

Legionella in drinking water, the need to improve understanding of pipe biofilm ecology

Dr Phil Bond

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1996-2016



**Advanced Water
Management Centre**

**Queensland Alliance for
Environmental Health Sciences**



**THE UNIVERSITY
OF QUEENSLAND**
AUSTRALIA

Queensland Alliance for Environmental Health Sciences

- Queensland Health seeking to broaden research activities in environmental health and science
- January 2016 – QH in conjunction with The University of Queensland, QAEHS was established
- Brings together expertise at QH and UQ for a centre to conduct research in environmental health sciences themes
- <https://qaehs.centre.uq.edu.au/>
- Research topic of interest: *Legionella* and biofilms in plumbing of health care facilities

QAEHS themes

Environmental toxicology

Environmental health epidemiology

Environmental health microbiology

Environmental health risk assessment

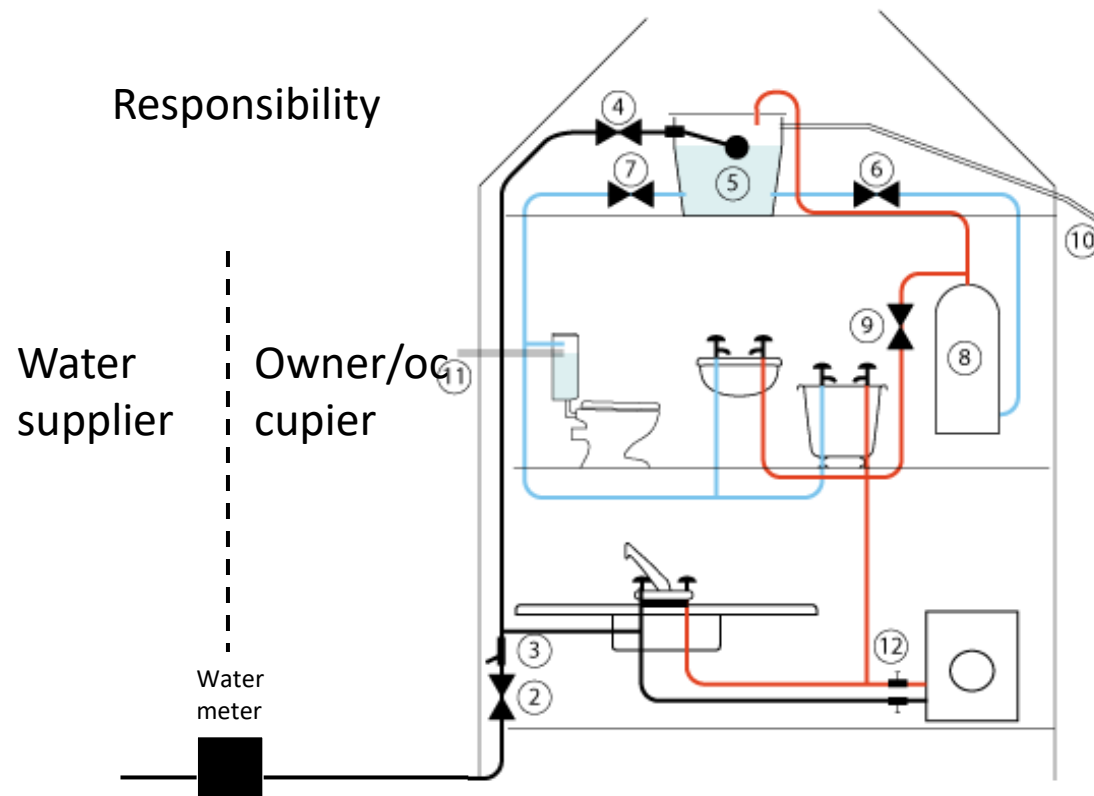
Environmental health risk communication

Emerging environmental health risks

Monitoring and analytical techniques, methodologies and technologies for environmental hazards and exposures

The twilight zone: the last metres before the tap

- Australia Drinking Water Guidelines and water supply utilities describe water quality to the water meter



Legionnaires Disease (Legionellosis)

- A form of pneumonia (estimated to be 10-15% of cases)
- Infection of lungs by the bacterium *Legionella pneumophila*
- Flu like symptoms (take 2-10 days to develop)
- Incidence of disease ~1.5/100,000 population, is rising
- 75% of cases are people over 50 years
- Antibiotic treatment is required
- ~ 70% of cases require hospitalization, ~ 5% are fatal
- Young, Old, and Immuno-compromised patients in hospitals at risk
- Transmission by aerosols that contain the bacteria from water sources such as cooling towers, shower heads, spa baths, taps...
- No human to human transmission of the disease



The Wesley Hospital legionellosis occurrences

- 2011 outbreak, 73 year-old patient died
- 2013 outbreak, one patient died, another in intensive care
 - Tracked source to contaminated water taps
 - Admissions and surgery shut down, no showers
- 2016, a patient tested positive for legionellosis
 - Ice machine was possible source
- Plumbing systems were treated post 2013 outbreaks
 - Series of flushing with hot water, and hyperchlorination treatments



Premise plumbing – good environment for *Legionella*.

Pipe conditions:

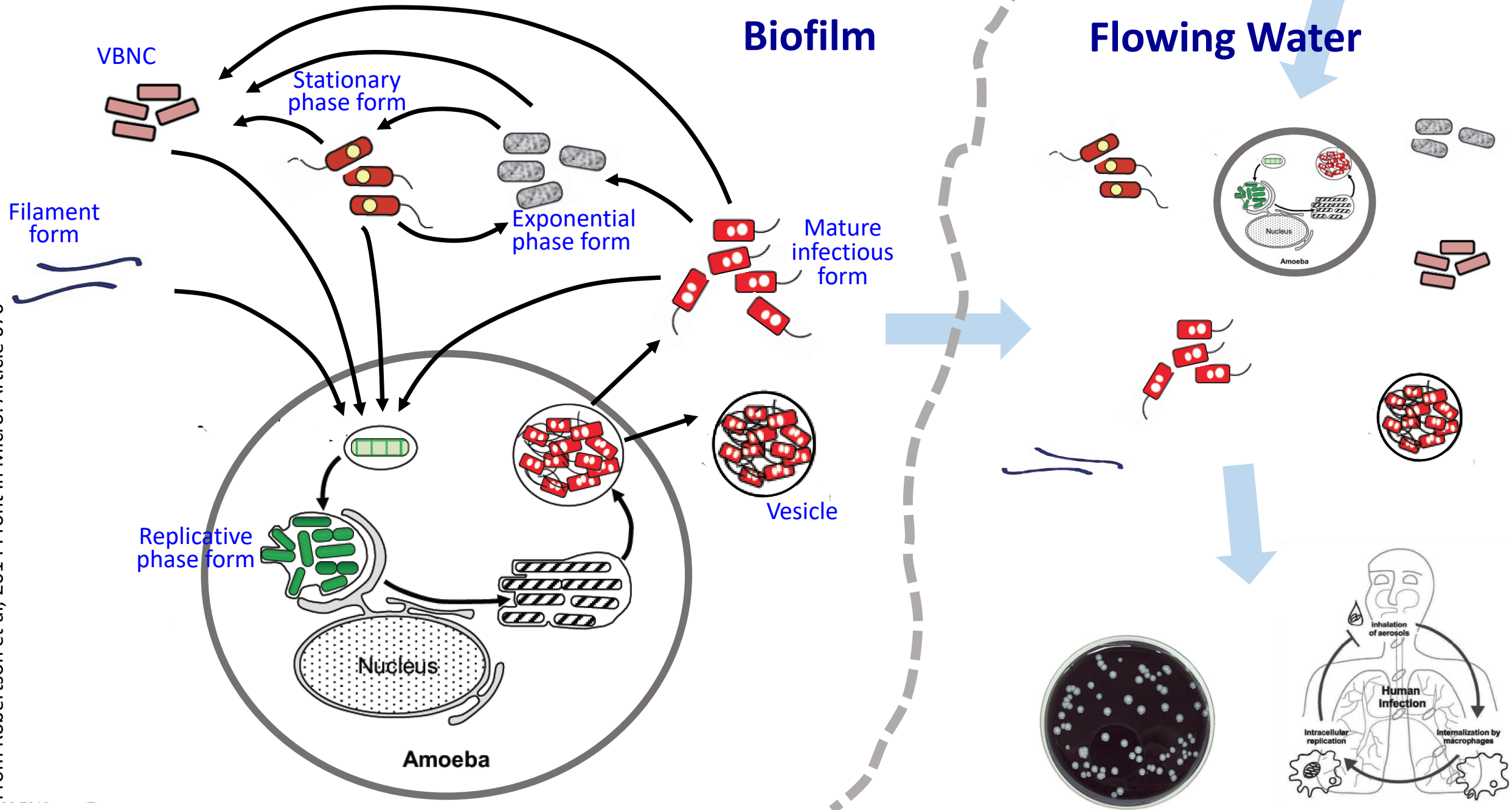
- Disinfectants levels vary
- Temperature ranges - warm
- Pipe material: copper, iron, synth polymers, fittings
- Low dissolved oxygen levels, low nutrient
- Flow variations: dead end pipes
- Connections to various devices, taps, showers, ice machines
- High surface area:volume

Legionella:

- Survive well in low nutrient conditions, viable but non-culturable state (VBNC)
- Biofilm ecology suits their many growth stages
- Biofilm provides protection



Legionella has various forms in its life cycle stages



Specific qualities of pipe biofilms

- Mixed microbial communities, diverse, interacting, structured
- Cells adhere by use of extracellular polymeric substances (EPS)
- Conditions within biofilm habitat varies
- Will support persistent/slow growing microbes
- Chemical disinfectants less effective
- Removal is difficult, if not impossible
- Difficult to monitor (inside pipes)
- Support regrowth of other bacteria (e.g. coliforms)

Biofilm ecology – we need to know more

- What microbial types are present in WDS pipe biofilms?
- How do *Legionella* proliferate and persist in biofilms?
- What conditions favor their growth or enrichment in biofilms?
- What causes their release from or disruption of the biofilm?
- Determine the biofilm response to disinfection
- Does occurrence of *Legionella* correlate with other microorganisms?
- Investigate microbial interactions: e.g. parasitic with amoeba, evidence of antagonism against *Legionella*

Talk about detection - Legionella pneumophila

- Gram negative bacteria, Gammaproteobacteria, bacillus with flagella
- Causes most (~90%) of all *Legionella* clinical cases
- Aerobic heterotroph – does well in conditions of low DO
- Prefers amino acids as substrate for growth
- Prefers warm temperatures 25-45 °C
- Standard detection method Australia: AS3896 1991
- Concentrate cells in the sample, heat and acid treat to eliminate most other microbes, inoculate BCYE agar (cyst and antibiotics),
count colonies after 10 days incubation!



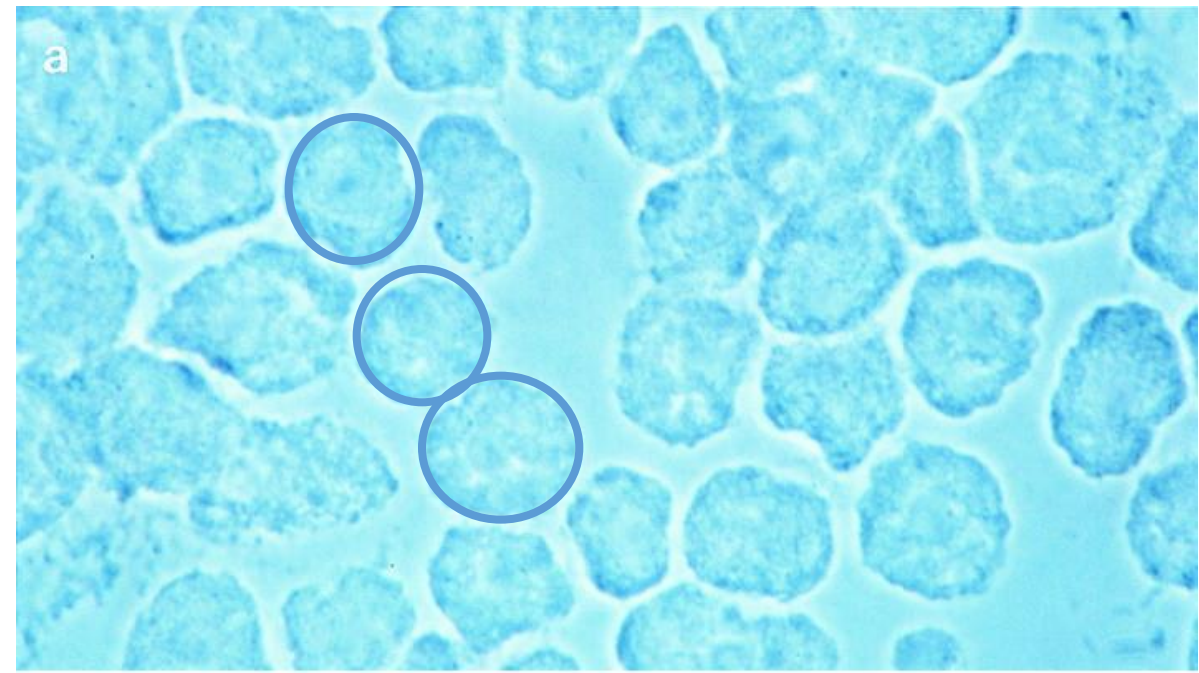
Legionella colonies on BCYE agar
biomerieux-culturemedia.com

Rapid methods for detection of *Legionella* spp.

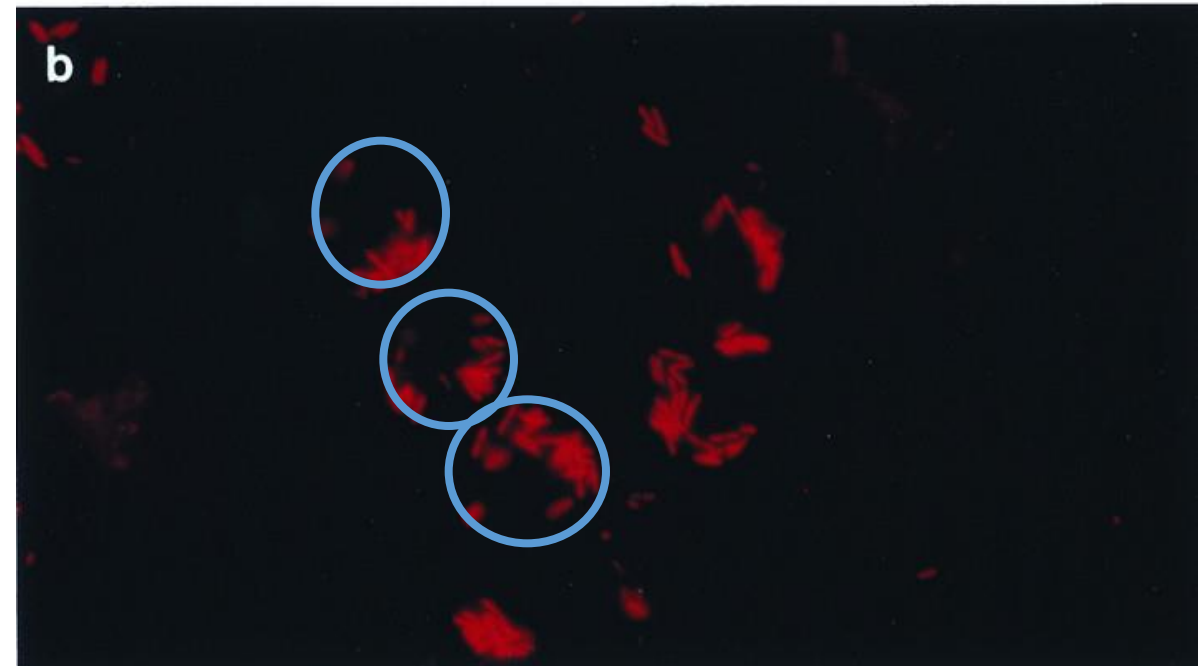
Method of detection/quantification	Time of analysis	Limit of detection	Comments
qPCR Filter 500 ml sample, Extract DNA, Perform Sybr green qPCR	1 day?	14 CFU/L	Detects LysR gene. Legionella species specificity Sensitive, but overestimates?
Fluorescence in situ hybridisation (FISH) Concentrate cells, hybridise with probes, detect and quantify by flow cytometry	4-5 h?	?	Difficult to detect due to low activity of Legionella
Cultivate-FISH-SPC Concentrate cells onto filter, incubate on media 48 h. Perform FISH on filter. Count microcolonies on filter using Solid phase cytometry	2 days	200 cell/L from 5 ml sample	Sensitive from small sample size (5 mL). Requires cultivation step
Immunogenic separation (IMS)-FCM 1L sample filtered. Use Legionella specific antibodies conjugate to magnetic microbeads, separate and detect by flow cytometry method (FCM)	120 min	40 cells/L	Specific antibodies available commercially
IMS-colormetric (qualitative commercial kit, Legipid®) 1 L samples conc by filtration, apply sample to IMS-magnetic microbead kit Apply reagents and read from chart.	60 min	93 CFU/L	Sensitive, rapid and very simple. Convenient kit Qualitative?
Microarray-IMS combined with adsorption filtration Concentrate 10L sample. Legionella antibodies on microarray. Apply cells to microarray. Read chemiluminescence.	90 min	400 CFU/L	Quick, but not so sensitive, and need to construct microarray

Using FISH probes for detection of *L. pneumophila*

Light microscopy:
shows Amoeba cells



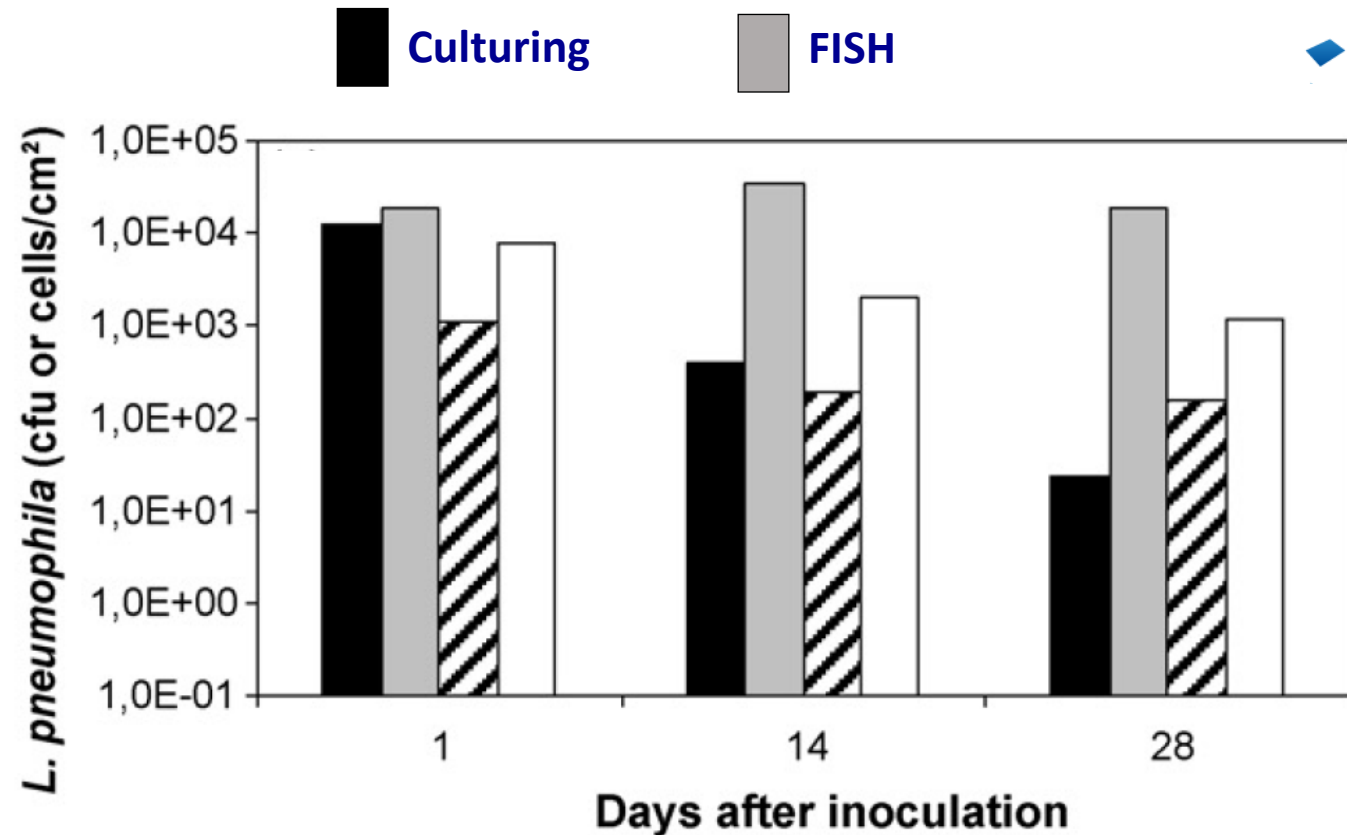
Fluorescence microscopy:
probe detects *Legionella*
within the Amoeba cells



FISH detects viable cells, may or may
not be culturable cells

Legionella counts on pipe biofilms

- Laboratory biofilms were inoculated with *Legionella*
- Cells detected by culturing and FISH
- Culture detected cells lowered over time
- FISH detected cells remained high



Suggests most *L. pneumophila* cells in biofilm going into VBNC state

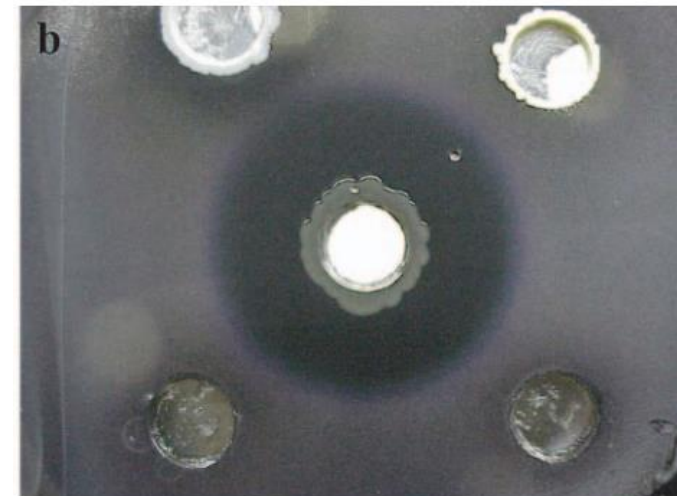
Biofilm ecology – we need to know more

- Certain bacteria may favor growth of *Legionella*, enable growth in biofilms
- Reproduces within amoeba – more infectious types
- *Pseudomonas putida* frequently detected with *Legionella*
 - may excrete organic compounds
 - May support necrotrophic growth (nutrients from dead cells)
- Certain bacteria produce molecules that are toxic to *L. pneumophila*.
 - *Bacillus* and *Flavobacterium* spp. produce bacteriocins

Need to look at whole microbial community of biofilm

Inhibition of *Legionella* growth due to microbial production of bacteriocin.

Messi et al. 2011. Biofouling, 27, 165–172



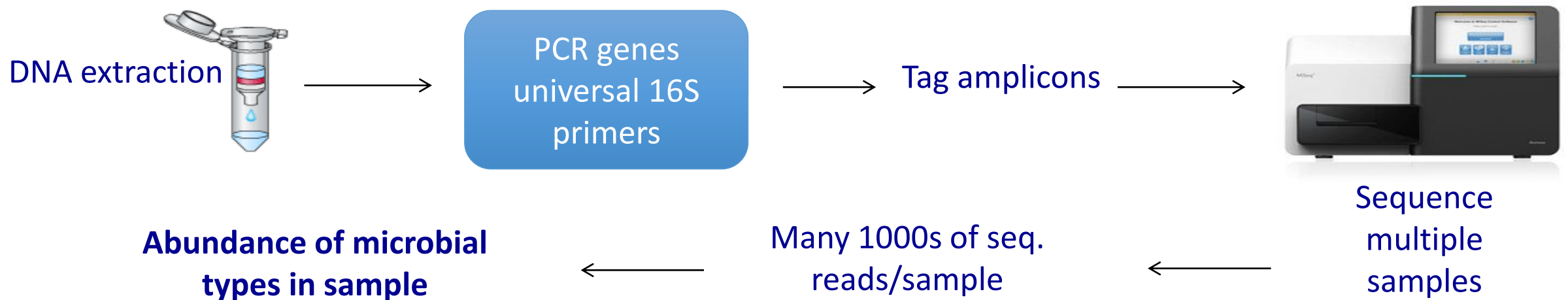
Correlate biofilm ecology with environmental conditions

- Disinfectants levels and methods
- Temperature ranges
- Pipe material: copper, iron, synth polymers, fittings
- Dissolved oxygen levels
- Flow: stagnation periods, dead end pipes
- Connections to various devices, taps, showers, washing machines
- Surface area:volume



Biofilm ecology – whole community analyses

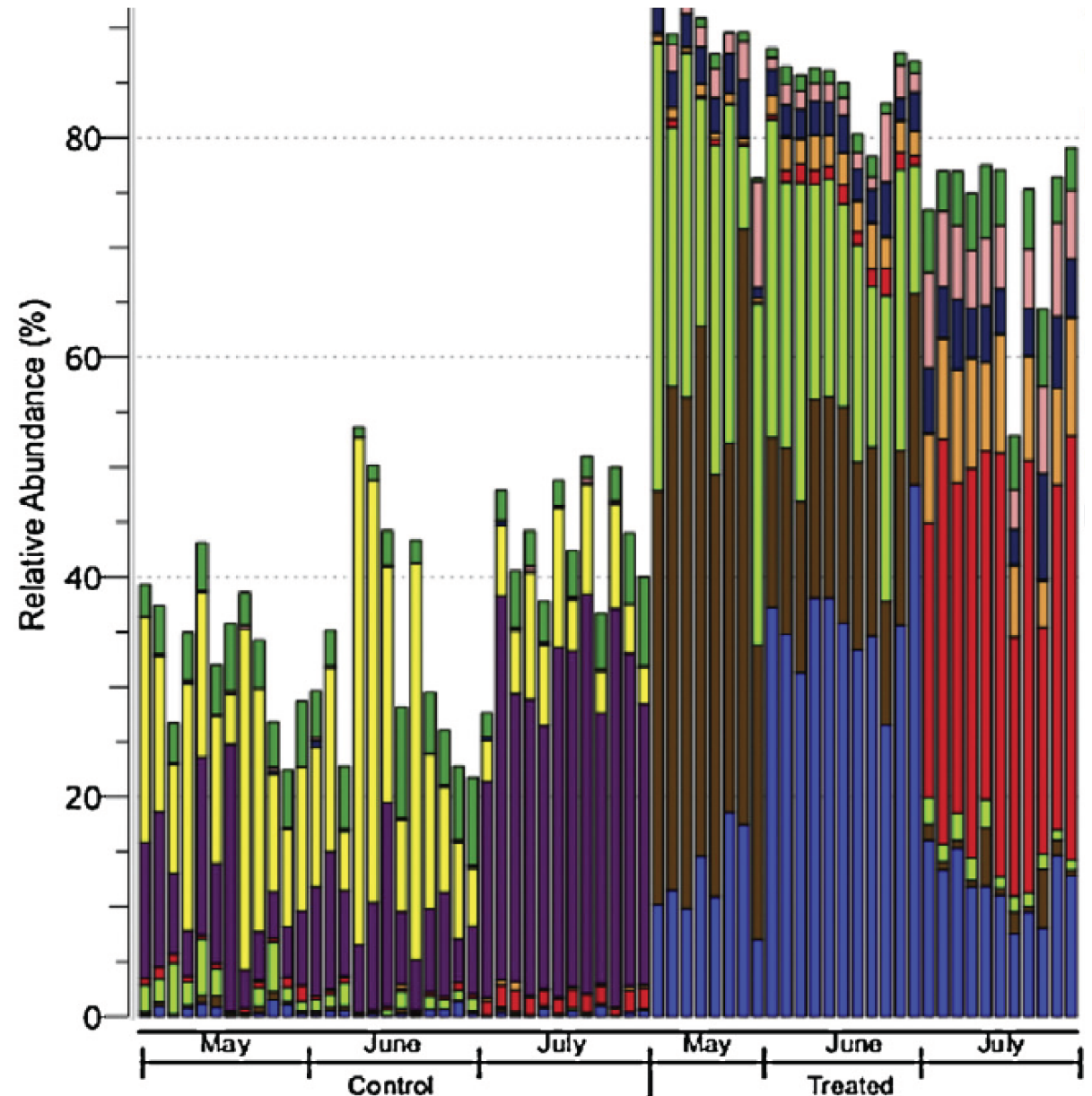
- High throughput, low cost DNA sequencing (last 7 years) has revolutionised microbial ecology studies – culture independent analysis
 - Based on sequencing PCR products (amplicons)
 - Focused on ribosomal SSU, 16S and 18S rRNA genes



- Other points: deep analysis , rare species, Id genus level at best, novel species
- Use sequences to design FISH probes, verify quantification using FISH

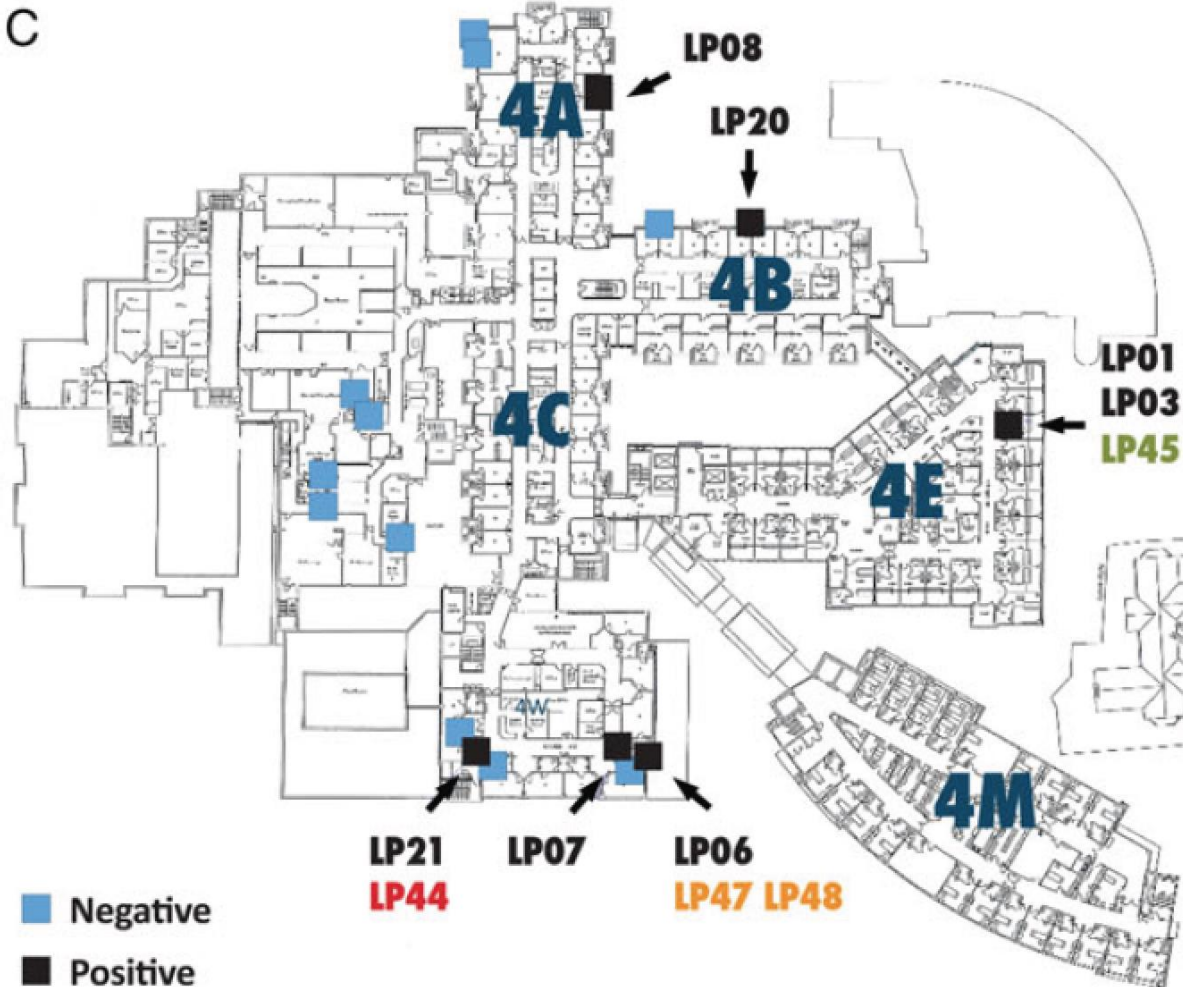
Bacterial populations in a hospital hot water system

- Used **DNA sequencing** to determine microbial populations in two hospital water systems
- One building had installed an on-site monochloramine generation system
- Dramatic differences in the bacterial composition and relative abundance
- Treated system had less diversity
- Cultured *Legionella* were always higher in the control building
- A failure in the treatment system coincided with a *Legionella* bloom
- Rapid colonisation of *Legionella* occurred when treatment failed
- Could be related to lower bacterial diversity



Genomic study of Wesley Hospital Legionellosis outbreaks

Wesley Hospital, Level 4 plan



- *L. pneumophila* isolates obtained from hospital tap water and patients
- Oct 2011, 73 year-old patient (LP44)
- 2013 patients (LP47, LP48, LP45)
- Very few differences between genome sequences of isolates
 - Eg. only one DNA base difference between LP44 (2011) and LP06 isolates (2013)!
- Persistent biofilms in plumbing contain clonal strains of *L. pneumophila* !

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Approach to understand and control *Legionella* levels in plumbing pipe biofilms.

Rather than eradication of biofilm – obtain preferential removal of *Legionella* from the biofilm.

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Approach to understand and control *Legionella* levels in plumbing pipe biofilms.

Detect and understand type of *Legionella* in pipe biofilm

- A range of methods can be used to detect *Legionella* in biofilms and determine the nature of the organism:
 - Specific detection of *Legionella*, e.g. standard culture method, FISH, qPCR, immunogenic separation,
 - Then general whole community analyses by DNA sequencing.
- Reveal the presence and nature of *Legionella* in the plumbing biofilms.
- Determine particular microbial-types and pipe conditions that either favour or are antagonistic towards biofilm *Legionella*.
- Does increased diversity disfavour *Legionella*?

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Approach to understand and control *Legionella* levels in plumbing pipe biofilms.

Compare the *Legionella* phenotypes and genome-types in the pipe biofilm and those in the flowing water phase.

- Compare isolated *Legionella* from the biofilm and water phases by genome sequence analysis.
- Compare the virulence of the isolates (examine by amoeba infection assay) to determine if this differs between biofilm and planktonic *Legionella*.
- To examine the questions:
 - are certain types of *Legionella* being released from biofilms?
 - are biofilm *Legionella* more infectious than water phase cells?

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Approach to understand and control *Legionella* levels in plumbing pipe biofilms.

Determine conditions that disfavour *Legionella* in pipe biofilms.

- Apply a range of antimicrobial agents and treatments to biofilms to detect the specific removal of *Legionella*.
 - real pipe biofilms
 - laboratory constructed biofilms.
- The range of treatments could include: chlorine, hydrogen peroxide, nitrite, copper-silver ions, silver nanoparticles, nano zero valent iron (NZVI), nitrous oxide, etc.
- From the treatments, detect *Legionella* specifically and perform whole community analysis.
- Look for treatment that diminishes *Legionella* abundance without lowering microbial diversity in the biofilm.

In conclusion

- Don't forget about water samples and culturing studies!
- Biofilm ecology in water supply pipes – only beginning to be explored by culture-independent techniques
- Understand physiological state of *Legionella* at different life cycle stages, especially in biofilm, VBNC, biocide resistant types...
- Are there particular microorganisms/conditions that enhance or reduce the presence of *Legionella* in pipe biofilms?
- Improve control of pipe biofilms – target *Legionella* removal
 - Trial a range of treatment conditions

