Review

Determination of viable legionellae in engineered water systems: Do we find what we are looking for?

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A B S T R A C T

In developed countries, legionellae are one of the most important water-based bacterial pathogens caused by management failure of engineered water systems. For routine surveillance of legionellae in engineered water systems and outbreak investigations, cultivation-based standard techniques are currently applied. However, in many cases culture-negative results are obtained despite the presence of viable legionellae, and clinical cases of legionellosis cannot be traced back to their respective contaminated water source. Among the various explanations for these discrepancies, the presence of viable but non-culturable (VBNC) *Legionella* cells has received increased attention in recent discussions and scientific literature. Alternative culture-independent methods to detect and quantify legionellae have been proposed in order to complement or even substitute the culture method in the future. Such methods should detect VBNC *Legionella* cells and provide a more comprehensive picture of the presence of legionellae in engineered water systems. However, it is still unclear whether and to what extent these VBNC legionellae are hazardous to human health. Current risk assessment models to predict the risk of legionellosis from *Legionella* concentrations in the investigated water systems contain many uncertainties and are mainly based on culture-based enumeration. If VBNC legionellae should be considered in future standard analysis, quantitative risk assessment models including VBNC legionellae must be proven to result in better estimates of human health risk than models based on cultivation alone. This review critically evaluates current methods to determine legionellae in the VBNC state, their potential to complement the standard culture-based method in the near future, and summarizes current knowledge on the threat that VBNC legionellae may pose to human health.

Contents

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1. The importance of legionellae for public health

Legionellae are ubiquitously present at low concentrations in natural aquatic ecosystems (Parthuisot et al., 2010). Due to their sessile mode of life and their preference for temperatures above 25 °C, man-made engineered water systems often select for legionellae, if they are not adequately managed. Among the more than 50 described *Legionella* species, *Legionella pneumophila* is one of the most important water-based bacterial pathogens in developed countries. Legionellae predominantly cause two kinds of respiratory tract infections, the severe and potentially fatal Legionnaires' disease and the mild, non-fatal, influenza-like illness Pontiac Fever (Hornei et al., 2007). Different serogroups (*L. pneumophila* SG 1, 6, 7) and species (*Legionella micdadei, Legionella feeleii, Legionella anisa*) have been reported to cause Pontiac Fever, but the underlying mechanisms that are responsible for causing either Pontiac fever or Legionnaires' disease have not been elucidated so far (Tossa et al., 2006; Wang et al., 2015). With the exception of Australia, New Zealand and Japan, where *Legionella longbeachae* infections can occur as often as cases of *L. pneumophila* infection (Whiley and Bentham, 2011), the majority (>95%) of all notified cases of Legionnaires' disease has been caused by *L. pneumophila* strains, most of them (>85%) belonging to serogroup 1 (ECDC, 2015). Especially Mab 3/1 positive strains — determined according to the Dresden panel (Lück et al., 1992) — and specific sequence types (ST) — determined according to the seven-gene sequence based typing scheme for *L. pneumophila* (Ratzow et al., 2007) — are more frequently involved in Legionnaires' disease, such as ST1, ST23, ST37, ST47, and ST222. This varies slightly among different countries (Cassier et al., 2015; Harrison et al., 2009; Kozak-Muiznieks et al., 2014). For the European Union and Norway, 5844 notified cases of Legionnaires' disease were recorded in 2013, corresponding to an average notification rate of 1.14 per 100,000 population (ECDC, 2015). Similar rates (1.15 per 100,000 population) have been reported in the United States of America for 2009 (CDC, 2011). An increasing trend in notification rates was observed in many countries until the end of the last decade (CDC, 2011; ECDC, 2015). Potential reasons for this increase were mainly (i) a continuous increase in engineered water systems suitable for *Legionella* growth like cooling facilities, (ii) the ageing society in developed countries as aged people are more vulnerable to *Legionella* infection and (iii) improved diagnosis and reporting. In any case, it was estimated that the number of notified Legionnaires' disease cases is just the tip of the iceberg and that the true incidence rates could be 10× (Parr et al., 2015) to 20× higher (Marston et al., 1997), in addition to the far more underreported Pontiac Fever (Parr et al., 2015).

2. Standard methods to detect and quantify legionellae in water

2.1. Cultivation dependent methods

Standard culture based methods are usually used on a routine basis to detect and quantify legionellae in engineered water systems suspected to harbour high concentrations of these bacterial pathogens (ISO, 1998). Following the standard method, 1000 ml of water (in case of hot water systems) or down to 100 ml (in case of cooling towers) is either concentrated on 0.22 μm or 0.45 μm pore-size polycarbonate or nylon filters, subsequently sonicated and resuspended in 5–25 ml of sterile diluent. Alternatively, 200 ml of sample is centrifuged and the pellet is resuspended in 2–20 ml sterile diluent. Only if the number of *Legionella* is expected to exceed 10^5 per litre, direct plating of liquid is permissive. Aliquots of the concentrate are treated with heat (30 min at 50 °C) and/or acid (buffered 0.2 M HCl for 5 min) in order to reduce background microbiota, in comparison to an untreated control. After seven to ten days of incubation at 36 °C on (GVPC) agar (buffered charcoal yeast extract (BCYE) agar plus supplements glycine, vancomycin, polymyxin B and cycloheximide), representative presumptive colonies (three per plate) are streaked on both BCYE and BCYE agar without cysteine, and checked for growth after 2 days at 36 °C. Alternative to BCYE agar without cysteine, blood-agar or nutrient agar can be used. Those colonies can be regarded as *Legionella* that grow on BCYE but fail to grow on cysteine free medium. Identification of *Legionella* species and serogroups may then be performed by a variety of methods (see ISO 11731:1998). Aside from the fact that this standard method needs up to 14 days for analysis, it often fails to detect legionellae in water samples despite the presence of viable cells (Kirschner et al., 2012; Parthuisot et al., 2011). Moreover, in many instances, clinical cases of legionellosis cannot be traced back to their respective contaminated water sources because no culturable environmental legionellae can be found. In the most recent report on Legionnaires' disease in Europe (ECDC, 2015) it

was stated that for four of the ten largest Legionnaires' disease clusters in Europe, no source could be identified, and that in 57% of 624 environmental investigations, no source of legionellae could be found. In only 9% of the 43% positive findings could environmental isolates be matched to clinical isolates. Several reasons might be responsible for the discrepancy between clinical cases and negative environmental findings: (i) there are too many potential sources and not all of them can be analysed; (ii) sources are not considered as a potential source as they are, for example, too far away from the site of infection (like cooling towers or air scrubbers spreading contaminated aerosols over several kilometres (Nygard et al., 2008; White et al., 2013)) or they are not recognised as a source because of missing knowledge (like in a recent outbreak in Spain caused by an asphalt paving machine (Coscolla et al., 2010)); (iii) a heterogenous *Legionella* population is present and the causative strain is not detected; (iv) the number of legionellae is too low to be detected by the standard procedure but the strain is highly virulent even at low numbers; (v) competitive microbiota inhibit growth of *Legionella* on the standard agar plate despite heat and acid treatment; and finally, (vi) the presence of viable but non-culturable (VBNC) *Legionella* cells may prevent successful detection with the culture based method, a topic that has been receiving increased attention in recent discussions and scientific literature (Ducret et al., 2014; Mustapha et al., 2015; Wingender and Flemming, 2011).

2.2. Cultivation independent methods

The only culture independent method that has achieved the status of a standard is quantitative real-time PCR. This standard NF T90-471:2010 was developed in France (AFNOR, 2010) and has been translated to ISO technical specification pre-standard ISO/TS 12869 (ISO, 2012). The procedure is based on water sample filtration, DNA extraction and real-time PCR quantification of *Legionella* spp. or *L. pneumophila* in clear water samples and has been carefully validated recently for spiked distilled, tap and sulphurous water by Omiccioli and co-workers (Omiccioli et al., 2015). The sample limit of detection (SLOD) was 100 genomic units (GU) per L, the sample limit of quantification (SLOQ) was 500 GU/L, when one litre of sample is analysed. Recovery rates depended on the water matrix and ranged from 38 to 75%. Due to the fact that according to ISO/TS 12869, a recovery rate >25% is needed, this method may not be equally suitable for cooling towers or other complex matrices, where high concentrations of substances that impact DNA extraction and inhibit PCR may be expected. On the market, there are several qPCR kits available for both *Legionella* spp. and *L. pneumophila* that have been validated in accordance to ISO/TS 12869 or NF T90-471:2010 and that achieve similar performances with respect to SLOD (~100 GU/L) and SLOQ (~500 GU/L). Among those are kits from Biorad (iQ-check; Biorad, Hercules, CA; (Ditommaso et al., 2015)), Minerva Biolabs (AquaScreen; Minerva Biolabs, Berlin, Germany), Pall (GeneDisc; Pall, New York, USA; (Lee et al., 2011)) or Diatheva (Diatheva, Fano, Italy; (Omiccioli et al., 2015)). It was reported that qPCR results were on average 28 times higher than results obtained by standard cultivation, with a range of 1—2 logs (Ditommaso et al., 2015; Lee et al., 2011). A recent review summarizing the results from 28 studies published between 2003 and 2013 pointed out that on a sample per sample basis, environmental water samples analysed concurrently by qPCR and culture were approximately 50% more likely to return a positive result by qPCR, with 2856/3967 (72%) of all samples positive by qPCR and 1331/3967 (34%) of samples positive by culture (Whiley and Taylor, 2014). Such large discrepancies are not surprising, as it is well known that — in contrast to cultivation — qPCR detects cells in the VBNC state but also detects dead cells and free DNA.

3. The VBNC state of legionella

There is on-going controversy about the VBNC state (Nystrom, 2003; Pinto et al., 2015) since its discovery in 1982 (Xu et al., 1982). Now, most researchers agree that the VBNC state can be regarded as a survival strategy of mostly Gram negative bacteria in response to stress (Li et al., 2014), comparable to spore formation of Gram positives (Roszak and Colwell, 1987). Accordingly, VBNC cells can be defined as living cells that have lost their ability to grow on routine media, on which they normally grow (Oliver, 2000). This definition is especially appropriate for bacterial pathogens like *Legionella pneumophila* as it separates them from the vast bulk of non-cultured bacterial species in the environment, for which no culturability has been proven so far. In this context, it has to be mentioned that within the *Legionella* genus, there are species that are obligate intracellular amoeba endosymbionts like *Legionella jeonii* (Park et al., 2004) that cannot be cultured on GVPC agar plates, and that can be considered as VBNC cells. However, as they fall out of Oliver's VBNC definition (Oliver, 2000) and because they are not relevant for human health, they were not specifically considered in this review. In contrast, the former *Legionella* like amoebal pathogens (LLAPs) like *Legionella fallonii*, *Legionella rowbothamii* or *Legionella drozanskii* (Adeleke et al. 2001) that are not culturable routinely on GVPC agar plates, have been related to human respiratory disease (Adeleke et al., 2001) and they have been frequently found in various water systems by culture independent methods (Huang et al., 2011; Wery et al., 2008). Despite not falling into Oliver's VBNC definition, they are potentially relevant for human health and their detection in engineered water systems would thus be desirable.

3.1. Stress induces the VBNC state of legionellae

If legionellae are exposed to stress (e.g. starvation, chemical disinfectants, UV light, or heat) they may switch to the VBNC state unless they die. It was claimed by Ducret et al. (2014) that during stress exposure, three different types of VBNC *Legionella* cells are produced. The first one is the damaged VBNC type, in which the cells are still alive but on the way to death and are thus not of public health concern. The second type is the injured VBNC type, in which the cells are injured to varying degrees and artificially lose their ability to grow on the culture medium. After repair of injury, they may resuscitate and thus they are of potential public health concern. The third type is actually the one that corresponds most to the above definition; these cells undergo a purposeful physiological adaptation to the exposed stress that requires a coordinated change in gene expression. From this state, they are resuscitable after the release from stress and, thus, they are of potential public health concern.

Until now, the pyramid of physiological processes and their underlying genetic or epigenetic regulation in response to different stressors has not been well known in *Legionella*. It has been shown that under nutrient deprivation, *L. pneumophila* accumulates the small non-coding RNA (p)ppGpp, a well-known pan-bacterial regulator (Wu and Xie, 2009) to fully induce a stringent response. In addition to relA and SpoT, two enzymes known to synthesize ppGpp in *L. pneumophila* in response to distinct nutrient conditions (Robertson et al., 2014), the sigma factor RpoS is a key factor of the regulatory network of the stringent response (Trigui et al., 2015). In contrast to starvation, other stressors may induce different physiological and regulatory responses and it is highly probable that different stresses may lead to different types of VBNC cells. The ability of *Legionella* cells to resuscitate from these different types of VBNC states may also be quite different and may require very specific conditions.

Table 1
Overview of the most important stressors and representative conditions that were shown to push Legionella into the VBNC state. Additional potential stressors are also listed.

Stressor	Conditions	Effects	Reference
Starvation	20 °C, tap water, 125 days	Resuscitation in Acanthamoeba castellani	(Steinert et al., 1997)
	45 °C, tap water, 45 days	20—69% VBNC cells	(Al-Bana et al., 2014)
	45 °C, distilled water, 18—30 days	5—87% VBNC cells	
Chemical disinfectants	Monochloramine 1 mg/L	30% VBNC after 15 days	(Alleron et al., 2008)
	Sodium hypochlorite 128—1024 ppm	Resuscitation in Acanthamoeba polyphaga	(Garcia et al., 2007)
	Bromo-nitropropandiol shock + isothiozolinone 1 mg/L/day	Regrowth of L. pneumophila in system observed with FISH	(Kirschner et al., 2012)
Heat treatment	70 °C, 30 min	10—25% VBNC, resuscitable in amoeba	(Allegra et al., 2008)
	70 °C, 30 min	Resuscitable in amoebae, infecting macrophage-like cells	(Epalle et al., 2015)
Other microbes	Acidovorax, Sphingomonas	Antagonistic effect on culturability, but not on viability	(Giao et al., 2011)
	Vermamoeba vermiformis	Antagonistic effect on culturability	(Buse et al., 2013)
UV irradiation	50—300 mJ/cm^2	reported for E.coli and P. aeruginosa, but doubted by experts	(Zhang et al., 2015) (Gehr, 2015) (Linden et al., 2015)
Metal ions	Copper, 10 μM, 24 h at 20 °C	shown for P. aeruginosa, 100% VBNC cells	(Dwidjosiswojo et al., 2011)
Ultrasound	Different conditions may apply	Not shown so far for legionellae	(Declerck et al., 2010)

The most important kinds of stress legionellae may experience during their life in engineered water systems are starvation, the presence of chemical disinfectants, and exposure to heat and UV light, but other stressors may also occur (Table 1).

3.1.1. Starvation

Bacteria have developed several strategies to cope with unfavourable environmental conditions like nutrient scarcity. In response to living in oligotrophic environments like (drinking) water distribution systems, a sessile mode of life and the formation of biofilms favour growth and survival of bacteria (including bacterial pathogens like *Legionella*) due to several benefits including, among others, inter-species communication, nutrient scavenging or nutrient digestion by exoenzymes (Flemming, 2015). Additionally, the transition to the VBNC state can be regarded as a purposeful physiological adaptation (see above (Ducret et al., 2014),) and survival strategy of (mostly) Gram-negative bacteria in response to nutrient starvation. The VBNC state of pathogenic bacteria may thus be "induced" in biofilms in oligotrophic drinking water systems (Wingender and Flemming, 2011).

Steinert et al. (1997) were the first who reported successful resuscitation of long-term starved *L. pneumophila* in *Acanthamoeba castellanii*. The time planktonic *Legionella* cells need to enter into the VBNC state during starvation is dependent on the water matrix and temperature. *L. pneumophila* cells suspended in distilled water entered the VBNC state more quickly (18—30 days) than when suspended in tap water (45 days) (Al-Bana et al., 2014). Temperatures lower than the 45 °C used in those experiments prolong the time needed to become VBNC (own, unpublished data). Comparing different *Legionella* strains and species, it could be shown that there are huge differences between the strains and that the time needed to become VBNC also depends on the growth status of the cells (own, unpublished data).

3.1.2. Chemical disinfection

Mono-chloramine was reported to quickly push *L. pneumophila* into the VBNC state (Turetgen, 2008). It was shown that above concentrations of 1 mg/L of mono-chloramine, about 30% of viable cells were still observed after 15 days in a biofilm, while no culturable cells could be detected (Alleron et al., 2008). Garcia et al. (2007) reported resuscitation of various VBNC *Legionella* strains in *Acanthamoabe polyphaga* after treatment with sodium hypochlorite. In a hospital cooling tower, a combination of biocides led to the immediate loss of culturability within a population of

6×10^5 CFU/L *L. pneumophila* (Kirschner et al., 2012). In contrast, the concentration of *L. pneumophila* cells, detected with fluorescence in-situ hybridization (FISH) in combination with epifluorescence microscopy (EFM), followed the pattern of biocide addition over several months. From this it was concluded that viable *L. pneumophila* cells were present throughout the investigation period and only the doubling of the initially used biocide concentrations was capable of reducing *L. pneumophila* concentrations down to undetectable levels (Kirschner et al., 2012).

3.1.3. Heat treatment

Temperature treatments of 70 °C are often used to combat *Legionella* colonization of hot water systems (Kim et al., 2002; Lin et al., 2011; Marchesi et al., 2011; Mouchtouri et al., 2007; Stout et al., 1986; Tablan et al., 2003). At this temperature, legionellae usually lose their culturability (Allegra et al., 2011b; Cervero-Arago et al., 2015; Stout et al., 1986). However, it was shown recently that different *L. pneumophila* strains were able to be resuscitated in amoebae after exposure to 30 min of heat (70 °C) (Allegra et al., 2008; Epalle et al., 2015). After resuscitation, they were able to infect macrophage-like or alveolar epithelial cells (Epalle et al., 2015). Only after 60 min of exposure, no viable and infectious cells were observed. Lower temperatures between 60 and 70 °C may also push legionellae into the VBNC state, but the time needed may be significantly longer to achieve unculturability.

3.1.4. UV-disinfection

No studies are so far available on the ability of UV disinfection to push *Legionella* cells into the VBNC state. Curiously enough, UV disinfection can be regarded as "the" method to produce VBNC cells, as it only harms the DNA preventing correct replication and transcription (Sinha and Hader, 2002). Thus, cellular functions may be kept for a variable time and during this time period, the cell can be regarded as viable but non-culturable. If the cell is damaged in a way that cellular functions stop completely and cell death occurs, these cells belong to the damaged VBNC type, after the definition of Ducret et al. (2014) and do not have any consequences for human health. If the UV-irradiated cells, instead, are able to repair and resuscitate, they will be of potential importance for human health. Such a scenario was recently reported for *Pseudomonas aeruginosa* and *Escherichia coli* (Zhang et al., 2015) and may in principle also occur with legionellae, despite that some doubts were expressed on the appropriateness of the study design (Gehr, 2015; Linden et al., 2015).

3.1.5. Other stress factors

Interestingly, the interaction with other microbes has also been shown to impact the culturability of *Legionella* cells. The two aquatic bacterial genera *Acidovorax* sp. and *Sphingomonas* sp. had an antagonistic effect on *L. pneumophila* culturability but not on their viability (Giao et al., 2011) as well as the culture supernatants of the amoebal host *Vermamoeba* (previously *Hartmannella*) *vermiformis* (Buse et al., 2013). Another potential stress that legionellae may experience is ultrasound (Declerck et al., 2010), but so far, no publications exist that demonstrate its potential to push legionellae into the VBNC state. Copper ions were shown to effectively induce the VBNC state in *P. aeruginosa* (Dwidjosiswojo et al., 2011), but have remained untested so far for legionellae.

3.2. The different life forms of legionellae result in different VBNC cell types

Legionellae are highly pleomorphic and exhibit a variety of different life forms (Robertson et al., 2014). In the extracellular environment like in a nutrient rich medium or a biofilm, actively replicating cells (not infectious, exponential phase forms) and stationary phase forms (non-replicating, infectious to host cells) can be distinguished (Byrne and Swanson, 1998). Likewise, in the intracellular environment of an amoeba or human macrophage, legionellae exist as a replicative phase form that is actively replicating in the *Legionella* containing vacuole and, once nutrients are depleted, differentiate into a mature infectious form (MIF) in amoebae (Garduno et al., 2002) or into an immature intracellular form in macrophages (Abdelhady and Garduno, 2013). A filamentous form was also described as early as in 1978 by Rodgers et al. (Rodgers et al., 1978) that can be present in response to various stresses extra- and intracellularly and that is infectious to host cells (Prashar et al., 2012). Additionally, *Legionella* cells may be ingested by ciliates like *Tetrahymena* sp. and expelled as unique developmental forms packaged in free spherical pellets (Berk et al., 2008). Moreover, among the many of these developmental forms, intermediate forms have been postulated and all of the described forms are in principle able to produce different VBNC cells (Robertson et al., 2014). Until now, it could be shown that VBNC cells derived from MIFs and stationary phase forms are morphologically distinct. Using an electron microscope, VBNC cells derived from MIFs showed an electron dense cytoplasm and an intact outer membrane, in contrast to VBNC cells derived from stationary phase forms, that had only one or two electron dense spots in the cytoplasm and an outer membrane producing vesicles (Al-Bana et al., 2014).

4. Methods to proof the viability of VBNC legionellae

In routine analysis, when looking for legionellae in a water sample, we want to determine the exact number of *Legionella* cells that are (i) living, (ii) able to proliferate, and (iii) infectious to humans. The cultivation-based approach is theoretically an excellent approach that is quantitative and able to fulfil all three requirements. When cells have proliferated to a colony of visible size they are counted and their infectious potential may then be determined either by species/serogroup specification, by infectivity testing or by the detection of specific virulence factors (e.g. via PCR). As outlined in detail above, a major disadvantage of cultivation for the detection of pathogens is that it overlooks cells that are viable, potentially proliferating and virulent, but non-culturable after stress. Alternative methods to proof viability of *Legionella* cells in water samples are therefore needed.

4.1. Resuscitation

Resuscitation of VBNC cells in amoebae has been demonstrated several times and is a perfect proof of viability, proliferation and infectivity (Ducret et al., 2014; Garcia et al., 2007; Hwang et al., 2006; Steinert et al., 1997). However, it is time-consuming and generally not performed on a routine basis. Interestingly, no successful resuscitation and growth of VBNC legionellae have been demonstrated so far in human cell lines or animal models (Ducret et al., 2014; Epalle et al., 2015) but only few studies have been reported. It has to be stated here, that resuscitation could be understood as both regaining the capability of growing on agar plates or/ and intracellular multiplication in amoebae or human cells, but this is still a matter of discussion.

4.2. Cell-based approaches

4.2.1. Membrane integrity

A frequently used unspecific cell-based approach to proof viability of bacteria (including legionellae) is the test for membrane

Table 2
Cell-based and nucleic acid based approaches to prove the viability of bacterial cells. The majority of the methods has already been applied for legionellae. For abbreviations see text.

Target	Method	Detection platform	Reference
Cell-based methods			
Membrane integrity	Sybr Green/PI	FCM	(Berney et al., 2008)
	Syto9/PI (BacLight® kit)	EFM	(Alleron et al., 2008; Stocks, 2004)
		FCM	(Allegra et al., 2008)
	Thiazole Orange/PI (Cell viability® kit)	FCM	(Alsharif and Godfrey, 2002)
Membrane potential	DiBac4	FCM	(Wang et al., 2010)
Enzyme activity	CFDA	EFM	(Yamamoto et al., 1996)
	Chemchrome V6	FCM	(Wang et al., 2010)
		SPC	(Parthuisot et al., 2011)
		EFM	(Delgado-Viscogliosi et al., 2005)
16S-rRNA	FISH	EFM	(Wilks and Keevil, 2006)
	CARD-FISH	EFM	(Kirschner et al., 2012)
Aminoacid uptake	MAR-FISH	EFM	(Alonso, 2012)
Electron transport system	CTC	EFM	(Creach et al., 2003)
Nucleic acid based methods			
DNA	EMA-qPCR	qPCR	(Delgado-Viscogliosi et al., 2009)
	PMA-qPCR	qPCR	(Ditommaso et al., 2014)
pre-rRNA	RT-qPCR	qPCR	(Cangelosi and Meschke, 2014)
mRNA	RT-qPCR	qPCR	Not performed so far for legionellae

integrity. A permanently compromised membrane is thought to be a clear sign of (near) death, as both the outer and the inner membrane of Gram-negative bacteria have indispensible vital functions, such as protection against unwanted external influences, mediation of substrate and nutrient transport processes via trans-membrane proteins, allocation of components of the electron transport chain and oxidative phosphorylation, and as attachment sites for chromosomal and plasmid DNA via the cytoskeleton structure (Strauber and Muller, 2010). The most commonly applied substrate that is used to show damaged bacterial cell membranes is propidium iodide (PI). It is a hydrophilic cationic molecule that is impermeant for intact membranes, but penetrates compromised membranes and binds to the DNA of those cells (Strauber and Muller, 2010) resulting in orange to red fluorescence when excited with 488 nm wavelength light. It has been used in combination with DNA dyes that enter all cells (cells with and without intact cell membranes) like Syto 9 or Sybr Green to distinguish between dead and viable *Legionella* cells either by EFM (Alleron et al., 2008) or flow cytometry (FCM) (Allegra et al., 2008; Keserue et al., 2012a) (Table 2). Several kits of various companies are available, e.g. the BacLight kit (Life-Technologies, Thermo Fisher, Waltham, MA, USA; (Stocks, 2004)) or the cell viability kit (Becton Dickinson, New Jersey, USA; (Alsharif and Godfrey, 2002)) combining PI with thiazole orange (Table 2). For reliable application, the protocol has to be specifically adapted in terms of incubation time and stain concentration and the appropriate controls have to be selected. Once a suitable protocol is set up, this method works well for starved (Chaiyanan et al., 2001; Trigui et al., 2015), heat-inactivated (Allegra et al., 2008; Nocker et al., 2011) or chemically disinfected cells (Alleron et al., 2013), but it is not a suitable technology for proving the viability of UV-disinfected cells, as UV disinfection does not harm the cellular integrity of the cell (see above).

4.2.2. Membrane potential

Similar to the test for membrane integrity, the green fluorescent probe bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC$_4$) is used to monitor the membrane potential of cells. The stain enters the cells only when the membrane potential is lost and it has been proven as a robust indicator of viability in cells (Jepras et al., 1995). It was used in combination with flow cytometry for different bacterial cells (Berney et al., 2008; Jepras et al., 1995) and has been applied once for the detection of viable *Legionella beliardensis* (Wang et al., 2010). There, it was concluded that that DiBAC$_4$-staining was not an optimal cell viability indicator for *Legionella* cells, because a high percentage of positive signals were already obtained with the viable control samples (Wang et al., 2010) (Table 2). Nevertheless, more *Legionella* species, especially *L. pneumophila*, and more experimental conditions have to be tested to judge the applicability of this stain. As above, it has to be considered, that this method is most probably not suitable for proving the viability of UV irradiated cells.

4.2.3. Intracellular enzyme activity

The detection of esterase activity is another widely used unspecific cell-based approach to prove the viability of bacterial cells. The most common substrates are carboxyfluorescein diacetate (CFDA; Life-Technologies, Thermo Fisher, Waltham, MA, USA) and Chemchrome V6, another modified fluorescein acetate (Biomerieux, Marcy l'Etoile, France). Both dyes are cell-permeant esterase substrates that indicate both enzymatic activity, which is required to activate their fluorescence, and cell-membrane integrity, which is required for intracellular retention of their fluorescent product. Upon hydrolysis by intracellular esterases, the substrate is converted by intracellular esterases into fluorescein that has a net negative charge at neutral pH (Parthuisot et al., 2000). CFDA

(Yamamoto et al., 1996) and Chemchrome V6 have been successfully applied for the determination of viable legionellae via EFM (Alleron et al., 2013, 2008; Delgado-Viscogliosi et al., 2005), flow cytometry (Wang et al., 2010) and solid phase cytometry (SPC) (Parthuisot et al., 2011) (Table 2). It can be considered to be a good indicator of viability during starvation and after heat or chemical treamtent, but not after UV disinfection.

4.2.4. Fluorescence in situ hybridization (FISH)

FISH is used to detect specific microbial taxa in complex environmental samples. Protocols for the specific detection of *L. pneumophila* and *Legionella* spp. in water and biofilm samples have been developed (Declerck and Ollevier, 2006; Declerck et al., 2003; Wilks and Keevil, 2006). In addition, 16S rRNA targeted FISH has been described as a suitable viability testing technique (Karner and Fuhrman, 1997). As reported more recently, *Legionella* numbers detected with catalysed reporter deposition-FISH (CARD-FISH) — an enhanced FISH to detect cells with low ribosome content — followed by the biocide treatment profile in a hospital cooling tower indicated that those cells were mostly viable (Kirschner et al., 2012) (Table 2). In contrast, after UV disinfection, FISH alone was not suitable for monitoring viability, and only a combination of FISH and cell elongation in response to nutrients in the presence of an inhibitor of cell division had the ability of differentiate life from dead cells (Villarino et al., 2000). There are also other conceivable scenarios after heat or chemical treatment, where the rRNA is still intact but the cells have compromised cell membranes, so that considering rRNA targeted FISH alone as a proof of viability of legionellae seems dubious.

4.2.5. Other cell based approaches

Other cell-based assays to detect viability of bacteria at the single cell level are the uptake of radiolabelled substrates like ^{3}H-leucine via micro-autoradiography (MAR) (Alonso, 2012) or the proof of the activity of the electron transport system via the uptake of the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Rodriguez et al., 1992). In contrast to MAR, which is a rather sophisticated technique, CTC is easy to perform and is a good estimator of cell viability (Creach et al., 2003) (Table 2). Until now, these two methods have not been intensively used for the assessment of viability of *Legionella* cells.

4.3. Nucleic acid based approaches

Viability PCR (v-PCR) is a recently developed approach with the aim to detect and quantify viable bacteria in environmental samples. Specific nucleic acid-binding dyes selectively enter bacterial cells with compromised membranes, which can be covalently linked to the DNA by subsequent photo-activation. By this method, only DNA of cells with intact cell membranes can be extracted and amplified with quantitative (real-time) PCR (qPCR). Initially, the nucleic acid stain ethidium-monoazide (EMA) was used (Nogva et al., 2003; Rudi et al., 2005), but due to its penetration into cells with intact cell membranes in some cases and its higher cytotoxicity (Cawthorn and Witthuhn, 2008; Yanez et al., 2011) the alternative dye propidium-monoazide (PMA) has become the substance of choice (Nkuipou-Kenfack et al., 2013; Nocker et al., 2006, 2007; Schnetzinger et al., 2013). For the quantification of viable legionellae both EMA (Chang et al., 2009; Delgado-Viscogliosi et al., 2009) and PMA qPCR have been applied (Ditommaso et al., 2014; Fittipaldi et al., 2011; Li et al., 2015; Yanez et al., 2011) (Table 2). The method was shown to also work well when performed directly on filters without the need for resuspension (Slimani et al., 2012). However, serious precautions have been raised concerning the validity of this approach, when biofilms are investigated (Taylor

et al., 2014) or high numbers of background bacteria are present in the sample (Ditommaso et al., 2015). To overcome this problem, the use of an algorithm for correct interpretation of the results of PMA qPCR was suggested (Ditommaso et al., 2015). Similar to the use of PI for cell membrane integrity assessment for cell-based approaches, influence of UV disinfection on the viability of *Legionella* cells cannot be assessed with this method.

As an alternative to v-PCR targeting the DNA, RNA could be a possible target for qPCR. Reverse transcriptase-qPCR (RT-qPCR) of species specific pre-rRNA targets after nutrient pulse was suggested by Cangelosi and Meschke (Cangelosi and Meschke, 2014) (Table 2). Pre-rRNAs in inactivated cells and free nucleic acids do not increase in numbers upon nutritional stimulation and therefore are excluded. At this point, it is not clear whether dormant and VBNC cells produce pre-rRNA in response to nutritional stimulation and this approach has not been tested with legionellae. Also the mRNA of *Legionella* specific genes could be a possible target for RT-qPCR amplification (Buse et al., 2015). DNA should only be transcribed to mRNA when bacterial cells possess a functional transcriptional machinery and an intact respective DNA segment. As mRNA is degraded very rapidly (seconds to hours (Deutscher, 2006)), a positive mRNA signal means that the cell has just produced this mRNA. It was shown recently that specific virulence genes may even be over-expressed in the VBNC state of *L. pneumophila*, among those the macrophage infectivity potentiator protein (mip), a virulence factor that is exposed on the bacterial cell surface, which is involved in the early stages of host cell infection and that plays an important role in the intracellular survival in macrophages and in protozoa (Alleron et al., 2013). The mRNA of such a protein may be a suitable target to assess both viability and potential virulence.

5. Are VBNC legionellae hazardous to humans?

The key question to answer is certainly whether VBNC legionellae are hazardous to human health and if so, to what extent. As mentioned above, there are several reports that VBNC legionellae could be successfully resuscitated in amoebae (Ducret et al., 2014; Epalle et al., 2015; Garcia et al., 2007; Hwang et al., 2006; Steinert et al., 1997) and it has been shown that *Legionella* cells after amoebae infection show enhanced virulence to human monocytes and enhanced growth in the lungs of mice (Cirillo et al., 1999). There is only a single published paper that showed that non-culturable *L. pneumophila* (serogroup 6) directly caused an outbreak of Pontiac fever in humans (Miller et al., 1993), but there might be a much stronger link between VBNC legionellae and Pontiac fever (Christian Lück, personal communication). Interestingly, no successful resuscitation and growth of VBNC legionellae could be

demonstrated so far in human cell lines or animal models (Ducret et al., 2014; Epalle et al., 2015). In this context, it has to be mentioned that many factors can influence the success of resuscitation, such as the strain used, the age of the VBNC cells, the conditions that induced the VBNC state, and the conditions provided for resuscitation (Li et al., 2014). It was proposed that different species and different strains do have a specific "resuscitation window", i.e. a time period during which successful resuscitation may take place (Pinto et al., 2011). As it is not easy to find out the perfect resuscitation conditions, a negative finding does not necessarily mean that resuscitation is impossible.

In a first attempt to quantify the number of resuscitable VBNC *Legionella* cells and to establish a kind of dose–response relationship for VBNC *L. pneumophila*, Al-Bana and et al. came up with a semi-quantitative approach using an *Acanthamoeba castellani* model that was challenged with a series of ten-fold serial dilutions of VBNC *L. pneumophila* cells (Al-Bana et al., 2014). For VBNC cells derived from stationary phase forms, only one to three per 100.000 VBNC cells were able to resuscitate in amoeba. It has to be stated that more quantitative experiments are necessary to prove these low ratios to be widely representative, especially because of the large variability of conditions, between strains and VBNC types.

5.1. Public health risk assessment for legionellae

If VBNC legionellae are of significant health relevance, their consideration in routine water investigations must result in better risk assessment models and must be reflected in epidemiological data. Setting up risk assessment for legionellae on solid quantitative data is needed. The fundamental steps of such approach are (i) comprehensive hazard identification, (ii) the characterization of human exposures and (iii) the establishment of suitable dose–response relationships. All of these steps have to consider differences in strain infectivity and the presence of VBNC cells. Current risk assessment models are prone to many uncertainties, including *Legionella* epidemiology, strain infectivity, the conditions under which legionellae are aerosolized and taken up by humans, the infection dose and current quantification methods (Whiley et al., 2014). Currently used legal control limits are based on general microbiological considerations and have evolved from experience (Armstrong and Haas, 2008) rather than being based on solid quantitative microbial risk assessment (QMRA). For all kinds of engineered water systems that are potential sources for legionellae like cooling towers, whirlpool spas and hot water distribution systems, the basis for *Legionella* control limits is not well documented, and a direct correlation between culturable *Legionella* concentrations and risk of legionellosis could not be demonstrated so far (Ditommaso et al., 2015). Table 3 shows a representative risk scheme and guideline values for *Legionella* concentrations in warm water systems, the Austrian Standard OENORM B5019 (OENORM, 2011), similar guideline values are used in other countries as well (WHO, 2007). The indicated values are derived from culture based determinations, and therefore do not consider VBNC legionellae and do not distinguish between species/strains of different virulence (e.g. specific highly virulent sequence types or Mab 3/1 positive strains). At least, it is recommended in the Austrian Standard that in case of a serious suspicion, also at densities lower than the guideline values (1000 CFU L^{-1} for risk groups 1–3 and 100 CFU L^{-1} for risk group 4), rehabilitation measures have to be tackled. In addition, *L. pneumophila* SG 1 should be classified more critical than other *Legionella* serogroups and species.

There have been recent efforts to apply QMRA to estimate the risk from legionellae in various water systems (Armstrong and Haas, 2008; Schoen and Ashbolt, 2011), but at present information is very rudimentary mostly because of the limited number of

Table 3
Risk scheme of Legionella spp. concentrations according to OENORM B5019:2011. Risk group 1: private housings and public buildings. Risk group 2: schools, sports and wellness facilities, hotels, barracks, risk group 3: hospitals, retirement homes, nursing homes, sanatoria; risk group 4: hospitals or hospital wards with immuno-suppressed patients.

Assessment	Classification	Risk group 1, 2, 3 (CFU/L)	Risk group 4 (CFU/L)
Not tolerable	Very high concentration	≥100.000	≥100.000
Not tolerable	High concentration	10.000–<100.000	10.000–<100.000
Not tolerable	Medium concentration	1.000–<10.000	100–<10.000
Tolerable	Medium concentration	1.000–1.500	100–150
Tolerable	Low concentration	<1.000	<100

dose—response and exposure studies (Ashbolt, 2015). In addition, none of the used models and dose—response relationships considered differences in the virulence of the *Legionella* strains, the differential expression of virulence factors in different growth phases and the fact that a significant sub-population of infectious legionellae may be in the VBNC state (Buse et al., 2012). Lee and co-workers performed an international trial to derive alert and action levels for the use of quantitative PCR (qPCR) in the monitoring of *Legionella* (Lee et al., 2011). The qPCR approach has the principle advantage of capturing a broader range of infectious legionellae, including cells in the VBNC state, but it may also overestimate the risk due to inclusion of inactivated or dead cells (Buse et al., 2012). In the aforementioned study (Lee et al., 2011), qPCR results were between 0.6 and 2 logs higher than culture results. Alert $(5 \times 10^3 \, \text{GU L}^{-1})$ and action levels $(5 \times 10^4 \, \text{GU L}^{-1})$ were derived for qPCR data, but these values were deduced from adjustment to the application of the European guidelines based on culture and they are not based on the true concentration of viable, infectious *Legionella* cells and their risk for human health.

6. Upcoming detection methods and research needs

Based on the many uncertainties with risk assessment and the potential hazard of VBNC legionellae to human health, as outlined in detail above, major research and action needs can be formulated, directed to more effective prevention of legionellosis in the future. Most importantly, a focus has to be set on more comprehensive hazard identification, considering VBNC cells and differences in strain virulence. For this purpose, the application of culture independent methods for the quantification of *Legionella* in water samples is necessary, complementing the use of the standard culture method. Moreover, studies on the characterization of human exposures have to be performed for different exposure types (showering, whirlpool, cooling towers, etc.) and more comprehensive and robust dose—response relationships have to be established, including those for VBNC legionellae.

6.1. Which methods should be used for Legionella quantification in the future?

6.1.1. Cultivation

For the time being, cultivation will remain the gold standard for quantification of legionellae in engineered water systems. Recently, there have been some attempts to improve the performance of the cultivation method. In order to significantly reduce the time of analysis to two days, a suitable alternative to the currently applied standard procedure (ISO, 1998), based on the detection of micro-colonies with a solid phase cytometer (SPC) was presented (Baudart et al., 2015). Both methods were compared according to ISO 17994 (ISO, 2014) and yielded comparable results over a wide range of *Legionella* concentrations $(10^3 - 10^6 \, \text{CFU/L})$. Due to the relatively small volume that could be used for analysis (5 ml), the SLOQ was 600 CFU/L in comparison to the standard method with 35 CFU/L (when a 1 L sample is investigated) and 350 CFU/L (when a 100 ml sample is analysed; see Table 4). In order to increase the recovery of legionellae, especially *L. pneumophila*, from environmental samples by cultivation, immuno-magnetic separation prior to cultivation was applied. With this pre-treatment, a significantly higher percentage of positive samples was achieved in comparison to the standard method (Allegra et al., 2011a). It should be emphasized here that the use of monoclonal antibodies is to be preferred over polyclonal antibodies provided that their application works well for all different strains and that the targeted epitopes are still expressed in the VBNC state (Sboui et al., 2015). Another promising approach seems to be the amendment of the plating medium with reactive oxygen species (ROS) scavengers. With the addition of pyruvate and glutamate, Ducret and co-workers were able to efficiently recover injured, previously non-culturable *Legionella* cells (Ducret et al., 2014). But, so far this method has not been used in a comparative trial according to ISO 17994.

6.1.2. Cultivation independent methods

An ideal cultivation-independent method used for quantification of *Legionella* targets all *Legionella* cells that are relevant for human health, i.e. they are viable, virulent and able to proliferate. For this, the method must have a low LOD/LOQ of approx. 100 measurement units per litre and it must deliver reproducible results with a variance of less than 30%. In addition, the method should be standardisable and usable by routine laboratories. It should have a short time to result (less than 2 days) and a short hands-on time and it should be of comparable costs as the standard culture method. Optimally, the method is also automatable, high throughput and utilizable in the field. Although it is quite clear that such a jack-of-all-trades device will most probably never exist, there are currently three promising approaches available that combine most of the above defined demands and that may have the potential to be used for future *Legionella* routine monitoring — flow cytometry, solid phase cytometry and qPCR — and all of them have the ability to include VBNC cells in their detection range. The potential application of biosensors, that would meet a lot of the expectations listed above, is additionally discussed. However, at the current state it is the author's feeling that they are too far away

Table 4
State-of-the-art and upcoming cultivation-based and cultivation independent methods for the quantification of legionellae in engineered water systems. For comparison, EFM based detection approaches were added. For abbreviations see text.

Detection platform	Method	Volume (ml)	SLOQ	Time to result (d)	Reference
Cultivation					
Agar plate	Colony	1000	35 CFU/L	10—14	(ISO, 1998)
		100	350 CFU/L	10—14	
SPC	Microcolony	5	600 mCFU/L	2	(Baudart et al., 2015)
Cultivation independent methods					
FCM	Antibody/IMS	1000	45—150 cells/L	Same day	(Keserue et al., 2012a)
		100	450—1500 cells/L		
SPC	Antibody	3×10	300 cells/L	Same day	(Parthuisot et al., 2011)
	CARD-FISH	10	400 cells/L	2	Kirschner et al., unpublished
qPCR	qPCR	1000	500 GU/L	Same day	(ISO, 2012; Omiccioli et al., 2015)
		100	5000 GU/L		
	PMA-qPCR	1000	500 GU/L	Same day	(Ditommaso et al., 2014)
	EMA-qPCR	1000	500 GU/L	Same day	(Delgado-Viscogliosi et al., 2009)
EFM	Antibody	100	5×10^3 cells/L	Same day	(Delgado-Viscogliosi et al., 2005)
	CARD-FISH	100	3.5×10^4 cells/L	2	(Kirschner et al., 2012)

from practical use (in comparison to qPCR, FCM, SPC), mostly because of too high detection limits.

6.1.2.1. Flow Cytometry. Flow Cytometry (FCM) is a fast, cost-effective, potentially automatable and high throughput technology that has attracted increased attention for the detection of microbial targets in the past decade (Hammes and Egli, 2010; Keserue et al., 2012b). It was recommended for detection of legionellae in water samples as early as in 1982 (Ingram et al., 1982). Low-cost, bench-top size machines are now available that allow application in routine laboratories. A four-step FCM protocol was presented by Thomas Egli's group for the rapid quantification of total *L. pneumophila* without a viability assay (Füchslin et al., 2010). The whole method allows detection of *L. pneumophila* in 180 min with a detection limit of around 500 cells L^{-1} and a recovery rate of approximately 50%. After concentration of *Legionella* cells on 0.45 μm filter membranes (filter membranes with smaller pore-size, e.g. 0.2 μm, may be better suitable for concentration, in order to include long-starved VBNC cells that may undergo size reduction (Harley et al., 1997)), the cells are resuspended in 5 ml of buffer and double-stained with two FITC- and Alexa-conjugated *Legionella* specific polyclonal antibodies. Subsequently, the cells are immunomagnetically caught and quantified by flow cytometry. Double staining was necessary as signals from single stained *Legionella* cells were covered by the huge background noise in drinking water samples and to minimize the risk for cross-reactions leading to false-positive results. It was stated that the use of monoclonal antibodies specifically targeted to naturally grown pathogens and the availability of *Legionella* specific antibody-coated microbeads for immuno-magnetic separation (IMS) may significantly improve and simplify the procedure (Füchslin et al., 2010; Keserue et al., 2012a). Based on this technique, this group advanced the application by combining it with the widely used viability assay for membrane integrity (see above) (Keserue et al., 2012a). The reported recovery rate was 85% in 1 L of tap water with detection limits around 15 and 50 cells L^{-1} and quantification limits (SLOQ) around 45 and 150 cells L^{-1} for viable and total *L. pneumophila*, respectively (Table 4). Very low standard deviations (5—10%) for results above the SLOQ were achieved. In a comprehensive comparative trial, 85 tap water samples were tested with both the FCM approach and the standard culture method (Keserue et al., 2012a). No correlation and a low strength of agreement were observed between culture and FCM results. In approximately 50% of the samples, FCM showed higher concentrations than culture (difference > 0.5 log), but in 20% of samples, higher plate counts were observed. These were attributed to the major draw-backs of the current approach—the polyclonal antibodies targeting only the serogroups 1—12 and the inability to detect *L. pneumophila* in amoebal cells. As a very low number of false positives was achieved with this protocol, the significantly higher values obtained by FCM in comparison to culture means that a significant amount of VBNC cells may have been present in these samples. Due to the fact that membrane integrity was used as the only proof of viability, the combination with another viability assay, like esterase activity, as outlined in detail above, is desirable. However, none of the viability assays currently available for cell-based detection on the FCM are able to prove viability after disinfection with UV-irradiation.

In conclusion, advances in the availability of suitable *Legionella* spp. or *L. pneumophila* specific monoclonal antibodies, optimally targeting epitopes of proteins or lipopolysaccharides that are well expressed in the VBNC state, and the combination of more than one viability assay, will significantly increase the potential of flow cytometry for routine determination of *Legionella* concentrations in tap water samples. Difficulties will remain with UV-disinfected samples and samples derived from more complex environments like cooling towers.

6.1.2.2. Solid phase cytometry. Solid phase cytometry (SPC) was developed at the end of the 1990s in order to enable cell-based quantification of low cell numbers on filters (Mignon-Godefroy et al., 1997). State of the art instruments consist of a laser scanning device that detects all fluorescent signals on a filter surface and a connected microscope equipped with a motorized stage, with which all detected signals can be subsequently verified by microscopic examination. For an average sample, the total analysis time (scanning and verification) is approximately 10 min. Unfortunately, there is only a single model commercially available (ChemScan RDI, Biomerieux) that is very expensive and equipped with only one (blue) LASER. The major technical drawback is that for a successful detection of a signal, the signal must be very bright with fluorescence intensities that are markedly above a standard FISH signal. Thus, the much more complicated CARD-FISH has to be applied in order to specifically detect and quantify *Legionella* cells via SPC (Kirschner, unpublished data) or fluorescent antibodies (Aurell et al., 2004). Recently, specific immunofluorescence staining using monoclonal antibodies in combination with the esterase activity marker Chemchrome V6 was applied to quantify total and viable *L. pneumophila* in 46 hot water samples where no legionellae were detectable by cultivation (Parthuisot et al., 2011). A cocktail of two antibodies was used, one for *L. pneumophila* SG 1 and one for *L. pneumophila* SG 2—15 (Microbiodetection, Commercy, France). An SLOD of 34 cells L^{-1} was reported, when three 10 ml subsamples were investigated, with a coefficient of variation (CV) generally falling below 30%. However, when critically reviewing the indicated SLOD, it turned out that Poisson distribution of bacterial cells in water samples had not been considered and the true SLOD was 100 cells L^{-1} (due to Poisson distribution, at least 3 cells in a sample have to be detected in order to be counted as positive at a probability level of 95%). In order to achieve a CV of 30%, at least 10 cells have to be counted via SPC (Stevenson et al., 2014), resulting in an SLOQ of approx. 300 cells L^{-1} (Table 4). The investigation of the 46 hot water samples revealed, that despite that all samples being negative with culture, 38 samples (83%; recalculated with the adjusted SLOD values) were positive for total *L. pneumophila*, while 8 samples (17%) yielded viable *L. pneumophila* concentrations of up to 1.3×10^{4} cells (Parthuisot et al., 2011). So, obviously in most cases, the total *L. pneumophila* cells detected with SPC were dead, but in a non-negligible number of samples VBNC cells were detected, that amounted between 0.2 and 90.9% of total *L. pneumophila* cells. It has to mentioned that the true esterase positive cell concentrations may be underestimated, as the recovery rates in artificially contaminated hot water samples was less than 10% (although erroneously reported to be 73.4% from a log/log regression curve) (Parthuisot et al., 2011). In this study, the method was also applied to thermal spring water, cooling tower water and freshwater samples and viable cell counts were in the range of the cultivation method or lower. Again, this observation could be a result of an underestimation due to the low recovery of living cells.

Summing up, the SPC approach is highly suitable for rapid (less than 2 days) micro-colony based detection of legionellae in comparison to 10 days needed for the standard culture method (Baudart et al., 2015) (see above, sub-chapter "Cultivation"). It is also an interesting approach to quantify total legionellae via immuno-fluorescence or CARD-FISH; its validity for quantifying viable *Legionella* cells remains somewhat dubious mainly due to the low recovery rates with the esterase activity assay. For this, an

advancement of the system with increased optical sensitivity and more LASERs would be necessary, in order to enable combination of different viability assays. Again, like for flow cytometry, UV irradiated *Legionella* cells would be detected as false positives.

6.1.2.3. Quantitative PCR. Currently, viability PCR using PMA seems to be the best available method for the nucleic acid-based quantification of viable legionellae in water samples (see chapter "Methods to proof the viability of VBNC legionellae"). With a sample volume of 1 L, qPCR can reach a sample limit of quantification of about 500 GU/L, comparable to FCM and SPC (Table 4). A big advantage of qPCR in comparison to the cell-based methods discussed above is that it can specifically target virulence factors or virulent strains. So it would be possible to design a multiplex PMA-qPCR specifically targeting the most important *L. pneumophila* sequence types manifested in clinical infections in a country enabling the rapid screening of suspicious sources for the presence of viable potentially highly virulent *L. pneumophila*. Problems with the interpretation of the results may arise when high background numbers of bacteria are present like in cooling towers (Ditommaso et al., 2015) and from the fact that membrane integrity is the only indication of viability with this method. The combination with a second viability indicator would therefore be highly desirable. Moreover, for UV-disinfected water, the PMA-qPCR approach is not suitable and alternative approaches have to be developed. Such alternatives could focus on the detection of pre-RNA (Cangelosi and Meschke, 2014) or mRNA (see chapter "Methods to proof the viability of VBNC legionellae"), but they are not available right now. Most recently, it was reported for *Salmonella enteritidis* cells that a combination of PMA with low concentrations of EMA differentiates cells with intact cell membranes and intact metabolism from cells with an intact membrane but without metabolism (Codony et al., 2015), an approach that remains to be tested for legionellae.

In conclusion, qPCR is a rapid and increasingly cheap method that can also be applied in routine laboratories, provided that skilled personal is present. Several commercial kits for the quantification of total *Legionella* spp. or *L. pneumophila* are available that can be easily combined with PMA or EMA treatment. However, one has to be careful with the use of PMA and especially EMA because of potential cytotoxic effects in living cells or cells with reversible membrane damage, leading to false negative results (Fittipaldi et al., 2012; Yanez et al., 2011). Further work is needed to improve the validity of the viability indicator function of PMA/EMA qPCR.

6.1.2.4. Biosensors. Different technical approaches of biosensors for the specific detection of *L. pneumophila* have been reported, including an immunosensor using surface plasmon resonance (Oh et al., 2003), an electrochemical genosensor (Miranda-Castro et al., 2007), an optical immunosensor (Cooper et al., 2009) or, most recently, an electrochemical immunosensor (Sboui et al., 2015; Souiri et al., 2014). While the earlier sensors had rather high detection limits in the range of 10^3/ml (Cooper et al., 2009) to 10^4/ml (Oh et al., 2003), the electrochemical immunosensor had a limit of detection in the range of 10–50 bacteria per ml of artificial water samples (Sboui et al., 2015; Souiri et al., 2014). This immunosensor is based on the immobilization of monoclonal anti-*L. pneumophila* antibodies on an indium-tin oxide electrode and was able to detect *L. pneumophila* cells in the VBNC state. However despite significant advances, this sensor is still two orders of magnitude less sensitive in comparison to FCM, qPCR or SPC, and its application has yet to be tested for real samples. In conclusion, immunosensors may be a promising technique in the future, but substantial efforts will be necessary to further lower the still high detection limits. Moreover, a widened target spectrum (not only *L. pneumophila*) based on the availability of appropriate antibodies, would be desirable.

6.2. Different scenarios demand different combinations of standard and complementary methods

Due to the availability of rapid methods for the quantification of legionellae, their application can bring useful advanced information for better hazard identification and a better assessment of public health risk. For a standard routine control analysis, when no indication of *Legionella* infections exists, it may be enough to stick to the cultivation-based approach alone. The results are not needed urgently and waiting ten days for the result may be appropriate. For large scale environmental screening programs, cultivation independent methods may be more suitable. A large number of samples from potentially critical control points can be rapidly screened with either qPCR or flow cytometry for total and viable *Legionella* spp or *L. pneumophila*, including VBNC cells, or solid phase cytometry, if available. Only in cases where hot-spots of legionellae are found, should cultivation then be used for confirmation and for typing purposes. Ideally, the qPCR has already covered the most important *L. pneumophila* sequence types. If low numbers of culturable legionellae and a high number of VBNC cells are observed, further measures have to be considered. In outbreak scenarios, a ten-day analysis period is much too long and culture-independent methods have to be applied immediately. As above, rapid screening of all potential sources should be done with qPCR, FCM or SPC, obtaining results within one or two working days. All positive sources should be checked in parallel with standard cultivation in order to (hopefully) isolate the causative strain. The availability of a multiplex PCR targeting the most important sequence types may help to direct efforts of source identification.

7. Conclusions

Due to the application of culture independent methods in the past decades, there has been increasing evidence that viable but non culturable legionellae are present in water systems at concentrations that are up to several logs higher than obtained by standard culture methods. Nowadays, there are culture independent methods at hand that can in principle distinguish between living and dead *Legionella* cells and that are ready to be used in routine laboratories. However, further improvements are necessary for a more reliable and robust determination of viability. The combination of at least two different viability assays is desirable and especially for UV-treated water, novel approaches have to be developed. In order to assess the public health threat emanating from the observed high concentrations of VBNC legionellae, accurate quantitative measurements are needed concerning the abundance of VBNC cells in engineered water systems and concerning dose–response relationships for VBNC legionellae considering differences in the virulence of different strains. Based on such data, more solid QMRA-derived guideline values for *Legionella* concentrations in different water systems may be derived for improved management of legionellosis.

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