Methodological approaches for monitoring opportunistic pathogens in premise plumbing: A review

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Opportunistic premise (i.e., building) plumbing pathogens (OPPPs, e.g., Legionella pneumophila, Myco-bacterium avium complex, Pseudomonas aeruginosa, Acanthamoeba, and Naegleria fowleri) are a signifi-cant and growing source of disease. Because OPPPs establish and grow as part of the native drinking water microbiota, they do not correspond to fecal indicators, presenting a major challenge to standard drinking water monitoring practices. Further, different OPPPs present distinct requirements for sampling, preservation, and analysis, creating an impediment to their parallel detection. The aim of this critical review is to evaluate the state of the science of monitoring OPPPs and identify a path forward for their parallel detection and quantification in a manner commensurate with the need for reliable data that is informative to risk assessment and mitigation. Water and biofilm sampling procedures, as well as factors influencing sample representativeness and detection sensitivity, are critically evaluated with respect to the five representative bacterial and amoebal OPPPs noted above. Available culturing and molecular approaches are discussed in terms of their advantages, limitations, and applicability. Knowledge gaps and research needs towards standardized approaches are identified.

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1. Introduction

Premise plumbing refers to the portion of potable water distribution systems beyond the property line and in buildings (NRC, 2006), including both hot water and cold water lines and devices such as water heaters, showers, faucets and filters (Fig. 1). Premise plumbing is generally characterized as a wet, warm, periodically stagnant environment with low disinfectant residual and high surface area, which creates ideal conditions for microbial growth. Accordingly, opportunistic premise plumbing pathogens (OPPPs) establish as part of the native microbiota, thereby presenting a major challenge for detection and monitoring since they violate the paradigm of traditional fecal indicator bacteria. As OPPPs are now the primary source of drinking water-related disease outbreak, particularly in developed countries (CDC, 2013; Anaissie et al., 2002; Craun et al., 2010; Pruden et al., 2013), there is an urgent need for reliable detection and monitoring strategies. In particular, the ability to monitor multiple OPPPs in parallel in a manner conducive to risk assessment and mitigation, as well as for research seeking to advance a fundamental understanding of their behavior in premise plumbing and inform better control measures, would be advantageous.

Here we focus on a representative cross section of OPPPs, including bacterial species, such as *Legionella pneumophila*, non-tuberculous mycobacteria (NTM), and *Pseudomonas aeruginosa*, and protozoans, such as *Acanthamoeba* spp. and *Naegleria fowleri*. These microorganisms were of particular concern due to their adverse health effects, involvement of public water systems as transmission routes, and complex ecological interactions with one another. Among these, *L. pneumophila*, and other *Legionella* spp. capable of causing Legionnaires' disease, have attracted the most attention as this deadly form of pneumonia accounts for the greatest proportion of OPPP-associated outbreaks in the United States (CDC, 2011; Hubbs, 2014). NTM is comprised of several pathogenic strains of mycobacteria, causing chronic pulmonary disease in an estimated 30,000 people in the U.S. (Winthrop et al., 2010). While first being

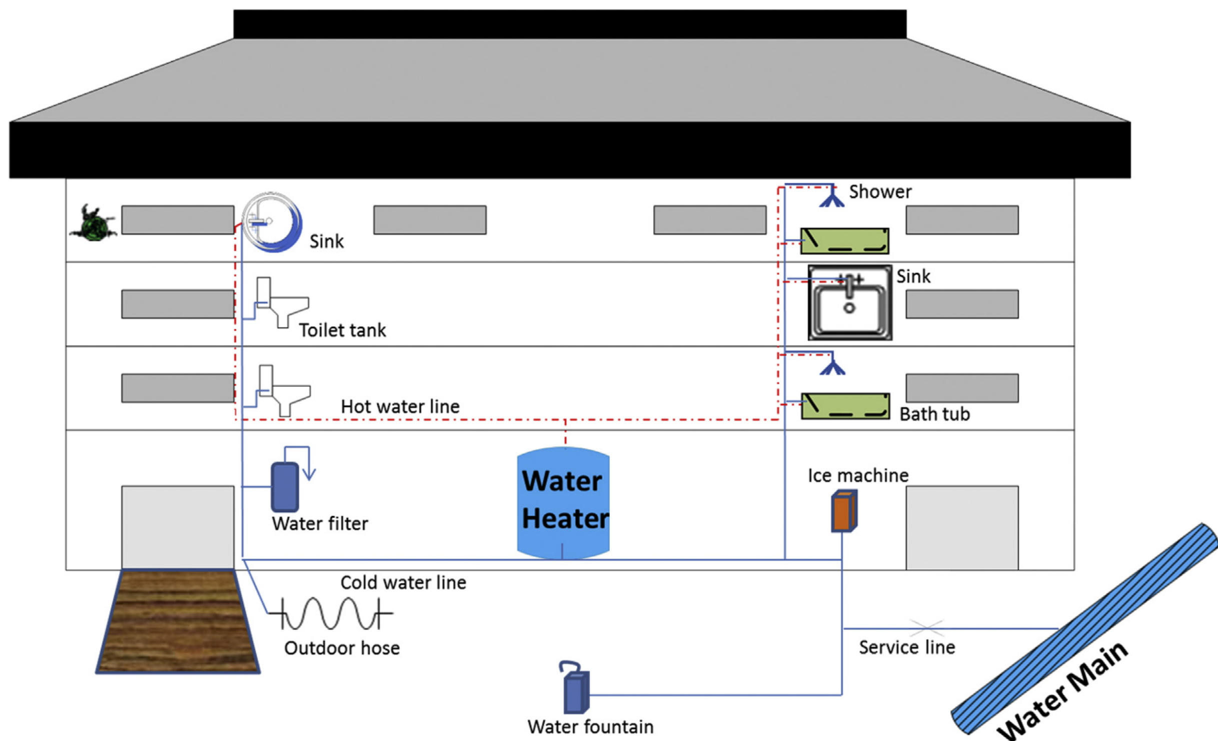

Fig. 1. Schematic of premise plumbing and potential sampling sites for OPPPs. Red dashed lines represent hot water lines. Blue solid lines represent cold water lines. Potential sampling sites include cold and hot taps, water heaters, shower heads, baths, sinks, water filters, ice machine, outdoor hoses, toilet tanks, public water fountains. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

noticed in immunocompromised populations, NTM also affects groups with other less-obvious risk factors (e.g., slender, elderly women) (Falkinham, 2009). *P. aeruginosa* is especially problematic as an agent of nosocomial (i.e., hospital-acquired) infection, particularly for neonates and burn patients, causing about 1400 deaths each year in the United States (Anaissie et al., 2002). *Acanthamoeba* can cause severe eye infections (i.e., keratitis) and granulomatous amebic encephalitis, the former being associated with poor contact lens hygiene (Kilvington et al., 2004). *Naegleria fowleri* is the only pathogenic member of its genus, known as the "brain-eating amoeba," causing the rare but highly fatal disease, primary amebic meningoencephalitis (PAM) (Yoder et al., 2012). Of these, *L. pneumophila*, *Mycobacterium avium* and *N. fowleri* have been included on the drinking water contaminant candidate list 3 (CCL3) (EPA, 2009). Moreover, protozoans such as *Acanthamoeba* and *Naegleria* species can serve as hosts for *Legionella*, mycobacteria, and *P. aeruginosa*, enhancing their survival in drinking water (Delafont et al., 2014; Greub and Raoult, 2004). However, a major impediment to advancing further policy action towards public health protection from OPPPs is the challenge that they pose to monitoring.

There is currently no consensus with respect to methodologies or protocols for monitoring OPPPs, particularly when seeking parallel detection of multiple OPPPs. From a policy standpoint, a major obstacle is that existing microbiological monitoring practices focus on the main distribution system and do not extend across the property line, where conditions are particularly susceptible to the multiplication of OPPPs (NRC, 2006). This is problematic because there is a need for insight into the relationship between the municipal water characteristics and the potential for regrowth of OPPPs, as has recently been illustrated in the case of corrosive water being associated with a major Legionnaires' outbreak in Flint, MI (Schwake et al., 2016). From a practical standpoint, premise plumbing is extremely complex and differs greatly among buildings, including diverse fixture types and materials, pipe surface to volume ratios, and water temperatures (NRC, 2006). Flow conditions within plumbing can also vary dramatically from one point to another depending on the building layout (Inkinen et al., 2014). All these factors result in heterogeneity within and between premise plumbing environments, making it difficult to identify common sampling plans that are representative of the actual contamination by OPPPs and risk for disease transmission. Important to recognize in identifying relevant monitoring points is that there are multiple OPPPs exposure routes, especially aerosol inhalation, aspiration, and skin contact. Finally, OPPPs themselves are characterized by complex microbial ecology and physiology, which has led towards highly specific protocols that are not necessarily compatible for monitoring of multiple OPPPs.

This work presents a critical review of various sampling and methodological approaches employed for monitoring OPPPs. Advantages, limitations, and applicability of current monitoring methodologies are discussed in the context of their value and potential for informing prevention and mitigation of risk, outbreak response, and research. Key knowledge gaps and urgent research needs are identified that must be addressed to improve the science and practice of OPPPs monitoring.

2. Sampling premise plumbing and sample handling

Given the complexity of premise plumbing, key components of sampling considerations include site selection and sampling frequency, sample type (i.e., biofilm, water) and size, and flow patterns (e.g., first-flush, post flush). Moreover, sample handling before analytical testing, such as sample preservation, transport, and pretreatment (e.g., concentration), can also impact the recovery efficiency of targeted OPPPs.

2.1. Site selection and sampling frequency

2.1.1. Site selection

Currently, there are no generally accepted protocols for choosing sampling sites or frequency for OPPPs (Lucas et al., 2011). The selection of sampling sites for routine monitoring of *L. pneumophila* in healthcare facilities and in response to outbreaks has been described most extensively (Barbaree et al., 1987; Allegheny County Health Department, 2014; U.K. Department of Health, 2006; FRML, 2010; VHA, 2014). Sampling points are chosen based on vulnerability of certain sectors of the building to pathogen proliferation (e.g., dead-ends or areas with infrequent use) (U.K. Department of Health, 2006) as well as the susceptibility of the occupants to exposure risks (e.g., frequently occupied areas, intensive care, neonatal, and burn units) (ASHRAE, 2015; FRML, 2010). To gain a full systems perspective, recommended sampling sites for *Legionella* in healthcare facilities have included incoming water mains, water softeners, holding tanks, water heaters (at the inflows and outflows), faucets, and shower heads (Fig. 1, CDC, 2015). Importantly, OPPPs commonly prefer warmer water and may be spread via aerosols (e.g., *Legionella*), rather than ingestion, making hot water systems particularly important monitoring targets. A minimal sample number has been required in some scenarios, e.g., sampling at least 10 outlets in the hot and cold water distribution system respectively for each building was endorsed by the U.S. Veterans' Health Administration (VHA, 2014). Another guideline published by the U.K. Department of Health suggests that samples should at least be taken from the cold water storage, the most distal outlet from the tank, the water heater flow (or the closest and furthest tap to the heater and associated recirculating systems), and drain valves, when available (U.K. Department of Health, 2006). Monitoring temperature and disinfectant levels of cold and/or hot water systems is a practical approach to identify points that are susceptible to OPPP colonization and candidate locations for testing. For example, the warmest point in a cold water system, or the coolest part of a hot water system are likely to pose the greatest risk to *Legionella* growth (U.K. Environmental Agency, 2005; U.K. Department of Health, 2006). Regardless, a detailed knowledge of water system layout together with a thorough understanding of conditions that are conducive to OPPP growth is essential to developing a sampling plan (ASHRAE, 2015).

Routine monitoring of OPPPs in non-healthcare related plumbing is generally not mandatory, except in France, where operators of all public buildings are required to complete regular monitoring of *Legionella* for any hot water distribution system (FRML, 2010). In general, cold water systems should not require routine monitoring for *Legionella* unless temperatures and stagnation times are elevated in the system (e.g., >25 °C, dead ends, storage tanks). However, it cannot be said that there is zero risk of *Legionella* contamination in cold water systems. In a cold water survey across the United States, nearly half the taps were positive for *L. pneumophila* sg1 in one sampling event, while 16% of taps were positive for more than one sampling event (Donohue et al., 2014). In Germany, a survey of public buildings revealed 5.4% of cold water samples exceeded the action value for *Legionella* (Volker et al., 2010). In hot water systems, it is recommended to sample water heaters set at low temperatures (e.g., <50° C), header tanks, water softeners, water heater drain-off points, and affected taps (pre-flush, post-flush) (U.K. Environmental Agency, 2005). For residential surveys of *L. pneumophila*, it has been recommended that at least 3 of 5 of the following sites be sampled: hot water tank, kitchen tap, bathroom tap, showerhead, and bath tub outlet (Stout et al., 1992). Although inclusion of more sampling sites has the advantage of obtaining comprehensive information of plumbing contamination, it is often impractical for routine monitoring

considering the cost of labor and supplies. Establishment of criteria for selecting a certain number of representative sites in premise plumbing is the first step towards effective monitoring of OPPPs and risk prevention, which is in need of further investigation.

Sampling plans are an essential aspect of responding to *Legionella* outbreaks, with the aim of identifying all potential sources of contamination and exposure. Cold water systems should be sampled at the incoming supply, the outlet of each reservoir, and outlets closest/furthest to the reservoir. Hot water systems should also be thoroughly investigated, including showers and taps used by people infected with Legionnaires' disease proximity. Any other sources of exposure, including those susceptible of generating aerosols, also need to be considered: ice machines, evaporative cooling systems, spas, humidifiers, decorative fountains, etc. (U.K. Environmental Agency, 2005).

Published guidelines for sampling site selection strategies for OPPPs other than *Legionella* are scarce. A recently published health technical memorandum by the U.K. Department of Health briefly suggested that water outlets supplying water for patients (direct and indirect contact) and staff hand-washing should be sampled for *P. aeruginosa* in augmented care units (U.K. Department of Health, 2013). With regard to NTM and amoebae, for which there are no surveillance guidelines for their monitoring in premise plumbing, recent studies followed general guidelines for standard microbiological sampling and testing. Taps and showers are frequent targets (Falkinham, 2011; Feazel et al., 2009; Ichijo et al., 2014; Kilvington et al., 2004; Thomas et al., 2006), with cisterns, filters, garden hoses and ice machines also sampled in some cases (Covert et al., 1999; Falkinham, 2010, 2011; Thomas et al., 2014). In an epidemiological and environmental investigation of a PAM death, a garden hose, service line hose bib, and toilet tank were also included in household samples, all of which were positive for *N. fowleri* (Cope et al., 2015). Absence of sampling guidelines for OPPPs other than *Legionella* poses a challenge to their routine monitoring for the purpose of informing risk mitigation and outbreak investigation. Accordingly, data collection on OPPP occurrence and distribution within premise plumbing are urgently needed for development of an integrated sampling plan towards risk assessment and control of multiple OPPPs.

2.1.2. Sampling frequency

There is no universal direction with regard to when and how often to sample premise plumbing for microbiological monitoring. Monthly, quarterly, semi-annual and annual samplings have been proposed for testing of *Legionella* and/or *P. aeruginosa* (U.K. Department of Health, 2013; Regierung, 2001; FRML, 2010; VHA, 2014). The U.S. VHA requires quarterly testing of their buildings' hot and cold water distribution systems for *L. pneumophila* (VHA, 2014); while annual sampling of hot water systems is generally recommended by German health care centers and residential monitoring programs (Regierung, 2001). Sampling selected taps every 6 months is suggested for *P. aeruginosa* monitoring in augmented care units in the U.K. However, more frequent tests or an expanded survey might be needed in the case of a positive test results or clinical evidence of contamination (U.K. Department of Health, 2013). In general, such proposed monitoring frequencies are largely empirical and may not be suitable for capture of seasonal variation (e.g., annual sampling) or baseline dynamics of OPPPs.

Sampling frequency can also differ based on water usage. For example, it has been suggested to perform one microbiological control per year per 100 beds, with a minimum of four controls per year for a healthcare facility in terms of monitoring *P. aeruginosa* and total coliforms in water for general use; while monthly monitoring of heterotrophic plate counts, total coliforms, *L. pneumophila*, *P. aeruginosa*, and *Staphylococcus aureus* is required for water used for spas, baths and showers (FRMHS, 2005). Sampling frequency is also associated with temperature variation and disinfectant levels in the plumbing. Daily or monthly temperature control has been prescribed for healthcare buildings in several European countries, a fundamental strategy for controlling *Legionella* exposure risks (U.K. Department of Health, 2006; FRMHS, 2005).

The reality is that any given sampling event does not capture the true dynamics of OPPPs in the system. A study examining the variability of *L. pneumophila* in 84 taps over 5 consecutive days in an Italian hospital revealed significant variation of the bacterial load from day to day, although the pattern was similar across the wards monitored (Napoli et al., 2009). Thus, repeated samplings might be required in order to gain sufficient statistical power, especially when the data are for the purpose of research, risk assessment, or mitigation. Ultimately, information gathered from repeated testing of OPPPs under different plumbing conditions would be valuable to justify optimal sampling frequencies in the contexts of routine monitoring, outbreak investigation and follow-up mitigation.

2.2. Sample type: bulk water and biofilm

Microbes and OPPPs reside within two general niches in the premise plumbing environment: the biofilm and the bulk water (Falkinham et al., 2008; Flannery et al., 2006; Moore et al., 2006; Thomas et al., 2006; Wang et al., 2012). Biofilm is thought to serve as the ideal habitat for OPPPs, providing food and protection from disinfectants and other harmful agents. Amoebae have been detected at a higher frequency in biofilm than bulk water (Stockman et al., 2011), as the biofilm consists of a diverse and concentrated source of bacteria to graze upon (Huws et al., 2005; Paris et al., 2007). Interestingly, it is a common feature among bacterial OPPPs to survive phagocytosis and experience enhanced growth and virulence within the amoeba digestive vacuole (Thomas and Ashbolt, 2011). Such a "Trojan horse" phenomenon has been confirmed for *Legionella*, NTM, and *P. aeruginosa* (Delafont et al., 2014; Greub and Raoult, 2004) and is thought to be obligate for *Legionella* replication under oligotrophic conditions (Declerck et al., 2009; Thomas and Ashbolt, 2011). However, the rich biofilm nutrient content may allow nutritionally fastidious *Legionella* spp. to multiply extracellularly (Taylor et al., 2009).

Even if biofilm is ultimately proven to be the most important ecological niche for OPPPs, sampling of which has the advantages of access to concentrated biomass and targeting specific zones of pathogen colonization, bulk water is more accessible than biofilm and remains the most targeted for OPPP investigation. Arguably, bulk water is also the most relevant to actual dissemination and exposure (Buse et al., 2012; Schoen and Ashbolt, 2011). As such, guidelines for *Legionella* and *P. aeruginosa* control are often based on bulk water bacterial loads (U.K. Department of Health, 2006; 2013). In a study comparing 3910 paired water/biofilm samples, concordant *Legionella* detection results were found among 81% of pairs, with only 2% of pairs only yielding positive detection in the biofilm (Ditommaso et al., 2010). Ecological niches of specific species might also affect their distribution in suspended versus attached phases. For example, across eight water distribution systems, higher detection frequency in biofilm relative to water samples was confirmed for *M. intracellulare*, but not *M. avium* (Falkinham et al., 2001). Thus, further systematic comparisons between biofilm and water samples are needed for other OPPPs to justify sampling plans. Specifically, quantitative assessment of partitioning of OPPPs between water and biofilm phases at genus and species level, as well as knowledge about how plumbing conditions (e.g., stagnation, flow rates, pipe materials) affect their partitioning processes, would be of value to formulating sampling

plans for different purposes (e.g., routine monitoring, outbreak investigation).

2.2.1. Sampling of water

Three consecutive steps are generally employed for sampling plumbing water: turning on a tap or valve, adjusting the flow rate, and collecting a prescribed volume of water using a sterilized container. Sodium thiosulfate is often used to neutralize residual chlorine and other halogens when the source water contains disinfectant (CDC, 2005). However, technical details during sampling and preservation beyond the basic steps are worthy of consideration, especially when parallel monitoring multiple OPPPs is the goal. Table 1 summarizes procedures for OPPPs sampling applied across several studies.

2.2.1.1. Sample volume. Sampling volumes for the detection of *Legionella* and *P. aeruginosa* in drinking water systems, as two representative bacterial OPPPs, have varied between 50 ml and 1L, depending on the anticipated concentrations of target organisms and the detection limit of applied methods (Ferroni et al., 1998; Halabi et al., 2001; Lavenir et al., 2008; van der Wielen and van der Kooij, 2013). Larger volumes (i.e., 5–10 L) were collected in case of low biomass level (e.g., distribution mains) in order to ensure sufficient recovery (U.K. Environment Agency, 2005; Barbaree et al., 1987). Even larger volumes, >100L, have been filtered for *N. fowleri* detection, yielding molecular- and culture-based detection when smaller volumes (0.7 L) did not (Cope et al., 2015). Large-volume sampling usually requires additional supplies, equipment and labor, which may not be practical for frequent monitoring. However, large volumes are extremely important for outbreak investigations that cannot afford false negative detections. In addition, for some analytes and water qualities, larger volume sampling might be associated with concentration of inhibitory substances that could affect downstream analysis, such as quantitative polymerase chain reaction (qPCR) amplification (Hata et al., 2011).

2.2.1.2. Pre-flushing or post-flushing. Complex water usage patterns typify premise plumbing, resulting in temporal and spatial heterogeneity and periodically stagnant water. Higher numbers of microorganisms are likely to be detected following stagnation or infrequent use, as a result of disinfectant decay and microbial regrowth (Lautenschlager et al., 2010). Thus, one approach to maximize the likelihood of OPPPs detection is to sample taps after a period of stagnation (e.g., first draw of water taken in the morning). Taking the first sample (pre-flushing) after at least 2 h of stagnation or during a period of minimum water usage has been recommended for *P. aeruginosa* detection in augmented care centers (U.K. Department of Health, 2013). Similarly, in a molecular survey of multiple OPPPs in building plumbing, imposing 8-h stagnation before sampling resulted in higher discrepancy between first-draw (pre-flushing) and post-flushing samples relative to sites without enforced stagnation (Wang et al., 2012). In case of a positive pre-flushing sample, a second round of testing is requested for *P. aeruginosa* monitoring by including a pre-flushing sample and a second sample collected after running taps for 2 min, in order to identify the source of contamination (U.K. Department of Health, 2013). Given these considerations, a major shortcoming of microbiological water quality monitoring, as recommended by U.S. EPA regulations and other common guidelines for distribution systems, is the practice of thorough flushing and disinfection of taps prior to sampling, which is unlikely to detect problems related to premise plumbing (NRC, 2006).

With regards to hot water systems, Western Pennsylvania Guidelines suggest sampling the first-draw (i.e., first 1 L) of hot water from the outlet valve and prior to cold water system sampling (Allegheny County Health Department, 2014). However, some researchers recommend flushing the tap for 1–2 min prior to sampling in order to exclude cold water (Bargellini et al., 2011; Borella et al., 2005). Since it is not easy to determine when cold water will be completely flushed out, reaching temperature equilibrium has been used as a criterion to determine when to collect samples (Moore et al., 2006; von Baum et al., 2010; Wellinghausen

Table 1
Sampling procedures for *P. aeruginosa and L. pneumophila* in premise plumbing among different protocols and studies.

		P. aeruginosa			*L. pneumophila*		
		Device	Doc Type	Ref	Device	Doc Type	Ref
Flow regime	First draw	Taps	Best practice guidance	(U.K. Department of Health, 2013)	Taps/Showers/ Water heaters	Study Protocol	(Martinelli et al., 2000) (Health Facilities Scotland, 2011)
	Brief period of flow (eliminate cold water)				Taps	Study	(Borella et al., 2004)
	1 min flow				Taps/Showers	Study	(Bargellini et al., 2011)
	2 min flow	Taps (post-flush)	Best practice guidance	(U.K. Department of Health, 2013)	Faucets	Protocols	(Barbaree et al., 1987; Health Facilities Scotland, 2011)
	Temperature at equilibrium					Study	(Flannery et al., 2006; Moore et al., 2006)
Volume of sample (mL)	100	Faucets	Studies	(Lavenir et al., 2008; Trautmann et al., 2001; van der Mee-Marquet et al., 2005)	Taps	Study	(Zhang et al., 2009)
	250	Faucets	Studies	(Berthelot et al., 2006; Rogues et al., 2007)			
	500	Faucets	Studies Protocol	(Halabi et al., 2001) (Health Facilities Scotland, 2011)			
	1000				General potable water system	Protocols	(Barbaree et al., 1987; Health Facilities Scotland, 2011)
					Residential taps/ showers	Study	(Flannery et al., 2006; Mathys et al., 2008; Moore et al., 2006)
	1000–2000					Standards	(APHA, 2012; ISO, 2004)
					Showers/Taps	Study	(Borella et al., 2004)
	10,000				Incoming water	Protocol	(Barbaree et al., 1987)

et al., 2001). In either situation, the sample will not include an important volume of hot water that was stagnant in the distal pipe and at optimal temperature for microbial growth. The volume of mixed hot and cold water in a tap is small (generally less than 100 mL) compared to the volume corresponding to a 1–2-min flush (3–12 L) or until temperature equilibrium is reached. Additional steps might be needed if the goal is solely to sample the hot water. For instance, one study surveying *Legionella* occurrence in hot water systems in German single-family residences removed all devices from the taps, heated taps with a gas burner, and discarded the first 5 L prior to collection (Mathys et al., 2008). ASTM guidelines and some researchers also suggest stratified sampling, which includes a first flush sample and a sample after temperature stabilization for *Legionella* in order to delineate the source of contamination (Bates et al., 2000; ASTM, 2008), which is an important step during outbreak investigations.

2.2.1.3. Aerator removal. Faucet aerators represent potential risks for colonization of *Legionella* and other pathogens (CDC, 2003). Whether to remove aerators before sampling depends on the objective. If the objective is to evaluate the risk and colonization of the outlet, the sample should be taken with the aerator and without disinfection. When the water flowing within the distribution system is the target, potential contamination from outlets should be minimized. CDC specifies that water samples for biological tests should be taken after removing aerators and disinfecting taps with sodium hypochlorite if the cleanliness of the tap is questionable (CDC, 2005, 2015). Other disinfection approaches for taps include ethanol or isopropanol (70% v:v), as well as flaming for metal taps (U.K. Environmental Agency, 2005; Mathys et al., 2008). However, in case of outbreak investigations, taking biofilm swabs from the inside of aerators is recommended (CDC, 2015). Care should be taken when removing aerators, as biofilm disruption and detachment might occur during disassembly.

2.2.1.4. Sample preservation and transportation. Shipping and handling can require a few hours to several days, during which samples should be preserved accordingly to minimize cell cultivability impairment and/or regrowth. Recommended preservation procedures for *Legionella* enumeration vary considerably in different documents in terms of storage temperature and holding time. For example, the International Standards Organization (ISO) requires refrigeration of samples that cannot be processed within 24 h (ISO, 2004); while the CDC and the American Society for Testing and Materials (ASTM) recommend refrigeration only if samples cannot be processed within 72 h (CDC, 2005), although protection from extreme heat or cold (i.e., <3 °C and/or >30 °C) is required at all times (ASTM, 2008). The U.K. Environmental Agency recommends maintaining samples between 6 and 20 °C, in order to prevent cells from entering a viable but not culturable (VBNC) state during shipping, and to commence concentration and incubation procedures within 48 h (U.K. Environmental Agency, 2005). Variation in preservation requirements likely stems from ambiguous knowledge regarding effects of temperature and holding times towards cell cultivability and regrowth potential. Two studies have investigated the effect of holding time on *Legionella* cultivability, reporting conflicting results. McCoy observed significant changes (>1 log_{10} unit) in 52% of water samples (n = 42) with 6–120 h holding time at room temperature (McCoy et al., 2012), while another recent study analyzing 159 samples found little effect of holding time on *Legionella* counts, with root mean squared error increased by only about 3–8% in samples held overnight at room temperature (Flanders et al., 2014). Variation of holding time applied in these two studies might contribute to the inconsistent conclusions. Further assessment of the effect of holding time on *Legionella* cultivability, especially for time frames and temperatures commonly referred to by guidelines (e.g.., 24–72 h at 4 °C or room temperature), is needed before drawing a solid conclusion.

On the other hand, water samples collected for *P. aeruginosa* culturing should be refrigerated (2–8 °C) within 2 h and processed within 24 h in order to prevent regrowth (U.K. Department of Health, 2013). On the contrary, little change in NTM numbers would be expected during shipping due to their slow growth and decay rates, as well as resistance to a wide range of environmental temperatures (Falkinham, 2009).

In terms of free-living amoeba, caution must be taken when handling and storing samples since common practices such as chilling and refrigeration will trigger the formation of cysts and VBNC state. *N. fowleri* trophozoites are known to become VBNC when the temperatures are <10 °C (Chang, 1978). Samples for *N. fowleri* cultivation should be stored and shipped at ambient temperature (i.e., non-chilled) (Cope et al., 2015) and with adequate headspace, since *N. fowleri* are thought to be highly aerobic (Kyle and Noblet, 1985). Thus, various shipping and handling requirements for each OPPP poses a significant challenge to parallel monitoring by culturing, likely requiring aliquoting of sub-samples to achieve optimal preservation conditions. However, further evaluation of influence of handling and preservation on OPPP cultivability would be valuable to simplify sub-sampling requirements by categorizing OPPPs according to similar requirements (e.g., overlapping acceptable temperature and time ranges).

2.2.1.5. Concentration methods. Sample concentration is typically required for detection of OPPPs, commonly ranging from 50 to 1000 ml premise plumbing water. Filtration, centrifugation and immuno-magnetic separation (IMS) are three common concentration methods. The first two are non-specific approaches widely used for microbial analysis, whereas IMS is able to select target microbes from background microorganisms by using antibody-coated magnetic media (Allegra et al., 2011; Bedrina et al., 2013; Mull et al., 2013).

Filtration is suitable for water samples with sufficiently low turbidity to avoid clogging. Generally, water is filtered through a 0.22 or 0.45 μm membrane (Bartie et al., 2001; CDC, 2005), which later is transferred directly to media for culturing (U.K. Department of Health, 2013) or used for cell resuspension by sonicating or vortexing the filter in a small volume of water (Bartie et al., 2001; CDC, 2005; Thomas et al., 2008). Membranes may also be shredded for DNA extraction. Membranes with larger pore-size (e.g., 1.2 μm) have sometimes been used for recovering amoebae, given the larger diameter of protozoans (Pernin et al., 1998), but more recently *N. fowleri* have been recovered from large volumes of water using ultrafiltration (Cope et al., 2015). Centrifugation can be applied for both clean and non-filterable water with higher turbidity. Typically, water samples are centrifuged at a speed of 3000 g for 30 min or 6000 g for 10 min (Bartie et al., 2001; Brindle et al., 1987), leaving pellets that can be resuspended in 2–20 ml of diluents after discarding the supernatants. Recovery efficiencies of filtration and centrifugation have been evaluated for *Legionella* and mycobacteria across several studies, with filtration demonstrating similar or higher specific recovery rates relative to centrifugation (Boulanger and Edelstein, 1995; Brindle et al., 1987; Ta et al., 1995; Thomson et al., 2008). However, centrifugation is a better choice for recovery of *Naegleria* from small-volume samples, as trophozoites may lyse during vacuum filtration (Pernin et al., 1998). Moreover, specific recovery rates can vary considerably when different kinds of membranes and centrifugation conditions are applied (Boulanger and Edelstein, 1995).

2.2.2. Sampling of biofilm

In premise plumbing, biofilm samples are often collected from faucets, shower heads, drains, hoses and water filters (Charron et al., 2015; Falkinham, 2010; Liu et al., 2012; Proctor et al., 2016; Thomas et al., 2014; Wang et al., 2012). Removal of anti-splash or spray nozzles from faucets and shower heads is recommended prior to biofilm sampling in order to access the inner area and obtain representative biofilm (U.K. Environmental Agency, 2005; Feazel et al., 2009). However, this is difficult to apply consistently in all cases since devices are often not designed for ready disassembly. Biofilm can be removed from surface by scraping a known surface area using a sterile knife or swab (Charron et al., 2015; Liu et al., 2012; Srinivasan et al., 2008). Thorough removal of biofilm may require multiple scrapings (Srinivasan et al., 2008) or further treatment, such as sonicating removable parts (e.g., faucet gaskets) in a cold bath (Liu et al., 2012). Although some studies used epifluorescence microscopy to quantify cell removal efficiencies (Percival et al., 1999), this technology is constrained for premise plumbing biofilm samples since most parts are not removable. Moreover, current biofilm sampling protocols have only been tested against bacteria (Gagnon and Slawson, 1999). It is unclear whether they can yield valid results for protozoa, such as *Acanthamoeba* and *N. fowleri*, since improper treatment of protozoa cells could trigger cell encystment and hinder recovery (Chang, 1978; Kyle and Noblet, 1985).

Representativeness of biofilm sampling is challenged by biofilm traits of spatial and physiological heterogeneity (Wimpenny et al., 2000). Most of the time, one biofilm sample is taken from the surface of a designated plumbing fixture with other water samples (CDC, 2015). Different choices in specific sampling location may lead to inconsistent results. For example, shower head biofilm samples can be taken from the inside of shower heads, the inside surface of joints, or deep, inner area of the connecting pipes, where different biofilm structures and compositions are expected. More research addressing influence of biofilm heterogeneity towards OPPP monitoring is needed in order to warrant biofilm sampling representativeness.

Biofilm sampling sequence may also affect representativeness. When both biofilm and bulk water are sampling targets, e.g., in case of outbreak investigations, biofilm samples should be taken first, as water sampling and flushing will dislodge biofilm microbes. However, sampling biofilm first might release bacteria into the bulk phase and affect the representativeness of subsequent bulk water sample if the goal is to take the first-draw samples.

2.3. Sample preservation strategies for culture- versus molecular-based methods

Different sample preservation strategies may be required when subject to culturing, molecular analysis, or both. Water samples concentrated by membrane filtration or centrifugation can be resuspended in media prior to culturing. Same (e.g., drinking water (Borella et al., 2005)) or similar non-nutrient media (e.g., phosphate-buffered saline (Leoni et al., 2001)) are usually used for resuspension, in order to minimize the influence of shifting conditions on regrowth potential and cultivability, with storage at 4 °C if plating is not performed immediately. When samples are intended for molecular analysis, membranes or resuspended media can be directly kept at −20 °C or lower until DNA/RNA extraction (Eichler et al., 2006). Similarly, culturing biofilm cells involves resuspension in appropriate media (Wullings et al., 2011); while cotton swab tips can be directly preserved in 70% ethanol (Feazel et al., 2009) at −20 °C or lower for molecular analysis.

As discussed above, there are multiple sampling, handling and preservation factors that can affect final monitoring results. Currently, determination of these factors (e.g., sample numbers, sampling sites and frequency, sample volume, holding time) is rather empirical and subject to discretion, particularly for OPPPs other than *Legionella*. Methodological inconsistence likely leads to variation in sampling results, making between-group comparison unreliable for different studies. A unified sampling guideline would be needed for different sampling purposes (i.e., routine monitoring, outbreaks), which can not only help streamline the sampling process, but also improve detection sensitivity and reliability. For OPPPs whose distribution and dynamics have not been comprehensively studied (e.g., *N. fowleri*), occurrence surveys would be necessary in order to provide supporting data for protocol development. Meanwhile, improved understanding of plumbing microbial ecology will assist in the refinement of sampling protocols.

3. Detection methods

3.1. Culturing of OPPPs

Culturing is used to verify and recover viable cells, remaining the "gold standard" for identifying and typing infectious and life-threatening pathogens. Thus, it is not a surprise that the majority of current standard methods, regulations, and action limits for OPPPs are based on conventional culture methods. However, culturing of OPPPs is often criticized as labor-intensive and time-consuming, requiring specialized expertise to correctly identify target cells and failing to provide timely information in urgent situations. Culture-based techniques are also sometimes limited in their quantitative capacity.

3.1.1. Legionella spp.

Legionella are fastidious, with highly-specific nutrient (e.g., iron, L-cysteine) and cultivation requirements. Laboratories around the world use different culturing protocols for *Legionella* recovery, with considerable variation noted in terms of pretreatment approaches, cultivation media, and incubation periods. ISO (ISO11731-2:2004) recommends use of buffered charcoal yeast extract (BCYE) agar containing L-cysteine or selective GVPC agar (BCYE supplemented with glycine, vancomycin, polymyxin B, cycloheximide) for recovery of *Legionella* from filtered water samples. The results are compared to that of BCYE agar without L-cysteine for specificity confirmation (ISO, 2004). Meanwhile, the CDC advocates simultaneous use of four different kinds of media, including BCYE base media, two selective BCYE agars (i.e., PCV (BCYE supplemented with polymyxin B, cycloheximide, and vancomycin) and GVPC) and PCV without L-cysteine as a negative control (CDC, 2005). Other versions of *Legionella* cultivation media include variation of antimicrobial supplements (Ta et al., 1995), dye supplements for colony staining (e.g., MWY medium) (Ta et al., 1995) or specific nutrients (e.g., ABCYE: BCYE with bovine serum albumin) to cater to the growth requirements of certain *Legionella* spp. such as *L. micdadei* (CDC, 2005).

Heat and acid treatments are routinely used to suppress growth of non-legionella species by taking advantage of *Legionella*'s tolerance of such extreme conditions. Heat treatment is typically performed between 50 and 59 °C for 3–30 min and pH treatment at ~2.2 for 3–15 min (De Luca et al., 1999; Reinthaler et al., 1993). Improved recovery had been observed for both pretreatment methods compared to no treatment (Reinthaler et al., 1993). However, inconsistent results have been noted among different studies in terms of optimal combinations of pretreatment approaches and cultivation media (Bartie et al., 2003; De Luca et al., 1999; Leoni and Legnani, 2001). Factors such as sample matrices, *Legionella* concentration, and commensal flora could possibly affect sensitivity and specificity of applied culturing techniques. However,

neither heat nor acid treatments are always 100% effective and they can also lead to underestimation of *Legionella* counts as a result of loss of cell cultivability (Leoni and Legnani, 2001).

Another approach for recovering *Legionella* in premise plumbing is to co-culture samples with amoebae (e.g., *Acanthamoeba* or *Vermamoeba*). This approach may improve recovery frequency and detection limits for samples with low *Legionella* counts and/or VBNC cells by increasing their numbers and resuscitating cultivability (Conza et al., 2013; Garcia et al., 2007).

3.1.2. *Mycobacterium spp.*

Mycobacterium spp. are a group of slow-growing, hydrophobic microorganisms ubiquitous in natural and engineered aquatic environments (Falkinham, 2009). Their cells tend to aggregate and adhere to surfaces, increasing difficulty for isolation and enumeration. Culturing techniques for *Mycobacterium* spp. typically include a pretreatment step to prevent bacterial and fungal overgrowth, use of nutrient-rich medium (e.g., M7H10 agar supplemented with 0.5% glycerol and 10% oleic-albumin enrichment) to support growth, and incubation at 35–37 °C for 10–21 days to allow enough time for recovery (Falkinham et al., 2001, 2008; Thomson et al., 2008). Although a variety of protocols have been used for mycobacteria recovery, they have yet to be standardized.

A range of bacterial and fungal inhibitors, including cetylpyridinium chloride (CPC), NaOH, formaldehyde, and oxalic acid had been used for sample pretreatment (Falkinham et al., 2001, 2008; Thomson et al., 2008; Torvinen et al., 2004). Decontamination with CPC was considered the best approach for treated water (Neumann et al., 1997) and has been widely applied in many drinking water-related studies (Falkinham et al., 2001, 2008; Torvinen et al., 2004). However, these pretreatment methods are not always effective. Decontamination efficacy can vary dramatically upon selection of different inhibitors, which might be partially ascribed to characteristics of the water matrix and presence of various mycobacterial species. Moreover, decontamination steps can also result in loss of viable mycobacteria (Thomson et al., 2008).

Employment of different culture media also leads to various recovery efficiencies. Neumann et al. suggested that Lowenstein-Jensen medium and Ogawa egg yolk medium are superior to Ogawa whole-egg medium containing ofloxacin and ethambutol in terms of mycobacterial recovery in surface and treated water (Neumann et al., 1997). Thomson et al. obtained similar results with Middlebrook 7H10 and 7H11, and Lowenstein-Jensen slants after 3-week incubation of water samples. However, Lowenstein-Jensen slants initially appeared to be less sensitive when examined earlier (Thomson et al., 2008). Other factors such as temperature (Neumann et al., 1997) and incubation period (Thomson et al., 2008) can also affect recovery.

3.1.3. *P. aeruginosa*

P. aeruginosa is a Gram-negative, oxidase-positive bacterium that usually produces pyocyanin and fluorescein and hydrolyzes casein (U.K. Department of Health, 2013). Standard culturing methods for *P. aeruginosa* in drinking and/or recreational water include the membrane filtration and multiple-tube techniques, both of which take advantage of its pigment-producing characteristics (APHA, 2012). M-PA agar can be used to recover *P. aeruginosa* from filter membranes, incubated at 41.5 ± 0.5 °C for 72 h for presumptive tests. Colonies with 0.8–2.2 mm in diameter, flat in appearance with light outer rims and brownish to greenish-black centers are scored as positive. Presumptive tests are followed by confirmation tests using milk agar for another round of cultivation at 35 °C for 24 h to assess atypical colonies, taking advantage of *P. aeruginosa*'s capability of hydrolyzing casein and producing a yellowish to green pigment. Another cultivation method widely used is ISO 16266:2006 (ISO, 2006). Incubation is conducted at 37.5 °C for 24–48 h. Volumes of 10 mL and 100 mL are filtered on 0.45-μm cellulose membrane in duplicate. Filters are placed on cetrimide agar with nalidixic acid. Multiple-tube techniques use asparagine broth for *P. aeruginosa* growth and green fluorescent pigment production in presumptive tests (35–37 °C for 24–48 h), followed by confirmation tests using acetamide broth or agar for 24–36 h cultivation. Acetamide can be deaminated by *P. aeruginosa*, resulting in the phenol red indicator shifting from yellow orange to purple.

As described above, standard culture methods for *P. aeruginosa* are highly time-consuming. An alternative most probable number (MPN) method, commercialized by IDEXX laboratories (Portland, ME), provides semi-quantitative results within 24–48 h by using a bacterial enzyme detection reagent. This method is reported to perform equivalently to the membrane filtration method for examination of hospital water (Sartory et al., 2015) and was recently listed as an alternative method for *P. aeruginosa* detection in swimming pool water testing by the German Federal Environment Agency (IDEXX, 2015).

3.1.4. *Acanthamoeba and Naegleria fowleri*

Spreading concentrated water samples or placing a membrane filter on non-nutrient agar plates containing a lawn of Gram-negative bacteria (e.g., *Escherichia coli* or *Enterobacter aerogenes*) as a food source is a common approach for recovering amoebae from drinking water (Delafont et al., 2014; Tyndall et al., 1989). Plates are typically incubated at 28–44 °C for up to one month (Delafont et al., 2014; Marciano-Cabral et al., 2010; Thomas et al., 2006; Tyndall et al., 1989) and observed at regular intervals by microscopy to confirm presence or absence. Assays specifically targeting *Naegleria* spp. favor a higher incubation temperature (i.e., 42–44 °C), since *Naegleria* are thermophiles (Ithoi et al., 2011; Marciano-Cabral et al., 2010; Mull et al., 2013; Tyndall et al., 1989). Identities of amoeba isolates are subsequently determined according to their morphology or using molecular methods (e.g., PCR) (Garcia et al., 2013). *Acanthamoeba* trophozoites and cysts have distinct features (e.g., double-walled cysts) that can be categorized into three different morphological groups based on cyst size and numbers of arms (Pussard and Pons, 1977). *Naegleria*-like trophozoites and cysts typically demonstrate eruptive-like formation of the pseudopodia and smooth walls, respectively (Tyndall et al., 1989). Further confirmation of *Naegleria* involves a test to induce trophozoites to flagellate by adding sterile distilled water and incubating 1–2 h (Ithoi et al., 2011; Visvesvara et al., 2007). However, full validation of either *Acanthamoeba* or *Naegleria* isolates to species level is not reliable using culturing alone, as morphology of trophozoites and cysts can be strongly affected by culturing conditions (Visvesvara et al., 2007). Therefore, other assays (molecular, enzymatic, or virulence tests) are needed for species identification.

3.2. *Molecular methods*

Molecular methods generally involve manipulation and analysis of DNA, RNA, or proteins. Compared to conventional cultivation methods, molecular methods have the advantage of low detection limit, high specificity and sensitivity, ability to detect viable but not cultivable (VBNC) cells, and short turnaround time (Girones et al., 2010). Such advantages are attractive for pathogen monitoring, especially when results are urgent and simultaneous detection and enumeration of multiple OPPPs is required.

A number of molecular techniques, including PCR, quantitative-PCR, and DNA sequencing, have been developed for identifying and quantifying OPPPs (Table 2). These methods are nucleic acid-based,

Table 2
Examples of available analytical methods for OPPP detection in environmental samples.

Targeting genus	Species(if any)	Method	Sample characteristics	Detection or quantification limit	Notes	Ref.
Legionella		Culturing	Pool water and shower water	LOD: 5 cfu/l or pool water and 10 cfu/l for shower water	Agglutination tests used following cultivation to distinguish *L. pneumophila* serogroup 1–6, *L. bozemanii, L. dumoffii, L. gormanii, and L. micdedei*	(Leoni et al., 2001)
		Culturing (ISO 11731)	Hot water from hotels	LOD:25 cfu/l	Agglutination tests were used to separate *L. pneumophila* isolates serogroup 1 and 2–14, as well as seven species of non- *L. pneumophila* legionellae	(Bargellini et al., 2011; Borella et al., 2005)
	Legionella spp. & *L. pneumophila*	Culturing, PCR, &q-PCR	Drinking water from treatment plants	LOD (*Legionella* spp., direct culturing, without concentration): 1000 cfu/l LOD (*L. pneumophila*, q-PCR): 1000 gene copies/L	Semiquantitative PCR used for concentration assessment. PCR products cloned and sequenced for genetic diversity exploration	(Wullings and van der Kooij, 2006)
	Legionella spp. & *L. pneumophila*	Culturing, PCR, &q-PCR	Hospital water	LOD (*Legionella* spp. culturing): 1 cfu/100 ml LOD (*Legionella* spp. q-PCR): 2.3 cfu/100 ml; LOQ (*Legionella* spp. q-PCR): 23 cfu/100 ml LOD and LOQ for *L. pneumophila*: <2.3 and 23 cfu/100 ml, respectively	Correlated q-PCR and culturing results ($P < 0.001$) with higher q-PCR numbers relative to culturing; Correlated results between genus- and species-specific assays	(Wellinghausen et al., 2001)
	Legionella spp. & *L. pneumophila*	Culturing & q-PCR	Cooling towers Hot and cold water	LOD for q-PCR and culturing: 750 GU/l for water samples from cooling tower and 190 GU/l for samples from hot and cold water systems.	Greater discrepancy between q-PCR and culturing for cooling tower compared to hot and cold water samples	(Lee et al., 2011b)
		q-PCR	Spa water	LOD: 40 GU/l LOQ: 1000 GU/l	Results revealed weak correlation between culturing and q-PCR	(Guillemet et al., 2010)
		Culturing, q-PCR, EMA-q-PCR	Hot water samples	LOD for EMA-q-PCR: 200 GU/ml for 1 ml of sample treated with EMA; 250GU/l for 1L of sample water	v-PCR counts were equal to or higher than those obtained by culture, and lower than or equal to conventional qPCR counts	(Delgado-Viscogliosi et al., 2009)
		Immunofluorescent labeling combined with solid-phase flow cytometry		LOD: 34 viable cells/L for *L. pneumophila*	Obtained numbers are higher than CFU counting	(Parthuisot et al., 2011)
Mycobacterium		Culturing	Drinking water samples	LOD: 10 cfu/l	After culturing, PCR amplification of the *hsp*-65 gene followed by enzyme restriction of the PCR product was used for identification	(Falkinham et al., 2001)
		q-PCR	Cooling tower water	LOQ: 500 cells/l		(Adrados et al., 2011)
		q-PCR and culturing	Drinking water and other environmental samples	LOD for q-PCR: 6 genome equivalents for *M. chelonae* LOQ for q-PCR:100 genome equivalents	Higher numbers but lower detection rates with q-PCR relative to culturing	(Radomski et al., 2013)
	Mycobacterium avium subsp. *paratuberculosis*	q-PCR	Drinking water and biofilm	Assay LOD: 1.8 gene copy		(Beumer et al., 2010)

P. aeruginosa	Pseudalert®/Quanti-Tray® MPN Test, Culturing method	pool water samples, artificially-contaminated samples		Comparable results between Pseudalert®/Quanti-Tray® MPN Test and ISO 16266 and MoDW Part 8 methods	(Sartory et al., 2015)
	q-PCR	Clinical and environmental isolates		Duplex q-PCR assay with two targeted genes (*ecf*X and *gyr*B), requires simultaneous confirmation of *P. aeruginosa* by two genes	(Anuj et al., 2009)
	q-PCR and culturing	Hospital faucets (water, aerator and drain swabs)		qPCR revealed 50% positivity for *P. aeruginosa* remaining in the water compared with 7% by culture	(Bedard et al., 2015)
	q-PCR, PMA-q-PCR, standard cultivation-based technique	Drinking water and process water	LOD of q-PCR and PMA-q-PCR:10^2~10^3 cells/l	>80% samples yield accordant results by q-PCR, PMA-q-PCR, and cultivation based method. PMA-q-PCR reduced 4% false positive rates when compared to q-PCR.	(Gensberger et al., 2014)
Acanthamoeba	Culturing followed by morphological identification	Household water		Fungal overgrowth of the samples occurred more often with biofilm than water samples.	(Stockman et al., 2011)
	Culturing followed by morphological identification and PCR/sequencing	Tap water			(Winck et al., 2011)
	q-PCR	Anthropogenic water and biofilms	Assay LOD (trophozoites): 3 cells for water samples, 10 cells for biofilm samples	Qvarnstrom assays outperforms Riviere assay for *Acanthamoeba* detection and quantification	(Chang et al., 2010)
	PMA-q-PCR	Culture suspension, eyewash station, cooling tower, wastewater treatment plant	Detection range: 5−1.5 × 10^5 cells		(Chang et al., 2013)
Naegleria fowleri	q-PCR and MPN methods	Cooling water samples	LOQ for q-PCR: 320 cells/l	Samples with concentration <200 cells require culture method analysis; high q-PCR estimated numbers compared to MPN method	(Behets et al., 2007a)
	q-PCR and NNA-*E. coli* culturing method	*N. fowleri* cells spiked into water and biofilm samples	LOD in water samples: 5 cells in 250 ml water for a 66% detection rate and 10 cells for a 100% detection rate; LOD in biofilm samples: 1 cell for a 66% detection rate and 5 cells for a 100% detection rate	Culturing is less sensitive compared to q-PCR	(Puzon et al., 2009)
	IMS-q-PCR	*N. fowleri* seeded lake water	LOD: 14 cells/l;	Average recovery rate of 46%	(Mull et al., 2013)
	ELISA	Environmental water samples	LOD: 2000 cells/l	Can detect three morphological stages of *N. fowleri*, with 97.4% sensitivity and 97% specificity	(Reveiller et al., 2003)

with DNA/RNA extraction as the first and the foremost step in analysis protocols. Obtaining sufficient high-quality DNA/RNA with minimal PCR inhibitors is prerequisite to downstream amplification, posing a particular challenge to drinking water given its inherently low biomass concentration. On the other hand, nucleic acid extraction efficiency can vary considerably across studies (Girones et al., 2010). Sample types and properties, cell characteristics, and extraction methods can affect the quantity and quality of nucleic acid yields (Hwang et al., 2012). Some OPPPs may demand special treatment during nucleic acid isolation. It has been reported that the structures of *Acanthamoeba* cysts are resistant to DNA extraction reagents, requiring incubation with protease K prior to extraction to improve yields (Goldschmidt et al., 2008). Some molecular methods do not require extraction of nucleic acids. For example, fluorescence in situ hybridization (FISH), flow cytometry, or combined methods (e.g., FISH-solid phase cytometry) utilize fluorescently-labeled DNA/RNA probes that can penetrate cells and bind to target nucleic acids without rupturing intracellular structures. The emitted fluorescence is captured by microscopy or cytometry. However, these techniques are often criticized for high detection limits, laborious procedures, and low resolution for aggregated microbial cells (Buchbinder et al., 2002; Nocker et al., 2009; Wullings and van der Kooij, 2006). Such techniques are rarely encountered, typically in research contexts employing inoculation of high numbers of OPPPs to simulated drinking water systems (Declerck et al., 2009; Lehtola et al., 2007). Below we focus more specifically on nucleic acid-based molecular techniques that are most widely practiced for drinking water monitoring (Girones et al., 2010).

3.2.1. PCR

PCR can selectively amplify signature genes (i.e., gene markers) from target microorganisms. Observation of PCR products following agarose gel electrophoresis can serve to verify the presence/absence of target organisms. Assay specificity depends on the specificity of primers and the stringency of the PCR (Nocker et al., 2009). PCR may also be combined with some downstream genotyping techniques for genetic diversity exploration or species identification (Huang and Hsu, 2011). For example, phylogenetic analysis of cloned and sequenced PCR-amplified *Legionella* 16S rRNA genes revealed a large diversity of uncultured *Legionella* spp. in water from water treatment plants, including *L. bozemanii*, *L. worsleiensis*, *L. quateirensis*, *L. waltersii*, and *L. pneumophila* (Wullings and van der Kooij, 2006). Some studies applied endonuclease restriction enzyme digestion of PCR products for mycobacterial isolate identification (Cheunoy et al., 2005; Chimara et al., 2008). However, this technique requires establishment of restriction patterns for a collection of known mycobacterial species. If the restriction pattern is atypical, other cultural, biochemical, and enzymatic characterization may also be necessary for further identification (Falkinham et al., 2001). Other variations of PCR-based methods, such as repetitive sequence PCR (rep-PCR) fingerprinting and multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA), can also be used for detection of novel genotypes or matching OPPP isolates between environmental and clinical samples (Falkinham, 2010, 2011; Kahlisch et al., 2010; Sobral et al., 2011).

3.2.2. Quantitative PCR (q-PCR)

q-PCR is advantageous relative to traditional PCR in that it provides quantitative information and a lower detection limit. q-PCR has been extensively applied for OPPP monitoring for both environmental and clinical samples (Adrados et al., 2011; Ahmed et al., 2014; Bedard et al., 2015; Behets et al., 2007a; Beumer et al., 2010; Bonetta et al., 2010; Madarova et al., 2010; Morio et al., 2008; Qin et al., 2003; Radomski et al., 2010). Given its high reproducibility and quantitative capabilities, q-PCR has potential to be accepted as a standard method for pathogen monitoring. For instance, the Association Française de Normalisation (AFNOR) and the ISO have developed standards NF T90-471 and ISO/TS 12869:2012, respectively, for detection and quantification of *Legionella* spp. and *L. pneumophila* (AFNOR, 2015; ISO, 2012).

A variety of q-PCR assays for monitoring *Legionella* spp. and *L. pneumophila* in environmental samples have been established for research (Behets et al., 2007b; Nazarian et al., 2008; Wellinghausen et al., 2001) or commercial (Yaradou et al., 2007) purposes. Genus level target genes for *Legionella* include 16S rRNA (Wellinghausen et al., 2001; Wullings and van der Kooij, 2006) and 23S rRNA (Nazarian et al., 2008) genes, which contain regions conserved for all *Legionella* species; while the macrophage infectivity potentiator (*mip*) gene, associated with its virulence, is the most common biomarker for differentiating *L. pneumophila* (Behets et al., 2007b; Wellinghausen et al., 2001).

q-PCR assays for the *Mycobacterium* genus often target the 16S rRNA gene (Adrados et al., 2011; Radomski et al., 2010) or other housekeeping genes (e.g., heat shock protein 65 gene *hsp65* (Tobler et al., 2006)). In order to develop a reliable q-PCR assay to detect mycobacteria in water, Radomski (Radomski et al., 2010) tested 18 pairs of previously published primers *in silico* and in house, finally identifying 110F/I571R as the best candidate. Verification of the newly-developed q-PCR method demonstrated improved specificity, but lower sensitivity relative to two previously-published q-PCR methods. An increasing number of mycobacterial whole-genome data can facilitate identification of alternative genes for improved mycobacterial identification. A q-PCR method based on the *atpE* gene that codes ATP synthase subunit C was recently proposed for mycobacteria detection and quantification in environmental and clinical samples. This assay was demonstrated to provide adequate specificity and sensitivity, yielding results that correlated well with the Radomski assay (2010) (Radomski et al., 2013). q-PCR was also used to target species level mycobacteria in drinking water, including *Mycobacterium avium* complex (Chern et al., 2015; Whiley et al., 2015), *Mycobacterium intracellulare* (Chern et al., 2015), and *M. avium* subspecies paratuberculosis (Beumer et al., 2010; Chern et al., 2015; Rodriguez-Lazaro et al., 2005).

Commonly used q-PCR-targeted signature sequences for *P. aeruginosa* identification that have been applied to both clinical and environmental samples include regions of the 16S rRNA, 23S rRNA, gyrase subunit B (*gyrB*), exotoxin A (ETA), *oprl* and *ecfX* genes (Anuj et al., 2009; Lee et al., 2011a; Qin et al., 2003; Schwartz et al., 2006). However, these assays have been criticized for false-positive and/or false-negative detection of *P. aeruginosa*, likely owing to wide sequence variation among *P. aeruginosa* strains and frequent genetic exchange with other microorganisms (Anuj et al., 2009; Choi et al., 2013; Qin et al., 2003; Schwartz et al., 2006). As a result, some researchers have proposed that a combination of two targets is more suitable for increased specificity and sensitivity of *P. aeruginosa* identification (Anuj et al., 2009; Qin et al., 2003). For instance, Anuj developed a duplex qPCR assay targeting the *ecf*X and *gyr*B genes. Although this assay can provide simultaneous confirmation of positive results, presence of only one gene might require supplemental tests with a third gene, as a single positive result might represent either a cross-reaction with a non-*P. aeruginosa* species or otherwise a *P. aeruginosa* presenting sequence variation within one of the target gene regions (Anuj et al., 2009). With advances in DNA sequencing technology and availability of increasing whole genome data, novel targets for *P. aeruginosa* may yet be identified. Choi et al. (2013) recently took advantage of comparative genomic tools and developed a new q-

PCR assay targeting the O-antigen acetylase gene. The specificity of this assay was tested against 6 *P. aeruginosa* isolates, 18 different *Pseudomonas* species and 23 other reference pathogens (Choi et al., 2013). However, tests against environmental samples would be needed for further validate specificity considering variation of background microorganisms in different sample matrices.

There are two commonly applied q-PCR assays for *Acanthamoeba*, the Riviere (Riviere et al., 2006) and Qvarnstrom (Qvarnstrom et al., 2006) assays, both of which employ Taqman probes and target a fragment of the 18S rRNA gene at the genus level. These two assays are reported to have high specificity and a comparable detection limits when monitoring *A. castellanii* trophozoites in water and biofilm samples. However, the Qvarnstrom assay is reported to have higher positive detection rates and quantification numbers in terms of environmental sample monitoring (Chang et al., 2010).

Several q-PCR assays for *N. fowleri* have been developed in recent years to complement traditional culturing and MPN methods for detection in clinical and environmental samples. These assays generally target the 18S rRNA gene (Qvarnstrom et al., 2006), 5.8S gene and internal transcribed spacer (ITS) regions (Mull et al., 2013; Puzon et al., 2009) or the MP2Cl5 sequence (Behets et al., 2007a; Madarova et al., 2010). Puzon developed and applied a q-PCR assay for *N. fowleri* monitoring in water and biofilm samples, demonstrating higher sensitivity relative to culture methods in terms of spiked cell detection in biofilm and water (i.e., 5 cells in 250 ml water or biofilm) (Puzon et al., 2009). A recent study (Streby et al., 2015) systematically evaluated four previously published q-PCR assays in terms of their themodynamic stability, sensitivity, specificity, detection limits, humic acid inhibition effects, and performances with seeded environmental matrices by standardizing the reaction conditions. Results demonstrated that Qvarnstrom (Qvarnstrom et al., 2006) and Mull assays (Mull et al., 2013) have better performance in terms of detection and amplification limits, but lower specificity (93%); while the Puzon assay (Puzon et al., 2009) was reported to provide 100% sensitivity and specificity, but a relatively higher detection limit. In other cases, q-PCR assays followed by a melt curve analysis were used for discrimination of *N. fowleri* and closely-related *Naegleria* spp. or other free-living amoebae (Behets et al., 2006; Robinson et al., 2006).

Head-to-head comparison of q-PCR and culture for OPPP detection in water and biofilm samples has been the subject of multiple studies (Table 2), with the general trend being higher detection frequency and cell numbers by q-PCR (e.g., *Legionella* (Wang et al., 2012; Wellinghausen et al., 2001; Wullings et al., 2011), mycobacteria (Hussein et al., 2009; Radomski et al., 2013), *P. aeruginosa* (Bedard et al., 2015), and *N. fowleri* (Behets et al., 2007a)). This phenomenon is likely associated with the higher sensitivity and lower detection limit of q-PCR. However, overestimation of OPPP numbers by q-PCR cannot be ruled out, as DNA from dead and lysed cells (i.e., extracellular DNA) may also be amplified. This possibility may be particularly applicable to samples with high levels of disinfectant residual (Girones et al., 2010). In addition, presence of multiple or varying genome copy numbers may also result in overestimation of cell numbers for q-PCR assays based on an assumption of one genome per cell (Einen et al., 2008). Although q-PCR is not able to distinguish live/dead cells, systematic application of qPCR at multiple monitoring points to inform a mass balance is one approach to discerning information regarding the behavior of viable cells in premise plumbing, which is particularly applicable for systems experiencing disinfectant decay and microbial regrowth. Another solution to decrease false positives resulting from dead cells is to target a longer DNA fragment instead of shorter pieces (McCarty and Atlas, 1993) or pretreat with nucleic acid-binding dye (see section 3.2.3 for more details). Positive correlations have been observed between q-PCR and culturing for *Legionella* under some circumstances (Guillemet et al., 2010; Wellinghausen et al., 2001), indicating the potential of using q-PCR as an alternative method for OPPP monitoring. However, in other cases, no correlations were found (e.g., *Legionella* (Wullings et al., 2011); mycobacteria (Hussein et al., 2009)). Discrepancies between q-PCR and culturing may also be associated with different sample types and characteristics (e.g., the number of VBNC cells and/or dead cells in samples). For instance, an international trial involving six participating countries demonstrated less discrepancy of log mean *Legoinella* number between q-PCR and culturing of premise plumbing water samples compared to cooling tower samples (Lee et al., 2011b). Nonetheless, appropriate interpretation q-PCR numbers remains a major impediment to broader application of q-PCR results for OPPP risk management. There is a lack of concrete data showing the relevance of q-PCR numbers to referenced culturing numbers in terms of OPPP infection risks and action limits. Establishment of molecular baselines of OPPPs gene copy numbers in premise plumbing would be a valuable first step towards informing risk prediction (e.g., identify a "hotspot" site) and mitigation, which requires extensive sampling of different plumbing systems and inclusion of sufficient number of samples in order to build statistical confidence.

Culturing is still sometimes more effective for detecting low levels of target pathogens than q-PCR, since DNA extraction can result in substantial cell loss, while culturing, under the right conditions, can facilitate amplification of dilute cells. For example, one study isolated *Mycobacterium* spp. from 76% of tap water samples, while q-PCR only yielded about 21–36% detection rates, depending on the DNA extraction method (Radomski et al., 2013). Another study monitoring *N. fowleri* in water also recommended MPN for samples with low *N. fowleri* numbers (i.e., <200 cells/l) and suggested that DNA extraction and PCR volume limitations contributed to a high q-PCR quantification limit (Behets et al., 2007a). Moreover, the presence of PCR inhibitors may also affect q-PCR readings (Levi et al., 2003). For example, disinfectant residues present in drinking water sample can inhibit PCR amplification (Lee et al., 2011a). An alternative quantitative method, droplet digital PCR, which is based on water-oil emulsion droplet partition technology, is promising for reducing PCR inhibition and increasing assay sensitivity (Hunter et al., 2017), yet it has not been widely applied for OPPP tests.

3.2.3. Viable PCR/q-PCR

The major disadvantages of PCR/q-PCR lie in their inabilities to distinguish DNA from live or dead cells. This is problematic for samples with a large amount of dead cells, which is likely to occur in drinking water systems where disinfectants are present or high temperatures exist (e.g., water heaters). Therefore, development of a molecular method capable of selectively amplifying nucleic acids from viable cells would be extremely valuable.

PCR/q-PCR combined with nucleic acid-binding dye (e.g., ethidium monoazide bromide, propidium monoazide bromide) pretreatment is being explored for selectively monitoring viable OPPPs in drinking water. These intercalating dyes are intended to enter damaged cell membranes and covalently bind to DNA after photo activation, preventing downstream PCR amplification of DNA from membrane-compromised cells. Several studies have indicated promise of an EMA/PMA-based qPCR method for *Legionella* (Adela Yanez et al., 2011; Chen and Chang, 2010; Delgado-Viscogliosi et al., 2009; Inoue et al., 2015; Mansi et al., 2014; Slimani et al., 2012), mycobacteria (Lee et al., 2015; Nocker et al., 2007), *P. aeruginosa* (Gensberger et al., 2013, 2014), and *Acanthamoeba* (Chang et al., 2013) cells by testing against heat-treated, chlorine-treated cells and/or environmental water samples (e.g. drinking

water, spa water, swimming pool water) as controls. In general, EMA/PMA-qPCR reduces the detection rates compared to conventional qPCR, but demonstrates equal or higher detection rates and cell counts relative to culturing due to inclusion of VBNC cells (Delgado-Viscogliosi et al., 2009; Inoue et al., 2015; Mansi et al., 2014). The effectiveness of EMA/PMA-qPCR is associated with a variety of factors, such as dye selection and dosage (Chen and Chang, 2010; Yanez et al., 2011), incubation time (Yanez et al., 2011), sample types (Inoue et al., 2015), target-cell loads and background flora (Gensberger et al., 2013; Slimani et al., 2012), as well as characteristics of target microorganisms (e.g., bacterial or eukaryotic cells (Fittipaldi et al., 2011)). Therefore, optimization is essential in order to adapt protocols for each sample and/or microorganism type prior to application of EMA/PMA-qPCR. In addition, it has been reported that PMA-PCR/qPCR assays with longer amplicons (e.g., 400 bp) may lead to better suppression of dead-cell signal compared to short-amplicons (e.g., 100 bp), since PMA-induced damage is more likely to occur in longer amplicons (Contreras et al., 2011; Ditommaso et al., 2015). Importantly, intercalating dyes may be limited in their application to biofilms because they can become absorbed by extracellular matrix, while signal from dye that does penetrate can be drowned out by dominant non-target organisms (Taylor et al., 2014).

3.2.4. High throughput DNA sequencing

High throughput DNA sequencing, also called next generation sequencing, is a catch-all term describing a variety of technologies that allow rapid and simultaneous sequencing of millions of nucleic acid fragments (Shendure and Ji, 2008). High throughput sequencing is a powerful tool just starting to reveal the astounding diversity of microbial communities inhabiting drinking water distribution systems across the globe (Chao et al., 2015; Delafont et al., 2014; Hong et al., 2010; Ji et al., 2015), with tremendous potential for understanding the microbial ecology of premise plumbing. A typical procedure for drinking water microbiome investigation includes application of universal primers targeting 16S rRNA genes for DNA amplification followed by high throughput sequencing, the result of which could demonstrate the presence or absence of genera containing potential OPPPs (Delafont et al., 2014; Gomez-Smith et al., 2015; Ji et al., 2015; Wang et al., 2013). Reported abundances of sequences belonging to these genera have varied considerably across different samples. Typically, lower abundances have been observed for *Legionella* (e.g., 0–2.1% and 0–0.48% for *Legionella* spp. in drinking water (Wang et al., 2014) and biofilm samples (Wang et al., 2013), respectively) compared to *Mycobacterium* spp. (e.g., 25–78% in water main biofilm samples (Gomez-Smith et al., 2015); ~20% in pooled dataset of municipal drinking water samples from different U.S. cities (Holinger et al., 2014)). However, due to the short read length, high throughput sequencing based on 16S rRNA gene PCR products have limited taxonomic resolution, which inhibits the ability to differentiate species and therefore cannot verify detection of actual pathogens. Thus, more specific primers (e.g., mycobacterial functional genes) have recently been applied prior to deep sequencing in order to increase taxonomic resolution. PCR amplification of a 461-bp fragment of the mycobacterial heat shock protein (*hsp*65) gene followed by Illumina MiSeq analysis were applied in water main samples contacting different pipe materials, demonstrating varying abundances of *M. frederiksbergense*, *M. aurum*, *M. hemophilum*, and *M. lentiflavum* (Gomez-Smith et al., 2015). A newer approach to achieve high-resolution identification is metagenomic sequencing, which is achieved by direct sequencing of DNA extract fragments, without prior amplification of DNA or the requirement to select target genes. Metagenomic sequencing has now been successfully applied to recover two draft genomes of mycobacterial species inhabiting hospital hose biofilms (Soto-Giron et al., 2016). This method is promising for simultaneous detection of multiple OPPPs at species or even strain levels, although the feasibility of latter may sometimes be challenged by high conservation of gene contents in closely related strains (Tu et al., 2014). Also, metagenomics sequencing remains costly and it can be challenging to obtain the necessary sequencing depth to definitively identify rare members of the community, such as OPPPs. High throughput sequencing can also be applied for characterization of whole genome of OPPPs following cultivation. For instance, Gomez-Valero et al. (2014) sequenced *L. micdadei*, *L. hackeliae* and *L. fallonii* (LLAP10), comparing them with existing *Legionella* genome data, and noted surprisingly dynamic genomes due to a large mobilome mainly comprising the type IV secretion system. Further, sequencing OPPP genomes can also aid in characterizing the "pan-genome," or the critical subset of core genes defining pathogenic strains, which in turn can be applied towards improving molecular detection of true pathogens (Pruden et al., 2013). High throughput DNA sequencing is particularly promising for research applications and it is beginning to be successfully applied for strain confirmation in outbreak investigations (Raphael et al., 2016).

3.3. Phenotypic assays

"Phenotypic assays" generally encompass techniques that take advantage of properties such as cell morphological, biochemical, serological, and physiological traits. Here we mainly focus on immunoassays applied for OPPP detection in environmental samples.

Immunoassays for OPPPs typically rely on genus or species-specific monoclonal or polyclonal antibodies to recognize and quantify antigens. These assays can have various formats; such as lateral flow (Helbig et al., 2006), enzyme-linked immunosorbance (ELISA) (Reveiller et al., 2003), or immunochromatography (Helbig et al., 2006), and can be combined with other detection methods (e.g., microscopy (Baba et al., 2012), cytometry (Fuechslin et al., 2010), sensors (Bekir et al., 2015; Enrico et al., 2013)). For instance, anti-*L. pneumophila* and anti-*Legionella* antibodies have been widely used in a number of immunoassays to detect *L. pneumophila* of various serogroups and/or distinguish *L. pneumophila* from other *Legionella* species (Delgado-Viscogliosi et al., 2005; Fuechslin et al., 2010). In general, *Legionella* antibodies are conjugated with reporter enzyme or fluorescent tags prior to specific antibody-antigen recognition processes, signals of which are captured by colorimetric reactions (Helbig et al., 2006), microscopy (Baba et al., 2012), solid-phase cytometry (Parthuisot et al., 2011) or flow cytometry (Tyndall et al., 1985). In addition, immunomagnetic separation assays, in which magnetic beads or particles are covered with antibodies, have been used to magnetically recover or concentrate *Legionella* (Bedrina et al., 2013). Recovered cells can be subjected to downstream analysis, such as q-PCR (Mull et al., 2013) or flow cytometry (Fuechslin et al., 2010; Keserue et al., 2012). Furthermore, co-staining with cell viability markers allows simultaneous detection of target microorganisms and live/dead discrimination. These techniques have recently been applied for live *Legionella* monitoring in drinking water samples (Delgado-Viscogliosi et al., 2005; Keserue et al., 2012). Other reported immunoanalytical methods include construction of miniaturized immunobiosensors (e.g., optic biosensor, impedimetric biosensor) with immobilized antibodies to provide rapid screening and on-site measurement of *L. pneumophila* and *P. aeruginosa* in water (Bekir et al., 2015; Enrico et al., 2013).

Advantages of immunoassays include rapid turnaround time (e.g., ~20 min to several hours), high specificity and sensitivity, and overall cost effectiveness (Lesnik, 2000). However, their applicability for premise plumbing remains challenged by the low

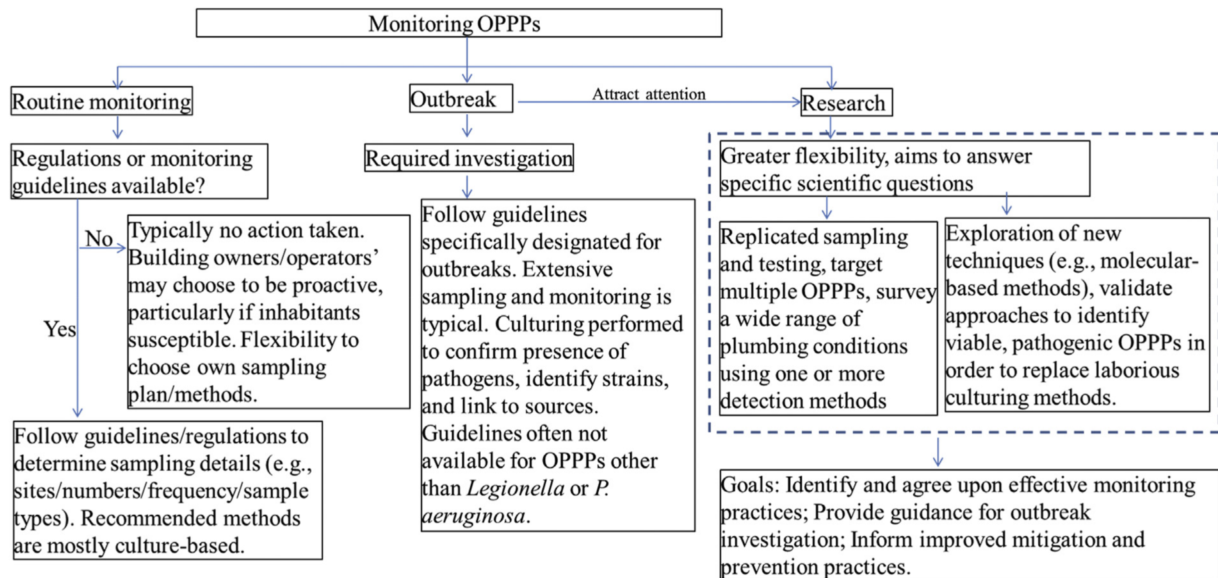

Fig. 2. Framework for selecting appropriate methods for OPPP monitoring in premise plumbing.

concentration of OPPPs and the need for highly specific and robust antibodies. Currently available antibodies might not be able to cover the whole range of serogroups or species (Keserue et al., 2012), while validation of specificity and sensitivity in environmental samples, which are inherently more complex and subject to interferences than clinical samples, is often lacking (Toriyama et al., 2015; Visvesvara et al., 1987).

4. Selecting appropriate methods for OPPP monitoring

A framework for guiding methodology selection for various purposes is proposed in Fig. 2. Appropriate methodologies for OPPP monitoring are dependent upon the intended goal. Given the high level of resolution needed to distinguish a true pathogen from related, non-pathogenic organisms, culture-based methods are likely to remain the gold standard, particularly for outbreak investigation requiring confirmation of virulent species. However, in the context of risk prevention, risk mitigation, and rapid screening during outbreaks to identify likely contaminated sites, molecular- and physiological-based methods hold the promise of supporting more extensive, timely, and economical evaluation of problematic sites that could be followed-up upon with culturing. Such an approach could help overcome challenges of developing and applying comprehensive and representative monitoring plans for complex premise-plumbing systems. For example, although regular application of q-PCR for monitoring *L. pneumophila* would not directly provide information about live/dead status, it can provide valuable characterization of the baseline and help identify "hot spots" that could be acted upon with culturing when there is suspicion of a problem. Essentially, such an approach could serve as a molecular "indicator", pinpointing the potential OPPP infection risks in PCR-positive sites. Threshold gene copy numbers indicative of risk have been proposed in some studies and would be valuable for guiding risk management towards a reasonable "non-zero" target (Lee et al., 2011a, 2011b; Schoen and Ashbolt, 2011; Schwake et al., 2016). Approaches for improving the ability of molecular methods for distinguishing live/dead and pathogenic status are currently under development.

In the context of research, molecular-based methods, particularly next-generation DNA sequencing, is beginning to revolutionize the understanding of OPPP microbial ecology in plumbing systems. As OPPP whole genomes continue to be sequenced, new insight is being gained into exactly what differentiates pathogenic from non-pathogenic forms. Thus, molecular-based methods can serve to identify improved markers for highly specific monitoring of virulent OPPP strains. Molecular methods will also improve understanding of their microbial ecology, including identification of key relationships and interactions that could be exploited for improved engineering control. In particular, the relationship between biofilm and bulk water and the role and importance of amoebal hosts in influencing replication and virulence of OPPPs would be extremely valuable. Eventually, molecular-approach based research would be a great help to identification of improved means for OPPP monitoring and control.

5. Conclusions

OPPPs pose a unique challenge to monitoring given that they do not correspond to fecal indicators and they establish as part of the native microbiota of premise plumbing systems, which are highly complex and variable from site to site. This review critically evaluates currently available approaches for OPPP sampling and monitoring with regard to their advantages, limitations and applicability under premise plumbing conditions, and identifies several research gaps towards improved OPPP monitoring practices. The foremost research need is to develop unified approaches to OPPP monitoring and detection, especially in the context of improving understanding of their behavior and informing proactive risk prevention, outbreak response, and effective monitoring and control practices. Identification and agreement upon effective monitoring practices, especially for multiple OPPPs, is vital to effective action towards development of policy aimed at preventing OPPP proliferation beyond the property line and protecting public health.

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