Legionella is an opportunistic premise plumbing pathogen and the causative agent of Legionnaires' disease and Pontiac fever. It specifically targets immunocompromised, immunosuppressed, and elderly people. Its prevalence in engineered water systems is an issue of increasing public health significance. Globally, the incidence of *Legionella* infections has been increasing. In the USA, *Legionella* is currently the leading cause of all drinking water related outbreaks. The persistence of *Legionella* in engineered water systems is associated with protozoan hosts, biofilms, failure of disinfection treatments, and water stagnation. Under unfavourable environmental conditions *Legionella* can transform into a viable but nonculturable (VBNC) state. VBNC *Legionella* are potentially pathogenic in nature, and under favourable conditions can transform back into the more pathogenic culturable state.

Globally, routine water testing for *Legionella* is recommended to manage hospital water systems to prevent outbreaks of Legionnaires' disease. The International Organization for Standardization (ISO) recommends two protocols for the detection and quantification of *Legionella* in engineered water systems. ISO11731:2017-05 detects only culturable *Legionella,* whereas ISO/TS12869:2019 is a quantitative PCR (qPCR) based method which detect its genomic DNA. However, both methods are unable to quantify and characterize VBNC *Legionella*. In this study, a culture-independent "viability-based flow cytometry-cell sorting and qPCR (VFC+qPCR)" assay was designed to detect and quantify VBNC *Legionella* from environmental samples. This was the first time that flow cytometry-cell sorting in conjunction with a qPCR assay has been used as a direct and rapid method to quantify VBNC *Legionella* from engineered water systems.

Protozoan hosts, specifically free-living amoebae, are natural hosts and reservoirs of *Legionella*. In this study, a systematic literature review identified that free-living amoebae, most commonly *Acanthamoeba* and *Vermamoeba vermiformis,* are the major hosts of *Legionella* in building water distribution systems. Furthermore, these two free-living amoebae play a significant role in the survival and persistence of *Legionella* in engineered water systems. Based on the findings from the systematic literature review, water and biofilm samples from Australian hospital and domestic water systems were screened for the presence of free-living amoebae and *Legionella*. Both culture-dependant and culture-independent approaches were used for screening and characterization of samples. Direct qPCR assays demonstrated that 41% of samples were positive for *Legionella,* 33% for *L. pneumophila*, 11% for *Acanthamoeba,* and 55% for *V. vermiformis*. Only 7% of samples were positive for culturable *Legionella* (which were *L. pneumophila* serogroup (sg)1, *L. pneumophila* sg2-14, and non-pneumophila

Legionella). In contrast, 41% of samples were positive for culturable free-living amoebae, which were identified as *V. vermiformis*, *Acanthamoeba*, *Stenamoeba*, and *Allovahlkampfia*. These culturable free-living amoebae were highly thermotolerant and osmotolerant and harboured strong broad spectrum bacteriogenic activity. Importantly, all *Legionella*/*L. pneumophila* positive samples were also positive for free-living amoeba, and this co-occurrence was statistically significant (Pearson's chi-squared, $p < 0.05$). Furthermore, using qPCR and fluorescence *in situ* hybridization it was identified that *V. vermiformis* and *Allovahlkampfia* harboured intracellular *L. pneumophila*. Importantly, this is the first time *Allovahlkampfia* and *Stenamoeba* have been demonstrated to be hosts of *L. pneumophila* in engineered potable water systems. In conclusion, the high frequency of free-living amoebae in Australian engineered water systems is a significant public health concern. Therefore, future water management methods should incorporate treatments protocols to control free-living amoebae and reduce the risk to end users.

Stagnation and flow dynamics are important parameters which affect the water quality in engineered water systems. In this study, a systematic literature review demonstrated that both permanent (i.e., dead ends and dead legs) and temporary (through intermittent water usage) stagnation promotes the growth of *Legionella* in engineered water systems. Based on the findings from the systematic literature review, a laboratory scale biofilm model study, and a real world (hospital water system) investigation into the effect of intermittent stagnation was conducted. In both of these studies the new method described above was used to quantify the VBNC *Legionella* present. In the laboratory scale study, a model plumbing system consisting of a water tank and two biofilm reactors was used to investigate the effect of stagnation, and intermitted usage, on *Legionella* and free-living amoebae. Initially, both biofilm reactors were left stagnant for 147 days to allow the formation of biofilm. This was followed by the operational phase during which one biofilm reactor was flushed once a day with 70 L of potable water, and the other biofilm reactor was flushed once a week. It was identified that once-a-day flushing for 28 days significantly (analysis of variance, $p < 0.001$) reduced the amount of biofilm-associated alive (potentially culturable based on VFC+qPCR assay) and culturable *Legionella* and increased the amount of VBNC *Legionella* compared with the once-a-week flushing. Furthermore, it was observed that the concentration of culturable *Legionella* (Spearman's ranking, $p < 0.001$) was positively correlated with heterotrophic plate count (HPC) and the concentration of VBNC *Legionella* (Spearman's ranking, $p < 0.001$) was positively correlated with the concentration of *V. vermiformis*. This laboratory scale study

demonstrated that a reduction of water stagnation, and an increase in usage/flushing, significantly decreased the population of total, alive and culturable *Legionella*. The effect of stagnation and flow dynamics was also investigated on water (n = 120) and biofilm (n = 46) samples collected from an Australian hospital water distribution system. The shower and hand basin water/biofilm samples were collected over 16 months from one hospital and water flushing data was obtained from Enware™ using their Smart Flow® monitoring system. The molecular analysis showed that 22% samples were positive for *Legionella* and 41% for *V. vermiformis* using qPCR. This investigation also suggested that temporary stagnation (< 2 hours water flushing per month) significantly (Kruskal-Wallis test, $p < 0.01$) increased the quantity of VBNC and total (genomic unit) *Legionella.* Moreover, it was also identified that high HPC load was significantly (Kruskal-Wallis test, $p < 0.01$) associated with increased concentrations of *Legionella* and *V. vermiformis* in engineered water system. These three studies demonstrate that stagnation arising through intermitted usage is an important factor influencing the risk of *Legionella* in engineered water systems.

Engineered water systems are a complex environment with a range of variables that can influence the growth and persistence of microbes, especially opportunistic premise plumbing pathogens. In this study, 16S rRNA sequence analysis was used to examine the prokaryotic communities present throughout a hospital distribution system. A total of 46 water samples from showers and hand basins collected during three different sampling periods were examined. The influence of temperature and water flow dynamics (number and total duration of flow events) for one week and six months prior to sample collection was examined. It was found that the hospital water primarily contained six bacterial phyla i.e., Proteobacteria, Actinobacteriota, Bacteroidota, Planctomycetota, Firmicutes, and Cyanobacteria. The diversity of prokaryotic communities present was significantly (Kruskal-Wallis test and PERMANOVA, $p < 0.05$) affected by sampling phase (month) and flow dynamics. Importantly, it was also observed that several biofilm forming (e.g., Pseudomonadales), corrosion responsible (e.g., Desulfobacterales), extremely resistant (e.g., Deinococcales), and potentially pathogenic (e.g., *Pseudomonas*) bacterial taxa were enriched in low flow regimes. This study showed that hospital water system consists of complex prokaryotic communities that is shaped by incoming water quality and the building flow dynamics. It was also identified that in this hospital, the water temperature (most probably, because temperature of hot and cold-water supplies was same) did not influence the composition of prokaryotic communities.

In conclusion, currently guidelines recommend routine monitoring for *Legionella;* however, this study showed that the standard methods fail to detect and quantify VBNC *Legionella* and that VBNC *Legionella* does not follow the same trends as culturable *Legionella*. This could explain long term persistence of *Legionella* contamination in engineered water systems. This study supports the use of HPC as an indicator for water quality, as it followed the same trends as culturable and alive (potentially culturable based on VFC+qPCR assay) *Legionella* but not VBNC *Legionella*. *Legionella* was always found in the presence of free-living amoeba hosts; therefore, future disinfection and control strategies need to address this and target the free-living amoeba. Stagnation had a greater influence of microbial water quality and *Legionella* concentration compared with water temperature. Importantly, this was not just long-term stagnation, but short-term stagnation arising through intermitted usage. This supports current guidelines recommending flushing of outlet to minimize the risk of legionellosis and more emphasis should be placed on this as a control measure. More research is needed to further examine this relationship and determine the optimum balance between the costs and water usage associated with increased flushing and the risk from *Legionella* to determine to optimum frequency of routine flushing.