United States
Environmental Protection
Agency

The impact of chlorine and chloramine on the detection and quantification of *Legionella pneumophila* and *Mycobacterium* spp.

Maura J. Donohue Ph.D.

Office of Research and Development
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PUBLIC AND ENVIRONMENTAL HEALTH MICROBIOLOGY

Impact of Chlorine and Chloramine on the Detection and Quantification of *Legionella pneumophila* and *Mycobacterium* Species

Maura J. Donohue,[a] Steve Vesper,[a] Jatin Mistry,[b] Joyce M. Donohue[c]

[a]U.S. Environmental Protection Agency, Cincinnati, Ohio, USA
[b]U.S. Environmental Protection Agency, Dallas, Texas, USA
[c]U.S. Environmental Protection Agency, Washington, DC, USA

ABSTRACT Potable water can be a source of transmission for legionellosis and nontuberculous mycobacterium (NTM) infections and diseases. Legionellosis is caused largely by *Legionella pneumophila*, specifically serogroup 1 (Sg1). *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium abscessus* are three leading species associated with pulmonary NTM disease. The estimated rates of these diseases are increasing in the United States, and the cost of treatment is high. Therefore, a national assessment of water disinfection efficacy for these pathogens was needed. The disinfectant type and total chlorine residual (TClR) were investigated to understand their influence on the detection and concentrations of the five pathogens in potable water. Samples (n = 358) were collected from point-of-use taps (cold or hot) from locations across the United States served by public water utilities that disinfected with chlorine or chloramine. The bacteria were detected and quantified using specific primer and probe quantitative-PCR (qPCR) methods. The total chlorine residual was measured spectrophotometrically. Chlorine was the more potent disinfectant for controlling the three mycobacterial species. Chloramine was effective at controlling *L. pneumophila* and Sg1. Plotting the TClR associated with positive microbial detection showed that an upward TClR adjustment could reduce the bacterial count in chlorinated water but was not as effective for chloramine. Each species of bacteria responded differently to the disinfection type, concentration, and temperature. There was no unifying condition among the water characteristics studied that achieved microbial control for all. This information will help guide disinfectant decisions aimed at reducing occurrences of these pathogens at consumer taps and as related to the disinfectant type and TClR.

IMPORTANCE The primary purpose of tap water disinfection is to control the presence of microbes. This study evaluated the role of disinfectant choice on the presence at the tap of *L. pneumophila*, its Sg1 serogroup, and three species of mycobacteria in tap water samples collected at points of human exposure at locations across the United States. The study demonstrates that microbial survival varies based on the microbial species, disinfectant, and TClR.

KEYWORDS chlorine, chloramine, drinking water, *Legionella*, *Mycobacterium*, total chlorine residual

Legionella and *Mycobacterium* are two genera of waterborne environmental bacteria that affect human health by causing the respiratory diseases legionellosis (Legionnaires' disease and Pontiac fever) (1) and nontuberculous mycobacterium (NTM) infections and diseases (2), respectively. Potable water is often suspected or identified as the cause of individual cases or a cluster of cases (an outbreak). Outbreaks for both genera

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Address correspondence to Maura J. Donohue, Donohue.maura@epa.gov.

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A Tale of Two Bacterium…

Legionellaceae

- Legionella (Genus)
  - Gram negative bacteria (Gammaproteobacteria)
  - Flagella rod (2-20 µm)
  - Slow grower (3 to 10 days)
  - Majority of species will grow in free-living amoebae
  - Aerobic, L-cysteine and iron salts are required for in vitro growth, pH: 6.8 to 7, T: 25 to 43 °C
  - ~65 species
  - Pathogenic or potentially pathogenic for human

Mycobacteriaceae

- Mycobacterium (Genus)
- Nontuberculous Mycobacterium (NTM)
- *M. avium-intracellulare* complex (MAC)
  - Gram positive bacteria
  - Rod shape(1-10 µm)
  - Non-motile, spore-forming, aerobic
  - Rapid to Slow grower (1 week to 8 weeks)
  - ~156 species
  - Some species capable of causing disease

NTM from Environmental Microorganism to Opportunistic Opponent

Genus | **156 Species** | **Disease**

NTM =Nontuberculous Mycobacteria   MAC = *M. avium* Complex

Mycobacterium spp.

Mycobacterium
Mycobacterium abscessus
Mycobacterium africanum
Mycobacterium agri
Mycobacterium aichiense
Mycobacterium algericum
Mycobacterium alsense
Mycobacterium alvei.
Mycobacterium angelicum
Mycobacterium anyangense
Mycobacterium arabiense.
Mycobacterium aromaticivorans
Mycobacterium arosiense
Mycobacterium arupense
Mycobacterium asiaticum
Mycobacterium aurum
Mycobacterium austroafricanum
Mycobacterium avium
Mycobacterium bacteremicum
Mycobacterium boenickei
Mycobacterium botniense
Mycobacterium bouchedurhonense
Mycobacterium bourgelatii
Mycobacterium bovis
Mycobacterium brisbanense
Mycobacterium brumae
Mycobacterium canariasense
Mycobacterium caprae
Mycobacterium celatum
Mycobacterium celeriflavum
Mycobacterium chelonae
Mycobacterium chitae
Mycobacterium chlorophenolicum
Mycobacterium chubuense
Mycobacterium colombiense
Mycobacterium conceptionense
Mycobacterium confluentis
Mycobacterium conspicuum
Mycobacterium cookii
Mycobacterium cosmeticum
Mycobacterium crocinum
Mycobacterium doricum
Mycobacterium duvalii
Mycobacterium elephantis
Mycobacterium europaeum
Mycobacterium fallax
Mycobacterium farcinogenes
Mycobacterium flavescens
Mycobacterium florentinum.
Mycobacterium fluoranthenivorans
Mycobacterium fortuitum
Mycobacterium franklinii
Mycobacterium frederiksbergense
Mycobacterium gadium
Mycobacterium gastri
Mycobacterium genavense
Mycobacterium gilvum
Mycobacterium goodii
Mycobacterium gordonae
Mycobacterium haemophilum
Mycobacterium hassiacum
Mycobacterium heckeshornense.
Mycobacterium heidelbergense
Mycobacterium hiberniae
Mycobacterium hippocampi
Mycobacterium hodleri
Mycobacterium holsaticum
Mycobacterium houstonense
Mycobacterium immunogenum
Mycobacterium insubricum
Mycobacterium interjectum
Mycobacterium intermedium
Mycobacterium intracellulare
Mycobacterium iranicum
Mycobacterium kansasii
Mycobacterium komossense
Mycobacterium koreense.
Mycobacterium kubicae.
Mycobacterium kumamotonense
, Mycobacterium kyorinense
Mycobacterium lacus.
Mycobacterium lentiflavum
Mycobacterium leprae
Mycobacterium lepraemurium
Mycobacterium litorale
Mycobacterium llatzerense.
Mycobacterium madagascariense
Mycobacterium mageritense,
Mycobacterium malmoense
Mycobacterium mantenii
Mycobacterium marinum
Mycobacterium massiliense
Mycobacterium microti
Mycobacterium minnesotense
Mycobacterium monacense
Mycobacterium montefiorense
Mycobacterium moriokaense,
Mycobacterium mucogenicum
Mycobacterium murale
Mycobacterium neoaurum
Mycobacterium nebraskense
Mycobacterium neworleansense
Mycobacterium nonchromogenicum
Mycobacterium noviomagense
Mycobacterium novocastrense
Mycobacterium pallens
Mycobacterium palustre
Mycobacterium paraense
Mycobacterium paraffinicum
Mycobacterium parafortuitum
Mycobacterium paragordonae.
Mycobacterium paraintracellulare
Mycobacterium parakoreense.
Mycobacterium parascrofulaceum
Mycobacterium paraseoulense
Mycobacterium paratuberculosis.
Mycobacterium parmense
Mycobacterium peregrinum
Mycobacterium phlei
Mycobacterium phocaicum
Mycobacterium pinnipedii
Mycobacterium porcinum
Mycobacterium poriferae,
Mycobacterium pseudoshottsii
Mycobacterium psychrotolerans
Mycobacterium pulveris
Mycobacterium pyrenivorans,
Mycobacterium rhodesiae
Mycobacterium riyadhense
Mycobacterium rufum
Mycobacterium rutilum
Mycobacterium salmoniphilum (
Mycobacterium saopaulense
Mycobacterium saskatchewanense
Mycobacterium scrofulaceum
Mycobacterium sediminis
Mycobacterium senegalense
Mycobacterium senuense
Mycobacterium seoulense,
Mycobacterium septicum
Mycobacterium setense
Mycobacterium sherrisii,
Mycobacterium shimoidei
Mycobacterium shinjukuense
Mycobacterium shottsii,
Mycobacterium simiae
Mycobacterium smegmatis
Mycobacterium sphagni
Mycobacterium stomatepiae
Mycobacterium szulgai
Mycobacterium terrae
Mycobacterium thermoresistibile
Mycobacterium timonense
Mycobacterium tokaiense
Mycobacterium triplex
Mycobacterium triviale
Mycobacterium tuberculosis
Mycobacterium tusciae
Mycobacterium ulcerans
Mycobacterium vaccae
Mycobacterium vanbaalenii.
Mycobacterium vulneris.
Mycobacterium wolinskyi
Mycobacterium xenopi
Mycobacterium yongonense

Clinically Relevant Species

M. avium, *M. intracellulare*, *M. fortuitum*, *M. chelonae*, *M. kansasii*, *M. abscessus*, etc.

- Pulmonary NTM lung disease
- Chronic bronchopulmonary disease
- Cervical or other lymphadenitis
- Skin and soft tissue diseases
- Disseminated infections
- Catheter-related infections

Legionellosis: Respiratory Disease

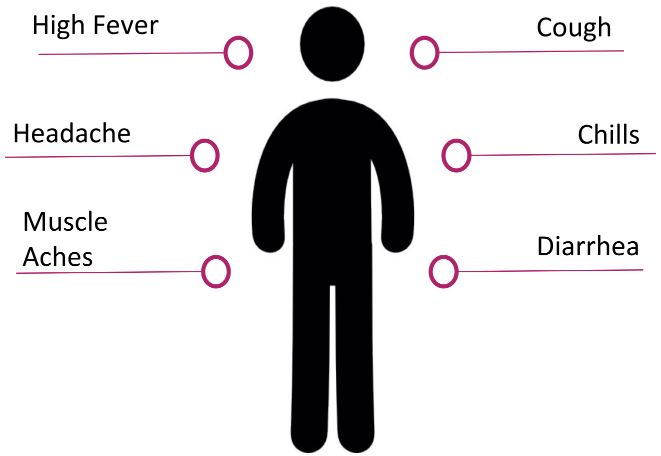
Disease

Legionellosis = pneumonia

- Legionaries' Disease (severe)
- Pontiac Fever (mild)

Signs/Symptoms

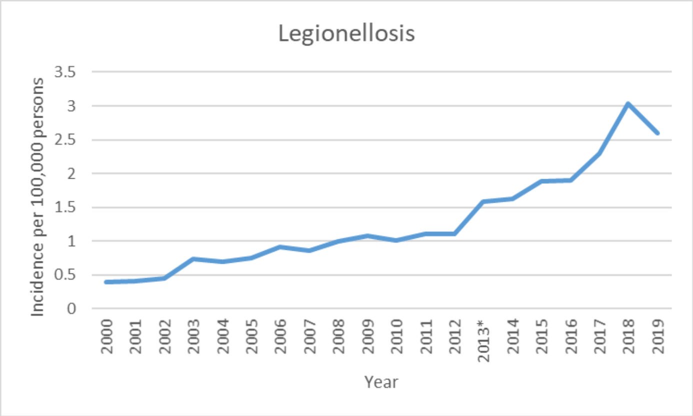
Pneumonia (Signs/Symptoms)



National Notifiable Disease Surveillance System (NNDSS)

2019 National Reportable Disease List: contained the names of over 110 Diseases/Microorganisms

Number of Cases Reported in 2019:
7,802 cases



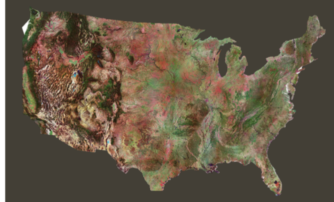
LEGIONELLOSIS.
Incidence,* by year — United States, 2000–2019, Source NNDSS

Annual Cost of Treatment in the U.S.

Number of Hospitalization/year
8,000-18,000 cases
avg(13,000) Marston, (1997)

Total Hospitalization Cost:
$433,758,000

Collier, S.A. et al 2012:
Direct healthcare costs of selected disease primarily or partially transmitted by water. Epidemiology Infection, 140, p2003-2013

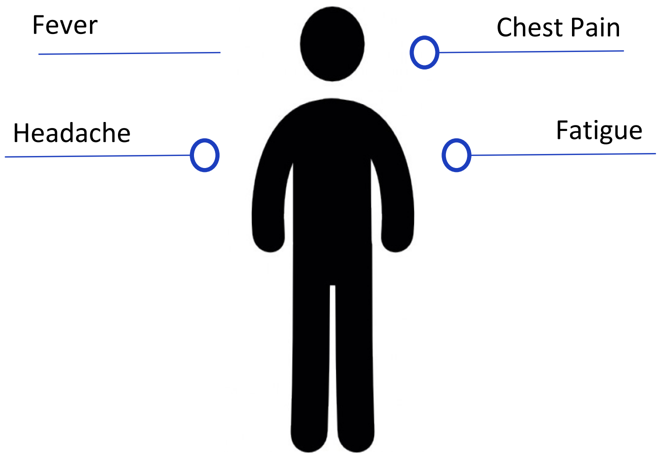


Nontuberculous Mycobacteria Infection/Disease: Primarily Respiratory Diseases

Infection/Disease

- Pulmonary NTM lung disease
- Chronic bronchopulmonary disease
- Cervical or other lymphadenitis
- Skin and soft tissue diseases
- Disseminated infections
- Catheter-related infections

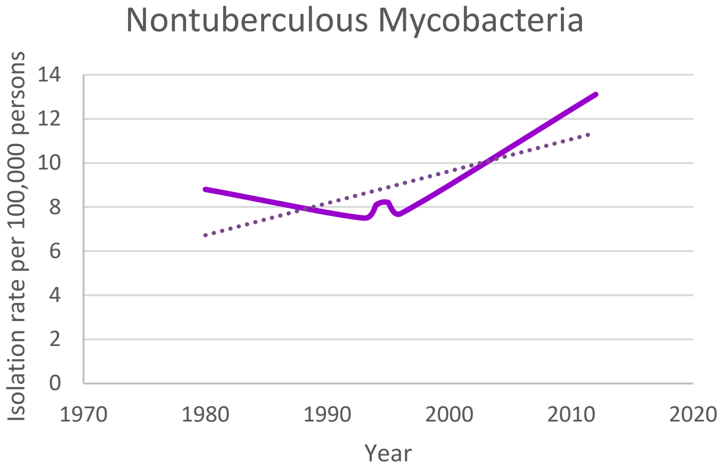
Signs/Symptoms



State Health Departments

Laboratory Reports
*Report is defined as the presence of culturable NTM from a human specimen (Lavage, Sputum, Blood, and/or Tissue)

Number of Cases Reported in 2010:
Est 86,244 cases



NONTUBERCULOUS MYCOBACTERIA.
Isolation Rate,* by year — United States, 1980–2013,
Sources: Good et al. (1980), CDC, NTM 1993-1996 report (1999), Donohue et al. (2016)

Annual Cost of Treatment in the U.S.

Number of Hospitalization/year
16,386 cases: 2007

Total Hospitalization Cost:
$425,788,469

Collier, S.A. et al 2012:
Direct healthcare costs of selected disease primarily or partially transmitted by water. Epidemiology Infection, **140**, p2003-2013

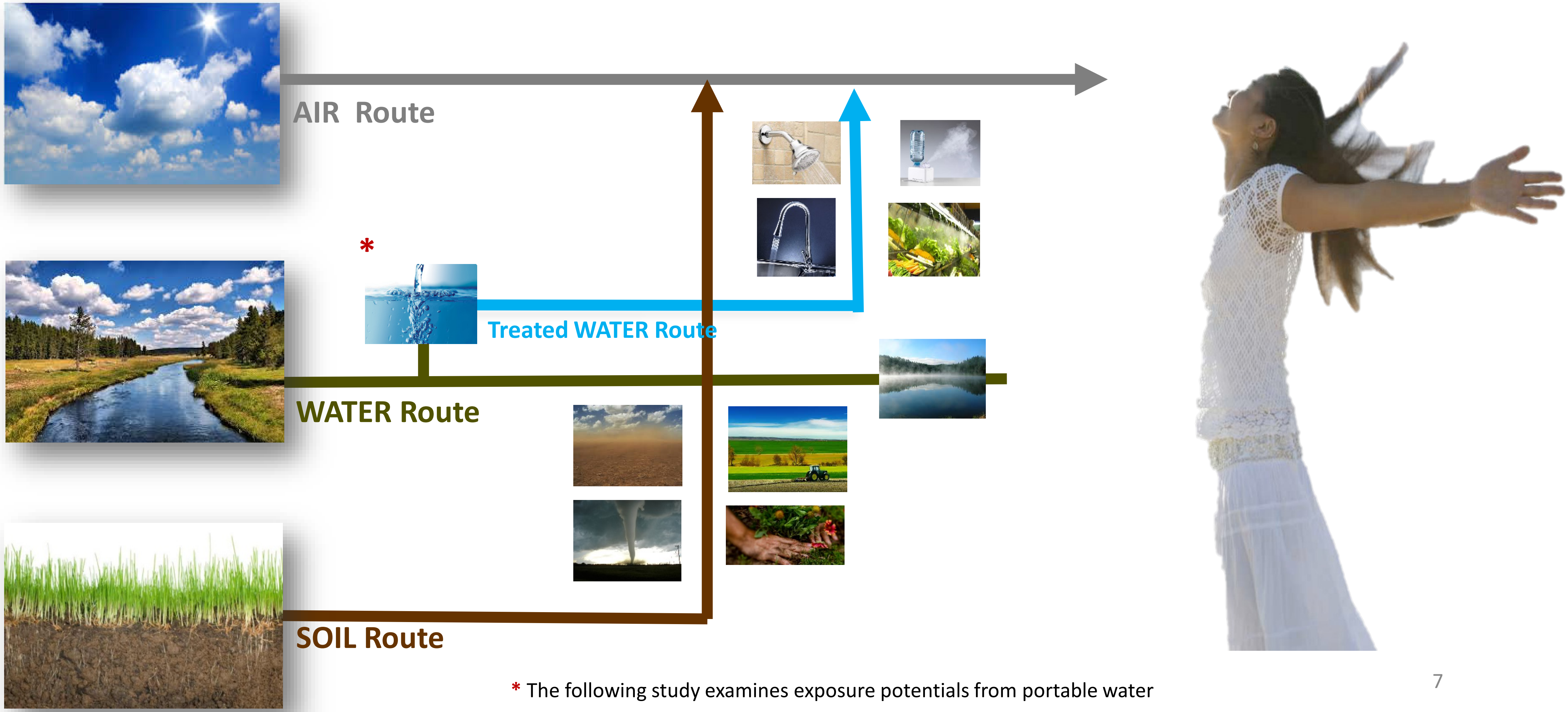
Number of NTM Cases
est 86,244 cases: 2010

Total Hospitalization Cost:
$815,098,690

Strollo S.E. et al 2015: The Burden of Pulmonary NOntuberculous Mycobacterial Disease in the United States. AnnalsATS, **12**, p1458-1464

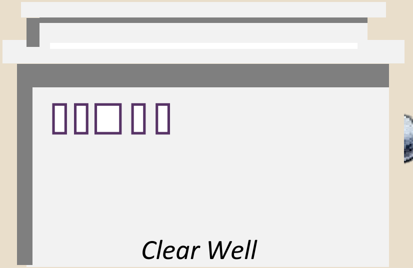
Exposure Routes: Environmental Sources

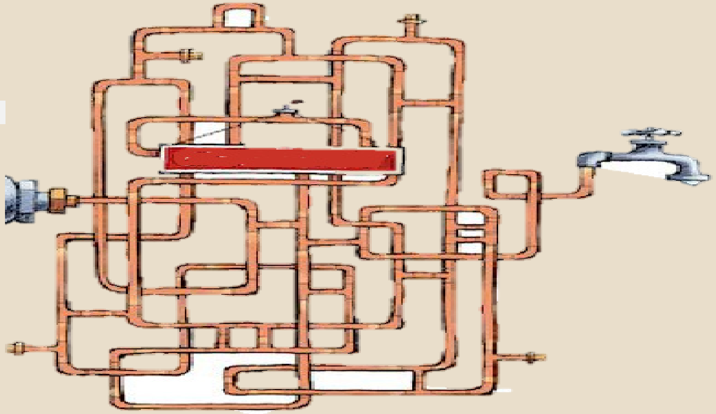
Legionella pneumophila and Mycobacterium avium are microorganisms of the natural environment found in soil and water.



* The following study examines exposure potentials from portable water

What Is the Purpose of this Study?





Houses/Building

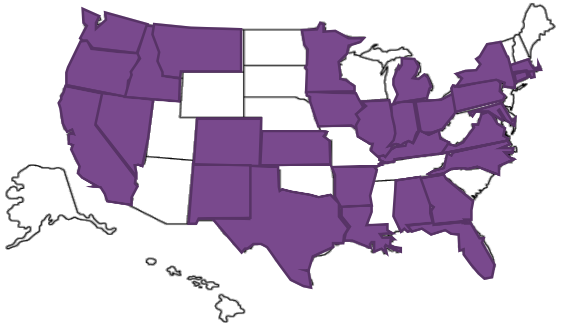
Source Water	Waterworks	Distribution	Point of Use
Lp = 25% (6/24) MA = 25% (6/24) MI = 25% (6/24)	Lp = 4% (1/24) MA = 25% (6/25) MI = 25% (6/25)	Y	X

King et al 2016: SOTEN

Donohue et al 2019: JAM & AEM

Study Design

46 States and Territories	Structure/ Point of Use 358 Samples	5 qPCR Assays	Water Quality Testing



Houses

Buildings

- *Legionella pneumophila*
  Donohue et al. 2014
- *L. pneumophila* Sg1
  Merault et al 2011l
- *Mycobacterium avium*
  Chen et al. 2015
- *Mycobacterium intracellulare*
  Chen et al. 2015
- *Mycobacterium abscessus*
  Steindor M. et al. 2015

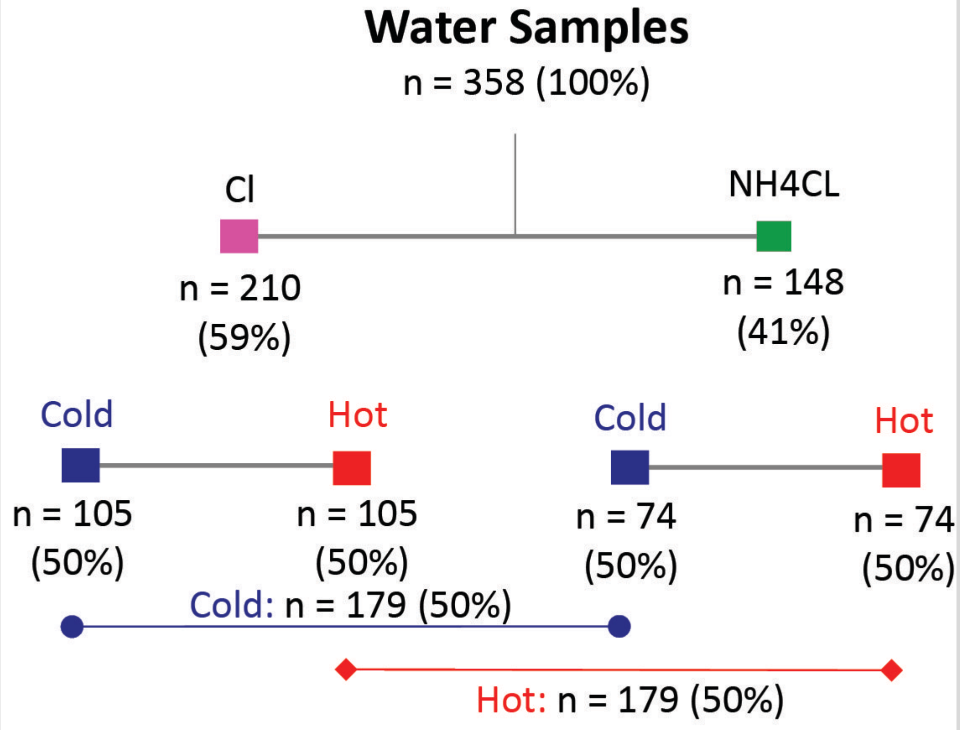
- Detection Frequency (FD)
- Persistence
- Concentration

- Total Chlorine
  DPD Total Chlorine Test
- Monochloramine
  Monochlor F Test
- Temperature
  Cold water line
  Hot water line
- Heterotrophic Plate Counts (HPC)
  Standard Method 9215

Sampling Time Frame : 2011 – 2017

Scope and Sample Design

- Chlorine (Cl) - Chloramine (CLM)
- Residual (Totatl Chlorine Test) – Temperature (Cold vs Hot)
- *L. pneumophila* (Lp) – Mycobacterium spp.(Myco)

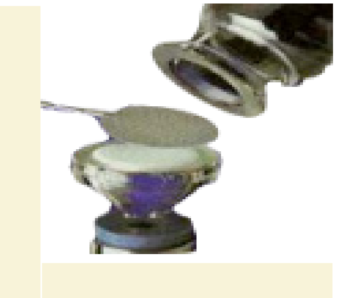


Molecular Testing: qPCR

Method

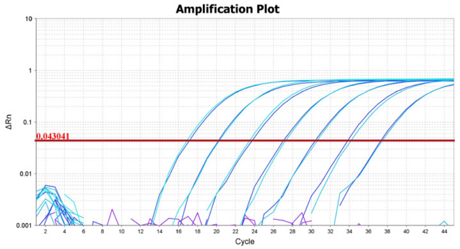
Water

Membrane Filtration
Polycarbonate 0.4 µm



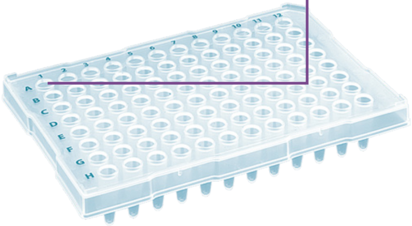
DNA Extraction
Bead Beating
DNA precipitation

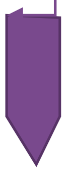
qPCR
40 cycles
1hr 45 min



Assays

1. **Genus: Gen-L (16S)**
2. **Species: Lp-16S (16S)**
3. **Species: Lp-Mip (Mip)**





1 Well = 1 Reaction

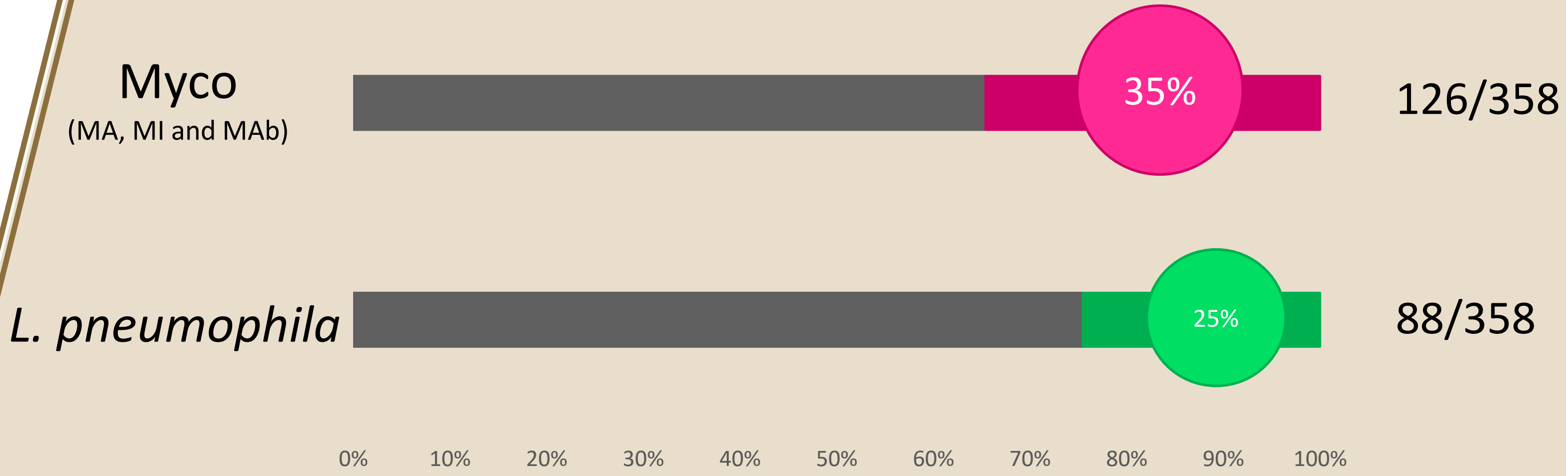
96 plate well: Well Format

- Assay/Mastermix
- Primer/Probes
- Template(DNA/ddH_2O)   10 µL reaction ~ 200mL
- Internal Control

Overview Of Occurrence (DF)

Point of Use

Houses/Building



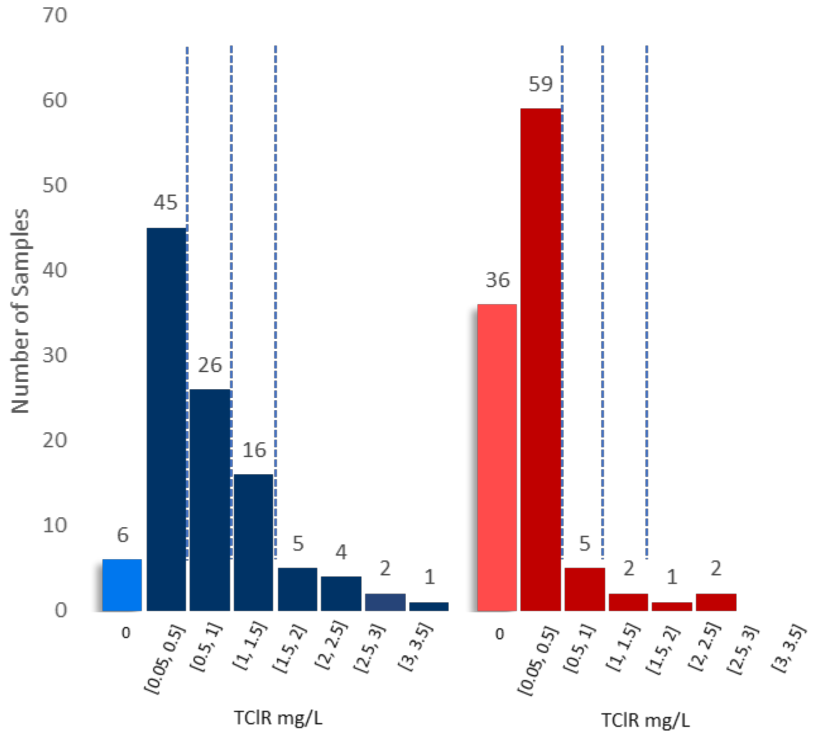
Is *L. pneumophila* *Mycobacteria spp.* Occurrence Influenced by Residual Choice?

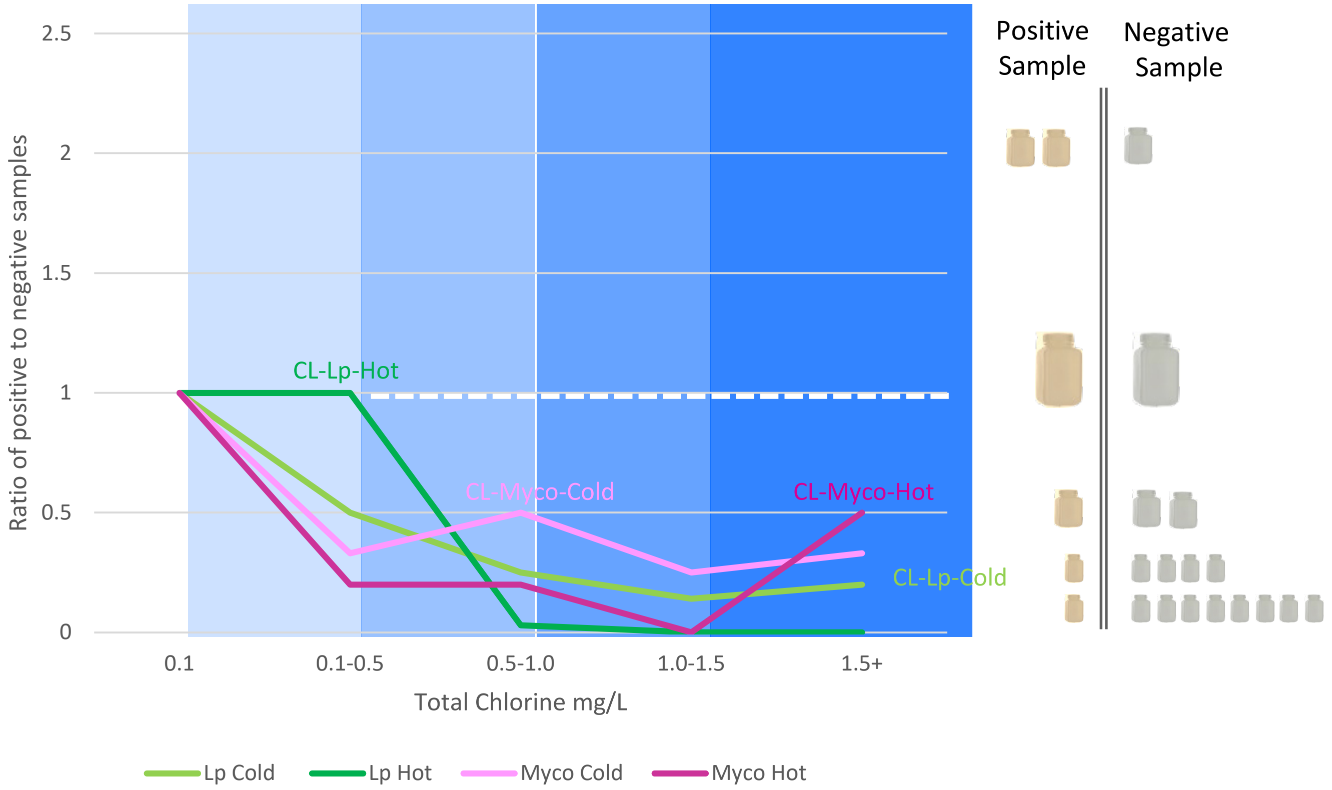
Detection Frequency

Species-Serogroup	Chlorine Number of Positive Samples (Percent)	Chloramine Number of Positive Samples (Percent)	Chi-Square p-value
All Samples	**N = 210**	**N = 148**	
L. pneumophila	55 (26)	32 (22)	NS
L. pneumophila Sg1	18 (9)	7 (5)	NS
M. avium	26 (12)	32 (22)	P = 0.02
M. intracellulare	44 (21)	29 (20)	NS
M. abscessus	19 (9)	25 (17)	P = 0.03
Cold Water Line	**N = 105**	**N = 74**	
L. pneumophila	29 (28)	17 (24)	NS
L. pneumophila Sg1	8 (8)	4 (6)	NS
M. avium	15 (14)	16 (22)	NS
M. intracellulare	21 (20)	16 (22)	NS
M. abscessus	11 (10)	13 (18)	NS
Hot Water Line	**N = 105**	**N = 74**	
L. pneumophila	27 (26)	16 (22)	NS
L. pneumophila Sg1	10 (9)	3 (4)	NS
M. avium	11 (10)	16 (22)	NS
M. intracellulare	23 (22)	13 (19)	NS
M. abscessus	8 (8)	12 (17)	NS

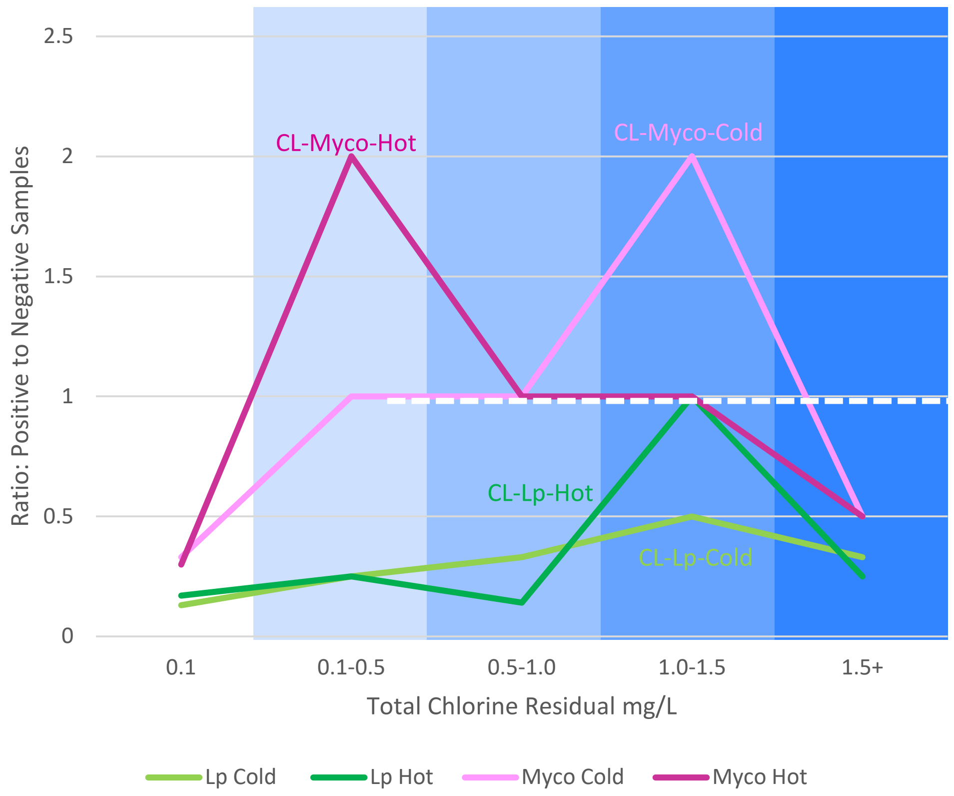
Yes, detection frequency for *M. avium* and *M. abscessus* were found to be significantly different between residual type.

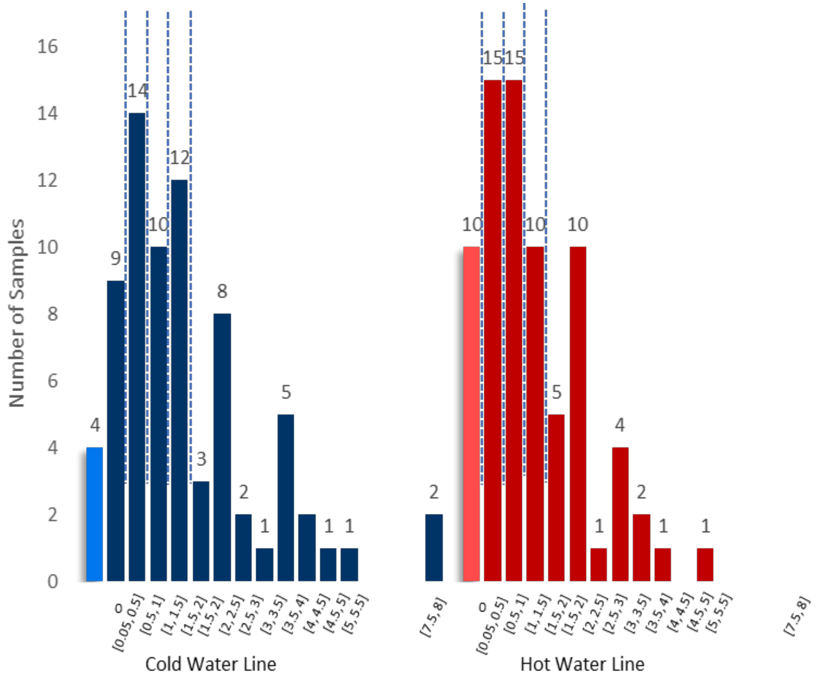
Chlorine: Does the Residual (TCLR) Concentration Influence *L. pneumophila /Mycobacterium spp.* Detection Frequency?





Chloramine: Does the Residual (TCLR) Concentration Influenced *L. pneumophila*/ *Mycobacterium* spp. Detection Frequency?



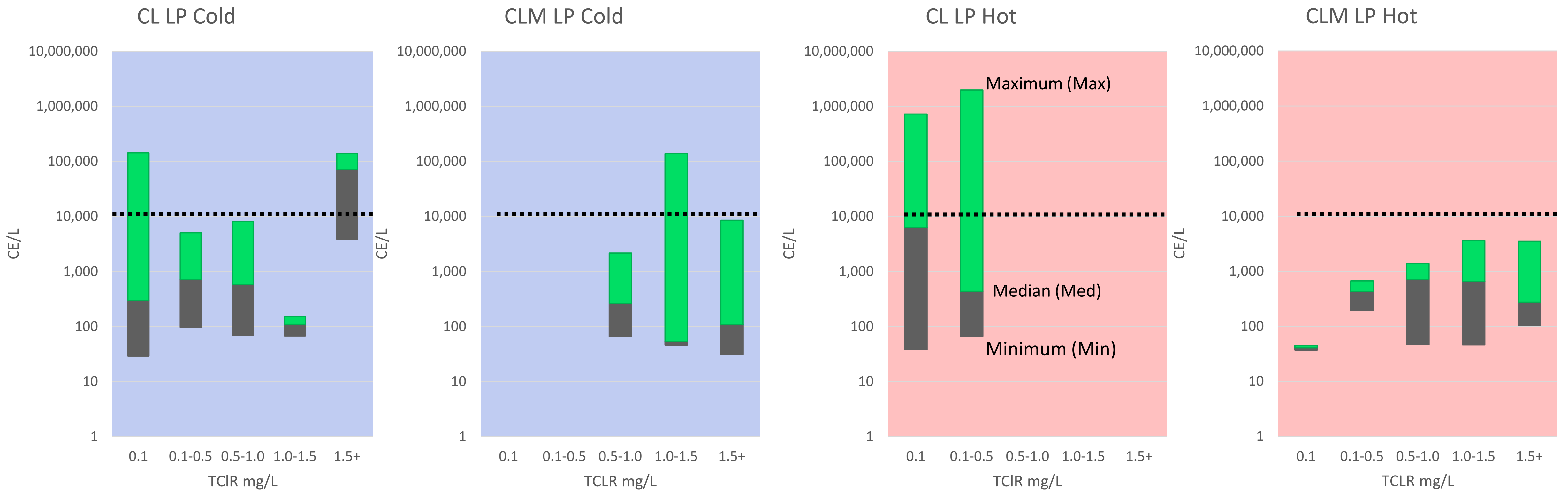


Does *L. pneumophila*/ *Mycobacterium* spp. Concentration Differ by Residual Choice?

Species-Serogroup	Chlorine	Chloramine	Mann-Whitney U
	Median (CE/L)	Median (CE/L)	p-value
All samples	**N = 210**	**N=148**	NS
L. pneumophila	581	132	P = <0.001
L. pneumophila Sg1	15,721	863	NS
M. avium	603	1,243	NS
M. intracellulare	487	661	NS
M. abscessus	1,339	2,157	NS
Cold Water Line	**N = 105**	**N = 74**	
L. pneumophila	341	82	P = 0.04
L. pneumophila Sg1	938	570	P = 0.05
M. avium	616	1,880	NS
M. intracellulare	359	928	P = 0.02
M. abscessus	1,113	834	NS
Hot Water Line	**N = 105**	**N = 74**	
L. pneumophila	4,201	187	P = 0.01
L. pneumophila Sg1	85,316	942	NS
M. avium	425	761	NS
M. intracellulare	542	602	NS
M. abscessus	9,048	17,304	NS

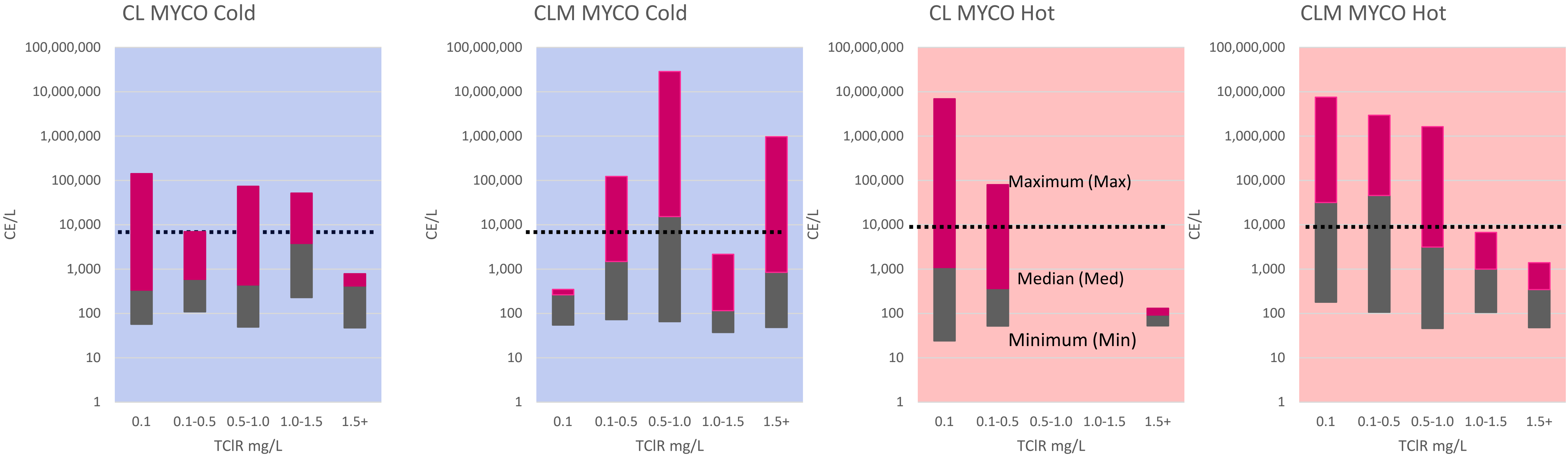
Yes, significant differences in concentrations were observed for *L. pneumophila* in both cold and hot water line samples and for *M. intracellulare* (cold water line samples).

Does Residual Concentration Influence *L. pneumophila* Concentration?



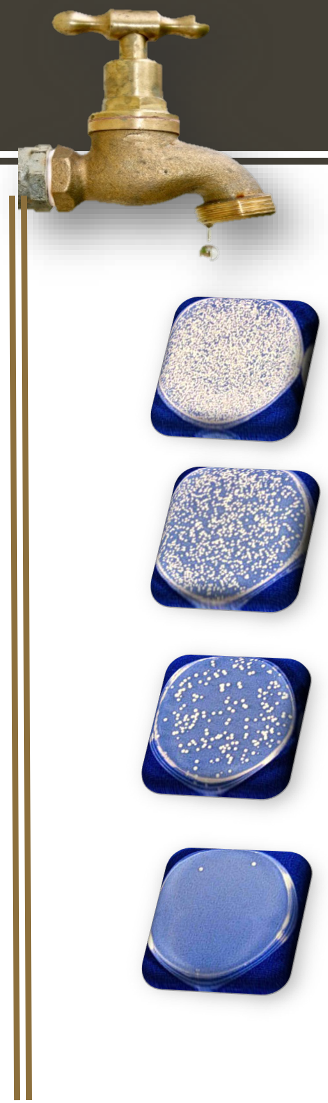
Each, residual type has its own impact on *L. pneumophila*. Chlorine (CL) impact on *L. pneumophila* concentrations is in a dose dependent manor. CLM impact on *L. pneumophila* isn't as does dependent.

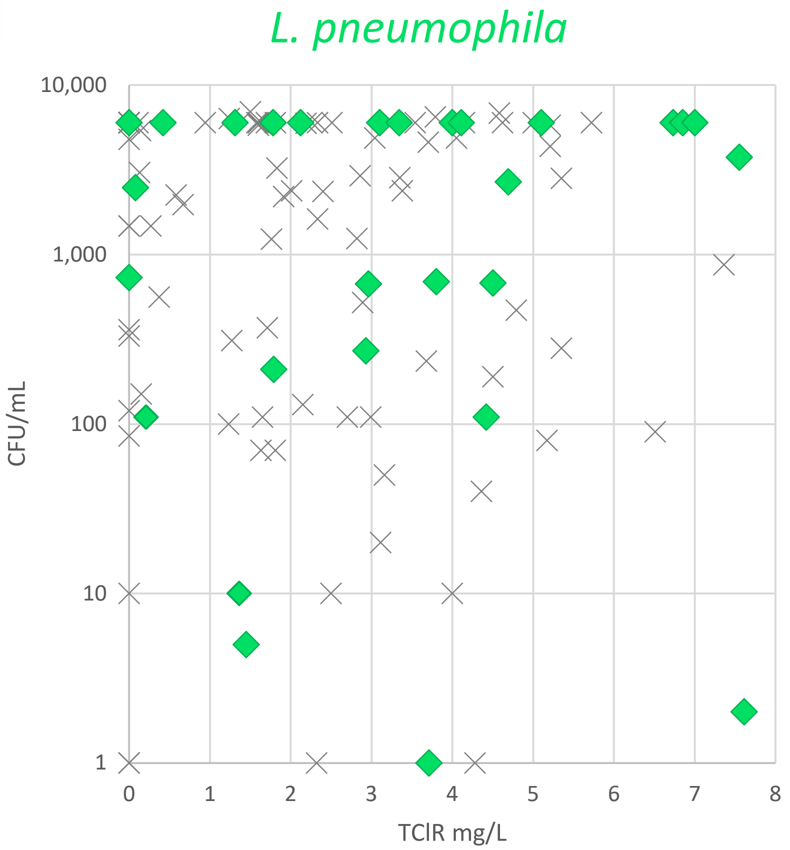
Does Residual Concentration Influence *Mycobacterium* spp. Concentration?

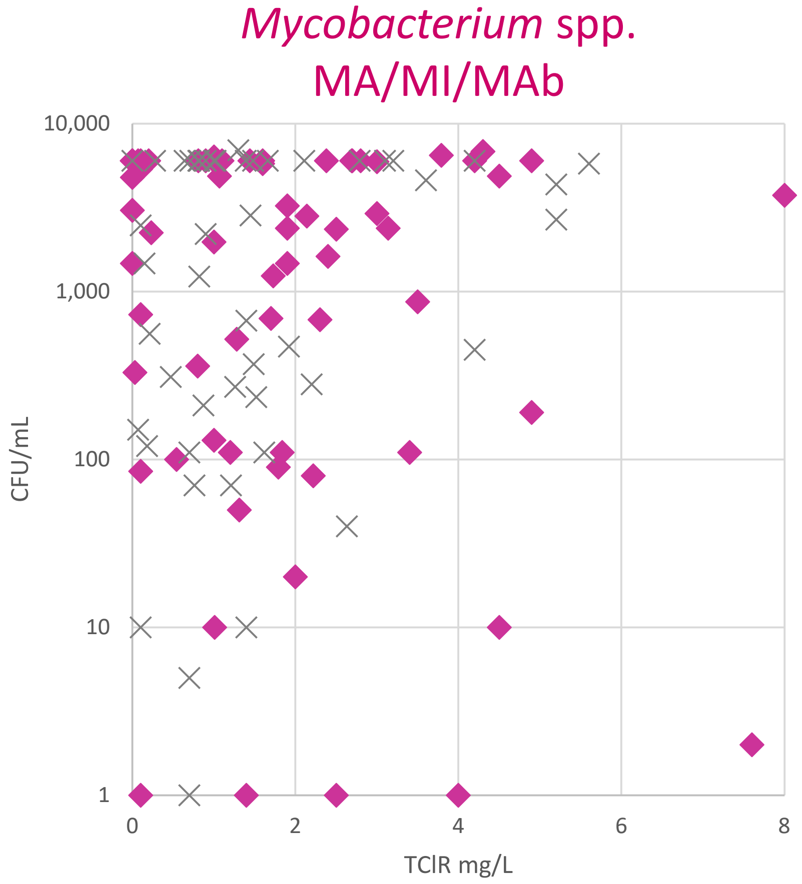


Each, residual type has its own impact on the *Mycobacterium spp*. Chlorine (CL) in cold water impact the *Mycobacterium spp* isn't does dependent. However CL+hot water does have a dose dependent impact on *Mycobacterium spp.* concentrations. CLM in cold water doesn't appear to impact on the *Mycobacterium spp.* species concentration. CLM+heat does impact *Mycobacterium spp.* in a does dependent manor.

Chloramine: HPC



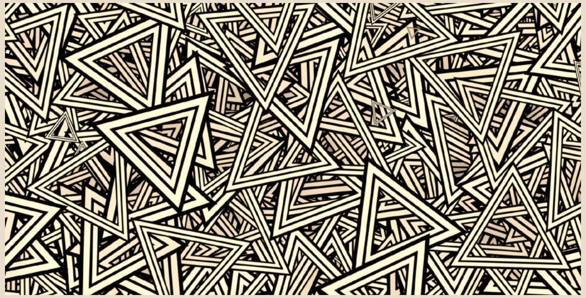
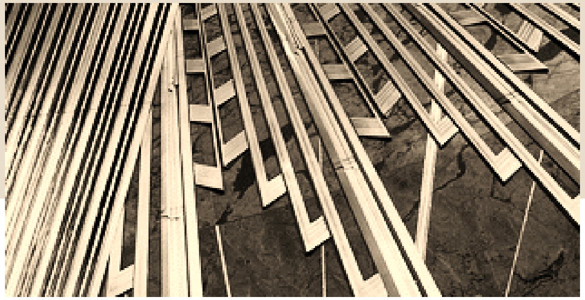




Lp/Myco positive Samples ◆ Negative samples X

Conclusions

- Residual type (Cl/CLM) does significantly influence MA and MAb occurrence patterns.
- Residual type (Cl/CLM) does significantly influence *L. pneumophila* and MI concentration.
- CLM is effective for controlling LP, but not MA/MI/Mab
- CL is relatively more effective at controlling MA/MI/Mab, than CLM.
- Temperature is both a stimulant for microbial growth but acts as a deterrent, especially if a residual is maintained in the hot water.

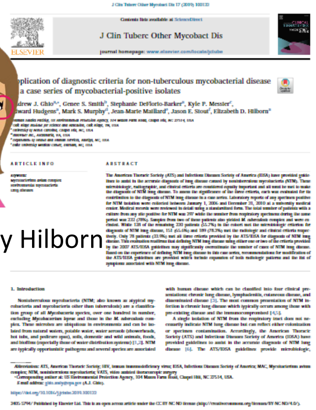
In the Larger Context

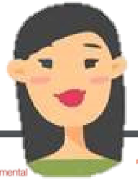
- JUST because your system uses CLM. Does NOT mean your water has MA/MI/Mab issues.
- JUST because your system uses CL doesn’t mean you have Lp issues.
- If you do have Lp issues and you’re a CL system is it most likely do to the lack of a active residual.
- A residual correction is not available for CLM systems.
- REMEMBER these observations are broad brush strokes which may or may not be applicable to your specific water system.
- Also REMEMBER, I talked today about just two water-borne bacteria and these observations do NOT take into account how other water pathogens will responds to our treatment and practices with.

Collaborators

- Jatin Mistry
- Stacy Pfaller
- Dawn King
- Steve Vesper

Dr. Betsy Hilborn

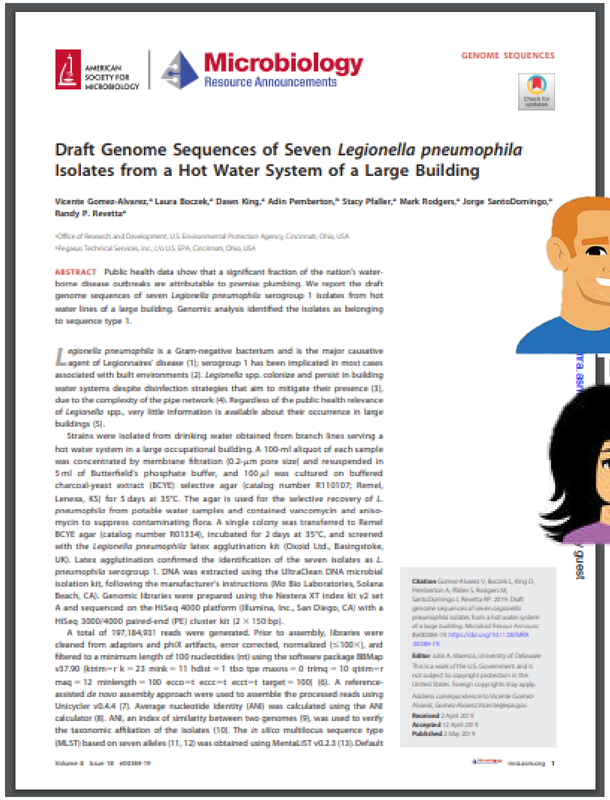




Dr. Helen Buse

EPA Researchers

Thank You!



Dr. Vicente -Gomez

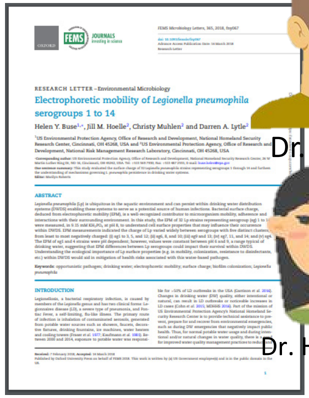
Laura Boczek

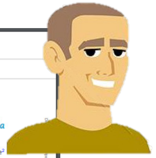
Maura Donohue

ORD/CESER/WID/BCB

Donohue.maura@epa.gov

513-569-7634





Dr. Darren Lyle

Dr. Helen Buse