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Mycobacteria in drinking water distribution systems: ecology and significance for human health

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Abstract

In contrast to the notorious pathogens *Mycobacterium tuberculosis* and *M. leprae*, the majority of the mycobacterial species described to date are generally not considered as obligate human pathogens. The natural reservoirs of these non-primary pathogenic mycobacteria include aquatic and terrestrial environments. Under certain circumstances, e.g., skin lesions, pulmonary or immune dysfunctions and chronic diseases, these environmental mycobacteria (EM) may cause disease. EM such as *M. avium*, *M. kansasii*, and *M. xenopi* have frequently been isolated from drinking water and hospital water distribution systems. Biofilm formation, amoeba-associated lifestyle, and resistance to chlorine have been recognized as important factors that contribute to the survival, colonization and persistence of EM in water distribution systems. Although the presence of EM in tap water has been linked to nosocomial infections and pseudo-infections, it remains unclear if these EM provide a health risk for immunocompromised people, in particular AIDS patients. In this regard, control strategies based on maintenance of an effective disinfectant residual and low concentration of nutrients have been proposed to keep EM numbers to a minimum in water distribution systems.

Keywords: Mycobacteria; Drinking water distribution system; Biofilm; Amoeba; Disinfection

Contents

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1. Introduction

The genus *Mycobacterium* consists of about 100 species, including pathogens and saprophytes, and may be divided into three groups on the basis of clinical significance [1]. The first group includes obligate pathogens in humans and animals, i.e., the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*, *M. caprae*, *M. microti*, *M. pinnipedii*, and *M. tuberculosis*), *M. leprae*, and *M. lepraemurium*, which are generally not found in the environment. The second group comprises mycobacteria that are potentially pathogenic to humans or animals. The majority of these species have been isolated from various terrestrial and aquatic environments and may cause disease under certain circumstances, e.g. skin lesions, pulmonary or immune dysfunctions and chronic diseases. Examples are *M. avium* and other members of the so-called '*M. avium* complex' (MAC). The third group consists of saprophytic species that are non-pathogenic or only exceptionally pathogenic. In literature, the second and third group are often referred to as 'atypical my cobacteria', 'anonymous mycobacteria', 'facultative pathogenic environmental mycobacteria', 'potentially pathogenic environmental mycobacteria', 'mycobacteria other than tuberculosis' or 'non-tuberculous mycobacteria'. However, the two latter terminologies are rather confusing because some members of these groups can also cause tuberculous-like lesions [2]. Also from the taxonomic point of view, the classification of mycobacteria in three groups has little or no value. Although research is focused mostly on the species *M. tuberculosis*, *M. leprae* and *M. ulcerans*, numerous studies have shown the clinical relevance of mycobacteria other than the three aforementioned species [see reviews [3–11]]. Environmental mycobacteria (EM) can cause infection or disease in individuals with predisposing (e.g., pulmonary dysfunction, low CD_4-lymphocyte cell count) conditions but it has also been shown that *M. avium* causes disease in persons without predisposing conditions [12,13]. The following main categories of clinical symptomes can be distinguished: pulmonary disease, hypersensitivity pneumonitis, (cervical) lymphadenitis, localized non-pulmonary lesions (e.g., cutaneous, soft tissue and bone disease), and disseminated disease. Furthermore, EM are often involved in nosocomial infections and pseudo-outbreaks [14–16] and must therefore be considered as an important group of bacteria showing increasing pathological importance. Unfortunately, official figures of infections due to EM cannot be given because they are mostly not incorporated in surveillance programs.

EM are found in dust, soil and water and they are mainly transmitted by ingestion, inhalation and inoculation from environmental sources rather than from person to person [7]. According to Collins et al. [17], most of the free-living mycobacteria cannot be regarded as truly aquatic microorganisms. Their principal habitat is more likely wet soil, or stagnant water in contact with soil, from which they are washed into rivers and lakes, and ultimately into the sea [17].

Several authors have demonstrated the presence of mycobacteria in public drinking water distribution systems (DWDSs) [18–42a,42b] hospital water distribution systems [22,43–89] and domestic tap water [20,22,40,43,46,50,67,73,76,90–98]. On the other hand, a number of authors failed to isolate EM from drinking water, probably due to absence or temporary presence

of EM in the piping, or the use of non-suitable isolation techniques.

The first reports of isolation of EM from DWDSs appeared in the early 1900s (reviewed by Collins et al. [17]). EM that are able to grow in DWDSs, have only for the last two decades been recognized as emerging pathogens [99–104]. Especially in the interest of immunocompromised persons (e.g., AIDS patients), increasing attention is paid to the ecology and human health significance of EM such as *M. avium, M. kansasii* and *M. xenopi*, and the possible role of water as transmission medium.

Interestingly, EM and *Legionella pneumophila* have a rather similar ecology including occurrence in water distribution systems, interactions with protozoa and formation of biofilms. The presence of EM and legionellae in the same water distribution system has been shown by several authors [38,40,79,87].

2. Prevalence and species diversity in water distribution systems

Testing drinking water quality comprises physical, chemical and microbiological analyses. In Europe, the quality standards for drinking water are described in the Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption [105]. An overview of the microbiological water quality standards according to Council Directive 98/83/EC is summarized in Table 1. Although the most common organisms found in drinking water systems are non-pathogenic heterotrophic bacteria, other organisms, like viruses (enteroviruses, Hepatitis A virus, norovirus), protozoa (amoebae, *Cryptosporidium parvum*, *Giardia* spp.), and emerging bacterial pathogens, such as *Aeromonas* spp., *Helicobacter pylori, L. pneumophila, Pseudomonas aeruginosa* and EM, have become increasingly important during the last decades [101–104]. Although each DWDS harbours its own mycobacterial flora, EM are generally not included as a parameter in standard microbiological analysis. Most EM are virtually never covered by the heterotrophic plate count because plates are usually incubated at 20 °C, 22 °C, 30 °C or 37 °C for 48 h, 72 h or 7 d (depending on the international standard followed), and the composition of the routine media such as R2A agar and plate count agar differs from those usually used to cultivate mycobacteria.

Table 1
Microbiological quality standards for water intended for human consumption supplied from a distribution network according to Council Directive 98/83/EC [105]

Microbiological parameters	Parametric value
Escherichia coli	0 per 100 ml
Enterococci	0 per 100 ml
Indicator parameters	**Parametric value**
Clostridium perfringens (including spores)	0 per 100 ml[a]
Coliform bacteria	0 per 100 ml
Colony count at 22 °C	No abnormal change

[a] This parameter need not be measured unless the water originates from or is influenced by surface water. In the event of non-compliance with this parametric value, the Member State concerned must investigate the supply to ensure that there is no potential danger to human health arising from the presence of pathogenic micro-organisms, e.g., *Cryptosporidium* spp.

2.1. Analytical methods

Water samples are analyzed after concentration of the mycobacteria by centrifugation or filtration. The pellet or filter is decontaminated in order to reduce the background flora of other bacteria and fungi. Several protocols have been described [106] but a standard protocol for the isolation of EM from drinking water is not available. Neumann et al. [107] suggested decontaminating treated water with 0.005% cetylpyridinium chloride for 30 min, filtration, rinsing of the filter with sterilized water, followed by incubation of the filter on Löwenstein–Jensen medium at 30 °C. Of course, it should be taken into account that the choice of a particular incubation temperature will select for those mycobacteria which only grow at the chosen temperature. For instance, *M. xenopi* will not be detected when the growth medium is incubated at 30 °C because its optimal growth temperature lies in the range 40–45 °C [108]. Other parameters such as incubation time, O_2/CO_2 ratio, and addition of micronutrients or specific growth factors to the medium, also play a role. Harsh decontamination procedures in order to isolate EM from environmental sources have probably lead to losses of numbers and in species or strain diversity [36,73]. Therefore, the failure to recover a particular species after isolation and identification, does not necessarily reflect its absence in the DWDS.

Identification of waterborne mycobacteria is mostly done after cultivation. Isolates are identified using phenotypic tests or applying chemotaxonomic techniques such as gas-liquid chromatography, high-performance liquid chromatography or thin-layer chromatography [109–112]. Due to the difficult growth requirements and rich species composition of the genus *Mycobacterium*, molecular methods are often the preferred techniques for identification. This can be achieved by species-specific PCR-tests, polymerase restriction endonuclease analysis (e.g., of the *hsp*65 gene) or 16S rRNA gene sequencing [106,110,111,113–116]. Commercial DNA probes (e.g., AccuProbe®, Gen-Probe Inc.; GenoType Mycobacterium, Hain Lifescience; INNO-LiPA Mycobacteria, Innogenetics N.V.) are quick tools to identify the most well-known mycobacteria [117–122]. However, it should be kept in mind that most of the above described identification methods are rather

narrowly focussed on clinically important mycobacteria. For example, 16S rRNA sequencing will not distinguish *M. abscessus* from *M. chelonae* [123,124]. A polyphasic approach, i.e., a combination of a wide range of phenotypic and genotypic techniques, remains the gold standard to identify known mycobacterial species and to describe new EM species [125,126].

2.2. Prevalence

Mycobacteria were isolated from 16 (38%) of 42 public DWDSs in the USA [33]. A total of 21.3% (42/197) and 72% (104/144) of the samples from a DWDS in Patras (Greece) and Paris (France), respectively, was positive for mycobacteria [34,36]. In tap water from a hospital, 20.4% (10/49) of the samples were positive for EM [81]. Interestingly, acid-fast bacilli other than mycobacteria were detected in 83.7% (41/49) of the tap water samples [81]. The occurrence of mycobacteria in drinking water from distribution systems that used groundwater was similar to distribution systems that used surface water: 31% and 36%, respectively [33]. *M. gordonae* was the only mycobacterial species recovered from drinking water samples from distribution systems that used groundwater as source [33]. These observations were not confirmed by Schwartz et al. [32], who could not detect mycobacteria in DWDS biofilm samples when groundwater was used as water source. However, the molecular techniques used by these authors did not cover all EM so it is possible that certain species were not detected. The isolation frequency of mycobacteria from Finnish DWDS samples was 35% and increased up to 80% at the most distal sites of the waterworks [41]. Higher mycobacterial numbers were encountered in systems using surface water and applying ozonation [41], which can be explained by a higher content of carbon source due to the oxidative breakdown of complex molecules. An overview of the mycobacterial numbers found in water distribution systems is shown in Table 2.

2.3. Species diversity

The species *M. avium, M. chelonae, M. fortuitum, M. gordonae, M. kansasii*, and *M. xenopi* are the most frequently reported mycobacteria occurring in drinking water (Table 3). This can partly be explained by the focus of some studies on particular species such as *M. avium, M. kansasii* and *M. xenopi*. As shown in Table 3, at least 33 of more than 100 taxonomically described species can be found in water distribution systems. It is clear that a complete overview of the species composition is hampered by the lack of suitable sampling and isolation methods, the limited ability of routinely used identification methods to also recognize non-clinical species, and the poor knowledge on the species' distribution in place and time in the pipes. For example, 57 of the 104 isolated mycobacteria from a DWDS in Paris could not be identified following 16S rDNA sequencing and *hsp*65 gene analysis [36]. Based on a polyphasic identification methodology, 126 of 351 isolates from drinking water and deposit samples could not be identified to the species level [41]. Given the relatively high number of unspeciated DWDS isolates in several studies, the description of new mycobacterial species is expected to increase in the coming years.

3. Growth and survival in water distribution systems

Routes through which (myco)bacteria can enter the distribution system include water treatment breakthroughs, leaking pipes, valves, joints and seals, cross-connections and backflows, finished water storage vessels, improper treatment of materials, equipment or

Table 2
Mycobacterial counts in water distribution systems

Counts	% of samples	Location/isolation medium	Reference
1–50 CFU l^{-1}	78	Drinking water	[36]
51–500 CFU l^{-1}	21		
>500 CFU l^{-1}	1		
1–20 CFU 500 ml^{-1}	>80	Drinking water	[33]
Median of 26 CFU l^{-1} (range: 4–1600 CFU l^{-1})	NR[a]	Cold water Sites	[69]
Median of 14 CFU l^{-1} (range: 4–400 CFU l^{-1})	NR[a]	Hot water sites	
Mean of 69 CFU l^{-1} (range <20–130 CFU l^{-1})	NR[a]	Tap water	[87]
Mean of 290 CFU l^{-1} (range 21–1400 CFU l^{-1})	NR[a]	Shower water from a hospital warm Water system	
Median of 7.0×10^5 CFU/g (range 4.9×10^2–1.4×10^6 CFU g^{-1} dry weight)	NR[a]	Old deposits in DWDS	[41]
Median of 3.9×10^5 CFU/g (range 1.0×10^5–2.1×10^6 CFU g^{-1} dry weight)	NR[a]	1-year old deposits	
Median of 140 CFU l^{-1} (range: 10–3500 CFU l^{-1})	NR[a]	Distal site of the waterworks	

[a] NR, not reported.

Table 3
Non-exhaustive overview of *Mycobacterium* species isolated from water distribution systems (domestic, public and hospital water supply)

Species/group	Reference
M. abscessus	[42b,50,59,71]
M. aurum	[31,35,36]
M. avium	[28,33,35,55,68,69,73,95,98]
M. avium complex (MAC)[a]	[23,27,30,40,46,49,50,54,56,66,67,73,76,84,89,90,94,95]
M. brumae	[40]
M. chelonae	[25,27,50,60][b], [34,36,69,72,86,92,127]
M. chlorophenolicus	[40]
M. flavescens	[27,31,34,40,69,72,92]
M. fortuitum	[21,24,31,33,34,36,40,42b,51,61,69,75,81,86,97,127]
M. fortuitum complex	[25,27]
M. gadium	[36]
M. gastri	[20,46,69]
M. gastri/kansasii[c]	[33,81]
M. genavense	[40],[74][d]
M. gilvum	[42b]
M. gordonae	[20,21,23–28,31,33–37,40–42b,46,47,50,54,57,62–66,69,72,77,79–81,83,84,86,92,93,97]
M. haemophilum	[35,40]
M. intracellulare	[33,35,36,40,44,73]
M. kansasii	[18,20–24,34,43,45,48,52,53,62,63,65,69,70,92,96]
M. lentiflavum	[41]
M. malmoense	[40,69]
M. marinum	[35]
M. mucogenicum	[33,86,88,127,128b]
M. nonchromogenicum	[36,40]
M. peregrinum	[33,36,88]
M. phlei	[34]
M. scrofulaceum	[31,33,54,81]
M. septicum	[42b]
M. shimoidei	[35]
M. simiae	[65,81,82]
M. smegmatis	[40,86]
M. szulgai	[40,81]
M. terrae	[24,31,34,35,58,72]
M. tusciae	[41,128]
M. vaccae	[40]
M. xenopi	[43,45,46,49,53,56,62,65,66,69,78,91,93]

[a] MAC (*M. avium* complex or *M. avium-intracellulare* complex) consists of the species *M. avium* and *M. intracellulare,* and isolates which cannot be assigned to the species level and which are often reported as 'Mx' or '*Mycobacterium* sp. X' (=positive for MAC by DNA probe but not for *M. avium* or *M. intracellulare*). *M. avium* consists of four subspecies: *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *silvaticum*.

[b] In these studies, *M. chelonae* was named *M. chelonei* according to the taxonomy of that time. *M. chelonei* has been renamed *M. chelonae* subsp. *chelonae*.

[c] Indistinguishable by 16 S rDNA sequencing [33] and RFLP [81].

[d] Could not be isolated from tap water by culture but was detected by PCR.

personnel before entry and inadequate distribution system security [129]. Once (myco)bacteria have gained access to the piping, these organisms can survive and persist provided that the environmental conditions are favourable. Physiological characteristics, such as growth at low nutrient concentrations, biofilm association, protozoal interactions, and chlorine resistance (see Section 7.2), are important survival factors.

3.1. Growth conditions

In general, growth of microorganisms in DWDSs and on pipe walls is infuenced by several factors, including the concentration of biodegradable organic matter (this parameter includes the biodegradable dissolved organic carbon and the assimilable organic carbon, AOC), microbially available phosphorus and nutrients, sediment accumulation, microbial interactions, concentration of free residual disinfectants, residence time, environmental factors (including pH, temperature and turbidity of the water), design of the network (presence of dead ends, diameter of pipes), hydraulics, and characteristics (composition, porosity, roughness) of the material covering the distribution pipes [129–140]. Because many of these factors (alone or in combination) can be detrimental for (myco)bacterial growth, it is acceptable

to conclude that results obtained by several studies cannot be easily compared.

3.1.1. Nutrition

Drinking water can be considered as a nutrient-poor medium under the conditions that AOC was removed during water treatment. The AOC is the fraction of the biodegradable organic matter which can be converted to new cellular material (assimilation) and is a measure of the growth potential in the drinking water. Starved bacteria, corrosion, sedimentation and oozing of organic matter will contribute to the increase of nutrients in drinking water. Prevention of regrowth of heterotrophic bacteria in drinking water is obtained when the AOC concentration is $\leqslant 10$ μg acetate-C equivalent l^{-1} [141].

Mycobacteria can survive in water containing trace amounts of nutrients. Proliferation of slowly growing mycobacteria appeared to be limited by the AOC levels in distribution system waters [35]. Unfortunately, these authors did not report a limiting concentration. In a model distribution system, it was shown that when no disinfection was applied, *M. avium* could be recovered from biofilms at an AOC level of >53 μg l^{-1} [142]. Carson et al. [143] showed that growth of *M. chelonae* (in their study assigned as *M. chelonei*) and *M. fortuitum* strains in commercial sterile distilled water at 25 °C was possible. *M. chelonae* was able to multiply in commercial sterile distilled water, attaining population levels of 10^5 to 10^6 CFU ml^{-1}. Over a period of one year, total viable counts in commercial sterile distilled water declined slowly. According to Carson et al. [143], *M. chelonae* utilized trace amounts of volatile or other nutrients which were inevitably present in commercial sterile distilled water. On the other hand, after three months, one log reduction of *M. intracellulare* in reverse-osmosis deionized water at 4 °C and 37 °C was observed [144].

Humic acids and fulvic acids stimulated the growth of *M. avium* [145]. Large numbers of mycobacteria (4.5×10^4–1.2×10^6 CFU g^{-1} dry soil) were found in boreal coniferous forest soils [146]. Drinking water from production units located in or nearby humic- and fulvic-rich environments (e.g., forests) can be rich of these compounds. Humic substances can act as the sole carbon and energy source for biofilm bacteria as was demonstrated in a reactor system [147]. Other conditions which stimulated growth of *M. avium* included low pH (pH as low as 4.0), low dissolved oxygen (4% of atmospheric levels) and high soluble zinc [145,148]. An association between soluble zinc and the recovery of *M. avium* from hospital water systems with galvanized pipes consisting of zinc alloys, has been made [148]. A positive correlation was found between the iron content of the water and the number of mycobacteria [41], although this does not necessarily points to a causal relationship. The higher iron content was probably due to corrosion of the pipe wall and it has been shown that iron reacts with free chlorine, resulting in a decrease of the chlorine content. Indeed, a minimal free chlorine content of 0.19 mg l^{-1} was found in the distal parts of the DWDSs [41].

3.1.2. Temperature

Water temperature can drastically affect the microbial diversity in aquatic environments. The group of EM contains a continuum of psychrophilic and mesophilic species, including a few species that are relatively heat resistant and can survive high temperatures. It has been suggested that *M. avium* subsp. *paratuberculosis* can survive high-temperature–short-time pasteurization (15 s at 72 °C) of raw milk although diverging opinions exist about this subject [149–151]. The water temperature in drinking water supply chains can fluctuate with the season while constant and higher water temperatures, even up to 60 °C, are found in distribution systems and hot water tanks in buildings [152].

M. avium was isolated from hot water systems (51.9–57.2 °C) in hospitals [55]. The number of organisms recovered by du Moulin et al. [55] ranged from 1 to 500 CFU 100 ml^{-1}. *M. avium* was found in hot tap and shower samples collected from two hospitals, in numbers ranging from 0.4 to 5.2 CFU ml^{-1} [68]. *M. intracellulare* was isolated from a shower sample (50.5 °C) [33]. 35% (13/37) of the samples from warm taps or showers were positive for mycobacteria [33]. Several authors made the association of *M. avium* or MAC with hot tubs, bath water systems or spas [153–162]. The predominance of *M. avium* or MAC in hot water was, however, not confirmed by Aronson et al. [73] and Covert et al. [33]. *M. kansasii* and *M. xenopi* were more frequently recovered from water distribution systems in buildings than from the main water system, probably because the higher temperatures in buildings are more favourable for growth [20,93].

A seasonal effect on mycobacterial recovery was observed by Kubálek and Komenda [31], who found a higher frequency of mycobacteria in potable water collected in spring compared to samples collected in autumn. These results could not be confirmed by Falkinham III et al. [35] who claimed that the choice of the incubation temperature influenced the recovery of mycobacteria.

3.2. Biofilms

Some EM may not be able to grow fast enough to overcome the dilution effect of the water flow but can persist in the DWDS through surface attachment. Several authors have demonstrated the capacity of myco-

bacteria to produce or live in biofilms in aquatic environments [32,35,39,42b,92,142,163–176]. A biofilm is a microbial community characterized by cells that are irreversibly attached to a substrate or interface or to each other, that are embedded in a matrix of extracellular polymeric substances that they have produced, and that exhibit an altered phenotype with respect to growth rate and gene transcription [177]. The formation of biofilms can be regarded as a universal bacterial strategy and optimum positioning with regard to available nutrients [178]. Biofilms, consisting of single or multiple bacterial species, can function as a reservoir of opportunistic and true human pathogens. Bacteria, including mycobacteria, embedded in a biofilm are more protected against antimicrobial agents than planktonic cells [169,170, 174,179,180]. Restricted penetration of the antimicrobials into the biofilm, decreased growth rate, and expression of possible biofilm-specific resistance genes are mechanisms which, alone or in combination, explain biofilm survival in a number of cases [179,180].

Biofilm formation can be described by a multi-step model [181–183], in which the following steps are distinguished: (1) formation of a conditioning film (often described as fouling of the surface), which alters the properties of the surface, which in turn influences bacterial adhesion, (2) reversible adhesion of the microorganism to the surface, (3) irreversible adhesion, (4) microcolony formation and (5) biofilm formation. Detachment ('sloughing') of the biofilm leads to the spread of bacteria further in the environment. The structure of a biofilm depends on many internal and external factors, and consequently, several models have been proposed [184,185]. Biofilm formation in DWDSs could be viewed as a successional process in structure and species composition [186]. Besides pathogenic bacteria, biofilms can harbour iron, sulphate and manganese bacteria which cause corrosion and water discolouration affecting taste, odour and appearance of the potable water.

The mechanism of biofilm formation by mycobacteria is not fully understood and has only recently been studied. Mycobacteria lack surface structures like flagella, pili, fimbrae, surfactants, slime or capsules as surface translocation modes. These structures are used by microorganisms to reach and to attach to the surface. Mycobacteria achieve spreading on surfaces by a sliding mechanism. This form of motility is likely to play an important role in the surface colonization in the environment as well as in the host [187]. The surface properties of cells, in particular determined through glycopeptidolipids, severely affect their ability to spread on solid surfaces [187–189]. Hydrophobic interactions between the exposed fatty acid tails of the glycopeptidolipids and the hydrophobic surface would mediate attachment and biofilm formation [188]. Conversely, the hydrophobic cell cannot interact with hydrophilic surfaces, resulting in a reduction of friction and sliding on the surface. Ridgway et al. [190] suggested that cell surface polypeptides, α-1,4- or α-1,6-linked glucan polymers, and carboxyl ester bond-containing substances (possibly glycopeptidolipids) may be involved in mycobacterial adhesion in their study of the adhesion of a *Mycobacterium* sp. to a cellulose diacetate membrane used in reverse osmosis. Limia et al. [191] hypothesized that one of the possible functions of the *secA* gene from *M. avium*, encoding a major preprotein translocase subunit associated with the secretion system of prokaryotes, was involved in the secretion of proteins or peptides associated with biofilm formation. The *upk* gene, encoding for undecaprenyl phosphokinase, seemed to be essential for biofilm formation of *M. smegmatis* [192]. Biofilm formation by *M. fortuitum* showed a sigmoidal growth curve on silastic rubber [167]. A similar growth pattern was observed in a *M. phlei* biofilm [169]. Microcolonies and exopolysaccharides formation in mature mycobacterial biofilms have been detected by scanning electron microscopy [169,170]. The presence of Ca^{2+}, Mg^{2+} or Zn^{2+} ions in water influenced *M. avium* biofilm formation on polyvinyl chloride, although biofilm formation was strain-dependent among five clinical *M. avium* isolates [171]. It should be stressed that all aforementioned studies were performed using one single mycobacterial species, under in vitro conditions and without the interaction of other microorganisms whereas biofilms in piped distribution systems will mostly be composed of multiple species.

Mycobacteria-containing biofilms have been found in water meters, which are usually placed at the distal site of the distribution system [35]. In this regard, the species *M. aurum*, *M. avium*, *M. gordonae*, *M. haemophilum*, *M. intracellulare*, *M. marinum*, *M. shimoidei* and *M. terrae* have been recovered from water meter biofilm samples. Among the 267 biofilm *Mycobacterium* sp. isolates, 131 were identified as *M. intracellulare*, four as *M. avium* and 30 isolates could not be assigned to the species level. The number of *M. intracellulare* in the mycobacterial biofilm was 1.3–2900 CFU cm^{-2}, with an average number of 600 CFU cm^{-2}. The number of slowly growing *Mycobacterium* spp. was between 1.4 and 4300 CFU cm^{-2}, with an average value of 820 CFU cm^{-2}. In a study of Schulze-Röbbecke et al. [92], the density of mycobacteria ranged between 10^3 and 10^4 CFU cm^{-2}, whereas *M. flavescens* was mainly found in domestic water system. Mycobacterial counts up to 4.6×10^5 CFU cm^{-2} were found in DWDS biofilms in South Africa [42b]. Falkinham III et al. [35] emphasized that the recovery of mycobacteria was an important parameter in their study. Mainly the growth of faster-growing bacteria influenced the results. In conclusion, the choice of the method (e.g., surface scraping, sonication, microscopy) to study the biofilm is an important parameter and the results in relation to counts and species composition between different studies need to be interpretated with care.

3.3. Protozoa

Another factor in the survival of mycobacteria is their association with free-living amoebae and other protozoa. Amoebae are present in potable water distribution systems [193,194] and hospital water systems [195] and it has been shown that several other microorganisms have an amoeba-associated lifestyle [196]. The exposure of humans to amoebae and amoeba-associated mycobacteria seems, therefore, not unlikely. Several species and strains of free-living amoebae show resistance to transient exposure to elevated temperatures [195,197]. Furthermore, it has been shown that *Acanthamoeba polyphaga* and *A. castellanii* produce small respirable vesicels which can contain living bacteria like *L. pneumophila* [198]. Due to their small size (2.1–6.4 μm in diameter), these vesicles can easily be inhaled. Although a limited number of studies has been published with regard to mycobacterial protozoal interactions, it is clear uptil now that in contrast to the so-called *Legionella*-like amoebal pathogens [199–202], some EM are associated with, but are not completely dependent on amoebae.

Survival of *Mycobacterium* spp. (including *M. avium*) in *A. castellanii* was already reported in 1978 [203]. *M. avium, M. fortuitum* and *M. marinum* were shown to grow intracellularly in *A. castellanii* Neff ATCC 30234, whereas *M. smegmatis* was not able to survive within this amoeba [204]. Survival or growth of *M. avium* was observed in other amoebae, such as *A. polyphaga* [205] and *Dictyostelium discoideum* [206], and it has been demonstrated that *M. avium, M. intracellulare* and *M. scrofulaceum* grew in the ciliate *Tetrahymena pyriformis* [207]. *M. avium* was also found within the outer walls of the double-walled cysts of *A. polyhaga* [205] and survived encystment of *T. pyriformis* [207]. *M. bovis* survived ingestion by *A. castellanii* under experimental conditions [208]. Survival and growth of *M. marinum* was observed in *D. discoidemum* [209]. All these studies suggest that free-living protozoa may be natural hosts and reservoirs of several *Mycobacterium* species, including *M. avium*. Amoebae may not only serve as reservoirs but also as vehicles for transmission [197]. Amoeba-grown *M. avium* displayed enhanced entry into epithelial cells and was more virulent in the macrophage in vitro models as well as in the C57BL/6 bg^{+}/bg^{+} beige mouse model of infection [204]. Although growth of *M. avium* in and interaction of *M. avium* with amoebae may have resulted in enhanced virulence, it is possible that *A. castellanii* transversed the mouse intestinal epithelium and could carry bacteria along. In addition, amoeba-grown *M. avium* have been shown to become less susceptible to rifabutin, azithromycin and clarithromycin [210].

As will be discussed further in this review (Section 4.4), special concern exists about dental unit waterlines (DUWLs). Biofilms inside dental instruments can considerably increase the concentration of free-living amoebae. Barbeau and Buhler [211] reported that the concentration of free-living amoebae was up to 300 times higher in dental unit water samples than in tap water even though the water source was the same. *Hartmanella* spp., *Vanella* spp., and *Vahlkampfia* spp. were the most frequently encountered amoebae species whereas *Naegleria* spp. and *Acanthamoeba* spp. were present in 40% of the samples. Through the exposure of dental workers and patients to aerosols containing amoebae, non-mycobacteria (e.g., *P. aeruginosa* and *L. pneumophila*) and EM, it cannot be ruled out that this can lead to clinical implications. Improvement of water quality by lowering bacterial and amoebal counts are, therefore, recommended.

4. Waterborne infections

EM can cause a broad range of infections and diseases. In some cases, colonization of 'healthy' individuals or individuals with no predisposing conditions has occurred, resulting in positive cultures without clinical signs. Furthermore, it should be highlighted that colonization (=an outcome of infection whereby a microbe is recovered from a non-sterile site at which no damage is clinically apparent), infection (=acquisition of a microbe by a host) and infectious disease (=a state of infection where host damage occurs as a result of host–microbe interaction) are distinct clinical concepts [212,213] that are occasionaly used in the wrong context in literature.

4.1. Typing of isolates

Molecular typing methods are important tools in recognizing outbreaks of infection, detecting cross-transmission of nosocomial pathogens, determining the source of infection, and recognizing particularly virulent strains of organisms [214]. It must be emphasized that molecular typing is a part of the epidemiological investigation and cannot replace information obtained by other techniques or information sources [215–217]. Multilocus enzyme typing and DNA fingerprinting methods such as randomly amplified polymorphic DNA (RAPD) analysis, restriction fragment length polymorphism (RFLP) analysis, and the widely used pulsed field gel electrophoresis (PFGE) technique have been used for typing EM [218].

4.2. Weakened immune system

In some cases, increased susceptibility of human hosts to EM has been observed due to mutations in five genes (*IFNGR1*, *IFNGR2*, *IL12RB1*, *IL12B* and *STAT1*)

[219–221]. Clinical treatments such as chemotherapy in cases of cancer treatment and organ transplantation, render these patient groups more vulnerable to infections. Other risk groups include neonates, intensive care unit patients, and elderly persons whose immune system became weakened. Most authors report infections in immunocompromised persons such as AIDS patients. This latter patient category has probably the highest risk for infection with EM.

Although EM have been recovered from potable water, there are only very few matches where such isolates were found to be genotypically related to patient isolates. By PFGE typing, a *M. avium* isolate from an AIDS patient was indistinguishable from an isolate from a hospital recirculating hot water system [68]. Another AIDS patient was infected with a strain of *M. avium* of which the PFGE pattern was indistinguishable from an isolate from his home water supply [98]. In a study using simian immunodeficiency virus-inoculated rhesus macaques (*Macaca mulatta*), six *M. avium* animal isolates displayed DNA fingerprints identical to that of an environmental isolate recovered from the facility's water distribution system [222]. In case of hospitalized immunocompromised patients, inexpensive but yet potentially effective measures such as boiling or filtering water and the use of disposable sterile sponges instead of showering are recommended [223,224]. The opinion that bathing is preferred above showering is not shared by all authors since the route of transmission is not fully understood [225].

Although isolation of waterborne strains from patients has been documented, there is no general agreement on the risk of exposure to environmental sources like, e.g., home water systems. In a study by Montecalvo et al. [94], it was shown that the patient's (HIV-positive) mean daily water consumption from taps that cultured positive for MAC was about three glasses. Rice et al. [226] estimated that approximately 1500 individuals with advanced AIDS, within a US HIV-infected population of 900,000, were exposed to MAC via tap water ingestion each day. The estimation was based on two MAC tap water exposure models, each resulting in a value of 650 and 2400 individuals. Horsburgh et al. [227] concluded that water or water aerosols due to daily showering were not risk factors for MAC disease in HIV-positive persons having <50 $CD4^+$ cells mm^{-3}. Similar observations were made by von Reyn et al. [98], who concluded that having a home water supply colonized with *M. avium* was not associated with risk of developing a disseminated *M. avium* infection. Other studies have shown that urban residence [76], consumption of raw or partially cooked fish or shellfish [76,228], swimming in an indoor pool [228], and history of bronchoscopy [228] are risk factors of infection or disseminated disease. Meunier et al. [37] advised not placing aquariums in units where imunocompromised patients are treated. Opening of the aquarium lid to feed the fish or replacement of the filter, may generate aerosols containing mycobacteria such as *M. fortuitum* and *M. peregrinum*. Clearly, there is no consensus today about the risk for AIDS patients ($CD4^+$-count < 100 mm^{-3}) or for healthy persons of exposure to drinking water, consumed as such, or through contact with water droplets (showering). Panic can have adverse effect on consumer confidence and drives them to home purification units which may adversely affect water quality when used without knowledge [229]. Rodgers et al. [230] showed in a laboratory experiment that *M. avium* can colonise point-of-use water filters and even exhibited resistance to silver. The percentage isolation frequency of acid-fast bacteria (no distinction was made between *Mycobacterium* spp. and other acid-fast bacilli such as *Nocardia* spp.) increased from 16.6% to 43.8% for tap water and point-of-use treated water, respectively [231]. These two observations were not supported by Sheffer et al. [232] who found that new point-of-use water filters completely eliminated *M. gordonae* from hot water. However, it should be stressed that these latter authors did not include other *Mycobacterium* spp. in their study.

Comprehensive epidemiological studies are needed to provide firm evidence that pathogenic mycobacteria are transmitted by the water route and contribute significantly to both morbidity and mortality [233]. In the end, it is the task of national governments to set standards in respect of public health and communicate these in the right way. Meanwhile, several organizations and patient associations have published warnings for the risk of waterborne infections with *M. avium*.

4.3. Nosocomial infections

Important water reservoirs in the hospital include potable water, bedside drinking-water carafes, water fountains, sinks, faucet aerators, showers, tub immersion, toilets, dialysis water, ice and ice machines, water baths, flower vases, eyewash stations and dental unit water stations [99,234]. Literature data on EM-associated nosocomial infections have been reviewed elsewhere [14–16]. Waterborne nosocomial infections caused by mycobacteria include wound infections, postinjection abscesses, surgery-related outbreaks, and infection after bronchoscopy or dialysis. The following sources can be distinguished [223]:

- rinsing with contaminated tap water of surgical devices and endoscopes/bronchoscopes after disinfection;
- contaminated ice and ice machines;
- inhalation of aerosols during showering.

Other sources of EM include biofilms inside equipment [64,235,236] and contaminated solutions used as skin disinfectant or for skin-marking [237,238].

4.4. Dental unit waterlines

Another concern consists of the development of respiratory infections in dental patients and dentists due to the presence of mycobacteria and mycobacterial biofilms in DUWLs [166,176,239–244]. The unique feature of dental chair waterlines is the capacity for rapid development of a biofilm on the dental water supply lines combined with the generation of potentially contaminated aerosols [239]. The plastic tubing utilized in dental equipment (e.g., polyvinyl chloride and polyurethane) generally favours bacterial adherence due to its hydrophobic character. The water column inside the small lumen moves in the center of the tubing leaving a thin layer of liquid virtually undisturbed against the walls. This physical state creates propitious conditions for water microflora to establish a biofilm [211]. Intermittent use patterns of dental lines leads to stagnation of the entire water column within the waterlines for extended periods during the day, thus promoting further undisturbed bacterial proliferation [239]. Microbial levels of water in dental unit water systems can exceed those considered as safe for drinking water, as established by international guidelines [239,240]. Numbers of EM in DUWLs can exceed those of drinking water by a factor of almost 400 [166]. Identified species were *M. chelonae*, *M. flavescens*, *M. gordonae*, and *M. simiae* [166]. High numbers of EM may be swallowed, inhaled or inoculated into oral wounds during dental treatment with the potential for colonization, infection or immunization [166,239]. At present, there is no evidence of a widespread public health problem from exposure to dental unit water. Nevertheless, the goal of infection control is to minimize the risk of exposure to potential pathogens, e.g., by using sterile water during surgical treatments and by creating a safe working environment in which to treat patients [239,243]. Personal protection such as the use of face seal masks and a rubber dam are measures that can be easily implemented [245,246]. Recently, the Centers for Disease Control and Prevention (Atlanta, USA) have published guidelines for infection control in dental health-care settings [247]. On the level of microbiological control, dental unit water should contain no more than 200 CFU ml^{-1} (aerobic heterotrophic plate count) as recommended by The American Dental Association [248]. The use of disinfectants such as chlorine was more effective than drying the DUWL [249]. Flushing the DUWL results only in a temporary reduction in bacterial load and has no or little effect on the biofilm [250]. Chlorine dioxide was a powerful chemical which had a positive result on controlling DUWL biofilm [251].

4.5. Cystic fibrosis

Cystic fibrosis (CF) is a genetic disease as a result of defects in the cystic fibrosis transmembrane conductance regulator gene. CF is characterized by abnormally viscous secretions, mucus plugging of the airways, intense inflammation, chronic airways infection, gastrointestinal/nutritional abnormalities, and early death due to progressive bronchiectatic lung disease [252]. Lungs of CF patients are often colonized or infected with bacteria, including *Burkholderia cepacia*, *Haemophilus influenza*, *P. aeruginosa*, and *Staphylococcus aureus* [253–256]. In the last decades, mycobacteria have been isolated from patients suffering from CF [257–259], including *M. abscessus* and MAC that are of particular concern. The role of mycobacteria as potential pathogens for CF patients remains unclear [254]. CF patients that are repeatedly positive in mycobacterial culture and that have persistent symptoms despite adequate conventional antimicrobial treatment should be evaluated for mycobacterial disease [260]. According to Griffith [261], the problem of EM lung infection will certainly grow because the life expectancy of CF patients continues to improve. Aging CF patients may be at greater risk because of increased exposure to antibiotics and immune-modulating drugs, chronic mucopurulent lung disease and diabetes mellitus [262].

To our knowledge, only one study so far has investigated the possible correlation between *Mycobacterium* spp. isolated from CF patients and drinking water. In this study [88], *M. mucogenicum* and *M. peregrinum* were isolated from water supply units from which the authors concluded that *M. abscessus* was not acquired nosocomially.

4.6. Repeated exposure

Healthy persons can become infected after repeated exposure. This was shown in a few studies where MAC caused mycobacterial disease after frequent bathing or use of hot tubs [153–162]. It is unclear if hot tub lung results from hypersensitivity to MAC or is associated with infection [263,264].

Picardeau et al. [70] found identical PFGE patterns for *M. kansasii* isolates from three HIV-negative patients from a single family living in the same household as for a shower isolate recovered from their home. A *M. avium* sputum isolate from a non-AIDS patient yielded an identical PFGE pattern as an isolate from a water sample isolated at home [73]. A similar observation was found between isolates from sputum samples of non-AIDS patients and hospital water isolates [73].

Mycobacteria can be found in stool of healthy persons [265], suggesting that drinking water and food [95,266,267] can be considered as sources of intake.

Swimming pools provide a suitable habitat for the survival and reproduction of mycobacteria [19,28,268,269]. Splashing in the water creates small water droplets which can be inhaled. Mycobacteria were detected in potentially respirable water particles (aerodynamic diameter of <10 μm) generated at whirlpools [270]. Until

today, no mycobacterial infections due to aerosolization of swimming pool water have been reported.

5. Mycobacterial drinking water risk assessment

It is clear that due to the presence of EM in drinking water, daily exposure cannot be excluded. People are exposed by drinking, eating, bathing, and inhalation of aerosols (showering, cleaning). The gut and respiratory tract are considered as the port of entry [271]. *M. avium* can survive the acidic conditions of the stomach [272]. It is also plausible that aspiration, rather than aerolisation, is a mode of transmission [273].

Microbial risk assessment (MRA) is a method to estimate the risk of infection from pathogens and is often used in food microbiology [274,275], but can of course also be applied in drinking water hygiene practices [276]. Essentially, microbial food safety risk assessment includes (i) hazard identification, (ii) exposure assessment, (iii) hazard characterization (or dose–response assessment), and (iv) risk characterization [274,275,277]. MRA is one of the three components of risk analysis next to risk management and risk communication [278].

It is the aim of MRA to predict the number of infected persons under non-outbreak conditions, to determine an acceptable risk and hence to define microbiological standards for the particular germ in drinking water, to determine the effectivenes of drinking water treatment, to optimize the disinfection process against microbial risks and to provide a defensive position for new and emerging agents in the absence of epidemiological evidence [276,279].

Although MRA would be a helpful tool to better understand the risk of EM in drinking water, there are at the moment no or few channels that can supply this information. Problems which are encountered include the lack of information with regard to the infectious dose of the pathogen, its relative distribution and abundance in the drinking water, putative virulence factors, and the choice of a 'model organism' and suitable mathematical model. In addition, water use, consumption patterns and diet of individual consumers can vary greatly and affect their potential exposure [229]. Information about the infectious dose of EM is scarce and is mainly concentrated on MAC and beige mice [280]. The oral infectious dose for *M. avium* as summarized by Rusin et al. [281] is 10^4–10^7 for mice. As shown above, a uniform distribution of EM cannot be given because their presence is depending on the DWDS itself.

It is clear that a MRA cannot be completed without the knowledge of fundamental parameters such as the infectious dose, daily intake or exposure. This explains why almost no MRAs applied to EM are available until today. In the risk assessment of Pankhurst [282], EM in DUWLs were considered as an overall low level of risk for healthy patients and staff. In a quantitative MRA, it was shown that biofilm detachment and the interaction of *Legionella* with acanthamoebae were two important ecological factors that increased the risk of legionellosis [283]. Because the ecology of EM and *Legionella* spp. shows interfaces, these parameters need to be taken into consideration as well in the set-up of an MRA.

6. Pseudo-infection diagnosis

Pseudo-infection diagnoses are false-positive cases exogenous to clinical samples, often traced to contamination which can occur at any stage in the laboratory procedure (sampling, transport, analysis) [284]. Because tap water can contain EM, it has been advised that patients may not rinse their mouths or gargle with tap water before sputum collection [77]. Furthermore, mouth rinsing with sterile water immediately before sputum expectoration or avoiding consumption of tap water and iced beverages for several hours before expectoration can be taken into consideration [77]. However, since sterilized water can contain dead mycobacteria, it is even recommended to filter the water in order to remove the cells [285]. In this way, false–positive results after cell staining are avoided. Stine et al. [54] suggested carrying out an in-line filtration of the water supply chain, with monthly filter changes, when water is used for obtaining and/or processing diagnostic specimens. Cooke et al. [286] recommended that a true bacterial 0.2 μm final filter should be used rather than a 0.2 μm particulate filter. Changing the filter at periodic time is absolutely necessary. Dizon et al. [287] showed that a deionizer filter was colonized with *M. gordonae* and subsequently contaminated deionized water which was used to prepare laboratory solutions. A similar observation was found by Kelly [285] and Heelan [288] who detected *M. gordonae* and *M. gordonae* and MAC, respectively, in distilled water prepared at the laboratory.

Pseudo-infection diagnoses may give rise to wrong conclusions followed by unnecessary chemotherapy. Clusters of pseudo-infections lead to pseudo-outbreaks or pseudo-epidemics.

7. Prevention of mycobacterial regrowth in DWDSs

It is clear that due to the size and complexity of a public water distribution network, the presence of EM cannot be fully excluded. Therefore, a step-by-step approach must be used in order to minimize the growth conditions of EM. This reduction has to occur at each level of the drinking water production and distribution chain. In addition, special treatment of drinking water can be considered in case of special patient groups.

General measures to control biofilm development are focussed on nutrient control, control of contamination from materials and equipment, control of mitigation of system hydraulic problems, cross-connection control and backflow prevention, disinfectant residuals and corrosion control [129]. In a model distribution system, it was demonstrated that reducing the biodegradable organic material in the water, control of corrosion of pipe material, maintenance of an effective disinfectant residual, and management of hot water temperatures can decrease the occurrence of *M. avium* [142]. Removal of nutrients and maintenance of a chlorine residual may be considered as the most important measures to minimize biofilm formation in drinking water systems. This section will discuss a number of suitable measures to reduce the mycobacterial regrowth in DWDSs.

7.1. Processing of the water

The source of the water can either be groundwater or surface water (e.g., from a river or lake). Because surface water is often more polluted than groundwater, more stringent treatments are needed such as coagulation, sedimentation, ozonation, sand filtration and granular activated carbon filtration in order to remove or breakdown pollutants.

Raw water densities of acid-fast organisms (no distinction between *Mycobacterium* spp. and other acid-fast bacilli such as *Nocardia* spp. was made) correlated with the level of raw water turbidity [289]. The positive association between *M. avium* CFU and raw source water turbidity suggested that *M. avium* cells were bound to colloidal or suspended particles [35]. Therefore, one approach to reduce the numbers of mycobacteria in drinking water would be the reduction of the particle count and turbidity of the raw water [290].

Treatment processes reduced the number of slowly growing *Mycobacterium* spp. (i.e., plant effluent CFU/raw source water CFU) with almost 2 log units (1.98), with a range of 1.96–4 log units, in five surface water systems [35]. Similar results were obtained by Le Dantec et al. [36], who showed that both rapid sand filtration and slow sand filtration removed mycobacteria. The number of mycobacteria was, however, higher after rapid sand filtration. No mycobacteria were found in most samples at the end of the water treatment processes, indicating that mycobacteria were efficiently removed from the raw water [36]. EM were more frequently detected in biofilms from bank-filtered drinking water than in biofilms from drinking water conditioned from ground water [32]. A higher mycobacterial count was found in systems using surface water and applying ozonation as an intermediate treatment or posttreatment than those distributing non-ozonated surface water, ground water, or mixed water [41].

7.2. Disinfection

Drinking water is disinfected through addition of chemicals (e.g., chlorine, sodium hypochlorite, chloramine, chlorine dioxide, ozone) to the water or by applying physical treatments (UV-irradiation or ionizing radiation) in order to reduce the survival and growth of microorganisms. The maintenance of a high residual concentration of disinfectants is obtained by prevention of corrosion and a low organic level in the drinking water.

Chlorination is the most common disinfection method and most chlorine-releasing agents are considered as mycobacteriocidal [291]. However, due to the complex cell wall, mycobacteria are quite resistant to several disinfectants. *M. chelonae* and *M. fortuitum* grown in commercial sterile distilled water were markedly resistant to chlorine, with up to 60% survivors at 60 min of exposure to 0.3 μg of free chlorine ml^{-1} and up to 2% survivors at 60 min of exposure to 0.7 μg of free chlorine ml^{-1} [143]. More than 80% survival was noticed for *M. chelonae, M. fortuitum*, *M. gordonae*, and *M. scrofulaceum* after 10 min exposure to 0.2 μg free chlorine ml^{-1} in a suspension test [292]. Free chlorine concentrations of 1.0 mg l^{-1} eliminated all the mycobacterial cultures (MAC, *M. chelonae*, *M. fortuitum*, *M. gordonae*, *M. intracellulare*, and *M. kansasii*; reported reduction of 100.000 CFU) tested within 8 hours of exposure whereas a concentration of 0.15 mg l^{-1} had virtually no bactericidal effect [293]. *M. avium* survived an exposure to chlorine at 4 mg l^{-1} for 60 min [294]. Susceptibility tests under standardized conditions have shown high chlorine resistance in comparison to *E. coli*. Chlorine $CT_{99.9\%}$ (concentration multiplied by the time required for 99.9% inactivation) values of 51 to 204 mg × min × l^{-1} for *M. avium* are 580 to 2300 times higher than those for *E. coli* [295]. $CT_{99.9\%}$ values of 100 and 135 mg × min × l^{-1} for *M. chelonae* and *M. fortuitum*, respectively, were calculated when cells were grown in 7H9 Middlebrook broth and tests were performed at pH 7 and 23 °C [296]. *M. aurum* and *M. gordonae* were 100 and 330 times more resistant to chlorine than *E. coli*, respectively [296]. The resistance of mycobacteria to chlorine, expressed as the *k* value (liters per minute per milligram), was as follows: *M. fortuitum* (0.02) > *M. chelonae* (0.03) > *M. gordonae* (0.09) > *M. aurum* (0.19) [296]. These data indicate that *M. chelonae* and *M. fortuitum* were more resistant whereas *M. aurum* appeared to be the most susceptible mycobacterial species to chlorine. Le Dantec et al. [296] calculated that a chlorination of 0.5 mg l^{-1} chlorine for 2 h could eliminate over 5 log units of *M. aurum*, 4 log units of *M. gordonae*, but only 1.5 log units of *M. fortuitum* or *M. chelonae*. Falkinham III [297] observed that the chlorine susceptibility of *M. avium* and *M. intracellulare* were similar whereas *M. scrofulaceum* was approximately threefold more

sensitive. Other factors that may influence chlorine susceptibility, are growth stage, growth rate, colony type, oxygen concentration, and growth temperature as observed for *M. avium* or *M. intracellulare* [297]. Moreover, Falkinham III [297] suggested that drinking water treatment with chlorine selects for transparant and unpigmented variants of *M. avium* and *M. intracellulare* because these variants are more resistant to chlorine, probably because they grow more slowly and are more hydrophobic than the isogenic opaque and pigmented strains. Mycobacteria grown in water were more resistant to chlorine than cells grown in culture medium [295,296]. Taylor et al. [295] suggested that a slower growth rate was responsible for a higher chlorine resistance. Chlorine dioxide was a better mycobacterial disinfectant than chlorine at equal concentrations. All tests mentioned above are mainly suspension tests, and it has been shown that the mycobactericidal efficacy of chlorine in suspension tests differs from carrier tests, with a lower efficacy noticed for carrier tests [298].

It can be assumed that mycobacteria in biofilms may be more protected from biocides than free-living cells. Key mechanisms of biofilm resistance to antimicrobial compounds include physical or chemical diffusion barriers (e.g., glycocalyx, exopolysaccharide matrix, waste products), slow growth of the biofilm owing to nutrient limitation, activation of the general stress response and the emergence of a biofilm-specific phenotype [179]. A concentration of 0.6 mg l^{-1} free chlorine had no effect while 10 mg l^{-1} free chlorine inactivated a mycobacterial biofilm of 10^6 CFU cm^{-2} [164]. The minimum biofilm eradication concentration (MBEC) was defined as the minimum concentration of the biocide in which there is no bacterial (biofilm) growth. A MBEC after 30 min for sodium hypochlorite of 26 mg l^{-1}, 125 mg l^{-1} and 2000 mg l^{-1} was found for *M. marinum*, *M. phlei* and *M. fortuitum*, respectively [169,170]. After 120 min, the MBEC was 26 mg l^{-1}, 63 mg l^{-1} and 500 mg l^{-1} for *M. marinum*, *M. phlei* and *M. fortuitum*, respectively.

EM were recovered from drinking water samples with free chlorine levels up to 2.5 mg l^{-1} and total chlorine levels of 2.8 mg l^{-1}; the mean free chlorine level of the EM positive drinking water samples was 0.7 mg l^{-1} [33]. Steadham [21] isolated *M. gordonae* from a potable water sample having a total chlorine level of 1.3 mg l^{-1}. EM were isolated from sites where the chlorine residual was >3.0 mg l^{-1} [40]. On the other hand, EM were not found in samples where the concentration of residual chlorine was higher than 0.5 mg l^{-1} [34]. In conclusion, these data show that insights obtained from (standardized) laboratory experiments do not necessarily reflect the situation to drinking-water supplies and that several environmental conditions (e.g., pH, temperature, chlorine decay, etc.) have an impact on the disinfection efficiency.

Chlorine was an effective disinfectant on non-corroded surfaces (copper and PVC) while monochloramine controlled bacterial levels better on corroded iron pipe surfaces [142]. An increase in *Mycobacterium*-positive samples was noticed when the disinfection regime was switched from chlorine toward chloramine [40].

7.3. Choice of the pipe construction material

Several materials are being used as piping material, including asbestos-cement, cement, iron, steel, lead, copper, polyethylene and polyvinyl chloride. A polyvinyl chloride (PVC) pipe would be recommended over iron or cement piping in order to limit bacterial regrowth and corrosion in a distribution system [134]. This recommendation was supported by one study, in which the number of EM in drinking water was significantly reduced by replacement of an old water distribution network using plastic pipes [34]. The results of Falkinham III et al. [35] did not confirm this observation and showed that the material supporting the biofilms did not significantly affect the frequency of recovery of mycobacteria. Mycobacteria were recovered from biofilms growing on brass or bronze (63%), galvanized (100%) or plastic (64%) surfaces, and *M. intracellulare* was isolated from 25% of brass or bronze, 43% of plastic surfaces but not from galvanized surfaces. It should be emphasized that the study of Tsintzou et al. [34] was conducted within 18 months after replacement, so it is possible that EM did not have the time to colonize the surface of the inner pipe wall.

In general, it can be accepted that old, corroded piping systems, containing dead ends and spaces favour (myco)bacterial growth. Regular inspection of the pipes and their replacement if necessary, are measures that should be considered.

8. Special measures

Although control factors such as reduction of the nutrient level of the water, removal of mycobacteria during sand filtration, and maintenance of a residual disinfectant level will result in a reduction or (temporary) elimination of the EM, it cannot be avoided that EM enter and proliferate in the DWDS (see Section 3). Other additional control factors which may be considered are heat inactivation and monitoring of the tap water quality in order to avoid nosocomial infections.

8.1. Heat inactivation

The heat resistance of bacteria is often expressed by means of the *D* value. The *D* value or thermal reduction time is the time to reduce 90% of the microbial population at a set temperature and under well-described

conditions. In general, the D value depends on many factors such as the temperature, the metabolic state of the microorganism (vegetative cells or spores), species and strain, age of the cells, growth conditions of the microorganism before and after heat treatment, the composition of the heating medium containing the microorganism, and the processing conditions of heating (batch or continuous flow). Therefore, it is necessary to record as many of these parameters as possible in order to correctly compare the different D values reported in literature. D_{65} values of 4 min or less and D_{70} values of 1.5 min or less were found for MAC (swine and human isolates) in phosphate buffer [299]. Schulze-Röbbecke and Buchholtz [300] found a D_{50} value of almost 17 h for *M. avium* DSM 43216 in sterile water with standardized hardness. At 55 °C, a D value of approximately 6 h and 53.5 min was found for *M. xenopi* NCTC 10042 and *M. avium* DSM 43216, respectively. The D_{60} value for *M. avium* DSM 43216 was 4 min. *M. xenopi* NCTC 10042 was the most heat-resistant strain, having a D_{60} value and D_{70} value of 33 min and approx. 23 s, respectively. Notably, the latter authors performed heat susceptibility tests mainly on laboratory strains. One water isolate of *M. kansasii* showed no pronounced differences in heat susceptibility in comparison with *M. kansassii* DSM 43224. Possibly, wild strains that are adapted to grow and survive in more stressful conditions (e.g., in biofilms in the neighbourhood of water boilers or hot water tanks) may exhibit a higher heat resistance. Two strains of *M. avium* and *L. pneumophila* were killed within 3 min during exposure to hot water at 70 °C [294]. This is in accordance with the results of Schulze-Röbbecke and Buchholtz [300], who found a D_{70} value for *M. avium* of 2.3 s. Schulze-Röbbecke and Buchholtz [300] concluded from their heat resistance study that thermal measures to control *L. pneumophila* may not be sufficient to control *M. avium*, *M. chelonae*, *M. phlei*, *M. scrofulaceum* and *M. xenopi* in contaminated water systems. In order to reduce the presence of legionellae in hot water distribution systems, it is recommended that hot water should be stored at 60 °C and distributed such that a temperature of at least 50 °C and preferably 55 °C is achieved within one minute at outlets [301]. However, it may be expected that the heat and flush method to eradicate *Legionella* spp. will also result in a dramatic reduction of EM, taking into account that several species do not tolerate temperatures >60 °C for several minutes. Drawbacks of the latter method include the risk of scalding and the fact that it is time consuming.

Norton et al. [142] showed that the effectiveness of the heat treatment depended upon the pipe material, the level of nutrients, and the effluent temperature. Hot water (>50 °C), in combination with copper pipes and a low-nutrient content influenced *M. avium* biofilm formation significantly in a model distribution system [142].

8.2. Monitoring the water quality of bronchoscope and endoscope washers

Special attention is paid to the effective disinfection of endoscopes and bronchoscopes during which alkaline glutaraldehyde is widely used. EM are generally more resistant to disinfectants than *M. tuberculosis*. It may be assumed that repeated exposure of EM to disinfectants may select for organisms with decreased susceptibility. Griffiths et al. [302] showed that *M. chelonae* subsp. *chelonae* NCTC 946^T was more susceptible than two endoscope washer disinfector isolates. A 5 log reduction was achieved when NCTC 946 was exposed for 1 min to 2% glutaraldehyde, while 60 min exposure was insufficient to achieve a >5 log reduction for the disinfector isolates. Griffiths et al. [302] recommended to disinfect machines with an agent 10,000 mg l^{-1} free chlorine followed by sessional disinfection with 1000 mg l^{-1} available chlorine, on the condition that the apparatus is not damaged through such high concentrations. High concentrations of free chlorine can be achieved by chlorine releasing agents such as sodium dichloroisocyanurate [303]. Special attention should go to cleaning of endoscopes and bronchoscopes in such a way that organic matter (e.g., blood, faeces, and respiratory secretions) must be removed before disinfection. Disinfection should be followed by thorough rinsing with sterile water instead of tap water. Several medical societies have edited guidelines for processing endoscopes and bronchoscopes [304–306].

9. Conclusions

EM are often isolated from potable water and can be considered as common inhabitants of public and hospital drinking water distribution systems. It is important to realize that each DWDS harbours its own (myco)bacterial flora as a result of the specific physico-chemical and microbiological properties of the water (including raw water and treated water), and the specific construction and condition of the DWDS. The complex cell wall is responsible for the relative resistance of EM to disinfectants such as chlorine, and some *Mycobacterium* spp. are resistant to or even multiply at high temperatures. In combination with other properties such as association or growth in biofilms and protozoa, EM seem difficult to eradicate from water distribution systems. Although most attention has been paid to *M. avium*, the ecology and health risk of other EM should not be neglected.

There still exists controversy about the public health significance and potential health risk of EM in drinking water. In case of severely ill or immunocompromised patients, reduction of exposure to EM in drinking water can be considered, but does not provide an absolute guarantee for prevention of infection. In this regard, a

number of simple safety measures including sterilization of water before consumption and washing instead of showering may already significantly reduce the risk of EM-associated infections. Regular inspection of shower heads is recommended because EM are often isolated from shower water samples, and special attention should go to the hygiene of clinical devices (e.g., bronchoscope washers) where EM can be concentrated due to (re)-growth and biofilm formation. For this reason, regular inspection of devices and replacements of sensitive parts of the medical equipment are highly recommended. If possible, hyperchlorination is applied at regular times. Contamination of medical instruments with EM after rinsing with tap water is not uncommon, and therefore rinsing water should be sterilized. Filtration of water in order to remove (dead) acid-fast bacteria will avoid false-positive results during microscopic investigation or PCR-amplification. Sterilized water should be prepared freshly because reservoirs (bottles, tubes, etc.) can become contaminated over time.

Molecular typing methods are excellent tools to trace back sources of EM contamination, but need to better integrated in epidemiological surveys. To this end, the construction of a database for central storage of molecular fingerprints, and well-structured data management are necessary for the early recognition of pseudo-infections or nosocomial outbreaks. Likewise, incorporation of EM as a group of target organisms in surveillance programs is recommended in order to obtain a better understanding of their health impact.

Further research needs to focus on mechanisms of biofilm formation and methods to prevent their development or enable their removal. A standardized method to investigate bulk water and biofilms needs to be developed in order to obtain a better insight in the mycobacterial species diversity and load of drinking water. Data obtained through risk assessments can be used to set guidelines for health workers. For this purpose, better insights in the distribution of EM in the DWDSs, their daily intake or exposure, the main routes of exposure, and the infectious dose of the most clinically relevant species (*M. avium*, *M. kansasii*, and *M. xenopi*) are necessary to sustain a MRA.

In case EM in drinking water present a noticeable health risk, redefinition of the microbiological quality standards for drinking water needs to be considered.

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