Efficacy of Inactivation of *Legionella pneumophila* In Premise Plumbing Applications

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Research Team

❖ USEPA

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– Mark Donhowe

Outline

- ❖Background information on *Legionella* study
- ❖Copper Silver Ionization Study
- ❖UV-LED technology for *Legionella* control in a premise plumbing system
  - Bench-scale CB tests by multiwavelength UV LED
  - Microbial inactivation mechanisms

2016 CDC Report on Legionnaires' Disease Outbreaks

Morbidity and Mortality Weekly Report

Vital Signs: Deficiencies in Environmental Control Identified in Outbreaks of Legionnaires' Disease — North America, 2000–2014

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FIGURE 1. Reported cases of legionellosis per 100,000 population, by year — United States, 2000–2014

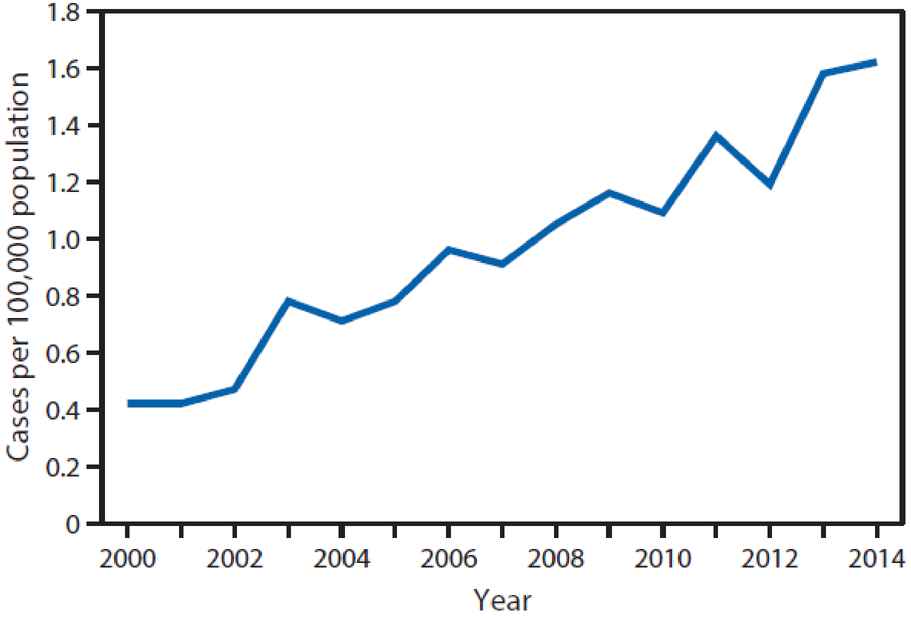
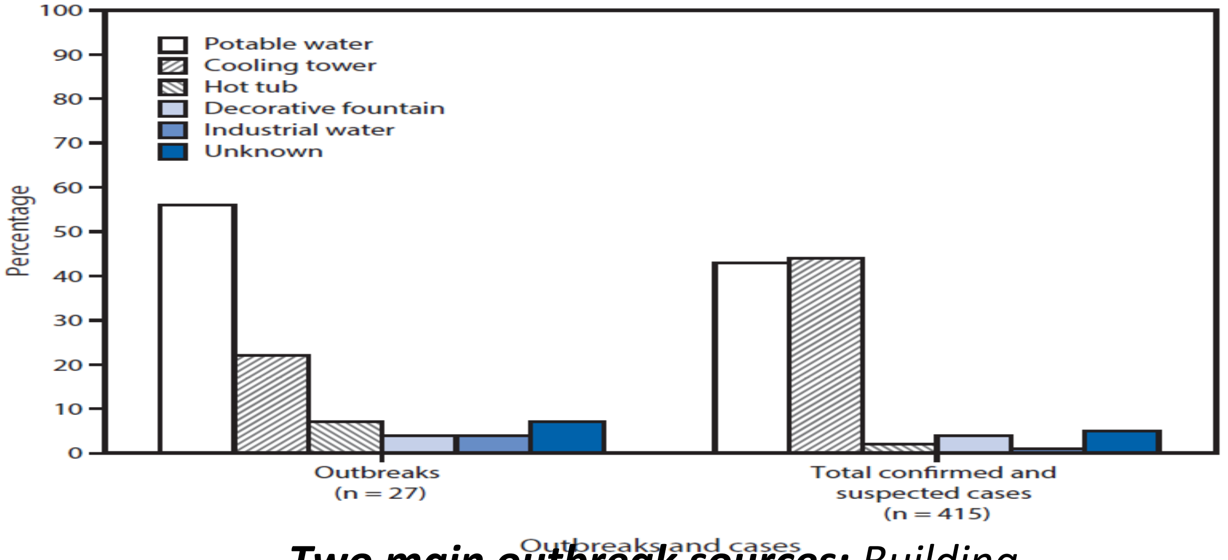


FIGURE 2. Percentage of outbreaks and cases of Legionnaires' disease, by environmental source — North America, 2000–2014



286% during 2000-2014

***Two main outbreak sources:** Building warm water systems & cooling towers*

Research Background

Legionellosis

- *Legionella pneumophila*, a waterborne pathogen, first caught the public's attention when an outbreak occurred in Philadelphia, PA in 1976 (Fraser et al. 1977).
  - 182 cases with 29 deaths

- Health departments reported about 6,100 cases of Legionnaires' disease in the United States in 2016 (https://wonder.cdc.gov/nndss/static/2016/annual/2016-table2h.html).
  - Estimated incident rate: 7.0 to 7.9 cases per 100,000 people

- One of the largest, most recent outbreaks occurred in Flint, MI.
  - 2014 outbreak: 45 cases, 5 deaths
  - 2015 outbreak: 46 cases, 7 deaths

Research Background

Premise Plumbing Disinfection Practices for Legionella

- Treatments that provide a disinfectant residual: Chlorine, Chloramine, Chlorine Dioxide and Copper Silver Ionization

- No disinfectant residual: Ozone and UV disinfection
  - Dependent variables of treatability: building-specific characteristics, water usage rates and water age
  - Water quality parameters – temperature, pH, turbidity, and DOC

- Emergency remediation
  - Superheat-and-Flush disinfection and Shock hyperchlorination

- Point-of-Use device
  - Filtration
  - **UV-LED (Ultraviolet-light emitting diodes)**

Cu-Ag Ionization (CSI)

- Treatment history
  - The 1st use of Ag ionization: Water disinfection by NASA for Apollo spacecraft (Albright et al., 1967)
  - Lin et al. (2011) documented CSI application controlling *Legionella* in hospitals worldwide

- Microbial disinfection efficacy
  - The bonding of the positively charged ions (Cu^{+2} & Ag^{+}) with negatively charged cell wall (Walraven et al., 2016)
  - In biofilm, CSI achieved 2- to 4-log less reduction of OPPPs compared to free-floating microbes (Shih and Lin, 2010)

Cu-Ag Ionization

- **Disinfection mechanism**
  - Positively charged copper and silver ions bond electrostatically with negative sites on bacterial cell walls and denature proteins.
  - Effectively disinfect biofilms: Higher copper concentrations found in biofilms after treatment with copper silver ionization, may be responsible for preventing biofilm formation. (Liu et al., 1994; 1998).
- **Field application**
  - *Recommended concentrations for Legionella eradication: Copper (200-400 ppb) and Silver (20-40 ppb) – lower concentrations after initial installation*
  - Monitoring: Copper (weekly with a colorimeter kit) and Silver (once every 2 months by AAS or ICP)
  - USEPA maximum contamination levels (MCL) for drinking water: 1,300 ppb for copper and 100 ppb for silver

Cu-Ag Ionization

- **Advantages**
  - Easy installation and maintenance
  - Limited oral consumption due to the installation into the hot water recirculation lines
  - Prolonged efficacy and biocidal activity at higher water temp.
- **Disadvantages**
  - Negative effect of high pH (>8.5) on biocidal efficacy of Cu, but no significant impact on Ag by pH
  - A phosphate compound to control corrosion may decrease the efficacy of ionization (Lin and Vidic, 2006)
  - Higher chloride concentration may decrease the availability of silver cations and reduce its biocidal potential
- **Synergistic inactivation with UV**
  - MS-2 bacteriophage study (Butkus et al., AEM 2004)

Materials & Methods

- Collect water sample from hot water faucet at local hospital that currently utilizes Cu-Ag ionization.

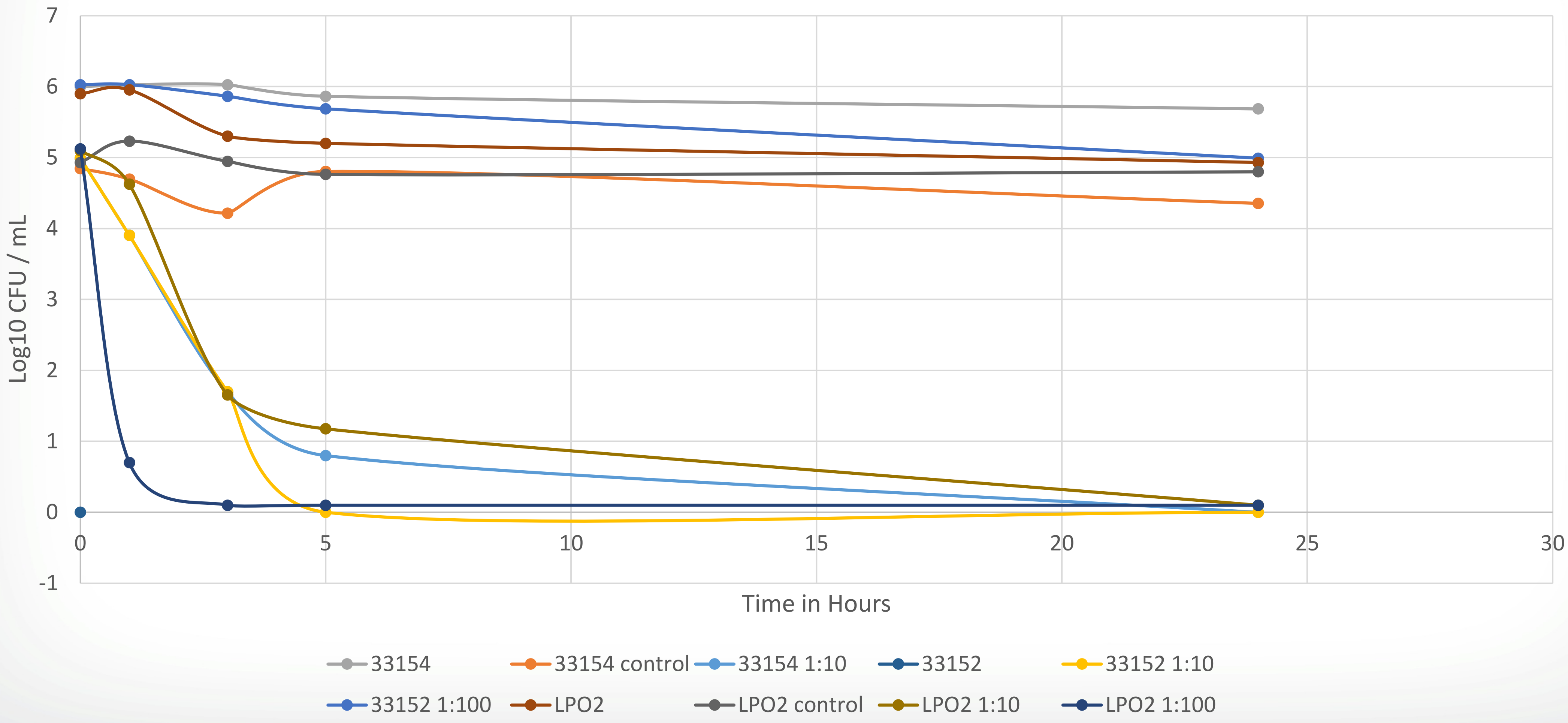
- Measure physical and other chemical properties of water sample.
  - pH (6.9 – 7.4)
  - Chlorine residual (Total 0.06 – 0.03ppm, Free 0.02 – 0.00ppm)
  - Copper / Silver concentration (Cu 380 – 397ppm / Ag 28 – 39ppm)

- Test bottles consisted of 100mL of Cu-Ag treated water spiked with a known concentration of *Legionella pneumophila*. Control bottle consisted of Cu-Ag treated water containing sodium thiosulfate, spiked with *Legionella pneumophila*.

- Test bottles were assayed at specific time intervals.
  - Time 0, 1, 3, 5, and 24 hrs for culture
  - Time 0, 3, and 24 hrs for PMA, PMAx, and EMA treated samples

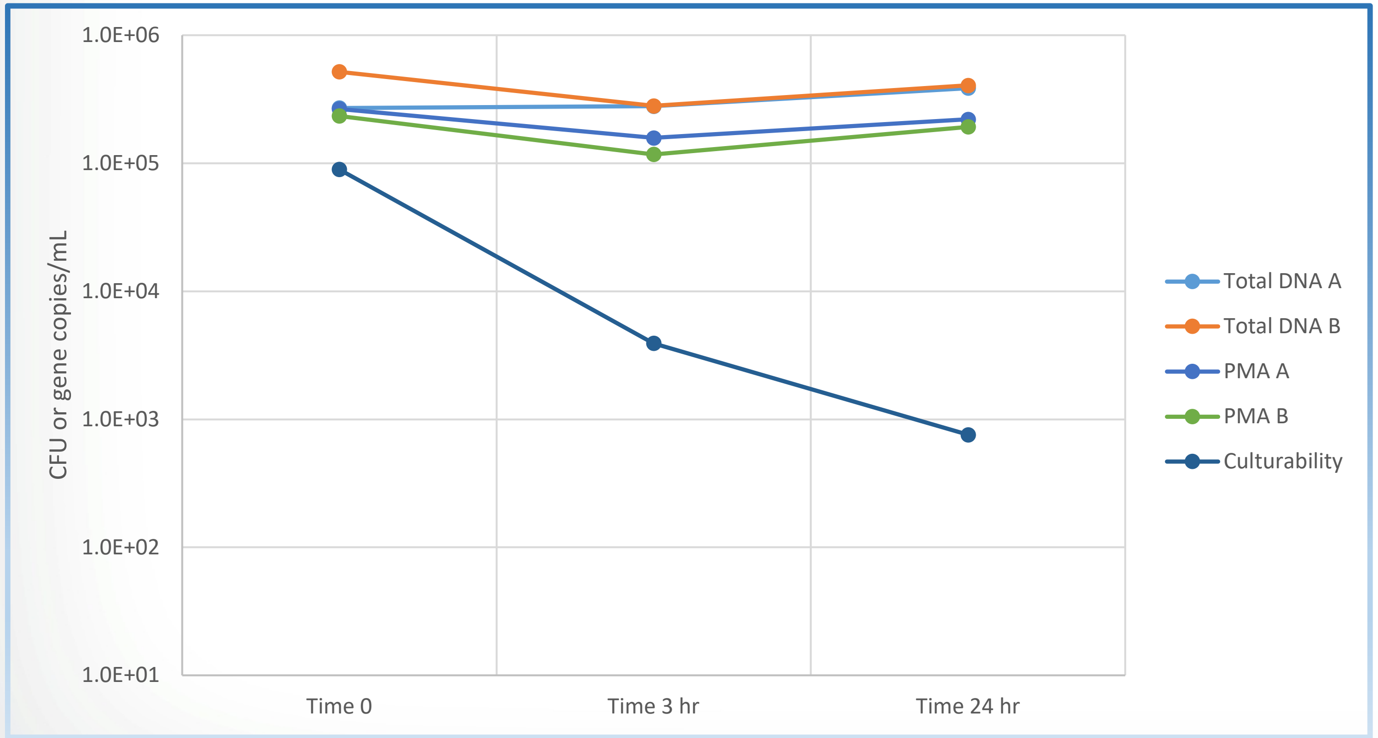
Results & Discussion

❖ **Varying Initial Concentrations of Legionella Cultures Exposed to Water Containing Copper / Silver Ions**



Results & Discussion

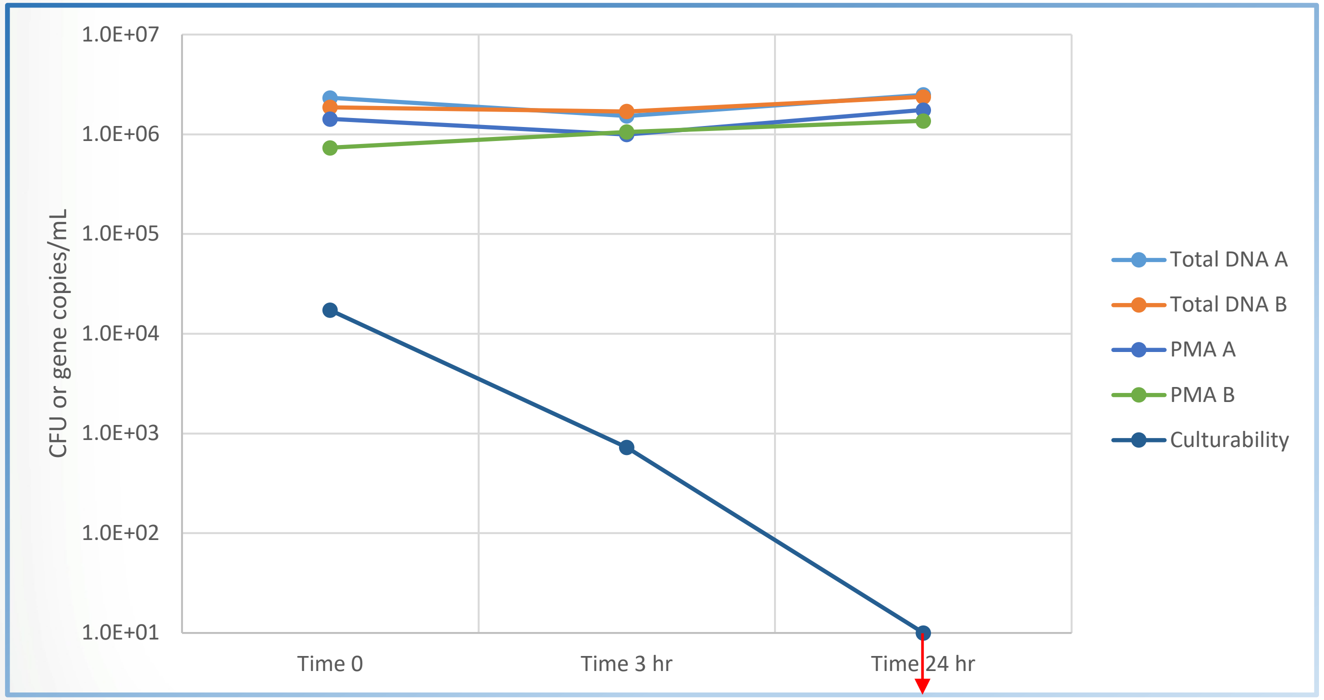
❖ Copper-Silver Ionization_Viablity vs. Culture with a spiking level of 10^5 CFU/mL



- ❖ qPCR (total) and PMA-qPCR (viability): relatively stable
- ❖ >2-log inactivation within 24 hours (culturability)

Results & Discussion

❖ Copper-Silver Ionization_Viablity vs. Culture with a spiking level of 10^4 CFU/mL



❖ Less spiking level (~10^4): more inactivation (>3-log)

❖ Viability assays: no significant changes within 24 hours

Molecular assays indicate cells retain membrane integrity after exposure to Cu/Ag ions.

Results suggest cells are viable but non-culturable.

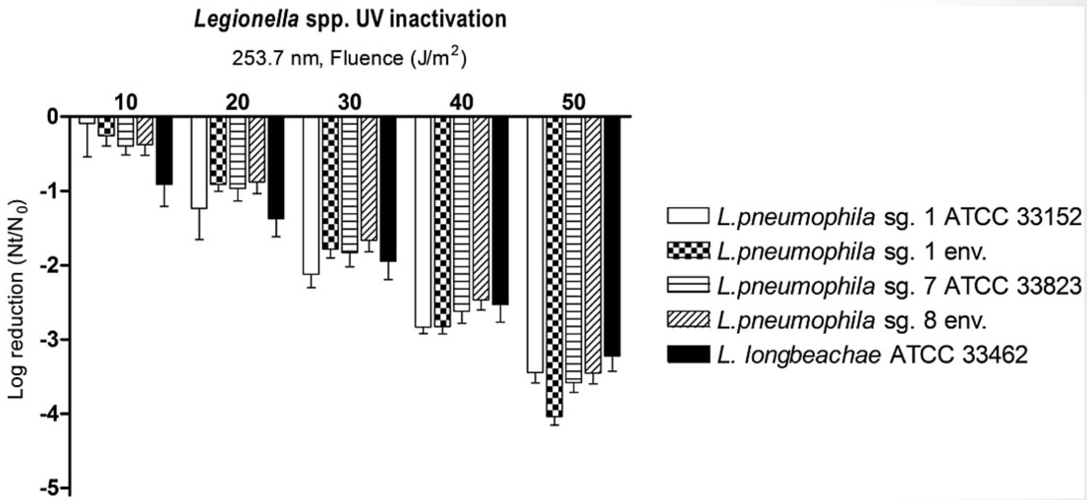
Summary

- **Optimum spiking concentrations of *Legionella* for PMA : 10^4 - 10^5 CFU/mL**
  - **Heat-killed *Legionella* was suitable for this viability evaluation experiments, resulting in a more than 90% reduction in amplifiable gene copy numbers.**
  - **Greater than 10^6 CFU/mL showed significant false-positive qPCR results.**
  - **Interestingly, inactivation rates of *L. pneumophila* in Cu-Ag ionization with >10^6 CFU/mL showed significant underestimation compared to lower spiking levels.**

- **Chemical penetration rate into cells: EMA>PMAx>PMA → EMA penetrates even into intact cells, suggesting its elimination for the further optimization experiments.**
  - **PMAx showed better resolution/differentiation of viable cells than PMA.**

Ultraviolet Light (UV) Disinfection

❖Ultraviolet (UV) light has been successfully used for treating a broad suite of pathogens in water.
- No carcinogenic DBPs formation
- Mechanism: causes pyrimidine dimers to form which prevents DNA replication



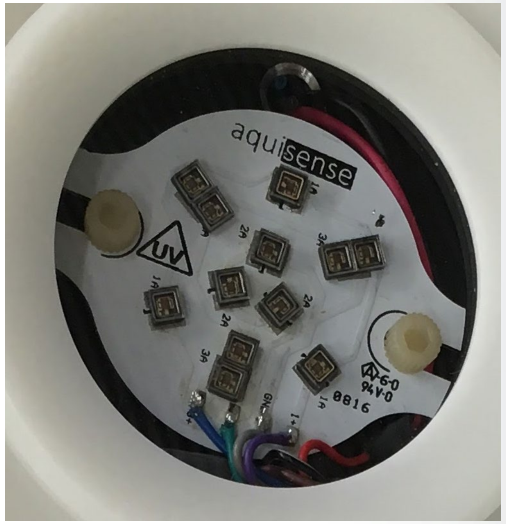
(Figure adopted from Cervero-Arago, S., et al. Water Research. 67(15), 299-309. 2014.)

- Microbial disinfection efficacy
  - Relatively low UV doses to inactivate *L. pneumophila* (Gilpin et al., 1985)

Research Motivation – UV LEDs

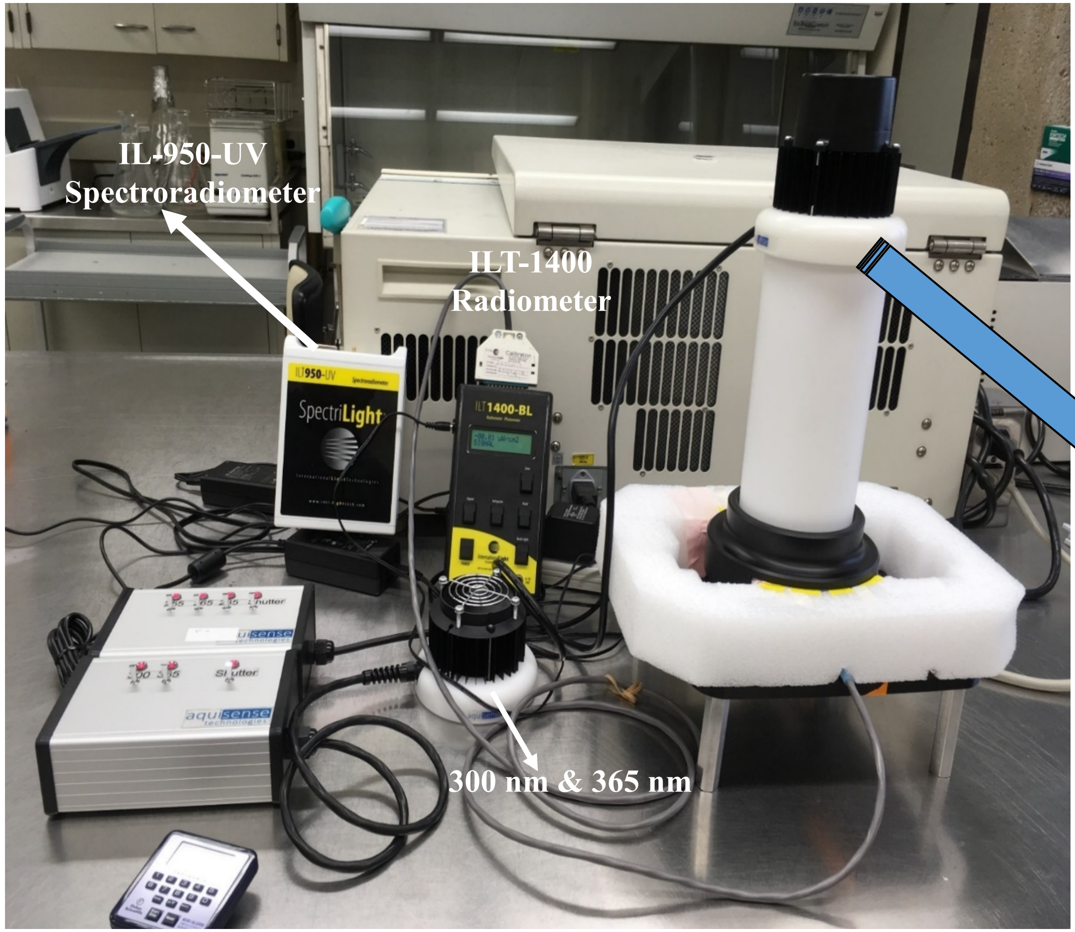
- However, conventional mercury UV lamps have some practical limitations in water treatment applications.
  - Inefficiency of energy consumption
  - Potential mercury contamination

- An emerging UV LEDs (light emitting diodes) technology has enormous potential and could eliminate the aforementioned limitations.
  - Smaller, lighter, less fragile, and mercury-free
  - Provides the capability to be turned instantaneously on and off

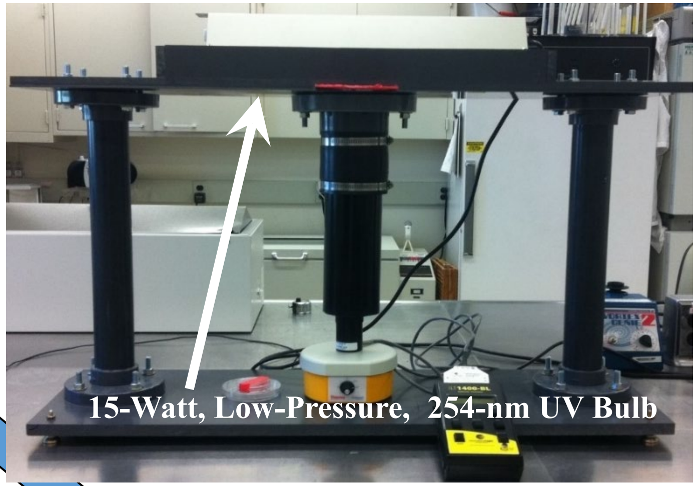


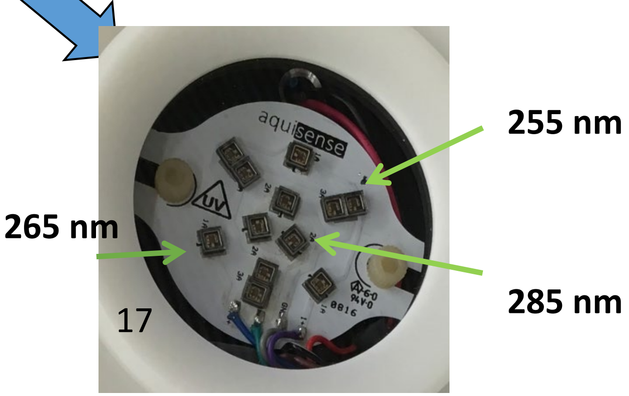
UV Collimated Beam Apparatus

Multiwavelength UV-LEDs



Low-pressure UV





Study Objectives

- We investigated the efficacy of multiple-wavelength UV LEDs for inactivating *Legionella pneumophila* in water.
  - ✓ Three major Opportunistic Premise Plumbing Pathogens (OPPPs)
    - Legionella *pneumophila*, *Pseudomonas aeruginosa*, & Nontuberculous mycobacteria

USEPA's Contaminant Candidate List (CCL) 4 microorganisms (2017)

Microbial Contaminant Name	Type	Diseases and Infections
Adenovirus	Virus	Respiratory illness and occasionally gastrointestinal illness.
Caliciviruses	Virus (includes Norovirus)	Mild self-limiting gastrointestinal illness.
Campylobacter jejuni	Bacteria	Mild self-limiting gastrointestinal illness.
Enterovirus	Viruses including polioviruses, coxsackieviruses and echoviruses	Mild respiratory illness.
Escherichia coli (0157)	Bacteria	Gastrointestinal illness and kidney failure.
Helicobacter pylori	Bacteria	Found in the environment capable of colonizing human gut that can cause ulcers and cancer.
Hepatitis A virus	Virus	Liver disease and jaundice.

Microbial Contaminant Name	Type	Diseases and Infections
Legionella pneumophila	Bacteria	Found in the environment including hot water systems causing lung diseases when inhaled.
Mycobacterium avium	Bacteria	Lung infection in those with underlying lung disease, and disseminated infection in the severely immuno compromised.
Naegleria fowleri	Protozoan	Parasite found in shallow, warm surface and ground water causing primary amebic meningoencephalitis.
Salmonella enterica	Bacteria	Mild self-limiting gastrointestinal illness.
Shigella sonnei	Bacteria	Mild self-limiting gastrointestinal illness and bloody diarrhea.

Materials & Methods

❖ *Legionella pneumophila* serogroup 1

- **Philadelphia 02 strain (Lp02) & two environmental strains: KMC strain and F7621 strain**
- Log phase cells generated by incubation at 37°C for 48 hours in buffered yeast extract broth

❖ Microbial Stock Preparation

- Step 1: Growth of the culture (overnight culture, followed by 2-day incubation)

- Step 2: Washing the culture and making the stock

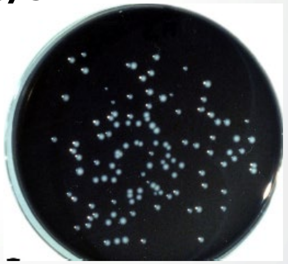
- Step 3: Sample preparation at 10^5 CFU/mL (10 mL per sample)

❖ Standard Culturable Method

- Step 1: Spread plating on BCYE agar

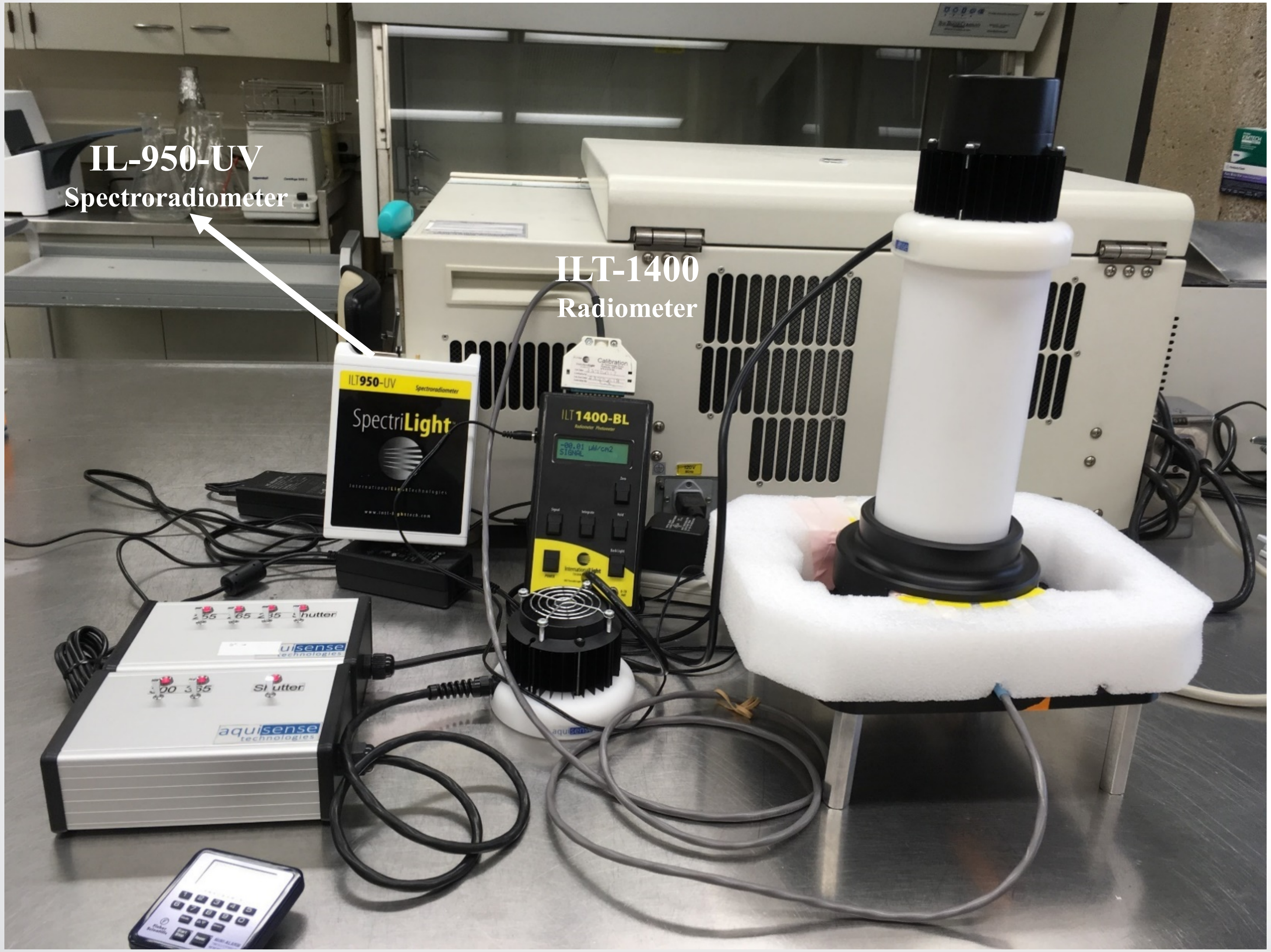
- Step 2: Incubate at 35°C for 5-7 days

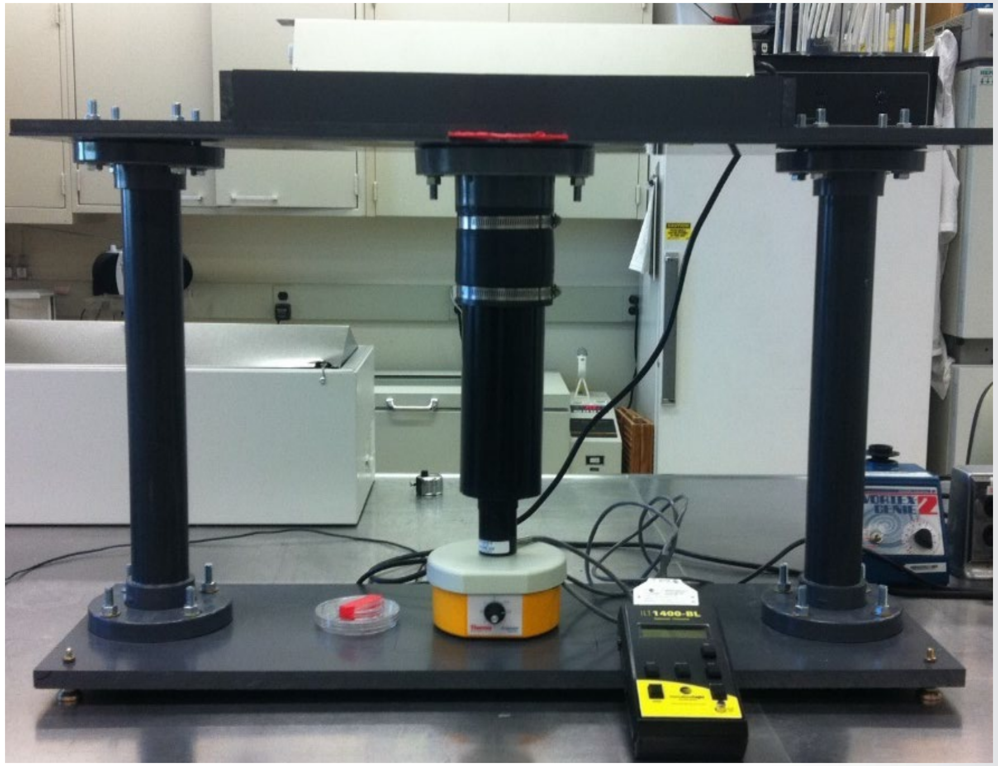
- Step 3: Counting colonies

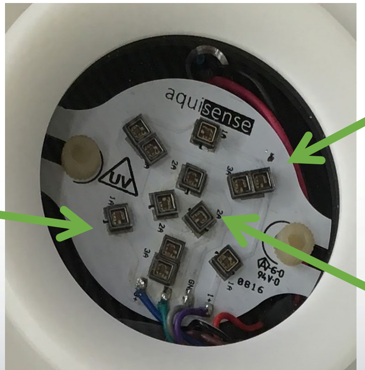


BCYE plate

UV Collimated Beam Apparatus







255 nm

265 nm

285 nm

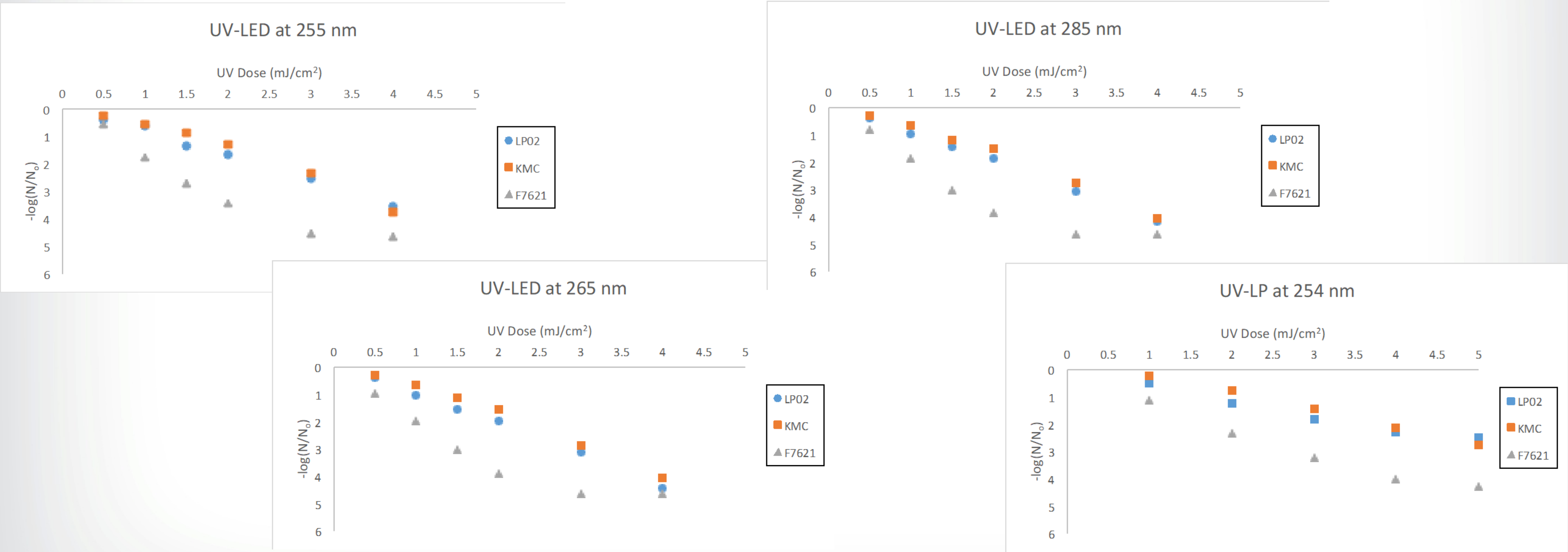
Materials & Methods

Experimental Design and Statistical Analyses

- For one experiment of a particular strain, three wavelengths of UV LEDs were tested (255 nm, 265 nm, and 285 nm) along with UV-LP (254 nm)
  - *-Three experiments for each strain, for a total of nine experiments*
- Linear Regression was performed to generate the inactivation coefficient, as the strains appeared to follow the Bunsen-Roscoe Reciprocity Law.
- 95% Confidence Intervals were generated using the previously calculated slope.
- ANCOVA was used to analyze the difference of the inactivation coefficients between wavelengths of a single strain.
- A factorial design was used to determine if a difference existed between strains for a single wavelength.

Results & Discussion

Comparison of the Three Strains



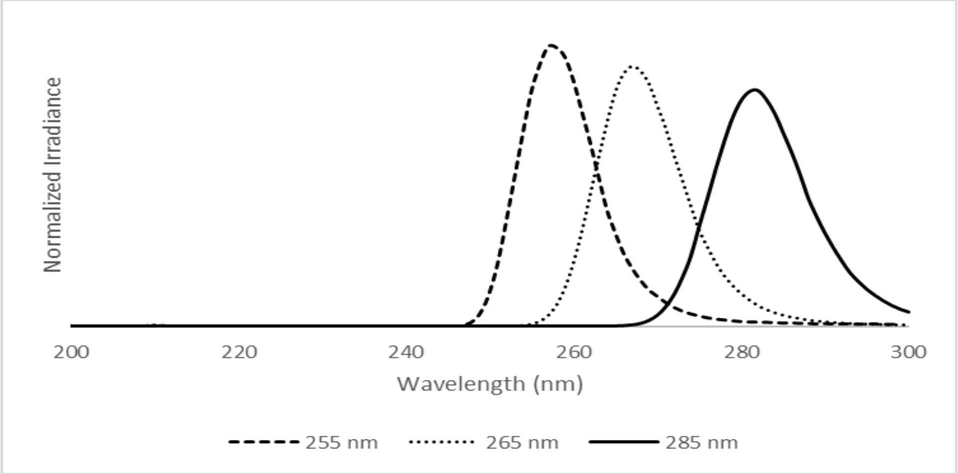
Overall Inactivation Efficacy: LP02 = KMC < F7621

Emission Spectra

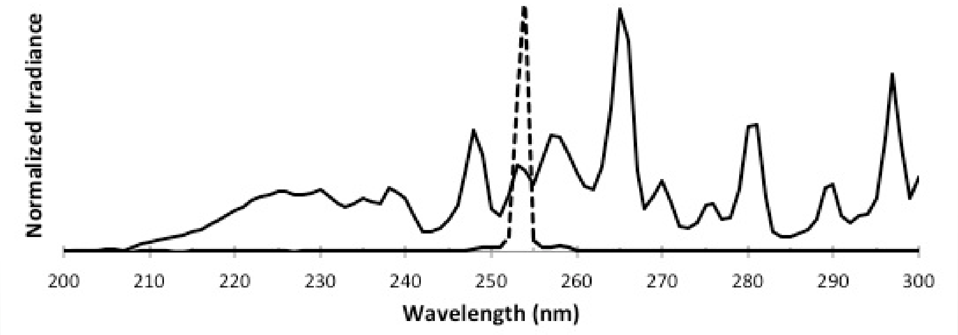
Peak wavelength emissions at 260.65 nm, 268.87 nm, and 282.98 nm with FWHM band widths of 10.5 nm, 11.7 nm, and 13.0 nm, respectively.

Emission spectrum for the low-pressure mercury vapor lamp (dashed)

LEDs (255, 265, & 285 nm)



LP & MP UV

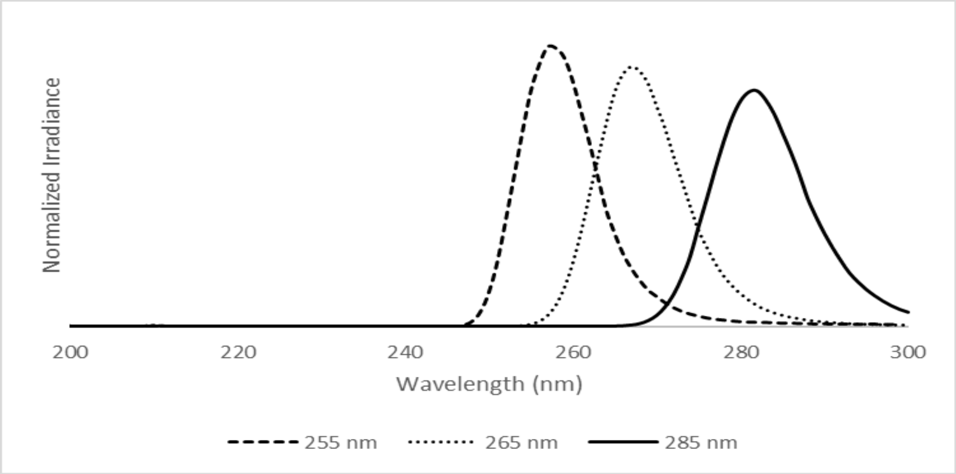


(adapted from Beck et al., 2017 Water Research)

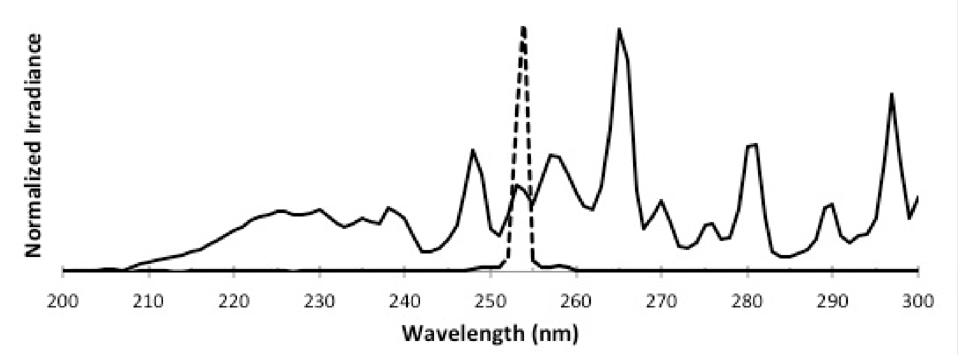
Summary/Conclusions

- All wavelengths of the UV LED outperformed traditional UV LP.
- When choosing a wavelength to inactivate *L. pneumophila* serogroup 1, 265 nm and 285 nm performed the best across all strains.
- Emission spectra most likely contributed to the differences observed between different wavelengths of LEDs and between LEDs and LP.

LEDs (255, 265, & 285 nm)

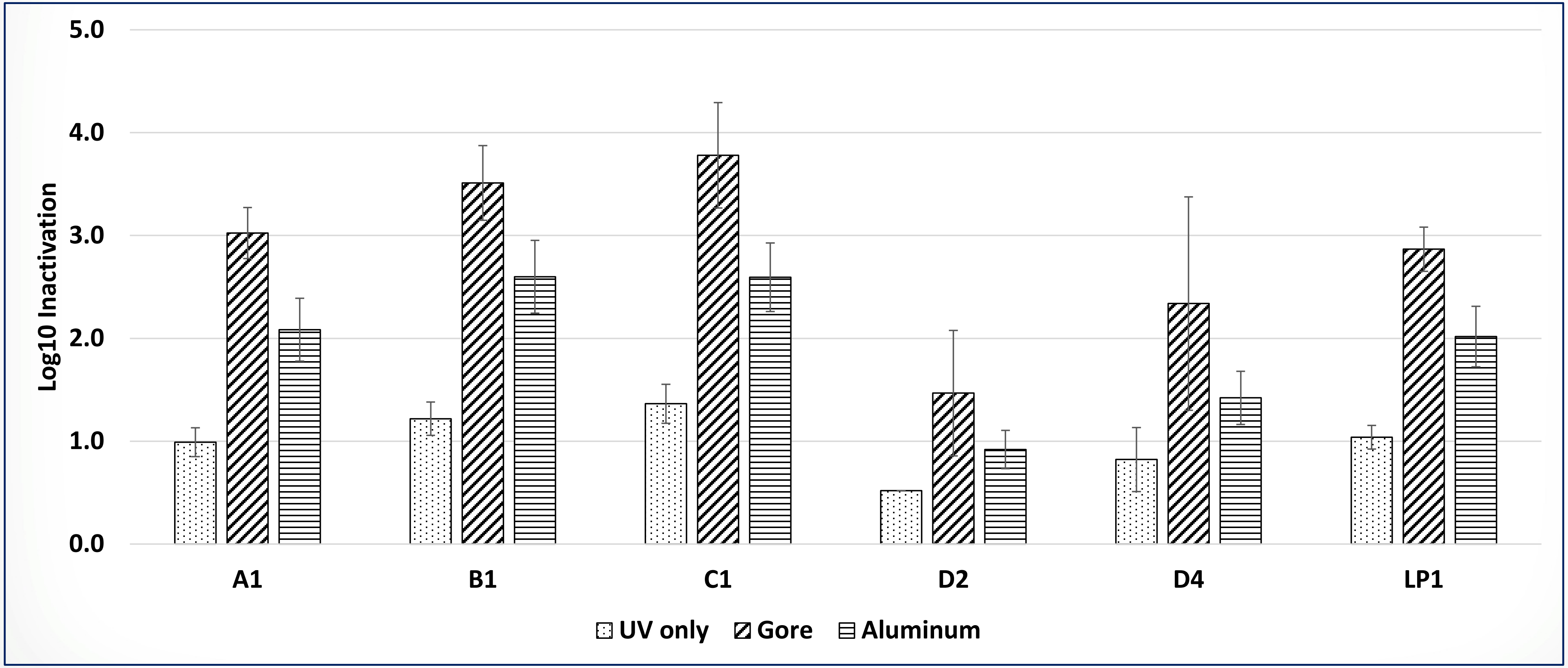


LP & MP UV



(adapted from Beck et al., 2017 Water Research)

Effect of Reflective Materials



A: 255 nm, B: 265 nm, C: 285 nm, D: 300 nm, LP at 254 nm

Further Study

- ✓ CCL microbes & other Premise Plumbing Pathogens – *Pseudomonas aeruginosa* & Nontuberculous Mycobacteria

- ✓ Synergistic effect of UV LEDs coupled with the Cu-Ag ionization – beneficial to hospital water systems

Questions?

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