD: 190 - 750 GU L⁻¹</td>
<td>Lee et al., 2011</td>
</tr>
<tr>
<td></td>
<td><em>L. pneumophila</em> serogroup 1</td>
<td>LOQ: 940 - 3750 GU L⁻¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>LOD: 80 GU L⁻¹</td>
<td>Merault et al., 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LOQ: 480 GU L⁻¹</td>
<td></td>
</tr>
<tr>
<td>vPCR w/EMA</td>
<td><em>L. pneumophila</em></td>
<td>LOD: 250 GU L⁻¹</td>
<td>Delgado-Viscogliosi et al., 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VBN: 99.9% reduction of non-viable cells</td>
<td></td>
</tr>
<tr>
<td>vPCR w/PMA</td>
<td><em>L. pneumophila</em></td>
<td>LOD: 660 GU L⁻¹</td>
<td>Yáñez et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VBN: 99.9% reduction of non-viable cells</td>
<td></td>
</tr>
<tr>
<td>IMS</td>
<td><em>L. pneumophila</em> serogroup 1</td>
<td>LOD: 10-100 cells L⁻¹</td>
<td>Füchslin et al, 2010</td>
</tr>
<tr>
<td>IMS w/Flow</td>
<td><em>L. pneumophila</em> serogroup 1</td>
<td>LOD: 500 cells L⁻¹</td>
<td>Füchslin et al, 2010</td>
</tr>
<tr>
<td>cytometry</td>
<td></td>
<td>52.1% recovery</td>
<td></td>
</tr>
<tr>
<td>IMS w/qPCR</td>
<td><em>L. pneumophila</em></td>
<td>LOD: 6.85 genomic copies</td>
<td>Yañez et al, 2005</td>
</tr>
<tr>
<td>Fluorescence</td>
<td><em>L. pneumophila</em></td>
<td>25-70% sensitivity and &gt; 99% specificity.</td>
<td>Health Protection Agency (UK), 2009</td>
</tr>
<tr>
<td>Microscopy</td>
<td></td>
<td>Does not accurately quantify.</td>
<td></td>
</tr>
</tbody>
</table>

Collection of aerosols for analysis of NTM contents is complex because of the need to collect the contents of a sufficient amount of air for analysis, typically hundreds to thousands of liters. Simple filtration of air is not effective because of flow rate limitations and electrostatic build-up that can result in filters becoming repulsive for bacteria (Pasenen, 2001; Willeke, 1995).
One popular method for collecting aerosols for culture is the Anderson Cascade sampler, with which air is drawn through size-graded chambers with culture plates (Anderson, 1958; Pasenen, 2001). This method, however, is not useful for molecular or chemical analyses. For studies such as these, fluid impingers offer more versatility (Lin et al., 1999). With fluid impingers, air is drawn through specially designed chambers containing a wetting fluid that scrubs particles from the air. Several types of fluid impingers, with varying efficiencies, are commercially available (Lin et al., 1999). Examples are the SKC BioSampler impinger®, which has high efficiency (>90% of bacteria-sized particles) but poor flow-rate (12 l/min); and the Scepter Industries Omni 3000, which has suboptimal efficiency (50-60% collection) but high flow rate (300 l/min). Regardless of the type of impinger used, it is critical that the device be amenable to or modified to be amenable to thorough internal cleaning because biofilms develop on plumbing required for such systems and can contaminate samples for analysis.

**Culture-Based Techniques**

A survey of the literature documents a wide range of methods used to isolate NTM from environmental samples; particularly soil samples. Although a variety of studies have compared methods (Brooks et al., 1984; Griffith et al., 2007), there is no consensus on the best approaches. For biofilm and soil samples, there is not yet a method to ensure recovery of attached NTM cells. Even with water samples, because of their hydrophobic quality NTM cells can be lost through irreversible attachment to pipette and tube surfaces. One way to minimize NTM cell losses during centrifugation and transfer operations is to collect cells in water or suspension on filters and place the filters on medium. However, the efficiency of recovery of colonies on filters has not yet been established and there is also the possibility that NTM may enter a viable but nonculturable (VBNC) state; it has been well established that NTM colony counts on laboratory culture medium are approximately 10% of microscopic cell counts. To date, there has been no systematic study of the possibility that NTM can enter a VBNC state, nor if some sample concentrates contain inhibitors of NTM growth and colony formation. Different media vary in their ability to support NTM colony growth and there is need for quality assurance/quality control (QA/QC) standards.

NTM cultivation techniques usually involve rich laboratory medium such as M7H10 agar that contains glycerol, oleic acid, albumin, and malachite green, an inhibitor of fungal growth and some bacteria. If the relatively small (0.5 mm diameter) NTM colonies that appear in 7-14 days are overgrown by other microbial colonies, the swab or suspension can be incubated in 0.005% cetylpyridium chloride (CPC) for 30 min to kill other microorganisms (Falkinham III et al., 2008). Although almost all other microbial cells, other than spores, are killed, approximately 10% of NTM survive CPC exposure.

Laboratory capacity for NTM isolation and identification differs among labs. While most laboratories successfully stain and evaluate respiratory specimens for the presence of acid fast organisms, thereafter, diagnostic capability differs greatly among labs. After a *Mycobacterium* spp. is successfully cultured from a specimen, the most commonly used identification technique among U.S. state public health laboratories in 1999 was the DNA probe (Hilborn et al., 2002). This method can quickly differentiate between *M. tuberculosis* and NTM, but introduces a second source of bias in identification of NTM species.

Identification of MAC, *M. avium, M. intracellulare, M. kansasii*, and *M. gordonae* are possible based on culture. Others are classified as “unidentified NTM” unless further analysis is
performed. Therefore, members of the *M. avium* complex, *M. kansasii*, and *M. gordonae* may be reported more frequently as these FDA-approved DNA probes are readily available, are inexpensive and easy to use in most laboratory settings (Griffith et al., 2007). Compared to laboratories with access to DNA probes, fewer clinical labs have the ability to perform High Performance Liquid Chromatography (HPLC) to characterize mycolic acid profiles and thereby identify *Mycobacterium* species. Methods for DNA sequencing and DNA fingerprinting are less prevalent among clinical laboratories. Therefore, other NTM species, even if present and successfully cultured from clinical specimens, may be less likely to be successfully identified and reported.

**Molecular Methods**

Although a great deal of information concerning NTM has originated from basic cultural, biochemical, and enzymatic tests, including taxonomic data, much analysis has been replaced by fatty acid- or PCR-based methods for identification following recovery of pure isolates (Griffith et al., 2007). Classical culture-based identification approaches, for instance, lack the ability to discriminate between related species; particularly, distinguishing *M. avium* from *M. intracellulare* and *M. abscessus* from *M. chelonae* (Falkinham III et al., 2001). These are challenging determinations even with molecular technology.

A variety of molecular techniques have been developed that have supplanted many culture-based methods. Two methods are widely employed for identification of isolated colonies. Fatty acid-based methods take advantage of the fact that NTMs exhibit species-specific patterns of fatty acids, particularly mycolic acids, separated by chromatography (Butler and Guthertz, 2001). Genus-specific PCR amplification of DNA, sometimes followed by restriction endonuclease digestion of PCR-amplified products or direct sequencing, can resolve some species (Steingrube et al., 1995). In general, however, rRNA based methods are limited by the high levels of sequence identity among NTM rRNA gene sequences. Consequently, recent focus for mycobacterial speciation has been on DNA sequences for several other genes, with more sequence variation. These include, for instance, genes for the 16S-23S rRNA gene internal transcribed spacer sequence, for hsp-65 (heat shock protein gene), rpoB (the major RNA polymerase subunit gene) and others (Adékambi and Drancourt, 2004; Devulder et al., 2005; Shin et al., 2007). Single nucleotide polymorphisms in entire or partial genome sequences promise higher resolutions of identification (Horan et al., 2006). However, few clinically relevant NTM genome sequences and no environmental NTM sequences are available on which to base analytical methods. As knowledge of the nature of the NTM “pangenome” grows, molecular methods based on PCR amplification or DNA sequencing will become increasingly powerful for resolving the taxonomy of NTM.

Circumventing culture altogether via direct molecular analysis is desirable given that NTM are extremely slow growing in nature, typically requiring about 2 weeks for colony formation. *Mycobacterium* specific PCR primers have been reported previously and widely applied (Wilton and Cousins, 1992). Unique problems surface when PCR is used for detection or enumeration (qPCR) of NTM in samples. Special considerations must be taken to ensure that the extracted DNA does not contain inhibitors of the PCR reaction. This can be particularly a problem in soil extracts and some water extracts due to poisoning by the presence of humic and fulvic acids in the sample (Radomski et al., 2010). Control for this is by conducting PCR reactions with test samples plus a known positive target. As is the case for culture-based
techniques for NTM identifications, it is important to develop and institute a quality assurance/quality control (QA/QC) program employing an array of internal standards and controls for both PCR and qPCR methods.

Other DNA fingerprinting methods employed include pulsed field gel electrophoresis (von Reyn et al., 1994), restriction fragment length polymorphism (RFLP) analysis of species-specific insertion sequences (e.g. IS1245, (Falkinham III et al., 2008)), and repeated interspersed sequence PCR (rep-PCR, (Falkinham III, 2011)). To date, however, there has been little comparison of any of the molecular methods with the same collection of NTM isolates or between clinical and environmental NTM.

**Methodology Gaps for NTM**

A summary of available methodology for detection of NTM is described in Table 4-2.

<table>
<thead>
<tr>
<th>Method</th>
<th>Target</th>
<th>Assay Performance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td><em>Mycobacterium</em> spp.</td>
<td>LOQ: 1 colony-forming unit</td>
<td>Jenkins et al., 1982</td>
</tr>
<tr>
<td>qPCR</td>
<td><em>Mycobacterium</em> spp.</td>
<td>LOQ: ≥ 500 cells L⁻¹</td>
<td>Adrados et al., 2011</td>
</tr>
<tr>
<td>qPCR</td>
<td><em>Mycobacterium</em> spp.</td>
<td>LOD: ≥100 cells ml⁻¹</td>
<td>Jacobs et al., 2009</td>
</tr>
<tr>
<td></td>
<td><em>Mycobacterium avium</em> and <em>Mycobacterium intracellulare</em></td>
<td>LOD: ≥ 100 cells ml⁻¹</td>
<td>Wilton and Cousins, 1992</td>
</tr>
<tr>
<td>IMS w/qPCR</td>
<td><em>Mycobacterium avium</em> ssp. <em>paratuberculosis</em></td>
<td>Detection sensitivity: 10 CFU L⁻¹</td>
<td>Whan et al., 2005</td>
</tr>
<tr>
<td>Acid-Fast Stain and Light Microscopy</td>
<td><em>Mycobacterium</em> spp.</td>
<td>LOD: ≥ 1,000 ml⁻¹</td>
<td>Smithwick, 1976</td>
</tr>
<tr>
<td>Auramine-Rhodamine Stain and Fluorescence Microscopy</td>
<td><em>Mycobacterium</em> spp.</td>
<td>LOD: ≥ 1,000 ml⁻¹</td>
<td>Smithwick, 1976</td>
</tr>
</tbody>
</table>

Methodological gaps and challenges for mycobacteria detection include the following:

1. Not all mycobacterial cells form colonies on laboratory media ("efficiency of plating" is low)
2. Mycobacterial cells are difficult to break open to obtain DNA for further analysis
3. Different Mycobacterial species cannot be distinguished well using 16S rRNA gene sequences.
4. Mycobacterial cells adhere to surfaces or particulates where they are difficult to isolate, enumerate and identify
5. Mycobacterial cells aggregate, thus confounding efforts to obtain accurate numbers

**PSEUDOMONAS AERUGINOSA**

**Sampling and Monitoring Considerations**

Historically, much of the research involving *P. aeruginosa* has been based on its significance in the clinical realm. However in more current research, *P. aeruginosa* has become the “model organism” with which to study the formation of biofilms (Ghafoor et al., 2011). In fact, ASTM Standard Methods for Biofilm growth have specifically been developed with respect to *P. aeruginosa* (ASTM E2562, 2007). Thus, appropriate biofilm sampling protocols are particularly critical. With respect to biofilms, much of the research is focused on the bacterium’s proteins, the genes that encode those proteins and drive its metabolic diversity, viability and role in biofilm formation (e.g. quorum sensing) as well as antibiotic resistance.

Coupons are commonly applied to directly attract *P. aeruginosa* biofilms in laboratory and field systems. Additionally, field biofilm samples are commonly gathered by swabbing or scraping. Biofilm samples may then be directly analyzed by culture-based, molecular-based, or other methods.

**Culture-Based Techniques**

Culture-based techniques take advantage of specific phenotypic properties of *P. aeruginosa*, including catalase positive, oxidase positive, indole negative, Methyl-Red negative, and Vogues-Proskauer negative response during incubation. Approved standard culture methods used in testing drinking water for the presence and enumeration of *P. aeruginosa* is membrane filtration and the multiple tube technique. Media and reagents for performing these analyses are readily available, relatively simple and inexpensive for a traditional microbiology laboratory.

The membrane filtration method involves filtration of the collected sample (typically 100-1000 mL) onto a filter, which is then emplaced on top of a medium selective for *P. aeruginosa*. According to Standard Method 9213E, the sample is first grown on M-PA agar for the presumptive test. Putative *P. aeruginosa* will appear as flat, 0.8-2.2 mm diameter brown to greenish black colonies. Presumptive *P. aeruginosa* are then transferred to Milk Agar for confirmation. This is based on the principle that casein is hydrolyzed by *P. aeruginosa*, producing a characteristic yellow/green pigment.

Standard Method 9213 F describes the multiple tube technique for *P. aeruginosa*, which is less laborious than the membrane filtration method, but only provides results in terms of presence/absence. For this test, asparagine broth is first applied for the presumptive test. After incubation for 24-48 hours, green pigment production is examined via long wave UV light, indicating a positive presumptive test. Presumptive *P. aeruginosa* are then inoculated into acetamide broth or agar for the confirmed test. Phenol red is added to the medium as an indicator, shifting to a purple color at alkaline pH induced by the presence of *P. aeruginosa*, thus confirming its presence.
Most Probable Number (MPN) and Phenotypic Assays

Recently, the Pseudalert® test has been developed and marketed by IDEXX Laboratories, Inc. and hit the market in early 2011. Pseudalert® is based on a proprietary enzyme-substrate reaction and currently is being primarily marketed to the bottled water industry. This method has the advantage of a simple platform, similar to the widely applied Colilert® test developed by the same company. Pseudalert® provides rapid presence/absence results for \textit{P. aeruginosa} within 24 hours. The Pseudalert® test can be adapted to both presence/absence and most probable number (MPN) outcomes, the latter providing semi-quantitative information about the level of \textit{P. aeruginosa} present. Pseudalert® is starting to be offered by commercial labs, which had traditionally offered membrane filtration analysis. Preliminary parallel analysis indicates consistent and comparable results (IDEXX Laboratories, 2010, 2011). IDEXX is currently seeking industry approval for this method, but is not yet recognized by Standard Methods.

Molecular Methods

Molecular detection of \textit{P. aeruginosa} is challenged by the high level of sequence similarity of the 16S rRNA gene and other gene targets shared among \textit{Pseudomonas} spp. This has been circumvented in one case through the development of a duplex qPCR assay targeting two different gene variants, \textit{ecfX} and the \textit{gyrB} (Anuj et al., 2010). While both gene variants must be present to confirm the presence of \textit{P. aeruginosa}, densities are estimated according to the least common denominator as applied to a standard curve. An alternative qPCR method has recently been reported targeting the \textit{opr1} gene highly conserved among \textit{Pseudomonas} (Rios-Licea et al., 2010). The addition of a TaqMan probe adds a layer of specificity to the method for \textit{P. aeruginosa}, however the method has only been applied to bronchoalveolar lavage, and has not been validated on environmental samples.

As noted in Chapter 3, the genome of \textit{P. aeruginosa} is extraordinarily large. However, it has also been fully sequenced, which opens the door to a variety of next generation methods for advancing \textit{P. aeruginosa} detection and quantification.

Methodology Gaps for \textit{P. aeruginosa}

Table 4-3 summarizes available methodology for \textit{P. aeruginosa}.
### Table 4-3

<table>
<thead>
<tr>
<th>Method</th>
<th>Target</th>
<th>Assay Performance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>1000 CFU mL⁻¹</td>
<td>Zemanick et al., 2010</td>
</tr>
<tr>
<td>qPCR</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>100 CFU mL⁻¹</td>
<td>Cattoir et al., 2010</td>
</tr>
<tr>
<td></td>
<td>ecfx gene</td>
<td>330 - 2300 CFU per PCR</td>
<td>Lee et al., 2011</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>100 CFU mL⁻¹</td>
<td>Wang et al. 2012</td>
</tr>
<tr>
<td></td>
<td>gyrB gene</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(ecfx/gyrB combined)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudalert®</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>1 CFU per 100 mL</td>
<td>IDEXX Laboratories Inc. 2010, 2011</td>
</tr>
<tr>
<td></td>
<td>culture and active enzyme assay</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Major challenges for *P. aeruginosa* detection and enumeration include:

1. Production of exopolymeric substances, which causes cells to clump and interferes with enumeration of CFU by culturing.
2. Limited known genetic variability to distinguish *P. aeruginosa* from related strains using molecular methods (thus the qPCR method requires two targets).

**ACANTHAMOEBA SPP.**

**Sampling and Monitoring**

*Acanthamoeba* spp. primarily inhabit biofilms, where they graze on bacteria, which emphasizes the importance of their sampling and monitoring (Rodriguez-Zaragoza, 1994). However, a recent review of literature on FLA in drinking water noted that biofilms and sediments have been under-sampled, relative to the bulk water (Thomas and Ashbolt, 2011). Standard biofilm sampling techniques generally apply to *Acanthamoeba* spp. For all FLA, it is critical to avoid stimulation of encystation during sampling, preservation, and shipment. Contrary to common practice, samples intended for quantification should not be chilled, as the cold shock triggers the formation of cysts. The cysts will especially be problematic for culture-based methods, resulting in a VBNC status.

**Microscopic examination**

As amoeba are much larger than bacteria, microscopy-based techniques are generally more common for their identification. Microscopy is the traditional method by which *Acanthamoeba* spp. have been identified. Acanthapodia are the primary morphological feature identifiable by microscopy that distinguishes *Acanthamoeba* from other FLA. An advantage of microscopy is the ability to distinguish between the cyst and trophozoite stage, and specialized
stains are available to aid in this. Hematocytometry has been used in concert with such stains to aid in direct cell counts. A limitation of microscopy is that specialized expertise is generally required for effective identification of *Acanthamoeba*, and certification for microscopic identification is encouraged for other protozoans, such as *Cryptosporidium* (EPA Method 1622 and 1623). Identification based on morphology alone has been noted to be frequently inaccurate (Smirnov et al., 2005).

**Culture-Based techniques**

Standard methods for the culture of *Acanthamoeba* spp. have been developed by the CDC (Barbaree et al., 1986; Bovee and United, 1979; Sawyer et al., 1977). As the preferred mode of growth for *Acanthamoeba* spp. is phagocytosis of bacterial cells, they are typically not grown directly on nutrient agar plates. Rather, non-nutrient agar plates are seeded with *Escherichia coli* or *Klebsiella pneumoniae* to provide a substrate for *Acanthamoeba* spp. (Kilvington and White, 1985; Visvesvara and Healy, 1975). The non-nutrient agar prevents over-proliferation of bacteria, while allowing them to grow sufficiently to serve as a food source for grazing *Acanthamoeba* spp. The selected bacterial food source is smeared or streaked over the agar surface and the plates are subsequently sealed, inverted, and incubated at 32°C under humid conditions. *Acanthamoeba* will migrate across the plate as they graze on the bacteria, leaving visible and measurable tracks. The tracks are typically visible within 48 hours, but occasionally longer incubation (up to two weeks) is needed (EPA, 2003; Illingworth and Cook, 1998).

*Acanthamoeba* are capable of uptake of dissolved nutrients for growth and do so under certain ecological constraints. This property is taken advantage of for the convenience of axenic (bacteria-free) media in the laboratory. Formulations for several complex liquid axenic media have been described by the American Type Culture Collection (Nerad, 1993). A disadvantage of axenic media is that it is not representative of the substrate conditions under which *Acanthamoeba* proliferate in drinking water systems. Since some species of amphizoic amoeba grow at mammalian body temperatures, many labs incubate replicate cultures at room temperature, 37°C to 45°C, or higher. MPN type approaches are also applied, in which amoeba are cultured with seeded bacteria.

Culturing techniques have also been applied to demonstrate the virulence of *Acanthamoeba* strains. One approach is to demonstrate virulence via infection of lab mice (Michel et al., 1998). Virulence can also be assessed by the observation apparent cytopathic effect of the growth of a suspected *Acanthamoeba* strain on a monolayer of rabbit corneal epithelial cells (Khan et al., 2001). Growth of potentially virulent *Acanthamoeba* strains on non-nutritive agar containing 0.5 to 1.0 M mannitol can provide a more accessible culture-based differentiation between pathogenic and nonpathogenic *Acanthamoeba* (Khan et al., 2001). Other molecular-based techniques can also differentiate between pathogenic and nonpathogenic strains.

A major limitation to culture-based methods is that encysted or damaged forms may not grow well and essentially contribute to VBNC status (Rivièere et al. 2006). Culture based methods have been observed to result in underestimation of density of free-living amoeba (Smirnov et al., 2004). Thomson et al. (2008) noted 4/168 ocular samples were culture-negative but positive according to DNA sequencing and two qPCR assays. One reason for this may be that sample processing during culturing can induce the VBNC status. For example, Khunkutti et al. (1997) reported lower plaque forming units when *A. castellanii* were subject to washing and
re-suspension in phosphate-buffered saline (PBS) solution. *Acanthamoeba* are more fragile than their bacterial counterparts, and care must be taken during vortexing, filter concentrating, etc., to avoid cell lysing and thus false negatives.

Overall, culture-based techniques can be of value for isolating *Acanthamoeba* and verifying the presence of live, virulent pathogens. However, the methods are cumbersome and the results are often ambiguous. The methods suffer from poor repeatability and poor quantitative capabilities, and overall tend to underestimate the density of *Acanthamoeba*.

**Molecular Methods**

Molecular techniques for *Acanthamoeba* spp. are of interest for addressing the limitations of culture-based techniques. However, there is not only a need for further development of molecular techniques but also to more widely apply them in order to gain an understanding of the perspective they provide relative to culture-based assessments. A recent review noted that a small minority of studies of free-living amoeba in drinking water systems to date have employed molecular methods (Thomas and Ashbolt, 2011). Only three out of 24 studies used PCR (Boost et al., 2008) and only two employed qPCR (Puzon et al., 2009; Valster et al., 2009). In addition to higher sensitivity and specificity, molecular methods are advantageous in that they are capable of detecting both cysts and trophozoites (Thompson et al., 2008). However, most approaches do not necessarily distinguish the two life stages.

DNA extraction could be of concern, especially when there is comprehensive interest in detecting both amoeba and bacterial pathogens. Given the distinct properties of these classes of microorganisms, it may not be possible to optimize DNA extraction for both. For example, more aggressive actions to extract bacterial DNA, such as bead-beating, may result in damage of the DNA of the more fragile amoeba (Goldschmidt et al., 2008). At the same time, the same aggressive actions may be necessary for cyst DNA extraction, at the expense of damaging trophozoite DNA. Various DNA extraction techniques have been tested in the clinical realm, where there is interest in rapid molecular diagnostic of disease. In one study, classic DNA extraction methods were reported to be ineffective for extraction of *Acanthamoeba* from corneal tissue, particularly when in the cyst form, as a result of the tight packaging of the DNA imposed by the cyst wall and nuclear proteins (Goldschmidt et al. 2008). Recently, a chellex resin approach was demonstrated to be affective for DNA extraction from cysts present in corneal scraping matrix, resulting qPCR detectability as low as 0.1 cyst/PCR reaction (Iovieno et al., 2011). However, the method has not been verified for simultaneous extraction of cysts and trophozoites. Another study found that a Proteinase K pre-treatment step combined with the commercially available MagNA Pure (Roche, Switzerland) extraction kit offered the best yields (Goldschmidt et al., 2008). It is uncertain if such treatments are effective or necessary in drinking water samples, where the nature of the protein matrix of the biofilm may be distinct from that of the corneal tissue. Efforts to optimize DNA/RNA extraction of both *Acanthamoeba* trophozoites and cysts from drinking water samples may be beneficial.

Once DNA is extracted, the next challenge is which gene to target and with which primers or probes. Corsaro and Venditti (2011) noted limited resolution is available to distinguish *Acanthamoeba* spp. based on 18S rRNA gene sequence, particularly when employing shorter sequences. For example, the 850 bp “Ami” sequence targeted by primers Ami6F and Ami9R has historically been used to identify eukaryotes to the genus level (Thomas, et al., 2006). While the full-length gene (greater than 2,200 bp) is sufficient to distinguish species in
most cases, caution is recommended in interpreting shorter gene fragments such as the Ami sequence (Corsaro and Venditti, 2011). Such distinctions are likely critical in distinguishing pathogenic from non-pathogenic strains. Mitochondrial DNA restriction endonuclease analysis is one potential alternative to the 18S rRNA gene target, and has been applied in some classification schemes (Yu et al., 1999).

Several PCR and qPCR methods have recently been reported for *Acanthamoeba* spp. Rivière et al., (2006) and Qvarnstrom et al., (2006) have reported two competing qPCR TaqMan assays targeting 18S rRNA genes at the genus-level. The Qvarnstrom assay was designed targeting 40 different *Acanthamoeba* species 18S rRNA gene sequences, representative of four genotypes. Thompson et al. (2008) compared the two assays for clinical samples, reporting 97% agreement between the two methods. However, Chang et al. (2010) compared the two assays for water and biofilm samples and noted several discrepancies. Considering that the Rivière et al. (2006) assay was only designed against six *Acanthamoeba* from a single genotype (T4), the Qvarnstrom assay generally possesses broader specificity and tends to yield a higher rate of positive detections, which was confirmed in side-by-side comparisons (Chang et al., 2010; Qvarnstrom et al., 2006; Thompson, Kowalski, Shanks, & Gordon, 2008). However, the Rivière assay was determined to have a slightly lower limit of detection than the Qvarnstrom assay (11.3 copies per 10 ul versus 43.8 copies/10 ul), with good detection of cysts (Thompson et al., 2008). Qvarnstrom assay provided equivalent results for both trophozoites and cysts, whereas the Rivière assay noted differences (Chang et al., 2010). These results indicate that while qPCR assays are promising for sensitive and rapid detection, they could still benefit from refinement to improve specificity.

Inhibitors of qPCR assays for *Acanthamoeba* have been noted for clinical samples. In particular, certain opthamic ointments can be inhibitory, especially polyhexamethylene biguanide (PHMB) (Thompson et al., 2008). This problem can be averted by administering such ointments after collecting samples for diagnostics. Studies investigating specific inhibitors of *Acanthamoeba* qPCR for water samples were not found. However, Thompson et al. (2008) successfully employed internal standards, which may aid in the identification of PCR inhibitors or other interferences and has been applied to both the Rivière and Qvanstrom assays.

Distinguishing live, dead, active, and inactive cells is an additional challenge for molecular methods, particularly in the case of *Acanthamoeba*, where the cyst and trophozoite life stages complicate matters. Recently, a PMA qPCR approach was developed by Fittipaldi et al. (2011), which allows selective quantification of viable *Acanthamoeba*. Consistent quantification of live cysts and trophozoites was observed using PMA qPCR, with discrimination of autoclaved and contact lens solution-treated *Acanthamoeba castellani*. However, optimization of the PMA treatment for each assay was recommended in order to avoid false positives. The study by Fittipaldi and colleagues represents the first demonstration of the feasibility of this approach with amoeba, and further efforts to adapt and validate such approaches for water could be beneficial. In particular, the PMA qPCR approach may prove extremely valuable for distinguishing cysts and trophozoites.

Flow cytometry is also a promising means of estimating densities of viable *Acanthamoeba*. Khunkitti et al. (1997) reported a flow cytometry procedure for assessing the live/dead proportion of *A. castellanii*. *A. castellanii* were subject to standard live/dead staining in which metabolically active cells hydrolyze fluorescein diacetate (FDA) to produce a green signal, whereas propidium iodide (PI) enters dead cells and stains nucleic acids to produce a red color. Flow cytometry consistently reported a higher fraction of live cells than standard plaque
forming assays (Khunkitti et al., 1997). This may be because cells are still able to actively metabolize but are in a VBNC state. Thus, further studies incorporating methods to distinguish live/dead cells may provide deeper insight into the relative distributions of cysts and trophozoites and the extent of their respective VBNC status. Immunological assays have also been applied to *Acanthamoeba* spp. For example, some taxonomic studies have employed isoenzyme (de Jonckheere, 1987).

**Methodology Gaps for *Acanthamoeba* spp.**

Knowledge gaps are summarized as follows:

- More thorough assessment and development of nucleic acid extraction techniques suitable for *Acanthamoeba* at various life stages specifically in water samples, and ideally also suitable for bacterial pathogens.
- Distinction of trophozoites and cysts.
- Distinction of live/dead cells.
- Combined surveys of various molecular as well as culture-based techniques. Can aid in assessing the ecological significance of the encysted status for *Acanthamoeba* and other FLA.
- Higher throughput methods capable of distinguishing multiple *Acanthamoeba* spp., especially known pathogenic strains. Metagenomic or micro-chip techniques may be beneficial here.
- Explore beyond *Acanthamoeba*, for detection and distinction of FLA capable of harboring pathogenic ARM.

Table 4-4 summarizes available methodology for *Acanthamoeba* spp..

**Table 4-4  
Summary of Available Methodology for *Acanthamoeba* spp.**

<table>
<thead>
<tr>
<th>Method</th>
<th>Target</th>
<th>Assay Performance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td>Growth on bacterial lawn</td>
<td>&quot;more sensitive than qPCR when applied to sediment samples&quot;</td>
<td>Hsu et al. 2009</td>
</tr>
<tr>
<td>qPCR</td>
<td><em>Acanthamoeba</em> spp.</td>
<td>1 cell per reaction (20 μL)</td>
<td>Qvarnstrom et al., 2006</td>
</tr>
<tr>
<td></td>
<td><em>Acanthamoeba</em> spp., <em>A. polyphaga</em></td>
<td>11.3 DNA copies per 10 μL</td>
<td>Thompson et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.3 +/- 1.7 trophozoites per 10 μL</td>
<td>Rivière et al., 2006</td>
</tr>
<tr>
<td>vPCR w/PMA</td>
<td><em>Acanthamoeba</em> spp.</td>
<td>5 cells per reaction (20 μL)</td>
<td>Fittipaldi et al., 2011</td>
</tr>
<tr>
<td>Fluorescence Flow cytometry</td>
<td><em>A. polyphaga</em></td>
<td>94% specificity</td>
<td>Flores et al., 1990</td>
</tr>
<tr>
<td>Light Microscopy</td>
<td><em>Acanthamoeba</em> spp.</td>
<td>50,000 cfu/mL</td>
<td>Methvin, 2009</td>
</tr>
</tbody>
</table>
NAEGLERIA FOWLERI

Sampling and Monitoring Considerations

The vast majority of environmental studies of *N. fowleri* have focused on detecting these amoebas in surface water and sediment from lakes, ponds, and rivers. Some studies have also been performed to detect *N. fowleri* in ground water. Few studies have focused on detecting *N. fowleri* in piped water systems. Sampling methods reported for *N. fowleri* have most often consisted of simple grab sampling to collect 1 L or less of water per sample, but large-volume water sampling methods (such as tangential flow microfiltration) have been reported (Rouby et al., 2000). From the literature on detection of *N. fowleri* in surface water, some research has indicated that *N. fowleri* can be detected in sample volumes as low as 1-10 mL and sampling larger volumes (~1 L) may actually be counterproductive (i.e., leading to false negative results after culture) (de Jonckheere, 1978; Pernin et al., 1998; Tyndall et al., 1989), but it is not clear how relevant these studies are for determining appropriate sample volumes for drinking water systems. Distribution system biofilm has also been sampled for *N. fowleri* using biofilm monitoring devices in which glass rings have been used as media for biofilm growth and recovery (Puzon et al., 2009). Premise plumbing systems may also be investigated for the presence of FLA, including *N. floweri*, using swabs. In addition, some research has been reported that indicates that swabs can be effective for detecting FLA in household water systems (e.g., shower heads, kitchen sprayers), and may yield positive detections for FLA when associated water samples are negative (Stockman et al., 2011). The data from Stockman et al. indicate that FLA may be more likely detected in samples collected from showers and kitchen sprayers, but the study detected *Naegleria* spp. in only 16 of 2,454 samples (0.6%). Reports indicate that hot water heaters may also be effective premise plumbing collection sites for detecting *N. fowleri* (Stockman et al., 2011; Yoder et al. 2012; LA DHH 2011). In addition, while there is little data available on the potential transport of *N. fowleri* via aerosols, it is likely that such a transmission route would not be useful to study during environmental occurrence investigations, as such a transmission route is not a likely exposure risk for PAM and it has been shown that *N. fowleri* trophozoites and cysts are quickly inactivated by drying in air (Chang, 1978).

After collection, swabs for *N. fowleri* testing should be kept moist as drying has been shown to cause *N. fowleri* trophozoites to become nonviable (Chang, 1978). Environmental samples (e.g., swabs, water samples) that will be processed for *N. fowleri* culture should not be chilled during storage and handling (as is typically done for samples collected for enteric microbe testing). *Naegleria fowleri* trophozoites are known to degenerate and become non-culturatable when subjected to temperatures below 10°C (Chang, 1978). In addition, because *N. fowleri* are thought to be highly aerobic (Kyle and Noblet, 1985), environmental samples should be stored and shipped with headspace (e.g., 1:1 water sample:air headspace) to maintain favorable conditions for culture.

Because of their relatively large size, water samples are often centrifuged (e.g., 4,6000 x g for 15 min for 1-L samples) to pellet *N. fowleri* trophozoites and cysts. Some researchers have reported filtering water samples through microfilters (e.g., 0.45-µm, 1.2-µm) to concentrate the samples prior to final processing by centrifugation (Ahmad et al., 2011) or directly culturing from the filters by inverting them on agar plates (Behets et al., 2007b). Research comparing established centrifugation and membrane filtration procedures indicated that centrifugation
procedures may be more effective at recovering *N. fowleri* trophozoites than cysts, with the researchers suggesting that the cytoplasmic membrane of trophozoites is likely more sensitive to damage during adverse conditions (e.g., stress during vacuum filtration through a filter) (Pernin et al., 1998). Pernin et al. (1998) did not report any significant differences in recovery of *N. fowleri* cysts between centrifugation and membrane filtration methods.

Field investigators tasked with conducting surveillance for *N. fowleri* in drinking water distribution systems or households should consider potential factors that could affect the recovery and detection of *N. fowleri*. Initially, these include questions such as where to sample in a water system and how to collect samples. Within buildings, it appears that *N. fowleri* may be more likely to be detected in the hot water system (hot water heater or shower/faucet when hot water is running). Swab sampling of shower heads and kitchen sprayers may also be useful. Sampling household water containers and point-of-use (POU) filter devices may also enable recovery of *N. fowleri*, as has been demonstrated in studies for other pathogens (Daly et al., 2010; Sedillo et al., 2008), but sampling refrigerator filters would likely only enable detection by molecular methods because cold temperatures are known to substantially reduce the culturability of *N. fowleri*. When collecting water samples for *N. fowleri* detection, it would also be useful to collect relevant water quality parameters, such as water temperature, disinfectant type and residual, and pH, as well as noting characteristics of the water system (e.g., hot water heater type, water system pipe material). If samples are handled and shipped appropriately (e.g., not chilled, testing within 24-36 hours), then culture-based test results can be used to evaluate the presence of viable *N. fowleri* in the water system. As for other microbial testing, positive PCR results for *N. fowleri* can be taken as evidence that *N. fowleri* was present in the system, but may not be sufficient as conclusive evidence that viable or culturable *N. fowleri* was present.

**Culture-Based Techniques**

To culture *N. fowleri*, concentrated water samples (or membrane filters through which water samples have been passed) are typically inoculated onto plates of non-nutrient agar (NNA) that have been seeded with *E. coli*. The agar plates are then incubated at 42-44°C for up to 14 days, and the plates are examined under an inverted microscope on a daily basis to look for characteristic amebic cells. Plates containing characteristic amoebas (Visvesvara et al., 2007) can then be scraped to recover the amoebas (or an agar piece can be excised from the plate) and a flagellation test performed by adding the material to distilled water. Amoebas belonging to the *Naegleria* genus will produce flagella within 1-2 hours in distilled water (Behets et al., 2003). After performing the flagella test, identification of *Naegleria* species is then most often performed using PCR. Culture-based methods have been effectively used to detect and study *N. fowleri* in diverse environmental media (e.g., water, sediment, air), but some research has suggested that the concentration of *N. fowleri* in environmental samples can be underestimated when the growth of other thermophilic amoebae interfere with the growth and detection of *N. fowleri* (Tyndall et al., 1989).

**Molecular Methods**

Traditionally, *N. fowleri* has been detected by culture, microscopy, and immunological assays (e.g., indirect immunofluorescence assay (IFA), ELISA) (Behets et al., 2003; Sparagano...
et al., 1993; Visvesvara et al., 1987), but more recently PCR assays have replaced immunological assays for detection of *N. fowleri*.

Prior to performing molecular testing of environmental samples for *N. fowleri*, a wide array of sample preparation and DNA extraction techniques have been reported. Some researchers have assayed concentrated water samples and *N. fowleri* cultures directly without DNA extraction (Bright et al., 2009; Jamerson et al., 2009; Marciano-Cabral et al., 2003). Others have reported using various lysis buffers and DNA purification procedures (Behets et al., 2007a; Lares-Villa and Hernández-Peña, 2010; Madarova, 2010; Pelandakis and Pernin, 2002; Puzon et al., 2009). For detection of *N. fowleri* in biofilm samples, a soil DNA extraction kit may be useful (Puzon et al., 2009) to remove biofilm-associated PCR inhibitors.

Numerous PCR techniques and assays have been reported for detection of *N. fowleri*, including conventional PCR, nested PCR, and real-time PCR. Conventional PCR assays reported in the literature include Pelandakis et al. (Pelandakis et al., 2000) and Lares-Villa and Hernández-Peña (Lares-Villa & Hernández-Peña, 2010), which modified the Pelandakis et al. (2000) PCR primers. A nested PCR assay originally reported by Réveiller et al (2002) has been used for multiple investigations of *N. fowleri* in well water and household water systems (Blair et al., 2008; Bright et al., 2009; Marciano-Cabral et al., 2003). Nested PCR has also been used by other researchers to detect *N. fowleri* in water (Ahmad et al., 2011). With the advent of real-time PCR, multiple real-time PCR assays have been reported for the specific detection of *N. fowleri*, including probe-based assays (Behets et al., 2007a; Madarová et al., 2010; Qvarnstrom et al., 2006) and intercalating dye, melting-curve analysis assays (Behets et al., 2006; Puzon et al., 2009). These real-time PCR assays have been used to detect *N. fowleri* in environmental samples, and some have been reported for quantification of *N. fowleri*.

Behets et al. (2007a) presented data on quantification of *N. fowleri* in water samples, but also suggested that Most Probable Number (MPN) culture would be more appropriate than their duplex real-time PCR assay for quantitation of *N. fowleri* present in water samples at concentrations < 200 cells/L. In general, nested PCR and real-time PCR assays for *N. fowleri* have been reported to have detection limits of 1-50 cells per reaction (Ahmad et al., 2011; Behets et al., 2007a; Puzon et al., 2009; Qvarnstrom et al., 2006), which roughly corresponds to 10-1,000 cells per sample for direct assay without culture (assuming that a PCR assay can be used to test approximately 1-5% of the volume of an environmental sample). In addition, Behets et al. (2007a) reported a quantitation limit (as opposed to a detection limit) of 320 *N. fowleri* cell equivalents/L.

In addition to specific detection of *N. fowleri* using PCR, molecular testing methods have also been reported to identify different *N. fowleri* genotypes. While no virulence differences have been identified in the detected *N. fowleri* types (De Jonckheere, 2011), typing *N. fowleri* strains using currently available methods is useful in understanding the global distribution and evolution of *N. fowleri*. Several genotyping targets have been reported, characterizing the genetic diversity of the 5.8S rRNA gene and internal transcribes spaces (ITS) (De Jonckheere, 2011; Pelandakis et al., 2000; Tiewcharoen et al., 2007) and the loci of mitochondrial small subunit rRNA (mtSSU rRNA) (Zhou et al., 2003). Using their genotyping system, Zhou et al. (2003) identified six *N. fowleri* genotypes. More recently, De Jonckheere has identified eight *N. fowleri* types using data from several research studies that used genotyping assays targeting the 5.8S rRNA gene and two ITS sequences (ITS1 and ITS2) (De Jonckheere, 2011). While available genotyping studies suggest that *N. fowleri* strains may be geographically segregated (De Jonckheere, 2002; 2011), the discriminatory power of available genotyping tools is not high and
these tools are limited in their usefulness for ecological investigations and disease surveillance in the U.S. (compared to, for example, genotyping and subtyping tools available for Cryptosporidium parvum).

Table 4-5 summarizes available methodology for *N. fowleri*.

### Table 4-5
Summary of Available Methods for *N. fowleri*.

<table>
<thead>
<tr>
<th>Method</th>
<th>Target</th>
<th>Assay Performance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture (NNAE)</td>
<td><em>Naegleria</em> spp.</td>
<td>2 MPN L⁻¹</td>
<td>Tyndall <em>et al.</em>, 1989</td>
</tr>
<tr>
<td>qPCR</td>
<td><em>N. fowleri</em></td>
<td>LOD: 3 cell eqi./L or 1 copy of <em>N. fowleri</em> plasmid control DNA/reaction LOQ: 16 cell eqi./L</td>
<td>Behets <em>et al.</em>, 2007a</td>
</tr>
<tr>
<td>Fluorescence Flow cytometry</td>
<td><em>N. fowleri</em></td>
<td>99.6% specificity LOD: 0.06 amoeba mL⁻¹ or in Solid State FC 200 to 500 cells L⁻¹</td>
<td>Flores <em>et al.</em>, 1990; Johnson <em>et al.</em>, 2007; Pougnard <em>et al.</em>, 2002</td>
</tr>
</tbody>
</table>

**Methodology Gaps for *N. fowleri***

Knowledge gaps for *N. fowleri* methodology identified in this section include:

- Appropriate sample size and processing methods for *N. fowleri* in premise plumbing.
- Immunomagnetic separation technique could help improve culture of *N. fowleri* (decreasing competition from other microbes).
- Use and effectiveness of biofilm sampling techniques for *N. fowleri*.
- An occurrence survey for *N. fowleri* in different drinking water systems (e.g., source water, disinfectant residual used, and geographic location) is needed to better understand the ecology and dynamics of *N. fowleri* in distribution systems; in addition, in-household occurrence testing would be useful to better understand the best locations for sampling premise plumbing.
- Non-MPN quantification and viability characterization methods (e.g., PMA-PCR) to decrease the time and cost associated with estimating *N. fowleri* concentrations in environmental samples.
- Higher discrimination genotyping methods to enable better linkage between *N. fowleri* detected in environmental sources and *N. fowleri* infecting humans.
CHAPTER 5: METHODOLOGY FOR SIMULATING DRINKING WATER SYSTEM ENVIRONMENT

The ability to reproducibly grow opportunistic pathogens in laboratory assays representative of real-world drinking water conditions remains as a serious barrier to understanding opportunistic pathogen behavior in premise plumbing systems. This section provides an overview of the specific challenges that opportunistic pathogens pose for relevant research, the approaches currently applied for their study. Strengths and weaknesses of each approach are discussed.

GENERAL LABORATORY ASSAY CHALLENGES FOR OPPORTUNISTIC PATHOGENS

A defined drinking water medium capable of sustaining growth of *Legionella* has yet to be identified. Typical growth media used in many prior studies, such as buffered charcoal yeast extract (BYCE), are highly eutrophic, and results obtained from such studies may not always be relevant to potable water. Also, opportunistic pathogens tend to reside in biofilms, which are challenging to establish consistently and also to sample effectively. Several factors have been noted to be potentially key with respect to successful growth of opportunistic pathogens in drinking water assays, in particular: temperature, ecology, strain adaptation, and biofilm characteristics.

Identifying appropriate incubation conditions is also challenging. Inconsistencies observed in response to temperature are especially acute in the case of *Legionella*. Most studies appear to assume 37°C to be the ideal growth temperature (Hwang et al., 2006; Wadowsky et al., 1991). However, a recent report indicates that lower temperatures (e.g., 25°C, 17°C, 12°C) are beneficial for maintaining *L. pneumophila* cultivability in tap water relative to 37°C (Soderberg, Dao, Starkenburg, and Ciacciotto, 2008). On the other hand, *Legionella* are frequently isolated from hot water at temperatures greater than 42°C (Borella et al., 2004; Mathys et al., 2008). Nonetheless, in a controlled study, even environmental strains of *L. pneumophila* failed to grow in tap water when the temperature was equal to or higher than 42°C (Wadowsky et al., 1985). These studies highlight inconsistencies commonly observed across studies with respect to temperature, particularly when results from the laboratory and field are compared.

The ecology that supports opportunistic pathogen growth under the oligotrophic conditions characterizing the premise plumbing environment is extremely complex. Thus, a major challenge for representative laboratory assays in simulated systems, such as annular reactors, simulated water distribution systems and simulated water heaters, is to representatively reproduce this ecology under controlled conditions. Thus, the inoculum, attachment substrate, and water composition will be key factors in obtaining representative and reproducible results.

OVERVIEW OF SYSTEMS FOR SIMULATING DRINKING WATER ENVIRONMENT

The purpose of this Chapter is to describe commonly used physical systems for investigating the behavior of biofilms under drinking water conditions. The focus will be on systems and operating conditions that can simulate to some extent full scale distribution systems and/or premise plumbing; it will not include unrealistic laboratory methods such as 96 well plates or other model systems used for studying the behavior of pure cultures. With the
exception of the large pilot-scale pipe reactors and side-stream devices, the systems described herein can be used with either defined populations or undefined mixed biofilms representative of the real world.

There are many potential model systems that can be used to simulate premise plumbing and distribution systems. All have limitations and restrictions on use. In spite of their limitations, reactor systems do allow the researcher to control desired variables so experiments can be designed around key questions. Another advantage is that pathogens can be studied without using a full-scale system and putting consumers at risk.

When selecting a model system to study pathogens, it is important to note that there is no universally accepted device or condition; experimental design requires setting some parameters and restricting the study to a set of known variables that can be manipulated. The researcher must be cognizant of the conditions that are important to the particular organism and the hypotheses/research questions to be addressed. The reactor system then serves as the framework for the experimental design. To select the best possible reactor option, the user must decide if the intent is to study an individual organism in pure culture, as part of a defined mixed population determined by the researcher, as added to an indigenous biofilm of drinking water organisms, or as part of a ‘natural’ biofilm where there is no specific inoculum added. The user must then determine physical/hydraulic and chemical variables of importance. Physical aspects include the type of material used for growing the biofilms and temperature. Another physical attribute is the flow or hydraulic condition. Critical parameters will include intermittent or continuous flow, scaling shear stress to a relevant system, residence time, and whether the system is best modeled as a continuous flow stirred tank reactor or as a plug flow reactor. Chemical parameters encompass basic water quality including the type and concentration of organic carbon for heterotrophs, inorganics for chemotrophs, electron acceptor(s), disinfectant(s), corrosion control chemicals, trace elements, and other compounds needed for balanced microbial growth. In some cases it may be important to use water quality that supports the growth of other organisms including grazing protozoa. When choosing a reactor the sampling strategy is also important. Since pathogens are often distributed heterogeneously across the biofilm and may be in low numbers, the level of detection of the analytical method will determine the amount of surface area needed for a representative sample. Statistical validity of the data will require a minimum number of replicate measurements, further increasing the amount of sample required. If the desire is to run the experiment for an extended period of time, the number of coupons/number of sampling opportunities will be important. The analytical technique will also determine if the reactor should allow for direct microscopic analysis or if the intent is to do destructive sampling followed by culturing, molecular methods, or microscopy. Another key aspect is the ease of operation. Plug flow systems may be more representative of the real world but typically require large volumes of influent and create problems with the disposal of pathogen contaminated effluent. Costs are also important, not only for the reactor itself but also in the time and effort to maintain the system and the operation costs.

With these considerations in mind, the following section on types of reactors was organized to provide an overview of the types of systems that have been used by various groups to study the behavior of pathogenic organisms in simulated drinking water systems. Reactors are typically defined based on their flow characteristics and how they are modeled. The three basic types are batch, continuous flow stirred tank reactor, and plug flow reactors. Each of these will be described in an overview followed by specific applications of the basic design.
BATCH REACTORS

Batch reactors have no flow in or out of the vessels. Batch systems are traditionally used to study pure cultures of organisms although they may also be used with mixed populations. They are commonly used in microbiology and are represented by test tubes and flasks. Because there is no flow, the organisms see changes in their environment over time as they utilize nutrients and produce waste products. The vessels can remain stationary or can be placed on a shaker or stir plate to induce some form of fluid motion. In these reactors, most of the growth of the organisms will be planktonic or in the bulk phase/liquid, although there can be the formation of a biofilm on the surfaces of the vessel, at the air/water interface, as flocs in the fluid, or as a pellicle at the surface. It is possible to make a very simple biofilm reactor by installing coupons or microscope slides within a flask (Valster et al., 2010). As a case in point, there are tests that where the ability of certain plumbing materials to support biofilm growth is tested. An example is the biomass production potential (BPP) test that is used in Europe to assess biofilm formation. In this method (van der Kooij & Veenendaal, 2001), materials used in drinking water systems are exposed to slow sand filtered water that is replaced weekly (semi-static batch) and the amount of ATP/cm² of the materials determined. The method was extended to Legionella (van der Kooij et al., 2005). The primary limitations of these reactors are that the organisms will see changing conditions with time, the organisms in the fluid could be the result of detached biofilm or growth of suspended cells, biofilm formation may not be favored over growth in the planktonic state, and they are not representative of most conditions in actual distribution systems. Advantages are that they are very simple to operate, can give preliminary results, and are reasonably easy to maintain with pure or define cultures of organisms since contamination can be minimized. Batch systems are most commonly used for laboratory scale experiments.

CONTINUOUS FLOW STIRRED TANK REACTORS (CFSTRS)

As the name implies, a CFSTR is continuously mixed or stirred and there is a constant feed rate of influent into the reactor. As a consequence, there are theoretically no gradients in any of the constituents in the bulk phase and the effluent reflects the conditions found everywhere within the vessel. In other words, the CFSTR should be operated so that it is at steady state where there is no net change in concentration or mass over time in the bulk fluid. During initial startup as a biofilm reactor, the CFSTR may not be at steady state until the biofilm reaches a thickness or organism concentration that is balanced between growth and detachment.

The time that it takes for the entire volume of the reactor to exchange once is known as the residence time (units of time). The dilution rate (D) is equal to the inverse of the residence time (1/t). When operated to grow organisms in the bulk phase, these are known as chemostats and the dilution rate must be smaller than the maximum growth rate ($\mu_{\text{max}}$) to prevent washout of the organism and the growth rate will equal the dilution rate. When the desire is to grow biofilms, CFSTRs are run so that the dilution is larger than the growth rate of the organism, thus ensuring that most organisms in the effluent of the reactor result from detached biofilms and not from growth in the planktonic phase. CFSTRs will also be run with a stir bar or other mixing mechanism to ensure that no short circuiting or non-ideal mixing occurs. To create a biofilm reactor, the surface area is usually increased by the addition of coupons or other removable surfaces. An example was described by Rogers et al. (1994b) where plumbing materials were...
suspended in two chemostats in series to investigate the importance of these surfaces on the growth of *Legionella pneumophila*.

A CFSTR is frequently chosen for growing biofilms because it is reasonably easy to operate, a flow rate that conserves influent volume can be set, coupons or other surfaces can be added for biofilm growth so that replicate samples can be evaluated, it is well mixed, a steady state can be reached which minimizes variability, and it can be modeled. A variety of CFSTRs that have been used to simulate water systems and the two most popular types are described below.

### CENTERS FOR DISEASE CONTROL AND PREVENTION (CDC) REACTOR

The CDC reactor is representative of a class of CFSTRs that modify a standard chemostat by the addition of coupons. The reactor was designed by Donlan et al. (2004) at the CDC and then modified by Goeres et al. (2005) at the Center for Biofilm Engineering. The CDC reactor has been commercialized and is available from Biosurface Technologies in Bozeman, MT. The reactor consists of a straight sided one liter glass vessel with an effluent port located approximately 1/3 of the distance from the bottom. The medium is supplied through a port on the top of the reactor which is made of polyethylene. The reactors also contain a vaned magnetic stir bar that provides mixing and generates fluid shear. The top also has ports that accommodate 8 polypropylene rods each containing three half-inch coupons. The coupons can be made of a variety of materials. These reactors have been incorporated into an ASTM standard method (ASTME2562, 2007). CDC reactors are laboratory scale devices. Advantages of these reactors are the large number of coupons that can be made of relevant materials, the existence of a standard method, the small size, and general ease of operation. The small volume is advantageous for reducing the amount of influent and for handling potentially contaminated effluent. Disadvantages include the inability to characterize the flow relative to annular reactors or plug flow models described below and the large surface area of materials other than that of the coupons (glass vessel and polypropylene rods and vane).

### ANNULAR REACTORS

Annular reactors are so named because they consist of inner and outer cylinders that create an annulus between them. Fluid flows in the annular space between the two surfaces. There are two styles of annular reactors; one developed and marketed in France (Propella®) and one developed and sold in the United States (rotating annular reactor).

The rotating annular reactor is characterized by a stationary outer cylinder and an inner cylinder that rotates. The inner cylinder contains four diagonal draft tubes to reduce the potential for the formation of Taylor vortices in the annular space by facilitating vertical mixing. The first descriptions of the use of annular reactors to simulate drinking water systems were by van der Wende (van der Wende, 1991). When equipped with a torque monitor to obtain measurements of hydraulic shear stress, they are called RotoTorques (van der Wende, 1989). Rotation of the inner drum controls shear stress and the selected speed is based on the Hazen-Williams coefficient for the specific material and scaled to a circular pipe of a desired diameter. As with other CFSTRs, the dilution rate can be controlled by the volumetric flow rate of the influent. The influent is supplied through a port in the top of the reactor and water level and volume (usually one liter) is controlled by manipulating the effluent tube with a vent port at the desired elevation.
commercially available autoclavable polycarbonate model from Biosurface Technologies will accept 20 coupons of a variety of materials on the inner cylinder. An option is a glass cylinder on the outside of the reactor that can be filled with circulating temperature controlled water. An alternative design is to use actual pipe materials for the outer surface with tapped, flush mounted coupons that can be removed periodically for analysis. A distinct disadvantage of this design is that the reactor must be drained prior to removing the coupons for analysis.

An alternative design is the Propella® reactor developed by Parent et al. (1996). Any pipe can be used on the exterior. There is an open cylinder fitted inside of the outer pipe with a defined annular space between them as well as at the bottom of the inner cylinder. A cap is attached to the top and bottom of the outer cylinder along with an inlet and outlet port at the top. Flow is provided via a propeller fitted at the top of the reactor that pushes water through the inner cylinder. Water then flows from the bottom of the inner cylinder into the annular space and up to the top of the system. For this reactor, coupons are tapped into the outer cylinder. As with the pipe annular reactor, the system must be partially drained for sampling. Annular reactors have a number of advantages for biofilm testing. It is possible to use actual distribution system pipes (larger diameter) for both the rotating annular reactor and Propella®. The devices can be used at the laboratory or pilot scale or as monitoring devices (side streams) for full-scale systems. The shear stress can be set independently from the dilution rate so that various points in a distribution system can be modeled. The systems are generally well mixed and can be modeled individually as CSTRs. There are multiple coupons so that replicate samples at one point in time or over an extended number of weeks/months. The reactors are much more compact than pipe loops (described below) and require less water to simulate extended water ages or residence times. When dealing with pathogens a desirable attribute is the ability to collect and disinfect the effluent. It is also possible to amend the influent water with a variety of chemicals or disinfectants. Disadvantages include a much larger surface area to volume ratio than found in pipes, the need for more skill in operating a system with more moving parts, and the need for flush mounted coupons to prevent undefined flow characteristics at the edges. It is also possible to have heterogeneous distribution of biofilm on the surfaces, depending upon operational conditions.

**PLUG FLOW REACTORS (PFRS)**

In a plug flow reactor the fluid moves through the system in the direction of flow (axial or X direction) with no axial mixing. Mixing occurs only in the radial or Y direction. Steady state assumes that the concentrations of substances are constant at any fixed point in the reactor, but the values will vary with location. In the case of a reactant, the concentration will decrease at distal ends of the system relative to the proximal end but at steady state any specific point the concentration will remain constant with time. A PFR is much different from a CFSTR when considering biofilm growth. In a PFR, environmental conditions will change progressively throughout the reactor. For example, for heterotrophic bacteria, the carbon source and terminal electron accepter will be at the highest concentrations at the influent end and lowest at the effluent. The opposite is true for waste products. In a CFSTR, the conditions are uniform throughout the entire reactor.

There are a variety of systems that can be characterized as plug flow reactors provided that they are operated in a once-through mode. These include flow cells, tubing reactors, the modified Robbins device, and pipe loops/rigs.
FLOW CELLS

Flow cells are small laboratory reactors designed so that direct microscopic examination of the biofilm can be accomplished non-destructively. When these devices are used to establish biofilms, it is important to consider entrance effects on the flow characteristics so that the data can appropriately interpreted. Typical configuration requires that the flow cell be connected upstream with some type of tubing; therefore, colonization of the tubing upstream of the flow cell should be considered. Care should be taken so that air bubbles do not form in the tubing as these can scour the biofilm from the surfaces of the flow cells. Another consideration is the type of pump that is used. Peristaltic pumps are most commonly used, but this can lead to pulses in flow and impacts on biofilm formation. Commercially available pulse dampers can help minimize bubble formation and pulsed flows.

One model of flow cells is the flat plate reactor where a coupon of desired material is used as the base and the channel is created with a gasket and a glass coverslip. The reactors have a small volume and due to the need for a short working distance for the microscope objective, the depth of the lumen is limited. The shallow lumen and thin glass coverslip, restricts flow regimes to laminar at low Reynolds number. In spite of these limitations, flow cells have been used routinely to investigate the behavior of individual organisms under a variety of environmental conditions, including disinfection. They are not generally used for the establishment of environmentally relevant biofilms and biofilm development time is usually limited to days since longer times/thicker biofilms will lead to plugging.

A second type of flow cell is the glass capillary. As with the chamber-style reactor described above, the advantage of these reactors is the ability to use a microscope to directly view biofilm formation and behavior. The glass capillaries are commercially available in square or rectangular configurations. For direct observation of biofilms with a microscope, the dimensions are often 1-3 mm. Entrance effects can be pronounced in these systems, requiring an adequate length to mitigate this problem.

Advantages of the flow cells are that the biofilms can be observed directly and in real time with a microscope. If the target cell (pathogen) is self-labeled with a fluorescent protein, initial attachment and proliferation can be tracked non-destructively. When image capture is combined with image analysis, the dynamics of the system can be documented and quantitative information can be obtained. In the flow cells, different substrata for biofilm formation can be used. In the case of glass flow cells, several can be run in parallel and fed with the same influent, permitting reasonably rapid replicate analyses at low cost. Disadvantages are restrictions on Reynolds number (limited to laminar flow), small volumes and tendency for plugging, short residence times, and breakage due to fragile glass coverslips and capillaries.

ROBBINS DEVICE AND MODIFIED ROBBINS DEVICE

The Robbins device (McCoy et al., 1981) was originally designed as a fouling test apparatus where individual plugs could be removed without draining the system, although flow must be stopped. It consists of a 1.422 cm inside diameter admiralty brass tube with screw-in flush mounted fittings topped with a sample surface. The devices were then modified for laboratory use by creating the flow channel inside an acrylic block with sampling ports on one side. The ports were filled with sampling plugs where different surfaces could be flush mounted.
with the interior lumen (Nickel et al., 1985). In both cases, the device is connected by tubing to a water or nutrient source and flow is provided by the system or a pump. If operated in a once-through mode, these units are characterized by plug flow. Advantages of these reactors are the ease of removing the plugs to sample the biofilms, the ability to use different surfaces, and the number of replicate samples that can be taken. For laboratory devices, pathogen behavior can be monitored, although sampling is typically destructive and requires scraping or otherwise dispersing the biofilm prior to analysis. Disadvantages are that gradients will develop along the length of the reactor (disadvantage of all plug flow systems) and that there can be leaking along the o-ring on the plug in the modified Robbins device. In the modified Robbins device, flow velocities are limited to those that do not dislodge the plugs, but this can also be an advantage if the effluent must be collected and sterilized prior to disposal.

**BIOFILM MONITOR**

The biofilm monitor was developed by van der Kooij et al. (1995) to assess the biofilm formation potential of drinking water. The device consists of a vertical glass column that contains rings of the desired test surface. As originally designed, the reactor is approximately 60 cm long and contains 40 1.5 cm long rings. Water flows downward and contacts both the inner and outer surfaces of the rings. Flow is controlled with a flow meter and pressure modified as needed. In the original documentation, empty column velocity was set at 0.2 m/s. Advantages of this design are many coupons with large surface area, the ability to use different surfaces, flow conditions can be set to represent realistic conditions, and actual source water can be used. A disadvantage is gradients will develop along the length (although no gradients were reported in colony counts in the original manuscript), and if pathogens are added, there is a large volume of effluent water to handle/disinfect.

**TUBING REACTORS**

Tubing reactors are simple devices where a length of flexible tubing with a small inside diameter (for example, size 16 silicon tubing, 1 m length has a volume of 7 ml). The tubing is directly attached to a nutrient supply and flow is controlled with a peristaltic pump. These reactors were initially described by Sauer and Camper (2001) where they were used to grow a pure culture biofilm. The system is inoculated by injecting the target organism into the tubing, followed by the introduction of flow. At desired time intervals, the biofilm is sampled by aseptically cutting sections of tubing to the desired length and then squeezing or otherwise removing the biofilm from the tubing surface. Experiments with these systems are usually short in duration (no more than two weeks). Advantages are that small volumes of fluid are needed, pure culture biofilms can be maintained if the system is protected from backwards migration of organisms from the effluent end, they are extremely simple to operate and inexpensive, and multiple replicates can be run simultaneously. Disadvantages are that they operate under low flow conditions (if the desire is to simulate turbulent flow in distribution systems), the tubing must be able to be cut for sampling, there may be gradients along the length, and long term experiments are often not feasible due to plugging.
PIPE REACTORS

There are a wide variety of pipe reactors varying in size from bench top to pilot scale, using plumbing materials or distribution system piping, new or old pipes, all with and without removable coupons or other means of sampling surfaces. Some operate under continuous flow while others are designed to mimic the intermittent flows found in premise plumbing or at the dead ends of distribution systems. Pipe reactors can be used to study non-biological interactions between water quality, hydraulic conditions and the pipe surface such as corrosion, or with biologically mediated surface and water quality as in the case of biofilms.

Pilot-scale distribution facilities are typically constructed to provide a platform for determining the influence of water quality parameters on full-scale performance. This is required when access to the full-scale system is limited, when the parameters to be tested may significantly alter the quality of water delivered to the consumer, or if more tightly controlled conditions are required (flow, water quality, pipe composition, etc.). Since pilot system results are often extrapolated to the distribution system, it is critical that the reactors be designed to simulate relevant operational conditions, including shear stress, pipe materials, temperature, disinfectant types and concentrations, and other water quality parameters such as organic carbon concentrations. Coupons for sampling surfaces should be flush mounted to reduce perturbations in the local hydrodynamics. Ideally, the reactors should be relatively compact, easily controlled, have minimal water demand, and be inexpensive to construct, maintain, and operate.

The system described by Haudidier, et al. (1987) was the first major pipe reactor used to investigate microbial water quality and biofilms. It was constructed in Nancy, France and consisted of six individual pipe loops composed of cement-lined pipe. This system was not operated in plug flow (see section 2.4 below) but rather in partial recycle mode. Test coupons of a desired material are inserted flush to the inner pipe wall and removed to assess biomass accumulation or other desired parameters. Additionally, the bulk fluid from that loop can be sampled at the same residence time. A similar system was designed and operated in Bozeman, MT and used to investigate coliform incorporation and survival in indigenous drinking water biofilms (Camper and Jones, 2000). Another example is the large pipe loop facility at the U.S. EPA in Cincinnati that has been used in a variety of experiments where biofilms have been of interest.

Because of the wide distribution in application and research questions, pipe reactor design is diverse. An overview of the different types of reactors and their design is presented in Eisnor and Gagnon (2003), as are guidelines for selecting the appropriate reactor based on physical, hydraulic, water quality, and study duration criteria. When considering the use of a large-scale pipe reactor, advantages are that relevant distribution pipes (composition and diameter) can be used, realistic hydraulic conditions can be set, and water quality can be altered to simulate conditions in actual distribution systems. Disadvantages are size, capital and operating cost, the inability to change pipe materials with the exception of coupons, and some designs have a limited number of coupons for sampling. Another distinct disadvantage is that large volumes of water and flow rates are needed to achieve realistic shear stresses in the pipes, and therefore the residence times are typically very short.

For the vast majority of researchers, the large pilot-scale pipe reactor is not necessary. More common is a smaller bench-scale system that uses relevant pipe materials. This is the configuration most often seen when the research question is focused on pathogens, so that smaller volumes can be used and sampling is more reasonably accomplished. As with the larger
systems, the pipes can be sampled directly, be equipped with coupons, and have continuous or intermittent flow. Temperature can be controlled and disinfectant can be added to select for specific organisms.

DIFFERENT MODES OF OPERATING CFSTRS AND PFRS

As stated above, CFSTRs have a set residence time determined by the dilution rate. If multiple residence times are of importance, CFSTRs can be operated in series, with the effluent of one reactor acting as the influent of the next one. With this configuration long residence times can be reasonably simulated, and if annular reactors are used, a realistic shear stress can be set in each reactor.

It is also possible to run PFRs in partial recycle mode. This configuration reduces the amount of influent water, and gives greater flexibility in setting the flow velocity and residence time. Pipe reactors run in partial recycle mode can be modeled as CFSTRs. If the experimental design benefits from varying residence times, pipe reactors operated in partial recycle mode can be run series as described in the previous paragraph on CFSTRs.

SIDESTREAM DEVICES

In some cases the best experimental reactor is one that is directly connected to an actual operating distribution system. These sidestream devices provide a “window” into the full-scale system that would be otherwise difficult to sample. Sidestream devices can be annular reactors, Robbins devices, biofilm monitors or pipe reactors. They can be once-through with water from the full-scale system flowing through the test device to waste, or they can be set up in partial recycle mode.

CONSIDERATIONS ON REACTOR SELECTION

In addition to the overview on reactor selection provided in the introduction, there are other considerations on the selection of the appropriate reactor selection. These may be as follows:

- In some cases, it may be important to know if the organisms in the bulk fluid are the result of detached biofilm. If so, batch reactors will not be appropriate.
- Specific mathematical models for growth and/or hydraulic conditions will require the use of the relevant type of reactor and reactor operation.
- Surface area to volume ratios will vary with reactor type. If this is an important parameter, the reactor must be sized and operated accordingly.
- Materials selection may be critical. For example, if the impact of copper surfaces is important, then it is obvious that copper must be used. However, if copper is only a small component of a reactor (as coupons), then sampling organisms in the bulk water will represent those that have also grown on other reactor surfaces. Conversely, even small quantities of iron surfaces will cause the release of corrosion products that will impact the biofilms present on the remainder of the reactor surfaces.
- Iron surfaces can be very difficult to work with. Iron corrosion products interfere with microscopic analysis, extraction of DNA, calculating surface area, and the
disaggregation of organisms for culturing and enumeration. If iron surfaces are important, then adjustments must be made in sampling and enumeration/detection methods.

- Ease of sampling must be balanced with system relevance. For example, coupons are much easier to remove and sample as opposed to actual pipe surfaces. However, in some situations actual pipe materials may be more appropriate for the experimental design. In either case the number of replicate samples, possible spatial heterogeneity in biofilm within the reactor (particularly important for plug flow reactors), and need for temporal analysis will help drive reactor selection.

- Accessibility and oversight of the reactor is a concern. Simulated reactors leak, pumps stop operating, and lines plug. These issues can be even more important if pathogenic organisms are involved. Therefore, the devices must be in an area that is accessible (especially important for side stream devices) and personnel should be appropriately trained to handle system failures. This is true if the reactor is a large pipe loop or a small flow cell.

- Cost and ease of operation should be balanced with relevance and experimental design. This includes pumping costs, time and costs for preparing the influent water for the reactor, the amount of effluent water to be properly disinfected and disposed, number of replicate experiments to be performed, etc.

CONSIDERATIONS ON SYSTEM OPERATION

Coupled with the decision of the type of reactor to use are the conditions under which they are operated. As with the type of reactor, the conditions should be selected so that they mimic the parameters most important for the pathogen of interest without unduly complicating the experimental design. In many cases this will require trade-offs between variables that can be manipulated and unknown real-world conditions that lead to the growth and/or persistence of the biofilm and pathogen. Inherent in this discussion is the variability that often exists in pathogen distribution and numbers in the biofilm and the problems that are encountered when the test organism is present heterogeneously and at numbers at or approaching the limit of detection.

PURE CULTURE VERSUS MIXED POPULATIONS

If the desire is to represent real-world conditions, then the pathogen will be added or recruited naturally into a mixed population biofilm. The pathogen will then become part of that ecology and subjected to either deleterious or beneficial effects from the other organisms. In this case the analytical method will require that the pathogen be distinguished from the other existing organisms. An advantage of this scenario is that no special efforts are required to prevent the introduction of organisms from the environment. If the intention is to simplify the system and remove the effects that may be introduced by the presence of other organisms in the biofilm, then it a pure culture biofilm of the pathogen is important. Unfortunately, for many environmental organisms, it may be difficult or impossible to grow them under drinking water conditions as pure cultures. This may be because they require the presence of other organisms to produce an environment conducive for growth, including the production of growth supporting substrates or the creation of a base biofilm that allows the pathogen to persist. Regardless, if the intent is to limit the experimental design towards exploring the behavior and response of a pathogen using
methods or data interpretation where only one organism is present, pure cultures are required. Operating reactors limited to single species is more difficult because of the chance for contamination.

**CHOICE OF THE BASE BIOFILM**

Related to the decision for using a mixed population biofilm is the choice of the source of the organism(s) that will be used along with the pathogen. If the ecology of the pathogen is understood, or if the desire is to have a simplified system for analytical reasons, then the reactor can be inoculated with organism(s) that will allow the pathogen to attach and persist. This defined culture/mixed population approach presents an intermediate solution for biofilm growth between a pure culture and an undefined indigenous biofilm. The other alternative is the undefined mixed population biofilm that originates from the water of interest. This may be from untreated source water, distributed tap water, or water in premise plumbing. In many cases the reactor is plumbed directly into a water supply or it could be that the water is collected in containers. The use of actual waters represents the most ‘natural’ situation for biofilm formation, but it will usually produce the biofilm with the most variable composition.

Replication of some opportunistic pathogens is dependent on an amoeba host. Thus, selection of a host amoeba to inoculate into laboratory assays will clearly affect the outcome of the study.

**INFLUENT WATER – CHOICE AND COMPOSITION**

Directly related to the decision on the type of base biofilm is the composition of the feed or influent to the reactor. The influent feed can vary from a completely defined medium/synthetic water to amended or treated source/tap water, to unamended waters. There are advantages and disadvantages to each of these. The defined influent is consistent and the composition understood, which may allow for more reproducible experimental conditions. Unfortunately, and especially with environmental organisms, the nutrients/trace compounds or other factors may not be present in these waters, and growth may be limited or inhibited. This choice also requires more labor and expense for preparation of the reactor influent. Another possibility is amending natural waters to provide some control over influent conditions that can affect either the pathogen or base biofilm. This would include treating ‘natural’ water by biological filtration or to remove disinfectants, subsequent amendment of biologically treated water with a desired level of carbon, the addition of nitrogen or phosphorus for balanced microbial growth, pH or alkalinity adjustment, addition of a disinfectant at a desired concentration, etc. In some cases it may be desirable to provide a mixture of a synthetic water and ‘natural’ water in a specific ratio to obtain influent reactor conditions that are reasonably reproducible. The other possibility is to use an unamended ‘natural’ water to most closely represent conditions that occur in the field.

**CHOICE OF PATHOGEN**

This may seem obvious – use the organism of interest in the experiment. However, this is not always simple or straightforward. There may be substantial differences in the response of an organism to conditions in a biofilm depending upon its source. An example would be the
behavior of a laboratory strain versus an environmental isolate of a specific organism; laboratory organisms tend to have much poorer survival and less realistic responses to real-world conditions than environmentally adapted organisms. The investigator must then decide if they wish to use a laboratory strain that may be very well characterized but less likely to represent an actual pathogen or an actual pathogen/environmental isolate that is not as well understood. Another alternative, particularly when the subject of interest is an opportunistic pathogen indigenous to water systems is to use the actual water from a system with that organism as the inoculum or feed water. In this case, the experimental system selects for the pathogen. A disadvantage of this option is that the desired organism may not become incorporated into the biofilm of the experimental system. Another limitation is that the recruited organism may not be ‘typical’ and detection/ enumeration may be hindered by the selectivity of the analytical methods.

**PATHOGEN INOCULATION**

In the case of bacteria, the way in which the organism is grown and prepared prior to inoculation will affect its ability to persist. Bacteria that arise from log phase, high nutrient conditions are less likely to persist in the biofilm due to nutrient shock and other phenomena. This may be a desirable scenario if the intent is to mimic direct fecal conditions. Note that if an inoculum from a rich medium is desired, the cells should be washed so that organic carbon is not transferred to the reactor. If the intent is to determine long term persistence of organisms exposed to the environment, it would be better to hold the inoculum under low nutrient conditions similar to those in the reactor before adding them to the reactor. Another consideration is the number of organisms introduced. There is a balance between a representative inoculum (what may occur in the environment) and the reality of the limit of detection. The end result is that the inoculum may need to be unrealistically high so that the organism can be enumerated over time. The method of introduction of the inoculum may also vary depending on the experimental design. In many cases, the organism is added to a biofilm system without flow but with mixing to allow for initial attachment. This could be desirable if the intent is to monitor long-term persistence or the response to other variables such as the introduction of a disinfectant, changing nutrient concentrations, etc. However, this method does not represent conditions in an actual system where flow may be continuous. Another consideration is whether the inoculum should be a slug dose or if the organism is added continuously. This choice will again depend upon the experimental design, but in most cases, slug doses are used.

**CONTINUOUS VERSUS INTERMITTENT FLOW**

Reactor operation under continuous or intermittent flow will depend upon the situation being simulated. If the desire is to represent full-scale distribution system mains, continuous flow is typically used. If the experiments have been designed to simulate premise plumbing or dead ends of distribution systems, intermittent flow is best. In the latter case there are choices to be made on the periods of stagnation versus flow. These options will once again depend on the situations to be simulated.
### BIOFILM SAMPLING TECHNIQUES

Table 5-1 provides an overview of biofilm sampling techniques, advantages, and limitations.

**Table 5-1**
Removal techniques found in the published literature and their associated advantages and limitations.

<table>
<thead>
<tr>
<th>Removal technique</th>
<th>No. of references; brief citations</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wash or Rinse or Application of Treatment</td>
<td>5; Morris et al., 1998; Augustin et al., 2004; Midelet and Carpentier, 2004; Antoniou and Frank, 2005; Vieira et al., 2005</td>
<td>Rinse removes loosely attached cells/clumps</td>
<td>Treatments can either completely remove or fix cells/biofilm to surface</td>
</tr>
<tr>
<td>Scrape</td>
<td>11; Gagnon and Slawson, 1999; Laopaiboon et al., 2002; Campanac et al., 2002; Marion-Ferey et al., 2003; Pitts et al., 2001; Pettit et al., 2005; Zelver et al., 2001; Vickery et al., 2004; Chen and Stewart, 2000; Pitts et al., 2003; Wood et al., 1998</td>
<td>Removal from a defined area</td>
<td>Requires time and thoroughness; no guarantee that cells are released from the scraper</td>
</tr>
<tr>
<td>Scrape/Vortex</td>
<td>1; Ntsama-Essomba et al., 1997</td>
<td>No rinsing or manipulations between samples</td>
<td>Removal of biofilm from all coupon surfaces</td>
</tr>
<tr>
<td>Scrape/Centrifuge/Sonicate</td>
<td>1; Cerca et al., 2005</td>
<td>Removal from a defined area</td>
<td>Injury to cells</td>
</tr>
<tr>
<td>Swab</td>
<td>2; Gagnon and Slawson, 1999; Wirtanen et al., 2001</td>
<td>Swab can remove biofilm evenly over surface</td>
<td>Most likely ineffective for rough surfaces. No guarantee that cells are released from swab material</td>
</tr>
<tr>
<td>Swab/Vortex</td>
<td>2; Donlan et al., 2004; Moltz and Martin, 2005</td>
<td>Swab can remove biofilm evenly over defined surface area</td>
<td>Most likely ineffective for rough surfaces. No guarantee that cells are released from swab material</td>
</tr>
<tr>
<td>Sonicate or Ultrasonicate</td>
<td>11; Harrison et al., 2005; Marion-Ferey et al., 2003; Ceri et al., 1999; Chavant et al., 2002; Lindsay and von Holy, 1997; Mermillod-Blondin et al., 2001; dos Santos Furtado and Casper, 2000; Salhani and Uelker-Deffur, 1998; Camper et al, 1985; Sreenivasan and Chorny, 2005; Oulahal et al., 2004</td>
<td>Aseptic, efficient, no rinsing or manipulations between samples. Ability to remove and disaggregate in one step</td>
<td>Death to injured and non-injured cells is a concern</td>
</tr>
<tr>
<td>Ultrasonicate with glass beads</td>
<td>1; Khammar et al., 2004</td>
<td>Aseptic, efficient, no rinsing or manipulations between samples. Ability to remove and disaggregate in one step</td>
<td>Death to injured and non-injured cells is a concern</td>
</tr>
</tbody>
</table>

(continued)
METHODOLOGICAL GAPS FOR SIMULATING DRINKING WATER ENVIRONMENT FOR LABORATORY STUDY

As described extensively above, each lab system for simulating premise plumbing has its advantages and disadvantages. It is therefore critical to choose the right system based on the question being asked. As noted in Table 5-1, each biofilm sampling approach also has advantages and disadvantages. In addition to the limitations for each system noted above, some broad methodological gaps are:

- Appropriate simulated drinking water media. At present, there is currently no known synthetic drinking water medium known to support the growth of Legionella. This makes it extremely challenging to vary water parameters in a controlled fashion in order to identify triggers for Legionella proliferation. This topic is explored extensively in Water Research Foundation Project 4251 Final Report.
- Ideal biofilm growth and sampling approaches. Drinking water biofilms can take years to establish stable populations (Martiny et al. 2003). The effect of repeated sampling of the same location and with time is poorly understood, and no biofilm recovery methods are 100%.
CHAPTER 6: POTENTIAL AND LIMITATIONS OF ENGINEERED CONTROLS FOR OPPORTUNISTIC PREMISE PLUMBING PATHOGENS

Ideally, regulatory and engineering controls could be implemented for community water systems that would eliminate occurrence of OPPPs for the entire population at all times. This ideal has been mostly achieved for control of conventional waterborne pathogens in the U.S. through decades of investment by regulated water utilities, with few noteworthy exceptions including treatment plant upsets, severely immunocompromised individuals (e.g., HIV and Cryptosporidiosis) and contamination events due to problematic infrastructure. This penultimate chapter describes why existing scientific understanding, practical engineering tools, and conventional regulatory approaches have not yet achieved a similar level of success for OPPPs, and further considers the types of solutions that must be implemented to protect consumers while addressing other important societal goals. Three sections provide a rational basis for identification and prioritization of OPPPs research by highlighting:

1. The key engineering and regulatory realities of OPPPs control versus that of conventional pathogens;
2. Shared responsibility model amongst parties with potential to address OPPPs; and
3. Engineered approaches that can reduce risk. Here, the present state of knowledge regarding likely solutions for each representative OPPP and discussion of acknowledged conflicts between OPPPs and other societal challenges such as water conservation, energy conservation, and infrastructure maintenance are discussed.

ENGINEERING AND REGULATORY REALITIES

The preceding chapters demonstrate that the occurrence of OPPPs at a given tap or shower of a community water system are influenced by numerous factors that are under utility control including:

1. The quality of water leaving the treatment plant as defined by temperature, pH, nutrients, seed microorganisms, type and dose of disinfectant;
2. The water distribution system design, operation, and maintenance including material type, storage facilities, water detention times, use of booster disinfectant facilities;
3. Types of materials in the service line connecting the water main to homes (galvanized iron, PVC, lead, copper); and
4. Corrosive and scaling propensity of water inherent in the source and as modified by treatment(s). At some point between the beginning and end of the service line, dependent on the specific city, responsibility for care of the water before it emerges from taps passes from the utility to the consumer (or building owner/operator).

Within buildings a multitude of factors also influence the level of OPPPs emerging from individual taps (Figure 6-1). Water use factors such as stagnation patterns, total flow and velocity can vary from building to building and within individual lines of individual buildings. The presence of home treatment devices can either add disinfectant to or remove disinfectant from water, or profoundly alter the basic chemistry and biology of the water through treatments
such as softening or GAC or particulate filtration. Different plumbing devices, such as faucets and valves, and different plumbing materials, such as copper, plastic, and galvanized iron pipe, can consume disinfectants and introduce an array of microbial nutrients and toxins dependent on material age and the specific water supply. Each building also typically has a hot water system. These systems can vary dramatically in terms of water storage volumes (tank vs. tankless), individual hot water temperature settings, frequency of showers or baths, temperature profiles of the distribution system, flow condition when the hot water system is not in use (stagnant, occasional recirculation, continuous recirculation), and the status/type of anode. Each of the above factors can strongly influence the concentration of disinfectant or OPPPs at a given tap on a given day.

It is instructive to compare and contrast the above realities of OPPPs (Table 6-1) to the control of conventional pathogens (Beach et al., 2009) via source water protection, particulate removal (sedimentation/filtration), and primary and secondary disinfection as currently regulated by EPA under authority of the Safe Drinking Water Act (SDWA) (EPA, 1998). In general, responsibility for control of conventional waterborne disease lies almost exclusively with water utilities. In cases of severely immunocompromised individuals or those seeking a higher level of protection, targeted public education (e.g., to boil tap water) or installation of advanced treatment devices and filters can reduce risk of conventional pathogens further still, and there is very little risk of interventions going astray and dramatically increasing risk of disease. OPPPs, in contrast, are generally not expected at high concentrations at the point of entry to distribution systems, and existing EPA regulation of Legionella at this location probably has little or no influence on incidence of Legionnaires’ disease. Many factors under the direct and indirect control of building operators can dramatically reduce or increase the likelihood of waterborne disease due to OPPPs, and public education of high risk groups is complicated by the greater number of potential risk factors (i.e., genetic predisposition, weight, smoking, age) and exposure pathways. Finally, there are a number of direct conflicts between control of OPPPs and other worthy goals such as scalding, disinfection by-products, energy conservation, water conservation and corrosion control (Table 6-2).

Overall, while much has been learned about the fundamental science of conventional pathogen control that is directly transferable to OPPPs (e.g., principles of disinfection, filtration and public education), from the perspective of managing and addressing the problem, there is relatively little direct overlap in terms of responsible parties, causal factors and solutions. New paradigms are currently evolving to address OPPPs through a shared responsibility model.
Figure 6-1  Overview of major factors under control of utilities and consumers (building owners) which can influence the presence of opportunistic premise plumbing pathogens at a given tap.
Table 6-1
Comparing and contrasting problems of traditional pathogens to opportunistic premise plumbing pathogens.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Traditional Pathogens</th>
<th>OPPPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplification (re-growth) in the distribution system</td>
<td>Generally no</td>
<td>Yes</td>
</tr>
<tr>
<td>Immune status, genetics and age a primary factor in disease</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Different risk profile for hot and cold water system in buildings</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Risk dramatically reduced by source water protection, primary disinfection, particle removal at water treatment plant</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Secondary disinfection and residual, location of building in distribution system, utility distribution system design and operation can strongly influence risk</td>
<td>Generally no</td>
<td>Yes</td>
</tr>
<tr>
<td>Homeowner actions and water use patterns, plumbing hydraulics, building design and operation, plumbing materials use, water heater settings can dramatically increase likelihood of disease.</td>
<td>Generally no</td>
<td>Yes</td>
</tr>
<tr>
<td>Route and mode of exposure.</td>
<td>Ingestion</td>
<td>Inhalation, ingestion, skin contact</td>
</tr>
<tr>
<td>Water nutrient levels (organic carbon, nitrogen, phosphorus, trace nutrients) influencing microbial ecology in distribution system</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Inherent direct conflicts between risk reduction and other goals</td>
<td>Some conflicts disinfection by-products (DBPs)</td>
<td>Major conflict with DBPs, Scalding, Energy and Water Conservation, Corrosivity</td>
</tr>
<tr>
<td>Impacts of premise plumbing treatment devices including GAC or filters</td>
<td>No effect or beneficial</td>
<td>Detriments/benefits possible</td>
</tr>
<tr>
<td>Microbial ecology in distribution system influential</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Influence of water chemistry (temperature, pH, dissolved oxygen) in main and premise distribution system important</td>
<td>Little or none</td>
<td>Yes</td>
</tr>
</tbody>
</table>

SHARED RESPONSIBILITY APPROACH

A "shared responsibility" conceptualization can help to guide research and interventions related to control of OPPPs in drinking water. Such approaches have been applied to address other important concerns for premise plumbing (EPA, 1991; Sarver and Edwards, 2011) such as the EPA Lead and Copper Rule (LCR), aesthetic complaints and materials failure (Table 6-2). For example, under the LCR utilities are responsible for controlling lead in the source water (generally not a concern), the corrosivity of the water supply as controlled by water chemistry, meeting targets for pH, corrosion inhibitors, disinfectant type and dose at the point of entry to buildings throughout the water system, and monitoring for lead at high risk homes to assess community risk. At the same time, it is explicitly acknowledged that there are many factors
beyond the control of water utilities which can dramatically increase and control risk of elevated lead in water (Figure 6-1). Therefore, it may not be realistic or even possible for utilities to meet a maximum contaminant level for lead at a given tap. The plumbing code and voluntary third party standards are used to control risk from plumbing materials such as leaded brass, and public education highlights the dangers of specific consumer behaviors deemed of high risk (i.e., ingestion of hot water, not flushing lines for up to two minutes before collecting water for cooking or drinking, use of tap water to mix formula).

This type of approach is logically extended to the problem of opportunistic pathogens in drinking water, explicitly acknowledging that a range of stakeholders including water utilities, consumers, building designers, plumbers, code setting organizations, and device manufacturers have critical roles to play in preventing and solving problem with OPPPs in buildings (Table 6-2). There is also a potential interplay, sometimes synergistic and sometimes antagonistic, amongst the different premise plumbing problems which must also be explicitly considered as discussed throughout this chapter.
Table 6-2
Analyses between shared responsibility models for general bacteria regrowth inorganics, taste and odor, premise plumbing pathogens, and materials failure.

<table>
<thead>
<tr>
<th>Regrowth</th>
<th>Inorganics (Pb/Cu)</th>
<th>Taste and Odor</th>
<th>Premise Plumbing Pathogens</th>
<th>Materials Failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Utility</td>
<td>Provision of disinfectant residual, microbial stability</td>
<td>Control corrosivity of water, provide public education for susceptible populations.</td>
<td>Control problems originating before the property line</td>
<td>Provision of disinfectant residual and microbial stability to property line</td>
</tr>
<tr>
<td>Building Operator</td>
<td>Stagnation/Flushing</td>
<td>Stagnation/Flushing</td>
<td>Stagnation/Flushing</td>
<td>Temperature settings, Maintenance</td>
</tr>
<tr>
<td>Building Designer</td>
<td>Proper sizing of pipes and consideration of water age</td>
<td>Specify materials and commissioning</td>
<td>Suitable materials</td>
<td>Hot water system, Consider control strategies (elevated temperature, disinfection)</td>
</tr>
<tr>
<td>Device Proprietor/Manufacturers</td>
<td>Warning regarding potential problems associated with removal of disinfectant</td>
<td>Treatments should not increase corrosivity</td>
<td>Potential contribution of microbes and plumbing to problems</td>
<td>Remove disinfectant with caution, use suitable materials</td>
</tr>
<tr>
<td>Plumbers</td>
<td>Proper commissioning and installation</td>
<td>Use certified materials</td>
<td>Proper commissioning and installation</td>
<td>Follow design and codes</td>
</tr>
<tr>
<td>Code Organizations</td>
<td>Consider guidelines for water age</td>
<td>Consider guidelines for water age</td>
<td>Consider guidelines for water age</td>
<td>Consider guidelines for disinfection and temperature</td>
</tr>
<tr>
<td>Consultants/Health Agencies</td>
<td>Need to develop background, basic scientific expertise, and public education guidance on all issues and their associated interplay.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ENGINEERED APPROACHES THAT INFLUENCE OPPPS

Although complexities (biotic/abiotic) and uncertainties associated with OPPPs described in the preceding sections are daunting, a large body of experience has been gathered regarding possible engineering approaches with potential to influence the incidence of OPPP waterborne disease (Table 6-3). While much of the previous research has focused on individual OPPPs and the effectiveness of each intervention is not known for all five representative pathogens targeted in this report or in all situations, an overview of current understanding is provided in this section. In theory, the incidence of OPPPs could be controlled at every point of the flow path from source...
water to the consumer tap (Figure 6-1), but the practical approaches described in the literature generally fall into categories of:

1. Source water treatment and distribution system maintenance;
2. OPPP control in buildings via temperature control;
3. Use of chlorine, chlorine dioxide, or chloramine disinfectants at the treatment plant or in buildings;
4. Use of other disinfectants in buildings;
5. Possible nutrient limitations/controls;
6. Microbial ecology interactions (positive and negative) with other microbes;
7. Plumbing system materials;
8. Premise plumbing water flow patterns and design;
9. Whole house, point of use and showerhead filters; and

Potential undesirable outcomes and conflicts with other important premise plumbing operational objectives are apparent (Table 6-1).

Source Water Treatment and Distribution System Maintenance

*N. fowleri*, *Acanthamoeba* and other FLA are readily removed by coagulation and filtration at treatment plants (Hoffman et al., 2006; Jeong et al., 2005; Loret et al., 2008; Schuster and Visvesvara, 2004; Thomas et al., 2008; Thomas et al., 2010). Even though periodic tank cleaning and biofilm control can help limit the proliferation of amoebae (Bontrager and Robinson, 2007), breakthrough events probably occur releasing high numbers into the distribution system if they are present (Thomas and Ashbolt, 2011). Maintaining a chlorine residual and periodic unidirectional flushing of water mains are considered useful controls (Bontrager and Robinson, 2007).

Distribution system chlorination and maintenance practices are believed to be important in controlling waterborne disease caused by *N. fowleri* and *Acanthamoeba*, perhaps by minimizing their inoculation into premise plumbing or by favoring the survival of FLA competitors. For *N. fowleri* main distribution system monitoring and inspection, especially before and during the summer months when temperatures are in the optimal range of 25-44°C, are important. In one study *N. fowleri* was only detected in summer months (Bontrager and Robinson, 2007; Thomas and Ashbolt, 2011). *N. fowleri* has been observed to be more susceptible to chlorine disinfectants than other FLA competitors (Cursons et al., 1980). A study by Joslin et al. (2006) in the Chicago area indicated that a marked increase in *Acanthamoeba* keratitis occurred at distances far from the treatment plant hypothesized to be due to changes in disinfection practices in response to disinfectant by-product regulations. But follow-up by Methvin (2009) indicated that the sites were less than 2 days from the treatment plant, and no change in disinfection practice occurred in Chicago during the relevant timeframe (2000-2006). Verani (2009) also concluded that water disinfection was relatively unimportant.

As a general rule, current literature emphasizes water treatment and distribution system operation much more for control of amoebic OPPPs than for *NTM*, *Legionella* and *P. aeruginosa*. While EPA standards mandate *Legionella* removal from water treatment plants, there is little reason to suspect that this significantly impacts the incidence of Legionnaires’
disease. These observations might reflect a greater propensity of waterborne disease originating from premise plumbing operation for *NTM, Legionella* and *P. aeruginosa*, or perhaps, less intensive monitoring and scientific understanding of *N. fowleri* and *Acanthamoeba*. Whether the true situation is primarily a function of the former or latter has yet to be elucidated.

**In-Building Temperature Control**

Many studies have found that water temperatures between certain thresholds are positively associated with OPPP colonization (Arnow et al., 1985; Borella et al., 2005; Flannery et al., 2006). Broadly speaking, maintaining hot distribution system temperature above 60°C in all hot water lines is a favored control strategy in Canada and Australia, and is recommended by the World Health Organization. This approach can be enforced and encouraged through the plumbing code (NRC, 2006b; WHO, 2007). However, in the U.S. this approach is considered to be at greater odds with goals of energy savings, scaling issues in water heaters, and scalding prevention. As a result, the U.S. EPA recommends much cooler temperatures of 48°C in residences, which are likely to be much less effective in pathogen control (Brazeau and Edwards, 2011; NRC, 2006b).

At the time of publication of this document, a new ASHRAE Standard 188 has been proposed for *Legionella* control in at risk U.S. buildings. Because the third revision of this standard is currently subject to public review at this time, the second revision is referenced in this document (there is no significant difference between the two relative to this discussion). This draft Standard mandates hot water temperatures at heater outlets above 60°C and temperatures above 51°C at all points of a building distribution system (ASHRAE, 2011). If implemented, this would also be expected to reduce the risk of all OPPP waterborne disease originating from hot water systems. But there is no mention in the ASHRAE 188 or Canadian standards (NRCC, 2010) of the need to resolve inherent conflicts with scaling or energy savings goals for premise plumbing, whereas WHO documentation explicitly acknowledges that the risk of “colonization, therefore, should be balanced with other risks linked to the choice of materials, such as dissolution, corrosion and scaling” (WHO, 2007). In certain potable waters it is simply not possible to raise hot water systems above approximately 45°C without softening water, or addition of anti-scaling chemicals, without permanently damaging the performance of water heaters and plumbing systems (Brazeau et al., 2011). The draft ASHRAE standard also requires cold water systems to have temperatures below 25°C at all locations, which is again a worthy goal, but may not be achievable in many U.S. buildings given that source water and in-wall temperatures can routinely exceed this threshold.

Attempts to obtain the benefits of thermal disinfection without the long-term detriments, have been attempted by briefly spiking the temperature of hot water systems above 60°C periodically, and such approaches are listed as an emergency measure by ASHRAE (2011) and numerous other documents (WHO, 2007). However, validation tests have revealed that *L. pneumophila* can rapidly proliferate after temperatures were lowered, presumably via microbial response to the nutrients released by the newly killed biofilm (necrotrophy). Heat-killed *Pseudomonas putida, Escherichia coli, Acanthamoeba castellani*, *Saccharomyces boulardii*, and a general biofilm sample caused rapid *L. pneumophila* growth, whereas heated-killed Gram-positive bacteria did not (Temmerman et al., 2006). This finding illustrates that disturbing the microbial ecology short-term may exacerbate pathogen regrowth in the long-term. On the other
hand, van der Mee-Marquet and colleagues (2005) found that heat shock of *P. aeruginosa* at metered faucets provided much more persistent benefits (greater than 6 months).

<table>
<thead>
<tr>
<th>Engineering approaches to control opportunistic premise plumbing pathogens.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Technique</strong></td>
</tr>
<tr>
<td><strong>Strategies Applied in Individual Buildings</strong></td>
</tr>
<tr>
<td>Maintain &gt; 60° C in all hot water lines</td>
</tr>
<tr>
<td>Temporarily increase temperature &gt; 60° C</td>
</tr>
<tr>
<td>Dosing of Cl₂, chloramine and chlorine dioxide in building</td>
</tr>
<tr>
<td>UV-radiation</td>
</tr>
<tr>
<td>Copper/silver ionization</td>
</tr>
<tr>
<td>Point of use filters</td>
</tr>
<tr>
<td>Plumbing material</td>
</tr>
<tr>
<td>Prevent stagnation</td>
</tr>
<tr>
<td>Avoid metered faucets</td>
</tr>
<tr>
<td><strong>Community Based Responses (i.e., could be applied at treatment plant by utilities)</strong></td>
</tr>
<tr>
<td>Chloramine</td>
</tr>
<tr>
<td>Remove AOC/BDOC at treatment plant</td>
</tr>
<tr>
<td>Maintain Distribution System Pipes/Corrosion Control</td>
</tr>
</tbody>
</table>
Disinfection with Chlorine/Chlorine Dioxide/Chloramine

Utility secondary disinfection with chloramine and/or chlorine can control many premise plumbing pathogens. In particular, shifting to chloramine is considered to be a key community-based intervention that will reduce risk of Legionnaires’ disease and, sometimes, of *P. aeruginosa* (Chen et al., 1993; Flannery et al., 2006; van der Wende, 1991). However, it is not yet clear how protective this strategy would be in certain situations where chloramine residuals may be absent such as at the end of distribution systems (Nguyen et al., 2011; Zhang and Edwards, 2009). Of greater concern, is the observation that shifting disinfection regimes has been observed to perturb drinking water biofilm populations, resulting in the emergence of *Legionella* and *H. Vermiformis* when the water is chlorinated, and *Mycobacteria* when chloramines are used (Pryor et al., 2004; Santo Domingo et al., 2003; Williams et al., 2005). Thus, while there is strong evidence that secondary disinfection is broadly protective and beneficial for control of most OPPPs, there is a potential risk that *Mycobacteria sp.* will have a contrary response due to its extreme chlorine resistance (Moore et al., 2006).

Building-level application of chemical disinfectants (chloramine, chlorine, chlorine dioxide) can be very effective in some situations including hospitals, and on-site disinfection may be required by ASHRAE 188 (ASHRAE, 2011). Building level application of chloramine has not been studied over a prolonged period (Lin et al., 2011), and in some cases can be implemented simply by dosing of ammonia to water with free chlorine. The in-building application of chloramine, and possibly chloramine, can cause serious pinhole leaks in copper (Sarver et al., 2011, Chord et al., 2011) and stress corrosion failure of stainless steel especially above 55° C (Cactussim, 2002). In one case study continuous chlorination increased the incidence of pinhole leaks by up to 30 times (Rutala and Weber, 1997), consistent with extensive recent laboratory work in soft higher pH waters (Sarver et al., 2011). Disinfectants can also be relatively ineffective against certain biofilms (Cloete et al., 1998; Davies, 2003; Gagnon et al., 2005; Gilbert et al., 2002; Zhang and Edwards, 2010) where pathogens are typically harbored (Banning et al., 2003). Successive chlorine absence/presence episodes might select for chlorine and even drug resistance of surviving OPPPs (Codony et al., 2005; Shrivastava et al., 2004). Little is known about possible corrosive effects of chlorine dioxide.

Other In-Building Disinfectants

Numerous other disinfectants have been tested in buildings with varying degrees of success dependent on the situation and specific OPPP. While an exhaustive review is beyond the scope of this document, for *Legionella*, chlorine dioxide (Walker et al., 1995; WHO, 2007; Y. Zhang & Edwards, 2009) and bromine (Thomas et al., 1999) have been reported to be partially or completely effective dependent on circumstance.

Copper-silver ionization has also been reported effective under some conditions (ACHD, 1997; Rohr et al., 1998; Liu et al., 1994). There has been some concern that, over the time span of years, microbial resistance to silver and disinfectants can develop (Rohr et al., 1998). *Legionella* outbreaks have recently been reported in buildings where copper-silver ionization systems were installed (Smeltz, 2012). The evidence supporting copper-silver efficacy has been primarily from field studies, and additional efficacy data is needed from controlled laboratory studies. On September 21, 2007 EPA issued a notice in the Federal Register (Vol. 72, No. 183) that clarified why copper-silver ionization systems are regulated under FIFRA as pesticides, and...
are not regulated as devices (EPA, 2007), and FIFRA applications need to be made with efficacy data to obtain long-term EPA approval. This same issue recently caused the EU to prohibit the use of copper-silver ionization for *Legionella* control (HSE, 2013).

Ozone and ultraviolet light also can be locally effective or at the point of use, but their reach within plumbing systems is severely limited due to a lack of a persistent disinfectant residual (Domingue et al., 1988; Heuner et al., 1997; Kim et al., 2002; Lehtola et al., 2005; Lin et al., 1998; Muraca et al., 1987). Similar data is available for these disinfectants with many other OPPP including *P. aeruginosa* (Durojaiye et al., 2011; Grobe et al., 2001; Harrison et al., 2005; Huang et al., 2008; Jungfer et al., 2007; Silvestry-Rodriguez et al., 2008; Teitzel and Parsek, 2003) and potential bioterrorism agents, such as anthrax, where chlorine dioxide in particular has shown promise (Hosni et al., 2009; Shane et al., 2011; Hosni et al., 2011). Other representative but less common and less effective disinfectants tested for *P. aeruginosa* and *Legionella* include peracetic acid (10%) and hydrogen peroxide (3%), non-oxidizing amines and other organics (Kim et al., 2002; McCall et al., 1999).

It is unclear the extent to which the above systems could be successfully applied in consumer homes, since most of the above experiences are with large buildings and hospitals in which better trained personnel can be dedicated to operating and maintaining systems. Also, the potential to increase corrosion and premature failures of iron, galvanized pipe, copper, and stainless steel components via deposition corrosion would be expected in some circumstances and has not been thoroughly researched (Cruse, 1971; Fox et al., 1986).

### Nutrient Control and Limitation

Consideration of biological control via nutrient limitation represents a possible approach to the control of premise plumbing pathogens. Reducing assimilable organic carbon (AOC) has been observed to limit HPC growth, particularly in water main distribution systems (Camper et al., 2003; EPA, 2002; LeChevalier et al., 1991; van der Kooij, 1992), and limiting the amount of organic carbon by treatments such as biofiltration has been utilized successfully in many European countries to safely distribute drinking water with little or no disinfectant. In a study of Dutch distribution systems, AOC levels less than 10 μg/L were deemed sufficient to limit HPC regrowth even in the absence of disinfectant residual. AOC levels below 50 mg/L have been cited as desirable to control coliforms in disinfected U.S. distribution systems, and if AOC rises above 100 mg/L, problems with HPC regrowth are sometimes observed (LeChevalier et al., 1991). Reasonable correlations exist between AOC and HPC bacteria levels in distribution systems not using chloramines (LeChevalier et al., 1991; van der Kooij, 1992).

However, it is much less likely that AOC limitation will control regrowth of bacteria and OPPPs in premise plumbing. There are several mechanisms of AOC generation in premise plumbing systems including leaching from plumbing, autotrophic growth, rendering of less biodegradable organic fractions more biodegradable, and leaching of organics from plastics (Bagh et al., 2004; Butterfield et al., 2002; Haddix and Lechevallier, 2003; Morton et al., 2005; Rogers et al., 1994a; Zhang and Edwards, 2010). These mechanisms can undermine or even overshadow the significance of AOC concentrations leaving the treatment plant. Limitations of OPPP via other nutrients such as phosphorus is difficult to achieve, although they may be a contributing factor in at least some rare cases (Rohner et al., 2004).
Microbial Ecology Controls

There is a very significant influence of microbial ecology on disease caused by enteric pathogens (Sherman et al., 2010; Neish, 2009), and the inoculation of susceptible individuals with fecal matter from non-susceptible individuals can sometimes prevent future infection (Chang et al., 2008; Young, 2009; Garner et al., 2009; Sherman et al., 2009). The critical role of microbial ecology has already been demonstrated in the context of corrosion (Videla and Herrera, 2005), and there is evidence that probiotic concepts may be applicable to control of OPPP in premise plumbing.

For example, work by Camper et al. (1985) supports the notion that inoculation is a viable means of inhibiting pathogens in drinking water environments, as colonization of GAC by *Yersinia enterocolitica* 0:8, *Salmonella typhimurium*, and enterotoxigenic *Escherichia coli* were all inhibited by addition of natural river water bacteria. In another study, growth of *L. pneumophila* and *S. typhimurium* were observed to exhibit varying responses on glass and PVC coupons subjected to sterile and un-sterile tap water, indicating that the precise microbial ecology merits further examination (Armon et al., 1997). Biological control of *L. pneumophila* has also recently been achieved by increasing the concentration of several bacteria in the water, including *Bacillus subtilis* BS 104 (Temmerman et al., 2007), *P. aeruginosa*, *P. fluorescens*, *P. putida*, *Burkholderia cepacia*, *Aeromonas hydrophila*, and *Stenotrophomonas maltophilia* (Guerrieri et al., 2008). In the former study, the primary mechanism of *L. pneumophila* inhibition was hypothesized to be via interference of replication within *Acanthamoeba*, while production of bacteriocin-like substances by competing strains was the primary hypothesis of the latter study.

Overall, microbial ecology is integral to drinking water systems, yet it is largely unaccounted for in current disinfection models (Berry et al., 2006) and there is some reason to believe it may even be an indirect OPPP control factor described previously for chlorine and chloramine (Springthorpe, 2011). Very little research on this promising subject has been conducted, perhaps because the approaches yielding landmark success for conventional pathogens focused on inactivation and control of microbial growth at every reasonable opportunity -- embracing a probiotic approach would therefore require a major paradigm shift. The much higher levels of microbes commonly encountered in premise plumbing (Nguyen et al., 2011; NRC, 2006a) versus water mains systems would suggest that such a shift may be worth exploring.

Plumbing Materials

Numerous studies have attempted to correlate OPPP occurrence in potable water systems to the presence of specific plumbing materials such as copper or plastic pipe. Copper in potable water is believed to exert an inhibiting action on *Legionella* (Leoni et al., 2005; Marrie et al., 1994; States et al., 1987; Zacheus and Martikainen, 1994), and other investigations found that copper did not encourage growth to the extent of other materials (Rogers et al., 1994b; van der Kooij et al., 2005; Zeybek and Cotuk, 2002). There is some evidence emerging that the effects of copper pipe are not long term, but results are also likely to depend on the concentration of Cu²⁺ ions in the water, which in turn depends on the specific water, pH, age, and other factors as has been elucidated recently for nitrifying bacteria in premise plumbing (Zhang et al., 2008; Zhang et al., 2009). In partial support of this hypothesis two surveys in Italy found copper levels above 50 µg/L were correlated with lower Legionella colonization (Borella et al., 2004; Borella
et al., 2005). Impacts of copper are not always beneficial (Mathys et al., 2008), which might be expected in some cases due to copper-accelerated decay of chlorine and chloramine disinfectant (Nguyen et al., 2011), the role copper plays as a trace micronutrient, or adaption to higher copper (Dwidjosiswojo et al., 2011; Teitzel and Parsek, 2003; Zhang and Edwards, 2009).

There are several studies that found plastic (PVC, PEX, elastomers) not only supported but sometimes encouraged growth of OPPPs for *Legionella* and *P. Aeruginosa* due to release of organics (Colbourne, 1985; Rogers et al., 1994b). Rubber coated valves, EPDM and polyurethane surfaces can also strongly encourage OPPP growth under at least some circumstances (Barbeau et al., 1996; Bressler et al., 2009; Kilb et al., 2003; Moritz et al., 2010; Rogers et al., 1994b; Tsvetanova and Hoekstra, 2010).

**Water Flow Patterns**

Water flow variations in buildings include the duration and timing of stagnation events, internal pipe velocities, presence of stagnation zones, and use of recirculation pumps. Flow patterns can influence biofilms and OPPP occurrence through shearing, transport of nutrients and disinfectants to biofilms, creation of micro-aerophillic niches, controlling rates of bulk water and biofilm surface disinfectant decay, and temperature profiles throughout the hot water distribution system (Brazeau et al., 2011; Brazeau and Edwards, 2011; Donlan et al., 1994; Liu et al., 2006). Given that higher flow would be expected to sometimes increase and sometimes decrease microbial growth dependent on circumstance, it is not surprising that effects of flow variations are also highly variable in the literature. Liu et al. (2006) acknowledge numerous articles implying that stagnation tends to increase *Legionella* occurrence, but also point out that unambiguous head to head testing data is generally lacking. These authors demonstrated that at room temperature in one system, *Legionella* markedly increased with more frequent flow. The Liu et al. (2006) observation is consistent with at least some other field observations suggesting a link between higher incidence of *Legionella* when hot water recirculating systems were present (Moore et al., 2006).

**Whole building, point of use and showerhead filters**

At least three types of filters including whole building GAC, end of faucet, and showerhead (Figure 6-1) could influence the incidence of OPPPs. Key potential impacts include:

1. Ability to physically remove OPPPs from flow;
2. Concentration of biofilms and nutrients increasing re-growth potential within the filter itself;
3. Depletion of disinfectant residual; and
4. Seeding of the plumbing system downstream from filters.

There is strong evidence to suggest that certain filters installed at the end of the system can reduce and remove *Legionella* (Sheffer et al., 2005). Application at the end of the system avoids removals of disinfectants from the premise plumbing system or the possibility of seeding plumbing surfaces downstream of filters. Although the approach is promising and potentially transformative if it proves successful, benefits of filters installed in showerheads is essentially un-researched. In some situations, point of use filters have been noted to sometimes increase the
occurrence of OPPP, especially *P. aeruginosa* (Chaidez and Gerba, 2004; Falkinham III, 2010; Trautmann et al., 2005; Williams et al., 2011). One recent outbreak of *Legionella* occurred in a building in which virtually all the chloramine residual was removed at the point of entry by a GAC filter, raising concerns about such treatments relative to OPPP control (Brown, 2011; Miami-Dade County Health, 2010).

**Specific Devices Such As Metered Faucets**

The electronic faucet (i.e., non-touch, eye, or hands-free faucet) is an example of an innovative engineering device installed in public and commercial buildings which has had surprising impacts on detected OPPPs such as *Legionella* spp. and *P. aeruginosa*. Originally installed as a public health measure to allow hand washing without touching surfaces to reduce exposure to bacteria, several studies have implied these devices are associated with increased incidence of OPPPs (Table 6-4). Head to head comparisons have not been conducted between metered faucets and research results are complicated in the current literature by the presence of new plumbing and/or construction when the studies were conducted. Amongst the suspected factors contributing to increased contamination are low flow and velocities through the device, how the magnetic valve functions, presence of specific materials such as rubber and PVC pipe, and contamination from the manufacturer (Assadian et al., 2002; Berthelot et al., 2006; Chaberny and Gastmeier, 2004; Halabi et al., 2001; Yapicioglu et al., 2011). Remedial actions have included hyperchlorination which reportedly has been ineffective in many instances, thermal shock which has been effective, and replacement of the devices with conventional faucets (Leprat et al., 2003; Merrer et al., 2005; van der Mee-Marquet et al., 2005).

**SYNTHESIS AND OVERVIEW OF ENGINEERING CONTROLS**

The above review has indicated that most existing strategies have unresolved complications or concerns that will require at least some additional research to resolve. Clearly, a single strategy does not exist to eliminate all five target pathogens that are the focus of this report, yet alone the growing list of other emerging OPPPs in drinking water. The microbial ecology of each pathogen is unique and interconnected with a host of biotic and abiotic factors, most of which remain undefined. Moreover, for some pathogens such as *Acanthamoeba* and *Naegleria fowleri*, it is unclear whether public education or engineering approaches would be most cost effective. Table 6-5 summarizes a hypothetical shared responsibility model for managing OPPPs.

**FUTURE COMPLICATIONS IN WATER QUALITY**

The range of conditions encountered in modern building plumbing systems is also becoming increasingly varied and complex, quite possibly in ways that could alter the likelihood of OPPP amplification. Specifically, as was observed with metered faucets, the increased use of green/sustainable design principles and water conservation can dramatically increase water age in premise plumbing with concomitant loss of disinfectant residuals and sometimes increased likelihood of microbial growth associated with use of non-traditional potable water sources in buildings. For example, an order of magnitude reduction in potable water demand and increase in water age for green construction at the University of North Carolina Chapel Hill was
associated with unusual problems with lead, disinfectant residual maintenance, microbial growth, and consumer taste/odor complaints (Effland et al., 2010; Nguyen et al., 2008; Nguyen et al., 2011). A wide range of green devices to save both energy and water are likely to be developed over the next few decades, with potentially significant impacts to OPPPs and which will require numerous stakeholders to detect, evaluate and mitigate unanticipated consequences.
Table 6-4 Summary of Prior Research Reports on OPPP in electronic faucets.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Location</th>
<th>Pipe age status</th>
<th>Number of EFs</th>
<th>P. aeruginosa growth</th>
<th>EF deficiency</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assaidian et al., 2002</td>
<td>Hospital</td>
<td>Not specified</td>
<td>18</td>
<td>0%</td>
<td>Distance from valve to outlet</td>
<td></td>
</tr>
<tr>
<td>Berthelot et al., 2006</td>
<td>Neonatal ICU</td>
<td>Tap replacement</td>
<td>Not specified</td>
<td>Yes</td>
<td>Magnetic valve</td>
<td>Letter to the editor. No contamination before magnetic mixing valve was observed. Several disinfections attempted.</td>
</tr>
<tr>
<td>Chaberny and Gastmeier, 2004</td>
<td>Hospital Kitchen</td>
<td>Renovation</td>
<td>27</td>
<td>8%</td>
<td>Low flow</td>
<td>73% of EFs exceeded 100 CFU/mL. None of the manual control faucets exceeded this reference value.</td>
</tr>
<tr>
<td>Halabi et al., 2001</td>
<td>Hospital</td>
<td>Tap replacement</td>
<td>23 without temperature control</td>
<td>74%</td>
<td>Low flow</td>
<td>Temperature Pipe material</td>
</tr>
<tr>
<td>Leprat et al., 2003</td>
<td>Hematology ward</td>
<td>Renovation</td>
<td>3</td>
<td>100%</td>
<td>None explicitly stated</td>
<td>Disinfection using chlorination attempted 6 times, all unsuccessful. Revert back to conventional taps yielded no contamination</td>
</tr>
<tr>
<td>Merrer et al., 2005</td>
<td>Hematology and ICUs</td>
<td>Not specified</td>
<td>92</td>
<td>39%</td>
<td>None explicitly stated</td>
<td>Took place in two separate hospitals. Overall, 1% of manually operated faucets were contaminated.</td>
</tr>
<tr>
<td>van der Mee-Marquet et al., 2005</td>
<td>Hospital wing</td>
<td>New construction</td>
<td>87</td>
<td>100%</td>
<td>None explicitly stated</td>
<td>Hyperchlorination was ineffective. Thermal disinfection (70°C flushing for 30 min.) was effective in eliminating <em>P. aeruginosa</em> contamination</td>
</tr>
<tr>
<td>Yapicioglu et al., 2011</td>
<td>Neonatal ICU</td>
<td>Tap replacement</td>
<td>Not specified</td>
<td>100% (cultured from filters on electronic taps)</td>
<td>Low flow</td>
<td>Low pressure Pipe material</td>
</tr>
</tbody>
</table>
### Table 6-5
Hypothetical Shared Responsibility Model for Management of Opportunistic Pathogens in Drinking Water

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Community Based Utility Response</th>
<th>In Building Treatment</th>
<th>Microbial ecology</th>
<th>Public Education/Other</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Legionella pneumophila</strong></td>
<td>Chloramine; Chlorine supplementation to assure residual in far reaches of system</td>
<td>Disinfection, Hot water heater temperature, Chemical/thermal shock</td>
<td>Limit biofilm growth in order to limit free living amoeba growth, and thus <em>L. pneumophila</em></td>
<td>Hot water heater temperature, limiting aerosol exposure in sensitive populations.</td>
</tr>
<tr>
<td><strong>Pseudomonas aeruginosa</strong></td>
<td>Higher disinfectant tends to limit risk</td>
<td>Regularly pasteurize faucets</td>
<td>Prevent inoculation of the system with <em>Pseudomonas</em> (e.g., spraying directly on drain)</td>
<td>Wound protection and care, educate sensitive groups (e.g., CF patients)</td>
</tr>
<tr>
<td><strong>Mycobacterium avium Complex</strong></td>
<td>Higher disinfectant residual ineffective and might actually select for MAC.</td>
<td>Uncertain effectiveness except high temperature.</td>
<td>Uncertain.</td>
<td>Hot water heater temperature, limiting aerosol exposure in sensitive populations.</td>
</tr>
<tr>
<td><strong>Acanthamoeba</strong></td>
<td>Uncertain</td>
<td>Uncertain</td>
<td>Uncertain. Limit biofilm growth to limit food source?</td>
<td>Perhaps greatest benefit from proper solutions and hygiene in contact lens.</td>
</tr>
<tr>
<td><strong>Naegleria fowleri</strong></td>
<td>Higher disinfectant tends to limit risk, bury pipes to avoid warming in sun.</td>
<td>Uncertain</td>
<td>Colder temperatures. Limit biofilm growth to limit food source?</td>
<td>Public education on neti pot use (use sterile water only)</td>
</tr>
</tbody>
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CHAPTER 7: REQUESTS FOR PROPOSALS (RFPS)

This final chapter summarizes the 20 RFPs that were developed at the expert workshop. The intention was to formulate key research topics that will fill critical knowledge gaps necessary in order to advance understanding of OPPPs in potable water and potential means by which their monitoring and control may be improved. Each RFP is encoded based on the theme from which it was originally derived (EPI- Epidemiology; METH- Methodology; ECOL- Ecology; and ENG- Engineering). The RFPs were drafted together with the participants at the workshop and voting on the ranking of relative priority was conducted at the workshop and by email following the workshop. Each RFP provides a basic rationale, outline of a suggested research study, and potential stakeholders that may be vested in funding such research, as well as an estimated budget. The RFPs are listed in ranked order.
#1–EPI 1: PREVALENCE, INCIDENCE AND TRENDS OF OPPP DISEASE.

Background:

(1) The prevalence and incidence of OPPP disease, with the exception of Legionnaires’ Disease is not reported and important public health information is lacking.

Problem:

(1) What is the prevalence, incidence, and trends of disease caused by OPPPs?

Approach:

(1) Focus on five OPPPs (i.e., *Legionella pneumophila*, *Mycobacterium avium* complex, *Pseudomonas aeruginosa*, *Acanthamoeba* spp. and *Naegleria fowleri*) as models for OPPPs and for other species within the genus.
(2) Develop a risk assessment framework (examining dose-response models and disease cases) and identify key data gaps.
(3) Request reporting from the Council of State and Territorial Epidemiologists (CSTE).
(4) Retrospective study based on existing databases (e.g., Kaiser Permanente, Medicare) of codes for OPPP diseases.

Risk of Approaches and Anticipated Rewards:

(1) Without CSTE required reporting, especially of *M. avium* complex disease, values will be underestimated.
(2) Available data on *L. pneumophila* of high quality.
(3) Knowledge of prevalence and incidence and calculation of disease trends of high value.
(4) These data could be used to address the appropriate dose-response models for water exposure pathways in further research as identified below.

Stakeholders:

(1) EPA, CDC, NIH, PHS, and Water Research Foundation

Estimated Budget:

(1) Single center, $100,000/yr for 3 years = $300,000
#2 RFP – METH 1: APPROPRIATE SAMPLING AND MONITORING STRATEGIES FOR OPPPS.

**Background:**

Standardized sampling and monitoring protocols have not been defined for OPPPs. OPPPs are distinct from pathogens of traditional concern, such as fecal source pathogens and those escaping drinking water treatment. OPPPs establish and thrive in the biofilms of pipes and other fixtures in buildings, making appropriate biofilm sampling of importance. At the same time, OPPPs released into bulk water may be of more direct concern for human exposure, making water sampling potentially equally important. First flush and flushed samples will yield different results and provide different information, and the volume of water may affect measurements as well. Some samples, such as water heater sediments, may have accumulated over years, making it impossible to replicate certain kinds of sampling events. Within building variation is also common and guidelines for ideal sampling locales and repeated sampling are lacking. Improved sampling and monitoring will enable better evaluation of the effectiveness of engineering controls and improved assessment of risk to humans posed by OPPPs in a given building.

**Problem:**

Appropriate sampling and monitoring protocols for OPPPs are needed in order to more precisely quantify their distribution in the biofilm and bulk water in the pipes and fixtures of buildings.

**Approach:**

1. Focus on a particular institution type (e.g., hospital) and conduct comprehensive sampling of a representative sub-set of buildings over several time points. The intent is to determine the variability within and between a particular building type and provide the basis for extrapolating a sampling and monitoring protocol for buildings in general (e.g., schools and homes).
2. Both biofilm and bulk water should be examined over a range of sampling and monitoring points, including hot and cold water pipes, showers, hot water heaters, eye wash stations, filters, and faucets. Working collaboratively with experts in other fields to develop a statistically-defensible sampling plan is highly encouraged.
3. A representative sub-set of OPPPs should be examined (i.e., *Legionella pneumophila*, *Mycobacterium avium complex*, *Pseudomonas aeruginosa*, *Acanthamoeba spp.* and *Naegleria fowleri*) as models for OPPPs and for other species within the genus.
4. Pathogen analytical techniques should include both culture-based and molecular techniques.
Risk of Approaches and Anticipated Rewards:

1. Appropriate sampling and monitoring protocols for OPPPs is a pre-requisite for accurately assessing exposure to humans as well as the effectiveness of potential engineering controls.
2. Without appropriate sampling and monitoring protocols, it is impossible to know the validity of the data and thus interpret and compare OPPP occurrence data within and between buildings.
3. Appropriate sampling and monitoring protocols will provide critical information and guidance to all future studies and assessments and therefore the anticipated rewards are viewed to be high.

Stakeholders:

EPA, CDC, NIH, NIST, AHA and Water Research Foundation

Estimated Budget:

Single center, $125,000/yr for 2 years = $250,000
#3 RFP –EPI 2: Sources and weights involved in OPPP disease and relationship between OPPP numbers and disease.

Background:

Although a variety of sources (i.e., premise and non-premise) have been shown to yield OPPPs, the weight of each source has not been determined.

Problem:

What are the sources and routes of transmission for each OPPP?

Approach:

(1) General:

(a) Focus on five OPPPs (i.e., Legionella pneumophila, Mycobacterium avium complex, Pseudomonas aeruginosa, Acanthamoeba spp. and Naegleria fowlerii) as models for OPPPs and for other species within the genus.
(b) Subdivide sources into premise (i.e., home and work) and non-premise (travel, golf).

(2) Experimental:

(a) Identify from available literature OPPP sources and map out routes of transmission.
(b) Weigh the contributions of the 3 sources (above) as sources of exposure, infection, and disease.
(c) Relate numbers of (virulent) OPPPs in each source to disease.

Risk of Approaches and Anticipated Rewards:

1. High probability for identification and characteristics of the 3 categories of sources (above) and routes of transmission for each OPPP.
2. Moderate probability establishing the weight of each of the 3 categories of sources (above) considering the routes of transmission.
3. Probability of success in relating OPPP numbers to disease requires knowledge of virulent/nonvirulent OPPP numbers for M. avium complex and Acanthamoeba spp. and known for L. pneumophila, P. aeruginosa, and N. fowlerii.

Stakeholders:

NIH, EPA, CDC, PHS, and Water Research Foundation

Estimated Budget:

Multicenter (4 OPPP categories) at $ 100,000/yr/center for 5 years = $ 2,000,000 = NIH (NIAID)
#4 RFP – METH 2: MOLECULAR-BASED SPATIAL AND TEMPORAL SURVEY OF OPPPS.

**Background:**

OPPPs are now considered to be the primary source of water-borne disease outbreak in developed countries. This is due in part to the success of water treatment plants in controlling the release of pathogens into the water distribution system. OPPPs pose the next generation of challenges to water-borne disease control in that they live and thrive within the water systems of buildings themselves. However, the baseline occurrence and distribution of OPPPs in building water systems has not been established and is entirely unknown for organisms such as *N. fowleri*. OPPPs appear to vary with respect to a variety of regional as well as building-level factors, including temperature, water chemistry, pipe materials, fixture type, and hydraulic regime. Information is required on the baseline occurrence level of OPPPs in order to establish and compare their abundance under a range of typical conditions encountered in a building. This information would also be useful for comparison with relative differences that are encountered in actual outbreak scenarios.

**Problem:**

Baseline occurrence data (who is there and how abundant) is required for OPPPs various building systems.

**Approach:**

(a) Ideally follow RFP Meth-1, or integrate Meth-1 as Phase I of this study.
(b) A longitudinal sampling plan that includes water distribution systems located in at least three distinct regions.
(c) A representative subset of comparable institutions and types should be selected within each region.
(d) Focus on five OPPPs (i.e., *Legionella pneumophila, Mycobacterium avium complex, Pseudomonas aeruginosa, Acanthamoeba spp. and Naegleria fowleri*) as models for OPPPs and for other species within the genus.
(e) Survey should primarily be molecular-based in order to enable high-throughput data collection and provide a broad survey of OPPPs.
(f) Data should also be collected on water chemistry, temperature, pipe material (when possible), hydraulic condition, fixture type, disinfectants used and concentrations, etc. Aim to identify “hot spots” with respect to these conditions and OPPP abundance.

**Risk of Approaches and Anticipated Rewards:**

1. The approach is generally considered low-risk in that the approach is clearly defined and would use existing molecular techniques.
2. Because OPPPs live and thrive in building water systems, detection of their molecular markers is expected to be more informative relative to direct monitoring following
disinfection at the treatment plant. Ideally, support of RFPs Meth-1 and Meth-3 can help establish the relationship between molecular occurrence and live OPPPs.

3. The reward is high because information on OPPP occurrence is lacking. Understanding of OPPP occurrence will aid in comparing background with outbreak scenarios and help establish an understanding of a “safe” level for such organisms that naturally inhabit water systems.

4. Identification of “hot spots” will also be extremely useful in targeting engineering controls.

**Stakeholders:**
EPA, CDC, NIH, NIST, AHA, and Water Research Foundation

**Estimated Budget:**

Single center, $125,000/yr for 3 years = $375,000. If combined with Meth-1 = $625,000
#5 RFP-ECOL 1: RELATIONSHIP BETWEEN DISTRIBUTION SYSTEM AND MICROBIOTA OF PREMISE PLUMBING.

Background:

OPPPs establish and grow within the water systems of premises (i.e., buildings) and therefore are beyond the water utility regulatory jurisdiction, which ends at the property line. However, the characteristics of the water delivered by the distribution system have the potential to affect the proliferation of OPPPs in premise plumbing. Disinfectant type and residual are likely to profoundly impact OPPPs in building water systems, but their precise impacts on OPPP occurrence is not yet well-defined. Other water quality characteristics may be critical as well, including temperature, pH, water age, pipe materials, and the use of corrosion inhibitors. Furthermore, it is unknown to what extent the microbiota of the distribution system is similar to, or impacts the microbiota of the premise plumbing. Establishing an understanding of the relationship between the water quality of the distribution system and occurrence of OPPPs in premise plumbing would be of great value for identifying potential management options that could be enacted by utilities to benefit premise plumbing.

Problem:

To what extent do the abiotic and biotic characteristics of the distribution system affect the occurrence of OPPPs in premise plumbing?

Approach:

(a) Could be conducted at laboratory or field-scale. Laboratory scale would enable examination of a range of water distribution system water quality characteristics and their effect on simulated premise plumbing fixtures under controlled conditions. For field-scale it would be beneficial to identify a utility partner with known hot-spots and a number of institutions interested in participating.  
(b) Extremes of conditions would be of particular interest, including stagnation, system upsets, distribution system materials, disinfectant residuals, and the use of corrosion inhibitors.  
(c) Focus on five OPPPs (i.e., Legionella pneumophila, Mycobacterium avium complex, Pseudomonas aeruginosa, Acanthamoeba spp. and Naegleria fowleri) as models for OPPPs and for other species within the genus.  
(d) Examine the relationship between water quality characteristics of the distribution system and occurrence of OPPPs in premise plumbing.

Risk of Approaches and Anticipated Rewards:

1. Medium Risk because it may be difficult to identify clear and consistent relationships between distribution system characteristics and the occurrence of OPPPs in premise plumbing.
2. High return because it will be of value to water utilities to know to what extent management actions may impact OPPPs in premise plumbing and which management actions may be most beneficial.

**Stakeholders:**

EPA, CDC, NIH, NSF, and Water Research Foundation, NSF. May be suitable for tailored collaboration program.

**Estimated Budget:**

Three years at $150,000/year for 3 years = $450,000
#6 RFP-ENG 2: FUNDAMENTAL FACTORS/MECHANISMS CONTROLLING DETACHMENT AND RELEASE OF OPPPS FROM BIOFILMS IN PREMISE PLUMBING.

**Background:**

Relatively little is known about premise plumbing events that would trigger release of biofilms and OPPPs to water, which is a possible critical first step for infection.

**Problem:**

Do high levels of OPPPs in premise plumbing biofilm invariably mean higher risk in water from fixtures/showers? Is there a relationship between water release of OPPPs and biofilm? Identify the key factors causing bio-film detachment and characterize size of released particulates.

**Approach:**

Use surrogates to examine mechanisms of biofilm detachment from hydraulic disturbances, disinfectant, flow reversal, water hammer, temperature, phosphate, materials, phosphate and orthophosphate corrosion inhibitors. Detached particulate quantity and size should also be characterized.

**Risk of Approach and Anticipated Rewards:**

This project has a high likelihood of success and represents a moderate contribution to knowledge and potential control strategies.

**Stakeholders:**

EPA, CDC, Water Research Foundation, WQA, Manufacturers, NSF

**Estimated Budget:**

$250,000 for 2 years
#7 RFP-ENG 1: IMPACT OF WATER TREATMENT ON CONTROL OF OPPPS IN PREMISE PLUMBING.

**Background:**

There are very few studies with head-to-head comparisons of premise plumbing control strategies for *Legionella pneumophila* and other pathogens. More information is needed on the strengths and weaknesses of each approach relative to *L. pneumophila*, and its ability to control (or possibly enhance) growth of other OPPPs.

**Problem:**

There is a need to establish the relative efficacy of treatments for the control of *L. pneumophila* and other OPPPs.

**Approach:**

1. Small scale pilot studies using pipe loops or reactors with established biofilms and water with established propensity to amplify *L. pneumophila* and possibly several other pathogens. Results need to compare and contrast, head-to-head, the ability of treatments to control OPPPS under conditions which are problematic in premise plumbing.
2. Treatments include high temperatures, UV, copper/silver, chlorine dioxide, chlorine and chloramine.

**Risk of Approach and Anticipated Rewards:**

1. Although results from one reactor type and one water may not directly apply to other situations, at least some baseline information is needed to establish a conventional wisdom regarding comparative performance.
2. There is concern that strategies that control *Legionella* might sometimes enhance other microbes, including *Mycobacterium avium Complex*.

**Stakeholders:**

EPA, CDC, Water Research Foundation, WQA, Manufacturers (of plumbing materials, water heaters, faucet types)

**Estimated Budget:**

$350,000 for 2-3 years
#8 RFP – ECOL 3: MECHANISMS OF CHLORAMINE AND CHLORINE IMPACT ON OPPPS.

Background:

Disinfectants represent a long-standing and effective tool for the control of a wide range of pathogens in drinking water. However, OPPPs pose a new generation of challenges for pathogen control. Chloramines are of growing interest as a promising community-level action effective against *L. pneumophila*. However, evidence available at present suggests that chlorine may be more effective for controlling *Mycobacteria* spp. Information on the precise mechanisms of action of drinking water disinfectants on OPPPs is vitally needed in order to appropriately guide utilities in the selection of disinfectants. Chloramines are of particular interest as they have shown early promise for some OPPPs and many utilities in the U.S. are switching to chloramines, but their mechanism of disinfection is still not well understood.

Problem:

What are the mechanistic impacts of chloramine on OPPPs, relative to chlorine?

Approach:

(a) Focus on five OPPPs (i.e., *Legionella pneumophila*, *Mycobacterium avium complex*, *Pseudomonas aeruginosa*, *Acanthamoeba* spp. and *Naegleria fowleri*) as models for OPPPs and for other species within the genus.

(b) Laboratory studies to examine mechanisms of disinfection and relative effectiveness for various OPPPs.

Risk of Approaches and Anticipated Rewards:

Low risk because studies under controlled conditions. High reward because chloramines have shown early promise for *L. pneumophila* and many utilities are switching to chloramines. It would be beneficial to better know the impacts as this switch is being considered or applied.

Stakeholders:

EPA, CDC, NSF, NIH, Water Research Foundation, chlorine chemistry council, certain industry sponsors (could draw from same application in cooling towers).

Estimated Budget:

$150,000/yr/center for 3 years = $450,000
#9 RFP – EPI- 3: COMMUNICATION OF OPPP PREVALENCE, INCIDENCE AND TRENDS.

**Background:**

The importance, significance, and impact of OPPP-diseases has not been communicated to stakeholders (e.g., federal and state agencies, foundations, physicians, and at-risk individuals).

**Problem:**

Who are the stakeholders and what information and data needs to be conveyed concerning OPPP disease?

**Approach:**

1. **General:**
   (a) Focus on five OPPPs (i.e., *Legionella pneumophila*, *Mycobacterium avium complex*, *Pseudomonas aeruginosa*, *Acanthamoeba* spp. and *Naegleria fowleri*) as models for OPPPs and for other species within the genus.
   (b) Subdivide sources into premise (i.e., home and work) and non-premise (travel, golf).

2. **Experimental:**
   (a) Identify at-risk individuals, HMOs, federal, state, and local agencies, corporations, and foundations who are stakeholders in knowledge of OPPP disease prevalence, incidence, and trends.
   (b) Identify information concerning OPPP disease:
      (i) prevalence, incidence, and trends
      (ii) routes of transmission,
      (iii) sources, and
      (iv) weights of different sources to be conveyed.
   (c) Identify possible vehicles for dissemination of information (above) concerning OPPPs.
   (d) Develop a method to assess the efficacy of dissemination of OPPP disease information.

**Risk of Approaches and Anticipated Rewards:**

1. High probability of success in identification of stakeholders.
2. High probability of identification of information to be disseminated to the variety of stakeholders.
3. Anticipated difficulty in identification of possible vehicles for dissemination of information for all possible stakeholders (e.g., non-electronic savvy).
Stakeholders:

At-risk individuals, HMOs, federal, state, and local agencies, corporations, and foundations (NTMir, Inc., CF Foundation), Water Research Foundation

Estimated Budget:

Single center, $100,000/yr for 2 years = $200,000
#10 RFP – METH 3: COMPARISON BETWEEN CULTURE-BASED AND MOLECULAR-BASED TECHNIQUES FOR OPPPS

**Background:**

Culture-based methods serve to define the basis for regulatory and action limits for pathogens in drinking water, including *L. pneumophila*. For OPPPs in particular, culture-based methods may not be the most appropriate for assessing their presence and abundance in water systems. As an example, the Environmental Legionella Isolation Techniques Evaluation (ELITE) program sponsored by the U.S. Centers for Disease Control and Prevention has revealed widespread inter-laboratory variability in the culture-based enumeration of this OPPP. For other OPPPs, such as *Mycobacteria* spp., standard culturing protocols are still not agreed upon and can require several weeks for results, increasing probability of culture contamination. OPPPs are especially challenging for culture-based methods because of their fastidious nature, susceptibility to a viable but non-culturable status (VBNC), and ineffectiveness of pre-treatment techniques for effectively eliminating non-target organisms. However, the primary advantage of culture-based methods remains their ability to confirm the presence of a live, virulent pathogen. On the other hand, advantages of molecular-based techniques include their specificity, sensitivity, and potential to produce high-throughput results. As expertise in molecular techniques is becoming more widespread, molecular techniques are beginning to be viewed as more convenient than culture-based techniques as well. Yet, lingering questions remain about the relevance of molecular detection to the presence of live, virulent pathogens and the relationship, if any, between molecular and culture-based assessments of OPPPs in water systems.

**Problem:**

Do molecular-based techniques represent a significant advance in their ability to assess live, virulent OPPPs in drinking water systems, relative to culture-based techniques? If so, which methods or combinations of methods are recommended?

**Approach:**

(a) Focus on five OPPPs (i.e., *Legionella pneumophila*, *Mycobacterium avium complex*, *Pseudomonas aeruginosa*, *Acanthamoeba* spp. and *Naegleria fowleri*) as models for OPPPs and for other species within the genus.

(b) For each OPPP, select a sub-set of culture-based and molecular-based methods to be compared. For molecular techniques, qPCR, treated and untreated with PMA or other approach to enhance detection of viable cells, is of interest. This will enable comparison of quantitative capabilities and assessment of live organisms. Multiple gene targets, including known markers for virulence, are also of interest, to aid in comparison of pathogenic organisms.

(c) Appropriate positive controls and internal controls for molecular methods highly desirable.

(d) Select a building or other site known to be a “hot spot” for OPPPs to sample and monitor.

(e) Include more than one lab for comparison.
Risk of Approaches and Anticipated Rewards:

1. High return because culture-based methods are insufficient to assess risk and new methods are needed. Would provide catalyst for molecular-based assessment, which provides greater sensitivity, specificity, and consistency.
2. Low risk because the methods to be compared are established.

Stakeholders:

EPA, Water Research Foundation, NIST, AHA, NIH.

Estimated Budget:

Single center, $125,000/yr for 2 years = $250,000
#11 RFP-ENG 5: OPTIMIZING THE DESIGN OF HOT WATER SYSTEMS TO ACHIEVE TEMPERATURE CONTROL OF OPPPS AND PREVENT AMPLIFICATION.

Background:

Impacts of flow duration, flow velocity, and presence of hot water recirculation can control transport of nutrients and disinfectants to biofilms. These factors also influence disinfectant concentrations and profiles in hot water systems, and temperature profiles of hot water pipe networks.

Problem:

It would be expected that the benefits/detriments of flow in practice will depend on the plumbing system configuration, OPPP control strategy, and other factors. Better understanding the interplay of these factors is important to developing sound control strategies for new and existing systems.

Approach:

(1) Synthesize design guidance from countries using thermal disinfection (> 60 C) for control of OPPPs.
(2) Conduct hot water distribution system testing, head-to-head, of flow duration impacts under different extremes of water heater set-points (48° C vs 60° C).
(3) Collect data on temperature profiles of main lines and distal arterioles/dead ends under reasonable extremes of design and water use.

Risk of Approaches and Anticipated Rewards:

This project has a very high likelihood of success and is of high importance.

Stakeholders:

Device manufacturers, Water Research Foundation, WQA, EPA

Estimated Budget:

$300,000
#12 RFP – ECOL 2: ROLE OF FREE-LIVING AMOEBAE (FLA) IN ENHANCING PERSISTENCE, SURVIVAL, AND GROWTH OF OPPPS.

**Background:**

FLA, including *Hartmanella* spp. and *Acanthamoeba* spp., are known to serve as important ecological hosts for *Legionella pneumophila* and may also be important for other OPPPs. Certain FLA have been demonstrated in laboratory assays to play a critical role in supporting intracellular amplification of *L. pneumophila*, enhancement of its virulence, and recovery from viable but non-cultur able (VBN C) status. FLA can also aid in protecting intracellular bacteria from disinfectants. However, the relationship between FLA, *L. pneumophila*, and other bacterial OPPPs in real-world premise plumbing systems is not well-established. In particular, the occurrence of FLA and other protozoans is widely unknown in premise plumbing. Further, it is not clear which are the most relevant, or potentially even obligate, hosts for OPPPs in actual premise plumbing systems and to what extent, if any, this information may be extrapolated from laboratory assays. This information is vital as it could support the hypothesis that controlling FLA and other protozoans may serve as an effective means for controlling OPPPs.

**Problem:**

What kinds of protozoa are present in premise plumbing systems, which are the most relevant hosts for bacterial OPPPs?

**Approach:**

(a) A field survey component is desirable in order to gain insight into the distribution of protozoa in a representative range of real-world premise plumbing systems.

(b) Methods for identifying bacterial OPPPs present within specific protozoan hosts, and their application, are of particular interest.

(c) A laboratory component may be beneficial to validate relationships observed in the field.

**Risk of Approaches and Anticipated Rewards:**

(a) Low risk and high reward in terms of likelihood of yielding new information about the distribution of protozoa in premise plumbing systems. It may be more challenging to establish precise relationships with amoeba-resisting OPPPs, making this aspect of the research medium risk.

**Stakeholders:**

EPA, CDC, NSF, NIH, Water Research Foundation. A more fundamental examination of ecological bacterial OPPP/protozoan host relationships beyond premise plumbing may be attractive to a broader range of sponsors.
Estimated Budget:

$150,000/yr/center for 3 years = $450,000
#13 RFP- ENG 3: UNDERSTANDING CHLORINE, CHLORINE DIOXIDE AND CHLORAMINE DEMAND IN HOT WATER PREMISE PLUMBING.

**Background:**

Many OPPP control strategies will rely on maintenance of chlorine and chloramine targets at the point-of-entry or within buildings. Practical monitoring data has demonstrated that chlorine levels in buildings vary by one or two orders of magnitude from one building to another.

**Problem:**

It is likely that plumbing materials, microbial reactions, abiotic chlorine demand, temperature and other factors control the observed rates of chlorine decay and residuals. Unfortunately, there is relatively little research on this important subject for premise plumbing.

**Approach:**

Conduct field and bench scale tests to identify the key factors controlling chlorine/chloramine/chlorine dioxide decay rates in premise plumbing. Examine impacts of corrosion control including corrosion inhibitor and pH control on rate of disinfectant decay.

**Risk of Approaches and Anticipated Rewards:**

1. This has a high likelihood of success and represents a moderately important contribution to knowledge.
2. There are very few studies examining disinfectant demand in building, and those that have been done suggest that stability of disinfectant is markedly lower than in main distribution systems. While results are unlikely to apply to all systems, at least some preliminary data is needed on this subject.

**Potential Stakeholders/Sponsors:**

Water Research Foundation, Homeland Security, Manufacturers, WaterRF, WQA, EPA, National Science Foundation, NSF International

**Estimated Budget:**

$300,000 and duration is variable
#14 RFP – ECOL 4: POTENTIAL FOR MICROBIAL ECOLOGICAL CONTROLS ON OPPPs.

**Background:**

OPPPs reside in the biofilms of building water systems and as such are subject to a complex array of ecological relationships, including commensalism, antagonism, and parasitism. In addition to offering physical protection from disinfectants and other stressors, biofilms are the host of numerous species of bacteria, protozoans, and fungi. Protozoans, in particular, are viewed to be of interest because they can serve as hosts to certain bacterial OPPPs, protect them from disinfectants, and enhance their amplification. While biofilms will be ever-present in any water system, no matter how “clean” the water, the potential to control the biofilm for the purposes of inhibiting the growth of OPPPs is of interest. In simple terms, this could be viewed as a “pro-biotic” approach in which a healthy microbiome is established, as has been recently and successfully been demonstrated in the medical sciences.

**Problem:**

Is it possible to beneficially shift the microbial ecology of premise plumbing systems in a manner that inhibits the growth of OPPPs?

**Approach:**

(a) A laboratory proof-of-concept study to investigate the relationship between the resident microbiome of simulated premise plumbing systems and the ability of OPPPs to establish, grow, and persist. These may be a function of abiotic factors (pipe material, disinfectant type, assimilable organic carbon concentration, etc.).

(b) Characterization the relationship between the microbiome and OPPPs using molecular tools.

(c) Identification of key characteristics of “healthy” premise plumbing microbiomes.

(d) Validation of approach using an array of candidate microbiome/OPPP combinations.

**Risk of Approaches and Anticipated Rewards:**

High risk because it may not be possible to successfully identify the key characteristics of a healthy premise plumbing microbiome that inhibits OPPPs or to practically implement it. The outcome, if successful, would be high return as it would offer an alternative method to control OPPPs beyond conventional disinfection and temperature control.

**Stakeholders:**

Water Research Foundation, EPA, CDC, NSF, NIH.

**Estimated Budget:**

$100,000/yr/center for 3 years = $ 300,000
#15 RFP-EPI 4: IS HARTMANELLA SPP. OR ACANTHAMOEBA SPP. OF MORE SIGNIFICANCE FOR BACTERIAL OPPPS?

Background:

Hartmanella spp. and Acanthamoeba spp. have both been shown to be common in premise plumbing and support the intracellular growth of the bacterial OPPPs.

Problem:

Is Hartmanella spp. or Acanthamoeba spp. of more significance for supporting the growth and survival of the bacterial OPPPs?

Approach:

(1) General:

Focus on the three bacterial OPPPs (i.e., *Legionella pneumophila*, *Mycobacterium avium* complex, and *Pseudomonas aeruginosa*) and fresh, non-laboratory-transferred isolates of Hartmanella and Acanthamoeba.

(2) Experimental: Premise Plumbing Survey on Prevalence

Isolate, identify and enumerate Hartmanella vs. Acanthamoeba in premise plumbing by either culture or qPCR.

(3) Experimental: Prevalence of Antibodies in Individuals Infected with Bacterial OPPPs

(a) Identify commercial antigen- or antibody-based diagnostic tests for evidence of infection by Hartmanella spp. and Acanthamoeba spp. in individuals.
(b) Develop antigen- or antibody-based serologic tests for detecting infection by Hartmanella spp. and Acanthamoeba spp. in individuals.
(c) Measure the prevalence of either anti-Hartmanella or anti-Acanthamoeba antigens or antibodies amongst bacterial-OPPP-infected patients and non-bacterial OPPP-infected patients.

Risk of Approaches and Anticipated Rewards:

(a) Prevalence: High likelihood of success and high reward in permitting focus in developing laboratory models.
(b) Antigen or Antibody: High likelihood of success unless expose and infection in the non-bacterial-OPPP-infected control population is high. High reward in establishing prevalence of exposure and infection by Hartmanella and Acanthamoeba, even if high in non-bacterial-OPPP-infected controls.
Stakeholders:

Water Research Foundation, EPA, CDC, NIH, and PHS

Estimated Budget:

Multicenter (4 OPPP categories) at $100,000/yr/center for 5 years = $2,000,000 = NIH (NIAID)
#16 RFP- ENG 4: DETERMINING WHY ELECTRONIC FAUCETS SOMETIMES HARBOR HIGH LEVELS OF OPPPS.

**Background:**

It has been suggested that possible benefits of electronic faucets, including improved sanitation and water/energy conservation, may be at least occasionally countered by much higher levels of OPPPs including *Legionella pneumophila* and *P. aeruginosa*. Unfortunately, head-to-head testing data is lacking, and the precise factors that may be involved in OPPP amplification have not yet been identified.

**Problem:**

Due to recent concerns and data, metered faucets have been removed due to their perceived higher potential for waterborne disease caused by OPPPs. Fully understanding and resolving this issue may be important to resolving other potential emerging conflicts between water/energy conservation and OPPPs.

**Approach:**

1. Bench scale testing of metered versus conventional faucets, and unambiguous isolation of potential causal factors for higher OPPP levels at individual taps.
2. Examine role of mixing valves in OPPP amplification.
3. Using surrogates for pathogens, such as non-pathogenic *Legionella* and *Mycobacterial* strains, culturing or qPCR are all viable strategies for examining this issue.

**Risk of Approaches and Rewards:**

This project has a very high likelihood of success and represents a high contribution to existing knowledge.

**Stakeholders:**

Water Research Foundation, WQA, EPA, Manufacturers

**Estimated Budget:**

$275,000
#17 RFP –EPI 5: CONTRIBUTIONS OF BACTERIAL, VIRAL, AND EUKARYOTIC MICROBIOTA TO OPPP DISEASE.

**Background:**

(Hartmanella spp. and Acanthamoeba spp. have both been shown to be common in premise plumbing and support the intracellular growth of the bacterial OPPPs. There is a lack of knowledge concerning the impact of the bacterial, eukaryote, and viral microbiota on disease caused by OPPPs.

**Problem:**

(1) What are the contributions of bacterial, viral, and eukaryotic microorganisms to disease caused by OPPPs?
(2) Does the dual presence influence delivery, infection, or disease presentation?

**Approach:**

(1) General:

Focus on five OPPPs (i.e., Legionella pneumophila, Mycobacterium avium complex, Pseudomonas aeruginosa, Acanthamoeba spp. and Naegleria fowleri) as models for OPPPs and for other species within the genus.

(2) Experimental:

Effect of single OPPP or co-exposure of OPPPs (i.e., representatives of the bacterial-, eukaryote-, and viral-microbiota of premise plumbing) on infection (e.g., expression of antibody response) and progression of disease (e.g., cell counts by culture or qPCR and pathology).

**Risk of Approaches and Anticipated Rewards:**

High likelihood of success and high reward in permitting focus in developing laboratory models.

**Stakeholders:**

Water Research Foundation, EPA, CDC, NIH, and PHS

**Estimated Budget:**

Multicenter (4 OPPP categories) at $100,000/yr/center for 5 years = $2,000,000 = NIH (NIAID)
#18 RFP – ECOL 5: POTENTIAL CONTRIBUTION OF DISINFECTION AND OTHER MANAGEMENT PRACTICES ON EMERGENCE OF MULTI-ANTIBIOTIC RESISTANT PATHOGENS?

**Background:**

Antibiotic resistance represents one of the most serious, and growing, human health challenges today. Bacterial OPPP infections, while serious, can most times be effectively treated by a regimen of antibiotics. However, as is the case for a growing number of bacterial infections, this option may begin to be diminished as antibiotic resistance becomes more widespread. Multi-antibiotic resistant *Pseudomonas aeruginosa*, in particular, have emerged as a major challenge in their colonization of water systems in hospitals. Of particular concern are several reports suggesting that chlorination can select for antibiotic resistant, and sometimes even virulent, pathogens. This phenomenon has only been explored in certain model strains, such as *E. coli*, and has not been examined specifically in the context of OPPPs. Information on the impacts of disinfectants with respect to the development of multi-antibiotic resistant OPPPs is important, especially considering that disinfection is widely viewed as a practical community-level and building-level management option. Ideally, impacts on antibiotic resistance should be investigated before wide scale implementation.

**Problem:**

Do disinfectants, such as chlorine, contribute to the selection of multi-antibiotic resistant, virulent OPPPs?

**Approach:**

Laboratory-scale studies to investigate the effect of chlorination and chloramination on the antibiotic resistance patterns of *L. pneumophila*, *M. avium*, and *P. aeruginosa*. Ideally, natural strains isolated from water systems should be tested.

**Risk of Approaches and Anticipated Rewards:**

Low risk because protocols for disinfection and multi-antibiotic resistance testing are well-established. Medium return because an effect may or may not be found, and if an effect is found, further investigation may be required to establish the extent of the phenomenon and potential alternative treatments.

**Stakeholders:**

EPA, CDC, NSF, NIH, Water Research Foundation.

**Estimated Budget:**

$100,000/yr/center for 2 years = $200,000
#19 RFP-ENG 6: SECONDARY IMPACTS OF IN-BUILDING DISINFECTION ON CORROSION OF GALVANIZED IRON, STAINLESS STEEL, COPPER, AND BRASS.

Background:

Emerging standards will require increased use of in-building disinfection for control of OPPPs. In-building disinfection can sometimes dramatically alter the corrosivity of water, resulting in reduced lifetime of premise plumbing systems and altering leaching of lead and copper to water.

Problem:

1. Both stainless steel and copper can be attacked by chlorine, chloramine, chlorine dioxide oxidants, higher chloride associated with continuous on-site disinfectant generators.
2. Systems relying on $\text{Cu}^{2+}$ or $\text{Ag}^{2+}$ may cause severe deposition corrosion and failure of less noble metallic components via deposition corrosion.
3. Anti-corrosion agents such as orthophosphate or higher pH, which might be applied to counter the higher corrosivity, can alter the effectiveness of chlorine or metallic disinfectants.
4. As with water mains, there is a substantial interplay between corrosion control and disinfection.

Approach:

1. Literature review of prior research on deposition corrosion and oxidant corrosion of common metallic in-building materials
2. Long-term (1 year or greater) laboratory scale testing of oxidant impacts and deposition corrosion for key plumbing materials.
3. Bench scale research and modeling to evaluate the impact of phosphate and higher pH on free $\text{Cu}^{2+}$ and $\text{Ag}^{2+}$ ion speciation.

Stakeholders:

Water Research Foundation, WQA, EPA

Estimated Budget:

$400,000
#20 RFP –METH 4: CHARACTERIZATION OF OPPP PANGENOMES FOR ADVANCING MOLECULAR TARGETS FOR OPPPS.

**Background:**

Recent advances in high-throughput sequencing have provided new insights into microbial ecology and is eroding past notions of the bacterial species concept. The term “pangenome” has been coined to describe the full complement of genes common to various strains of a bacterial “species” of interest. The pangenome concept is particularly relevant to pathogens and can be said to define what it is to be a pathogen. Particularly in the case of OPPPs, such as *Legionella* spp., *Mycobacterium* spp., and *Pseudomonas* spp., there tend to exist several related strains, some pathogenic, and some not. Characterizing the pangenomes of key OPPPs can serve to define the critical subset of genes (and proteins) common to pathogenic strains. These molecular targets, in turn, may serve to improve the future development of molecular techniques for detecting and quantifying virulent pathogens of interest and distinguishing them from their non-pathogenic counterparts.

**Problem:**

What is the critical subset of genes (the pangenome) that defines the pathogenic strains of the parent OPPP genera and can these serve to improve molecular detection of virulent pathogens?

**Approach:**

(a) Five OPPPs (i.e., *Legionella pneumophila*, *Mycobacterium avium* complex, *Pseudomonas aeruginosa*, *Acanthamoeba* spp. and *Naegleria fowleri*) are of particular interest.

(b) High throughput sequencing of several members of each genus of members known to be pathogenic relative to those established to be non-pathogenic.

(c) Identification of key molecular markers for enhancing detection of virulent pathogens and distinguishing them from non-pathogenic relatives.

**Risk of Approaches and Anticipated Rewards:**

1. High return because verifying that molecular targets truly represent pathogens stands as a major roadblock to the application of molecular tools for drinking water monitoring.

2. Medium risk because of cost of sequencing, but the costs are decreasing. Costs aside, the success of the approach is low risk.

**Stakeholders:**

Water Research Foundation, EPA, NSF, NIH, DOE.

**Estimated Budget:**

Single center, $ 200,000/yr for 3 years = $600,000
GLOSSARY

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Acanthamoeba keratitis</td>
<td>Infection of the cornea of the eye by an amoeba belonging to the taxonomic genera Acanthamoeba.</td>
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<tr>
<td>Acanthapodia</td>
<td>Spike-like pseudopodia projecting from the amoebal cell over the engulfed bacterial cell; defining morphological feature of Acanthamoeba species.</td>
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<tr>
<td>Alveolar proteinosis</td>
<td>Disease state in which surfactant accumulates inside the alveoli of the lungs.</td>
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<tr>
<td>Alveoli</td>
<td>Tiny sacs in the deepest reaches of the lung that serve as the primary sites of gas exchange for respiration.</td>
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<tr>
<td>Amoeba</td>
<td>Carnivorous unicellular protozoan that locomotes without flagella or cilia by using pseudopodia.</td>
</tr>
<tr>
<td>Amoeba-resisting Microorganism (ARM)</td>
<td>Microorganisms that are able to survive and sometimes even proliferate inside of phagocytic amoeba. Strategies employed to evade digestion are also associated with virulence to humans and can be taken advantage of for selective culturing (e.g., acid pre-treatment).</td>
</tr>
<tr>
<td>Amplified fragment length polymorphism (AFLP)</td>
<td>DNA fingerprinting method for determining differences between closely related organisms. Restriction enzymes are first used to digest genomic DNA, adaptors are applied to capture the fragments and select fragments are amplified by PCR. Migration of amplified fragments are electrophoresed on a denaturing polyacrylamide gel to compare the fingerprints.</td>
</tr>
<tr>
<td>Arbitrarily primed (AP) polymerase chain reaction (PCR), also called “RAPD”</td>
<td>Amplification of target genomic DNA with random or arbitrary primers.</td>
</tr>
<tr>
<td>Assimilable organic carbon</td>
<td>The fraction of total organic carbon (TOC) that can be utilized most easily by heterotrophic bacteria for growth. Because this fraction is heterogeneous in composition it cannot be directly measured by chemical means and instead is assessed by a standardized bacterial growth assay (Standard Method 9217 and derivatives).</td>
</tr>
<tr>
<td>Bacillus</td>
<td>A Gram-positive (no outer membrane), spore-forming rod-shaped bacterium.</td>
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<tr>
<td>Bacteremia</td>
<td>Bacterial infection of the blood.</td>
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<tr>
<td>Bacteria</td>
<td>Member of the domain Bacteria, one of the three main branches of the tree of life (Archaea, Bacteria, and Eukarya).</td>
</tr>
<tr>
<td>Batch reactor</td>
<td>One of three fundamental reactor configurations (batch, continuous-flow stirred tank reactor, and plug-flow reactor) with no flow into or out of the vessel as the defining feature. May be applied to biofilm research.</td>
</tr>
<tr>
<td>Bioaerosols</td>
<td>Suspended, airborne microscopic particles or droplets. May contain microorganisms and thus are a potential route of inhalation exposure.</td>
</tr>
<tr>
<td>Biofilm</td>
<td>A complex aggregation of microorganisms and extracellular matrix polysaccharide residing on a solid substrate.</td>
</tr>
<tr>
<td>Biofilm monitor</td>
<td>A biofilm research apparatus that consists of a vertical glass column that contains rings of the desired test surfaces.</td>
</tr>
<tr>
<td>Biosensor</td>
<td>A sensitive biological element harnessed to provide a signal indicative of the conditions in a system. The biological element may be coupled to or in itself serve as a transducing element that produce a readily measureable signal.</td>
</tr>
<tr>
<td>Bone and joint infections</td>
<td>Disease state caused by entrance of microorganism(s) and their proliferation with or without toxin production in bone or joint.</td>
</tr>
<tr>
<td>Bronchiectasis</td>
<td>Destruction of muscle and elastic tissue resulting in permanent dilation of portions of the bronchial tree of the lung.</td>
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<tr>
<td>Cell-mediated immunity</td>
<td>A many-pronged immune response that is one line of defense to an infectious agent that is separate (but linked) to antibody production.</td>
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<tr>
<td>Cervical lymphadenitis</td>
<td>Infection of the lymph nodes in the head and neck</td>
</tr>
<tr>
<td>Chemostat</td>
<td>(See continuous flow stirred tank reactor)</td>
</tr>
<tr>
<td>Chemotrophs</td>
<td>An organism that oxidizes chemicals (organic or inorganic) to obtain energy (as opposed to a phototroph).</td>
</tr>
<tr>
<td>Cilia</td>
<td>Slender, projecting organelles of respiratory epithelium that work together to expel particles greater than about 2 microns in size from the respiratory system.</td>
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<tr>
<td>Conjugation</td>
<td>The introduction of genetic information from one bacterial cell to another via cell-to-cell contact and mediated by the presence</td>
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<tr>
<td>Continuous flow stirred tank reactor</td>
<td>One of three fundamental reactor configurations (batch, continuous-flow stirred tank reactor, and plug-flow reactor) in which the system is continuously mixed, with a constant influent flow rate (equivalent to the effluent flow rate) maintained.</td>
</tr>
<tr>
<td>Copiotrophic</td>
<td>Describes organisms that thrive in environments rich in organic matter.</td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>Synthetized chemical compounds that resemble and induce a similar physiological response to naturally produced steroid hormones, usually used to reduce inflammation.</td>
</tr>
<tr>
<td>Coupon</td>
<td>A section of material of prescribed dimensions used as a substrate for microbial adherence and biofilm formation.</td>
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<tr>
<td>Cyst</td>
<td>A morphological state in which a protozoan drastically alters its biochemical process in order to conserve energy and secretes a protective membrane in response to potentially lethal environmental conditions.</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>Inherited human disease manifested by thick secretions in the lung and repeated pneumonia caused by an impaired ability to transport chloride ions.</td>
</tr>
<tr>
<td>Cytometry</td>
<td>Methodology enabling direct, physical count of cells of interest. One common variation, flow cytometry, employs various light detection methods to count separately labeled cells one at a time as they flow past a sensor.</td>
</tr>
<tr>
<td>Density gradient centrifugation</td>
<td>Technique that is used to separate cell fractions based on mass to volume ratios (buoyancy).</td>
</tr>
<tr>
<td>Dilution rate</td>
<td>The inverse of residence time, which represents the rate at which particles, including suspended microorganisms, are washed out of a vessel with the effluent.</td>
</tr>
<tr>
<td>Direct immunofluorescence assay</td>
<td>A diagnostic technique that utilizes fluorescently labeled antibodies to detect the presence of specific antigens.</td>
</tr>
<tr>
<td>Disinfection</td>
<td>The process of killing or inactivating pathogenic organisms in water or on surfaces, usually via physical (e.g., U.V. or heat treatment) or chemical (e.g., chlorination or chloramination) means.</td>
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<tr>
<td>Dissecting microscope</td>
<td>Low powered stereoscopic microscope that allows resolution of small features on macroscopic specimens.</td>
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<tr>
<td>Dissolved organic carbon (DOC)</td>
<td>The portion of total organic carbon that is able to pass through a 0.45 μm filter.</td>
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<tr>
<td>Distribution system</td>
<td>The network of pipes that transport water from water treatment plants and/or storage tanks to residences and other buildings.</td>
</tr>
<tr>
<td>DNA extraction</td>
<td>Procedure of breaking open cells, isolating, and concentrating intact DNA molecules, devoid of other cell components or sample impurities.</td>
</tr>
<tr>
<td>Ecology</td>
<td>The study of the relationships between living things and their environment.</td>
</tr>
<tr>
<td>Effluent</td>
<td>The fluid that exits a reactor or a vessel.</td>
</tr>
<tr>
<td>Electron acceptor</td>
<td>An electronegative chemical or elemental species that has an affinity for accepting electrons from other chemical species. In biological systems, electron acceptors enable respiration (e.g., O₂ is the electron acceptor for humans).</td>
</tr>
<tr>
<td>Endemic</td>
<td>Referring to an infection that occurs at a steady, constant rate within a population.</td>
</tr>
<tr>
<td>Engineering controls</td>
<td>Proposed, tested, or implemented methods aimed at abolishing or mitigating safety risks, including exposure to pathogens.</td>
</tr>
<tr>
<td>Enzyme-linked immunosorbent assay (ELISA)</td>
<td>A diagnostic tool utilizing one or more antibodies to recognize a specific antigen, indicating the presence or absence of an analyte of interest.</td>
</tr>
<tr>
<td>Epidemiology</td>
<td>The study of the causes of health-related states or events (including disease), their distribution in the population, and the application of this study to the control of diseases and other health problems.</td>
</tr>
<tr>
<td>Eutrophic</td>
<td>An aquatic habitat with relatively abundant organic material, which can result in depleted dissolved oxygen.</td>
</tr>
<tr>
<td>Extent of colonization</td>
<td>The percentage of sample sites testing positive for a microbe compared with the total number of sample sites.</td>
</tr>
<tr>
<td>Fluorescence in situ hybridization (FISH)</td>
<td>Technique that utilizes fluorescent oligonucleotide probes that attach to specific structures within a cell, usually mRNA, which allows for the specific detection of intact cells and cellular structures, without isolation of a pure culture or DNA.</td>
</tr>
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<td>Term</td>
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<tr>
<td>extraction</td>
<td>Target cells may then be detected by epifluorescence microscopy of flow cytometry.</td>
</tr>
<tr>
<td>Flagella</td>
<td>Tail-like projections from the cell membranes of prokaryotes and eukaryotes, which are used for motility and have a characteristic propeller-like motion.</td>
</tr>
<tr>
<td>Floc</td>
<td>Aggregation of particles and/or bacterial cells suspended in a fluid.</td>
</tr>
<tr>
<td>Flow cell</td>
<td>Small laboratory reactors designed so that direct microscopic examination of the biofilm can be accomplished non-destructively.</td>
</tr>
<tr>
<td>Flow regime</td>
<td>Broad parameter of a fluid system that is often characterized by the Reynolds number and describes the way in which the forces influencing a fluid system tend to influence particular phenomena.</td>
</tr>
<tr>
<td>Fluid impinger</td>
<td>Air sampling apparatus that removes particles from the air by drawing the air through a wetting agent and collecting the resulting particle containing droplets in vessel.</td>
</tr>
<tr>
<td>Folliculitis</td>
<td>Inflammation of one or more hair follicles.</td>
</tr>
<tr>
<td>Free-living amoeba</td>
<td>Amoeba that do not require association with a parasitic host in order to survive.</td>
</tr>
<tr>
<td>GI infections</td>
<td>Disease state caused by abnormal proliferation of and/or toxin production by natural or foreign flora within the gastrointestinal (GI) tract.</td>
</tr>
<tr>
<td>Gram-negative</td>
<td>Bacteria with a thin peptidoglycan cell wall and have an outer membrane that fails to retain the dye crystal-violet dye upon exposure to acidic-alcohol.</td>
</tr>
<tr>
<td>Gram-positive</td>
<td>Bacteria that lack an outer membrane and have a thick peptidoglycan cell wall that retains crystal-violet dye.</td>
</tr>
<tr>
<td>Granulomatous amoebic encephalitis</td>
<td>A central nervous system infection caused by specific types of amoeba, including some species in the taxonomic genera Acanthamoeba.</td>
</tr>
<tr>
<td>Hazen-Williams coefficient</td>
<td>A dimensionless unit that adjusts for the effects caused by the type of pipe material and the internal condition of the pipe on the calculated loss in fluid pressure due to friction.</td>
</tr>
<tr>
<td><strong>Healthcare-associated disease</strong></td>
<td>Infection or other disease state acquired while receiving treatment for a different disease state within a healthcare facility (also referred to as nosocomial infections).</td>
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<tr>
<td><strong>Heterotroph</strong></td>
<td>An organism that utilizes organic carbon for growth.</td>
</tr>
<tr>
<td><strong>Hydrotherapy</strong></td>
<td>Use of water as a medical treatment, usually administered as a bath or shower of prescribed temperature and pressure.</td>
</tr>
<tr>
<td><strong>Immunocompromised</strong></td>
<td>The state of having an immune system that has been suppressed by aging, disease or immunosuppressive suppressive therapy.</td>
</tr>
<tr>
<td><strong>Immunosuppression</strong></td>
<td>Impairment of the immune system by a means of drug or radiation treatment or to infection and disease.</td>
</tr>
<tr>
<td><strong>in vitro</strong></td>
<td>Refers to an experiment conducted in isolation from the living organism or native environment, rather than with the living organisms itself within its native state.</td>
</tr>
<tr>
<td><strong>Indirect immunofluorescence assay</strong></td>
<td>A diagnostic technique that utilizes fluorescently labeled antibodies to detect specific antibodies that are associated with specific antigens.</td>
</tr>
<tr>
<td><strong>Influent</strong></td>
<td>The fluid that enters a reactor or a vessel.</td>
</tr>
<tr>
<td><strong>Infrequent-restriction-site PCR</strong></td>
<td>A procedure developed to amplify large fragments of genomic DNA, and then subsequently digest these amplicons with very few restriction enzymes, thus reducing the need for the extraction of large amounts of high-molecular-weight DNA from a sample.</td>
</tr>
<tr>
<td><strong>Isoenzyme analysis</strong></td>
<td>A method of determining genetic differences between cell cultures based on the separation of isoenzymes (enzymes with similar or identical specificity) using pulsed-field gel electrophoresis.</td>
</tr>
<tr>
<td><strong>Lateral flow immunoassay</strong></td>
<td>Diagnostic test in which a sample flows along a solid surface containing analyte-specific test reagents. If the analyte being targeted is in the sample, it will bind to the test reagents (typically possessing characteristic color) and yield a visually positive output.</td>
</tr>
<tr>
<td><strong>Latex agglutination</strong></td>
<td>Diagnostic test in which a sample is added to a mixture of latex beads, which have been coated with antibodies. Presence/absence of a target, such as a microbe, in a cell is indicated by the visualization of latex bead clumping caused by</td>
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<td>Term</td>
<td>Definition</td>
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<tr>
<td>binding of the antibodies to a specific antigen.</td>
<td></td>
</tr>
<tr>
<td>Legionella-like amoebal pathogens (LLAP)</td>
<td>Probable Legionella species able to multiply within an amoebal host, but have not been cultivated on laboratory media.</td>
</tr>
<tr>
<td>Legionnaires’ disease</td>
<td>A severe form of pneumonia caused by Legionella pneumophila and other Legionella species.</td>
</tr>
<tr>
<td>Macrophage</td>
<td>A type of white blood that ingests (takes in) foreign material. Macrophages are key players in the immune response to foreign invaders, such as infectious microorganisms.</td>
</tr>
<tr>
<td>Magnetic immunoassay</td>
<td>Diagnostic test in which an analyte is immobilized on a solid surface by association with antibodies. The analyte is then treated with magnetic beads, which associate with the analyte and enable detection based on the resulting change in the strength of the magnetic field.</td>
</tr>
<tr>
<td>Malignancy</td>
<td>Unrestrained growth on animal or plant cells of a particular tissue with the possibility of appearance of the same cell type in other tissue (metastatic).</td>
</tr>
<tr>
<td>Maximum grow rate</td>
<td>Kinetic parameter defining the highest possible growth rate of an organism (units of $t^{-1}$ or mass/t). Maximum growth rate is typically a function of the organism and the organic carbon or nutrient type and concentration, although environmental factors (e.g., temperature, pH) can impose further boundaries on this parameter.</td>
</tr>
<tr>
<td>Methodology</td>
<td>The procedures that have been developed to obtain accurate and reproducible data directed at answering specific question.</td>
</tr>
<tr>
<td>Microbial ecology</td>
<td>The study of the relationships of microorganisms with each other and their environment.</td>
</tr>
<tr>
<td>Mitochondrial DNA</td>
<td>Circular highly conserved DNA found within the mitochondria of eukaryotes that encode for many genes whose products are involved in electron transport and respiration. Mitochondrial DNA is responsible for “maternal or cytoplasmic effects” in inheritance and genes show homology with some bacterial genes as a result of the mitochondrial origin as an endosymbiont.</td>
</tr>
<tr>
<td>Monoclonal antibody (MAb) subtyping</td>
<td>Procedure that indicates the presence of specific subtypes of microorganism in a sample by the detection of attachment of</td>
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<td>Term</td>
<td>Definition</td>
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<tr>
<td>subtype specific antibodies.</td>
<td></td>
</tr>
<tr>
<td>Mycobacterium avium complex (MAC)</td>
<td>Genetically related Mycobacteria including <em>Mycobacterium avium</em>, <em>Mycobacterium intracellulare</em>, and <em>Mycobacterium avium</em> subsp. paratuberculosis.</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>Diagnostic statistic that describes the ratio between the number of truly negative results and the total number of negative diagnoses.</td>
</tr>
<tr>
<td>Nontuberculous Mycobacteria (NTM)</td>
<td>Bacteria belonging to the <em>Mycobacteria</em> genus that are not the causative agents of tuberculosis.</td>
</tr>
<tr>
<td>Oligotrophic</td>
<td>Environmental conditions in which there is relatively little organic matter or nutrients to support the growth of heterotrophic organisms.</td>
</tr>
<tr>
<td>Opportunistic premise plumbing pathogens (OPPPs)</td>
<td>Group of microorganisms, including <em>L. pneumophila</em>, <em>M. avium</em>, <em>P. aeruginosa</em>, some Acanthamoeba spp., <em>Naegleria fowleri</em> and others, that proliferate in and/or are transmitted to humans from building plumbing and fixtures. Immunocompromised individuals are thought to be primarily at risk, but precise risk factors vary and may include a broader portion of the population.</td>
</tr>
<tr>
<td>Optical waveguide lightmode spectroscopy</td>
<td>Versatile characterization technique that can be adapted for real-time detection of specific biomaterials through continuous analysis of the refraction of a He-Ne laser that changes based on the absorption of biomaterials to a grating within the instrument's waveguide.</td>
</tr>
<tr>
<td>Oropharyngeal</td>
<td>Describes area of the pharynx that extends from the uvula to the level of the hyoid bone.</td>
</tr>
<tr>
<td>Oxidative stress</td>
<td>An imbalance in the abundance of reactive oxygen species within a cell, and the ability of a cell to detoxify those elements.</td>
</tr>
<tr>
<td>Pangenome</td>
<td>The superset of genes common to all strains of a given species. The pangenome concept addresses the significant genetic variation being revealed among individual species and subspecies of Bacteria and Archae through the recent application of metagenomic techniques.</td>
</tr>
<tr>
<td>Pasteurization</td>
<td>Temporarily heating a beverage or foodstuff (e.g., 80°C for 10 min), to reduce the number of viable microorganisms that could cause disease or spoilage.</td>
</tr>
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<td>Term</td>
<td>Definition</td>
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<tr>
<td>Pellicle</td>
<td>Aggregation of particles at the air-liquid surface of a fluid.</td>
</tr>
<tr>
<td>Physiology</td>
<td>The study of the functions of living organisms and their parts.</td>
</tr>
<tr>
<td>Pipe reactor</td>
<td>Biofilm research apparatus that utilizes plumbing materials or distribution system piping, new or old pipes, all with and without removable coupons or other means of sampling surfaces as a substrate.</td>
</tr>
<tr>
<td>Planktonic</td>
<td>Microorganisms that grow freely suspended in the fluid of an aquatic environment, rather than attached to a surface.</td>
</tr>
<tr>
<td>Plasmid analysis</td>
<td>Bacterial typing method in which extrachromosomal, autonomously replicating DNA molecules (i.e., plasmids), are isolated from cells and compared by either size, sequence or restriction endonuclease fragments.</td>
</tr>
<tr>
<td>Plug flow reactor</td>
<td>One of three fundamental reactor configurations (batch, continuous-flow stirred tank reactor, and plug-flow reactor) in which the fluid moves through the system in the direction of flow (axial or X direction) with no axial mixing. Plug flow reactors are ideal for simulating the pipe biofilm environment.</td>
</tr>
<tr>
<td>Pneumoconiosis</td>
<td>Lung disease associated with inhalations of dust that is common in miners.</td>
</tr>
<tr>
<td>Point-of-use filter</td>
<td>A device installed at the point-of-use of water (e.g., tap or showerhead) that can prevent the passage of certain molecules or particles present in water by binding depending upon its design.</td>
</tr>
<tr>
<td>Polymerase Chain Reaction (PCR)</td>
<td>A technique for selectively amplifying a target segment of DNA by several orders of magnitude in order to enhance detection and other downstream analysis. A forward and reverse oligonucleotide primer are used complementary to the forward and reverse strands of DNA to be amplified. Amplification is accomplished in vitro using polymerase enzyme and a series of temperature cycles that drive amplification.</td>
</tr>
<tr>
<td>Pontiac fever</td>
<td>A nonpneumonia respiratory illness manifested by flu-like symptoms and caused by Legionella pneumophila, the same bacterium that is responsible for Legionnaires’ disease. Children, in particular, tend to be more prone to Pontiac fever than Legionnaires’ disease.</td>
</tr>
<tr>
<td>Premise plumbing</td>
<td>The portion of potable water distribution systems within the property line and in buildings (businesses, schools, private</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>Prevalence</td>
<td>The number of individuals with a particular characteristic (e.g., infected or not) per total population.</td>
</tr>
<tr>
<td>Primary amoebic meningoencephalitis</td>
<td>Disease state caused by abnormal proliferation of <em>Naegleria fowleri</em> within the central nervous system.</td>
</tr>
<tr>
<td>Proteomics</td>
<td>Study of the suite of proteins expressed by a cell.</td>
</tr>
<tr>
<td>Protozoa</td>
<td>Free-living or parasitic, nonphotosynthetic, single-celled, eukaryotic microorganisms, such as paramecium or amoeba.</td>
</tr>
<tr>
<td>Pruritic</td>
<td>Extreme itchiness.</td>
</tr>
<tr>
<td>Pseudopod</td>
<td>A cell membrane projection from eukaryotic cells that expands and contacts and enables cell motility and prey capture.</td>
</tr>
<tr>
<td>Pulmonary disease</td>
<td>Disease developed due to an infection of the lung’s airways.</td>
</tr>
<tr>
<td>Pulsatile lavage</td>
<td>Wound cleansing technique where an irrigating solution is forced into a wound under pressure in order to remove microorganisms and debris.</td>
</tr>
<tr>
<td>Pulsed-field gel electrophoresis (PFGE)</td>
<td>Method that uses alternating voltage gradient to enhance separation of large DNA fragments or protein according to variations in molecular weight, shape, and net charge.</td>
</tr>
<tr>
<td>Pure culture</td>
<td>A population of cells originating from a single cell type.</td>
</tr>
<tr>
<td>Quantitative microbial risk assessment (QMRA)</td>
<td>A science-based framework for estimating the risk of disease transmission within an exposed population. Each step of a microbial exposure pathway is evaluated quantitatively, from the first release of a pathogen to the actual human infection.</td>
</tr>
<tr>
<td>Quantitative polymerase chain reaction (Q-PCR)</td>
<td>PCR technique in which target amplification is monitored in real time with each amplification cycle, which enables quantitative detection of the target gene. Calibration curves are generated as the threshold cycle (Ct) (where PCR product is detectable above a defined threshold) versus the standard concentration.</td>
</tr>
<tr>
<td>Raman spectroscopy</td>
<td>Spectroscopy technique that is used to characterize unique signatures of molecules or cells through analysis of the inelastic scattering of a focused, monochromatic light beam.</td>
</tr>
<tr>
<td>Repetitive element PCR (REP-PCR)</td>
<td>Strain-specific molecular fingerprinting technique that uses primers designed to PCR-amplify conserved repetitive...</td>
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<tr>
<td>Term</td>
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<tr>
<td>Residence time</td>
<td>The average time required for all fluid in a vessel or reactor to be displaced by inflowing media.</td>
</tr>
<tr>
<td>Respiratory infections</td>
<td>Disease state caused by abnormal proliferation of and/or toxin production by microorganisms in the respiratory tract, including the organs involved in the transport of air into and out of the body.</td>
</tr>
<tr>
<td>Restriction endonuclease analysis</td>
<td>Application of restriction endonucleases (enzymes that recognize specific DNA sequences and fragment the DNA at that location) to generate unique array of fragments varying in length. The resulting fragments may then be separated by gel electrophoresis to generate a fingerprint unique to a pure or mixed culture.</td>
</tr>
<tr>
<td>Restriction fragment length polymorphism (RFLP)</td>
<td>A fingerprinting technique that detects differences in homologous DNA sequences amplified by PCR. PCR products are subject to restriction digest and the variability in the length of resulting DNA fragments is determined by gel electrophoresis and visualized by staining or probe hybridization (Southern blotting).</td>
</tr>
<tr>
<td>Reynolds number</td>
<td>A dimensionless value denoting the ratio of inertial forces to viscous force, which is used to characterize the flow regime of a given system.</td>
</tr>
<tr>
<td>Ribosomal RNA (rRNA)</td>
<td>Ribonucleic acid (RNA) that serves as a structural and catalytic component of both the large and small subunits of ribosomes. Ribosomes drive protein synthesis and thus are present in all domains of life. RNA sequences provide sufficient conserved and variable regions for classifying organisms according to evolutionary history (phylogenetics).</td>
</tr>
<tr>
<td>Safe Drinking Water Act Amendments of 1996</td>
<td>Amendments to legislation that set health-related standards for drinking water in the United States which included new and more rigorous approaches for preventing contamination, requirement of consumer information, regulatory improvements, and the creation of the Drinking Water State Revolving Fund.</td>
</tr>
<tr>
<td>Serogroup</td>
<td>A group of microbes producing an antigen (e.g., protein, lipid, or polysaccharide) that binds to a characteristic antibody or suite of antibodies.</td>
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<tr>
<td>Term</td>
<td>Definition</td>
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</tr>
<tr>
<td>Serotype</td>
<td>A subdivision of a species that is characterized by a unique pattern of binding by a single or set of antibodies.</td>
</tr>
<tr>
<td>Sidestream device</td>
<td>One of various types of biofilm research apparatuses that allow a researcher a &quot;window&quot; in to the biofilm characteristic of a larger-scale system.</td>
</tr>
<tr>
<td>Silica exposure</td>
<td>Respiration of quartz, cristobalite, and tridymite particles usually as a consequence of one’s work environment.</td>
</tr>
<tr>
<td>Silicosis</td>
<td>Lung disease caused by chronic silica exposure.</td>
</tr>
<tr>
<td>Small subunit ribosomal RNA gene</td>
<td>DNA sequence that encodes the small RNA segments that are part of the ribosome complex. The 16S rRNA gene is conserved throughout all prokaryotes; therefore it is a convenient marker for the presences and enumeration of bacteria.</td>
</tr>
<tr>
<td>Soft tissue infections</td>
<td>Disease state caused by abnormal proliferation of and/or toxin production by the natural or foreign flora of the skin and its underlying layer.</td>
</tr>
<tr>
<td>Source water</td>
<td>The body of water from which water utilities first extracts it eventual product before water treatment and/or its conveyance to the consumer.</td>
</tr>
<tr>
<td>Spread plating</td>
<td>Microbial enumeration technique in which sterile instruments are used to spread a prescribed volume of a suspension of cells on the surface of a sterile medium surface of a growth supportive medium to induce colony formation.</td>
</tr>
<tr>
<td>Subtyping</td>
<td>Process of separating species into secondary groups based on distinct characteristics.</td>
</tr>
<tr>
<td>Synthetic water</td>
<td>Aqueous media used to simulate oligotrophic conditions, in which the concentrations of all the substitutes usually found in a real-world water environment are controlled by the researcher.</td>
</tr>
<tr>
<td>T-cell</td>
<td>Lymphocyte which mature in the thymus and play a central role in cell-mediated immunity.</td>
</tr>
<tr>
<td>Terminal restriction fragment length polymorphism (T-RFLP)</td>
<td>Mixed sample microbial community fingerprinting technique in which fluorescent primers are used to amplify the DNA in a sample, the PCR product is digested by specific restriction enzymes, and a DNA sequencer is used to build a profile of the</td>
</tr>
<tr>
<td><strong>Glossary</strong></td>
<td><strong>variable length of restriction enzyme fragments.</strong></td>
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<tr>
<td>Total Organic Carbon (TOC)</td>
<td>The density of the bonded carbon molecules within a water sample which are amenable to oxidation.</td>
</tr>
<tr>
<td>Transduction</td>
<td>The transfer of DNA into a bacterial cell with the DNA carried by a virus.</td>
</tr>
<tr>
<td>Trophozoite</td>
<td>Protozoan or amoebal stage in which the protozoan actively feeds.</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>Often fatal infectious pulmonary disease characterized by formation of calcium-rich granulomas in the lung by members of the genus Mycobacterium. Tuberculosis caused by members of the Mycobacterium tuberculosis complex are highly transmissible from person-to-person or animal-to-animal, or animal-to-person.</td>
</tr>
<tr>
<td>Tubing reactors</td>
<td>Simple devices where a length of flexible tubing with a small inside diameter is used as a biofilm research apparatus.</td>
</tr>
<tr>
<td>Typhoid</td>
<td>A gastrointestinal infection caused by Salmonella enterica serovar typhi and is commonly acquired by ingestion of food or water contaminated with the feces of an infected person.</td>
</tr>
<tr>
<td>Urinary tract infections</td>
<td>Disease state caused by abnormal proliferation of and/or toxin production by natural or foreign flora in the urinary tract, which includes the organs involved in the concentration and removal of nitrogenous waste from the body.</td>
</tr>
<tr>
<td>Viable but non-culturable (VBNC)</td>
<td>A protective state entered by some microorganisms, including Legionella pneumophila, in response to environmental stressors that is characterized by the absence of colony formation.</td>
</tr>
<tr>
<td>Virulence</td>
<td>The capability of a group or species of microorganism to cause an infectious disease. In some cases specific genes activating virulence are involved.</td>
</tr>
<tr>
<td>Water aerosols</td>
<td>Aqueous droplets that have condensed around nuclei such that they are suspended and can travel through the air.</td>
</tr>
<tr>
<td>Waterborne Disease and Outbreak Surveillance System (WBD OSS)</td>
<td>Collaborative database maintained by CDC, EPA, and the council on state and territorial epidemiologists for collecting and reporting data related to occurrences and causes of waterborne-disease outbreaks and cases of waterborne disease. The system is dependent on public health departments to provide complete and accurate data.</td>
</tr>
<tr>
<td><strong>Water treatment</strong></td>
<td>The process of using physical, chemical, or biological means to produce a product with desired water quality standards at the point-of-use.</td>
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<tr>
<td><strong>Wound debridement</strong></td>
<td>Referring to various techniques in which unhealthful tissue is removed from a patient.</td>
</tr>
</tbody>
</table>

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ACHD. (1997). Approaches to prevention and control of Legionella infection in Allegheny County health care facilities: Allegheny County Health Department.


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<table>
<thead>
<tr>
<th>ABBREVIATIONS</th>
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<tr>
<td><strong>ACHD</strong> - Allegheny County Health Department</td>
<td><strong>BU</strong> - burn unit</td>
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<tr>
<td><strong>AFNOR</strong> - Association Française de Normalisation</td>
<td><strong>CCL</strong> - Contaminant Candidate List</td>
</tr>
<tr>
<td><strong>AK</strong> - <em>Acanthamoeba</em> keratitis</td>
<td><strong>CDC</strong> – U.S. Centers for Disease Control and Prevention</td>
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<tr>
<td><strong>ANSI</strong> - American National Standards Institute</td>
<td><strong>CFSTR</strong> - continuous flow stirred tank reactors</td>
</tr>
<tr>
<td><strong>AOC</strong> - assimilable organic carbon</td>
<td><strong>CFU</strong> - colony forming unit</td>
</tr>
<tr>
<td><strong>AP-PCR</strong> - arbitrarily primed polymerase chain reaction</td>
<td><strong>CLABSI</strong> - Central line-associated blood stream infection</td>
</tr>
<tr>
<td><strong>ARB</strong> - amoeba resisting bacteria</td>
<td><strong>CLSM</strong> - Confocal laser scanning microscopy</td>
</tr>
<tr>
<td><strong>ARM</strong> – amoeba resisting microorganism</td>
<td><strong>CPC</strong> - Cetylpyridium chloride</td>
</tr>
<tr>
<td><strong>ASHRAE</strong> - American Society of Heating, Refrigerating, and Air Conditioning</td>
<td><strong>DFA</strong> - Direct immunofluorescence assay</td>
</tr>
<tr>
<td><strong>ASTM</strong> - American Society for Testing and Materials</td>
<td><strong>DNA</strong> - Deoxyribonucleic acid</td>
</tr>
<tr>
<td><strong>ATP</strong> - adenosine triphosphate</td>
<td><strong>DOC</strong> - Dissolved organic carbon</td>
</tr>
<tr>
<td><strong>BCYE</strong> - buffered charcoal yeast extract</td>
<td><strong>DOE</strong> - Department of Energy</td>
</tr>
<tr>
<td><strong>BDOC</strong> - Biodegradable dissolved organic carbon</td>
<td><strong>ELISA</strong> - enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td><strong>BLS</strong> - bacterocin-like substances</td>
<td><strong>ELITE</strong> - Environmental <em>Legionella</em> Isolation Techniques Evaluation</td>
</tr>
<tr>
<td><strong>BPP</strong> - biomass production potential</td>
<td><strong>EMA</strong> - ethidium monoazide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MPN</td>
<td>Most probable number</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial deoxyribonucleic acid</td>
</tr>
<tr>
<td>mtSSU rRNA</td>
<td>Mitochondrial small subunit ribosomal ribonucleic acid gene</td>
</tr>
<tr>
<td>NICU</td>
<td>Neonatal Intensive Care Unit</td>
</tr>
<tr>
<td>NRC</td>
<td>National Research Council</td>
</tr>
<tr>
<td>NTM</td>
<td>Nontuberculous <em>Mycobacteria</em></td>
</tr>
<tr>
<td>OPPP</td>
<td>Opportunistic premise plumbing pathogens</td>
</tr>
<tr>
<td>OWLS</td>
<td>Optical waveguide lightmode spectroscopy</td>
</tr>
<tr>
<td>PAM</td>
<td>Primary amoebic meningoencephalitis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PFGE</td>
<td>Pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>PFR</td>
<td>Plug flow reactor</td>
</tr>
<tr>
<td>PHMB</td>
<td>Polyhexamethylene biguanide</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PICU</td>
<td>Pediatric intensive care unit</td>
</tr>
<tr>
<td>PMA</td>
<td>Propidium monoazide</td>
</tr>
<tr>
<td>POU</td>
<td>Point-of-use</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinyl chloride</td>
</tr>
<tr>
<td>QMRA</td>
<td>Quantitative microbial risk assessment</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>rDNA</td>
<td>Ribosomal deoxyribonucleic acid</td>
</tr>
<tr>
<td>RE-PCR</td>
<td>Repetitive element polymerase chain reaction</td>
</tr>
<tr>
<td>RFP</td>
<td>Request for proposal</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphisms</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>SBT</td>
<td>Sequence based typing</td>
</tr>
<tr>
<td>SDWA</td>
<td>Safe Drinking Water Act</td>
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<tr>
<td>ssrDNA</td>
<td>small subunit ribosomal ribonucleic acid gene</td>
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<tr>
<td>T3SS</td>
<td>Type III secretion system</td>
</tr>
<tr>
<td>TOC</td>
<td>Total organic carbon</td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary tract infection</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VAP</td>
<td>Ventilator-associated pneumonia</td>
</tr>
<tr>
<td>VBNC</td>
<td>Viable but non-culturable</td>
</tr>
</tbody>
</table>
VHA - Veteran’s Hospital Administration

WBDOSS - Waterborne disease and outbreak surveillance system

WHO - World Health Organization