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Review

Occurrence of filamentous fungi in drinking water: their role on fungal-bacterial biofilm formation

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ABSTRACT

Water is indispensable to life and safe and accessible supply must be available to all. The presence of microorganisms is a threat to this commitment. Biofilms are the main reservoir of microorganisms inside water distribution systems and they are extremely ecologically diverse. Filamentous fungi and bacteria can coexist inside these systems forming inter-kingdom biofilms. This review has the goal of summarizing the most relevant and recent reports on the occurrence of filamentous fungi in water distribution systems along with the current knowledge and gaps about filamentous fungal biofilm formation. Special focus is given on fungal-bacterial interactions in water biofilms.

1. Introduction

Water is indispensable to life. Therefore, every effort should be done to achieve drinking water as safe as possible. The United Nations Sustainable Development Goal 6 (SDG 6) reinforces that "While substantial progress has been made in increasing access to clean drinking water and sanitation, billions of people – mostly in rural areas still lack these basic services. Worldwide, one in three people do not have access to safe drinking water, two out of five people do not have a basic hand-washing facility with soap and water" [1]. This becomes even more dramatic in times of SARS-Cov-2 where access to clean water and handwashing with soap are crucial to controlling the individual and communitarian pandemic disease of COVID-19. Clean water is still a luxury for poor regions. For this reason, water companies have the main goal of delivering microbiological safe water to the consumers, adequate in quantity and delivery pressure and satisfactory in terms of taste, odour and appearance [2]. This objective can be questioned when microorganisms are present in excess as their growth may affect the organoleptic properties of the water. These microorganisms can be found either as in planktonic forms inhabiting bulk water or as biofilms growing on pipes surfaces [3]. Biofilms can be considered the main source of microorganisms in drinking water distributions systems (DWDS) and are responsible for serious effects, such as changes in the taste, turbidity, colour and odour of the water, corrosion of metallic pipes, disinfectant demand, potential accumulation and dispersion of pathogens and production of toxins [4,5]. Primary and opportunistic pathogens are found in DWDS because they can survive water disinfection. Special protection is provided to microorganisms embedded in biofilms such as sharing of nutrients and metabolic products and increased resistance to environmental stresses, such as hydrodynamic shear forces and disinfection [6]. Under natural conditions, true monospecies biofilms are rare, and consequently, they are usually considered as complex communities [7]. The ecology of a biofilm is a complex function of prevailing growth conditions, hydrodynamic shear forces and presence of microbial metabolites and molecules, such as cell–cell signalling communication molecules, excreted by its inhabitants [8]. This diversity leads to a variety of complex interactions between the microorganisms that are present. Findings into the microbial ecology of DWDS have shown that resistance of microorganisms to disinfectants, particularly chlorine, is affected by this microbial diversity [9]. This information, has, however, been mainly obtained from studies with bacteria. Studies regarding fungi, in particular filamentous fungi, have been gaining attention due to their biofilm formation ability, however, their interaction with bacteria in fungal-bacterial biofilms is still poorly understood.

This review has the goal of summarizing the most relevant and recent reports on the occurrence of filamentous fungi in water distribution systems along with the current knowledge and gaps about filamentous fungal biofilm formation. Special focus on

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fungal-bacterial interactions in biofilms is provided given the latest findings on this topic.

2. Fungi and filamentous fungi biofilms

Fungi are a diverse and abundant group of organisms belonging to the kingdom *Eumycota* [10]. The most recent classification of this kingdom comprises seven recognised phyla: *Basidiomycota*, *Ascomycota*, *Glomeromycota*, *Microsporidia*, *Blastocladiomycota*, *Neocallimastigomycota* and *Chytridiomycota* [11]. Fungal classification is, however, very dynamic, as shown by the recently proposed phyla *Cryptomycota* [12]. As a practical approach to the classification of fungi, division regarding their cellular organisation has been made. They range from microscopic single-cell species (yeasts) to species with massive mycelia. This latter large group of organisms can be characterised by hyphal growth supporting macroscopic sexual reproductive structures (e.g., truffles, mushrooms) and microscopic sexual or asexual reproductive structures, known as moulds or filamentous fungi [13]. Fungi are ubiquitous and some of them, belonging to the phyla *Chytridiomycota,* are particularly adapted to aquatic environments. These fungi are known for producing zoospores morphologically apt to propagate in running waters. Filamentous fungi from other phyla in *Eumycota* are, however, mostly adapted to the terrestrial environment, such as soil and anything in interface with air, as they generally need a solid substrate for spore dispersal [14]. Although DWDS are not considered natural habitats for these filamentous fungi, they can often be introduced into these environments from different pathways, such as physical openings in storage facilities, treatment breakthroughs, leaking joints and adapters, cracks in pipelines and/or during maintenance or mains installation. Sammon et al. [15] demonstrated that airborne spores can be an important external source of filamentous fungi propagules in a DWDS. Once inside these systems, fungi can survive the oligotrophic conditions by scavenging nutrients from the substrate which they colonize or the water in which they are inhabiting. Consequently, their presence may then cause additional problems to the water quality (e.g., unpleasant appearance with flocs and earthy pungent odours, the presence of pigments, pipe blockage, a source of potentially pathogenic and allergy-causing fungi and the presence of mycotoxins) [5,6,16–19]. To maximize nutrient uptake, filamentous fungi will form hyphal mats. Due to their absorptive nutrition mode, secretion of extracellular enzymes that digest complex molecules and apical hyphal growth, filamentous fungi have a high ability to grow on surfaces, thus forming biofilms [20]. Fungal survivability and proliferation in DWDS are believed to be related to the ability to form biofilms [21].

Harding et al. [22] proposed a six-step pioneer model for filamentous fungal biofilm formation based on models for bacteria and yeasts: 1) propagule adsorption, 2) active attachment to a surface, 3) microcolony formation I, 4) microcolony formation II (or initial maturation), 5) maturation and, 6) dispersal (planktonic phase). Recently, Fernandes et al. [23] updated this model by adding an initial step – surface conditioning – where the surface hydrophobicity and charge of spores and the substratum play key roles in the adhesion process. This initial physical contact can result in reversible adhesion, followed by irreversible adhesion with the secretion of adhesive substances by germinated spores and active germlings [22]. Spore germination will ensue if suitable environmental conditions are met [23]. After germlings start to form, they secrete hydrophobins that mediate adhesion and hyphae–substratum interaction [24]. Subsequently, hyphal differentiation produces a complex hyphae net (mycelium) that grows in all directions enclosed within a polymeric extracellular matrix, where quorum-sensing molecules, similarly to bacterial biofilms, are present [25]. The maturation stage for DWDS biofilms should mainly occur in reservoirs due to the requirement of a stable air–water interface for aerial growth and subsequent spore formation and air dispersion [10]. Finally, the dispersion stage occurs through the release of spores or different propagules in response to environmental stresses or biological stimuli [22]. In drinking water, propagules can be dispersed by water flow, which can then establish new biofilms, further spreading the presence of filamentous fungi in drinking water [15,23,26].

3. Occurrence of filamentous fungi in DWDS

Since the 70s, several works have reported the presence of filamentous fungi in WDS worldwide. Table 1 lists and focuses only on the most relevant reports from the last two decades. A wide diversity of filamentous fungi has been isolated/detected from drinking water. The most frequent recovered species belong to the genera *Aspergillus, Cladosporium and Penicillium* (Table 1). This might be related to their ability to secrete a pigment called melanin, which confers protection to spores against a variety of stresses, providing these microorganisms with a competitive advantage and greater resistance to water treatment [6]. In addition, due to the hydrophobicity property of the spores from these genera, further protection is offered against water disinfection as spores tend to aggregate between each other and other particles [6].

Among the isolated filamentous fungi, potentially pathogenic, allergenic and toxigenic species have also been found. This is particularly concerning since several reports on the presence of these fungi have been obtained from hospital water systems [29,30,32,44,45,49,51,53]. In some cases, the presence of pathogenic species (e.g. *Aspergillus fumigatus; Fusarium solani*) in drinking water, has led to the hypothesis of hospital water systems serving as transmission routes for fungal infections. These results indicate that hospital water contains high fungal diversity, including potential pathogens. Many of the fungal species found in drinking water have also allergenic potential [5]. Fungal species from the main genera recovered from drinking water have also been investigated towards their implication with asthma and other respiratory problems, regarding indoor environments [60]. Some of these health adverse effects may arise not only from the fungi itself but also from the production of secondary metabolites and volatile organic compounds (VOC's). Several species from both *Penicillium* and *Aspergillus* genera are known mycotoxin producers. Mycotoxins cause a variety of health problems and are known to be carcinogenic and capable of impairing the immune system in both humans and animals [61]. Of all the different mycotoxins that can be produced, aflatoxins (*Aspergillus* spp.) and zearalenone (*Fusarium* spp.) are some of the most relevant and have been detected in drinking water [5,62]. The concentration of mycotoxins in drinking water is likely to be very diluted and, for the time being, has not been identified as the source of symptoms attributable to mycotoxins.

For an in-depth understanding of the occurrence, ecology and physiology of fungal contaminants in drinking water, Novak Babič et al. [61,63] recently compiled this information in reviews. It focuses on reports from European water sources in the last 30 years, including surface-, ground- and tap-water intended for human consumption.

4. Methodology progression to study fungi in drinking water: emphasis on biofilms

The methodology used to study fungi in drinking water is key to fully understand the real dimension and significance of their occurrence. A high variation on how the analyses are performed is a major issue when trying to compare different studies. This is due to

Table 1
Most relevant filamentous fungi surveys in drinking water from the last two decades.

Location, Date	Water source	Isolation method	Most frequent fungal genera	References
United Kingdom, 1996	Surface water and network	Membrane filtration, centrifugation, direct plating	*Aspergillus, Cladosporium, Epicoccum, Penicillium* and *Trichoderma*	[27]
United States of America (Springfield, MO), 1997	Municipal water supply system	Pipe coupons (Biofilm)	*Aspergillus* and *Penicillium*	[28]
Greece (Thessaloniki), 1998	Hospital and community tap water	Membrane filtration	*Acremonium, Aspergillus* and *Penicillium*	[29]
(Haemodialysis units)	Municipal water supplies of haemodialysis centres	Membrane filtration	*Aspergillus* and *Penicillium*	[30]
Germany (North Rhine-Westphalia), 1998-99	Drinking water	Pour-plating	*Acremonium, Exophiala, Penicillium* and *Phialophora*	[31]
Norway (Oslo), 1998-99	Hospital tap and shower water	Membrane filtration and swabs from water-related surfaces	*Aspergillus, Cladosporium, Paecilomyces* and *Trichoderma*	[32]
United States of America (Little Rock, AR), 1997−2000	Water distribution system of a Hospital	Membrane filtration, swab applicators	*Alternaria, Aspergillus, Paecilomyces* and *Penicillium*	[33,34]
(Houston, TX), 2000	Water distribution system of a Hospital	Membrane filtration, centrifugation, swab applicators	*Fusarium*	[35]
Greece (Thessaloniki, Athens and Heraklion), 2000	Hospital tap water	Membrane filtration	*Acremonium, Fusarium, Paecilomyces* and *Penicillium*	[36]
Poland (Warsaw), 2000-02	Municipal water supply system	Membrane filtration	*Aspergillus, Cladosporium* and *Fusarium*	[37]
Norway, 2002-03	Drinking water (surface and groundwater)	Membrane filtration	*Aspergillus, Penicillium* and *Trichoderma*	[16,38,39]
Turkey (Istanbul), 2003	Water distribution system of a Hospital	Membrane filtration	*Acremonium, Aspergillus* and *Penicillium*	[40]
Portugal (Braga), 2003-04	Tap water	Membrane filtration and swabbing	*Acremonium* and *Penicillium*	[41]
Belgium (Liège), 2005-06	Water distribution system of a Hospital	Membrane filtration	*Aspergillus, Fusarium, Paecilomyces* and *Penicillium*	[42]
Austria, 2006	Drinking water and groundwater	Membrane filtration and plating	*Cladosporium* and *Penicillium*	[43]
Brazil (São Paulo), 2006	Water distribution system of a haemodialysis unit	Membrane filtration	*Aspergillus, Cladosporium, Fusarium* and *Trichoderma*	[44]
	Water distribution system of a haemodialysis centre	Membrane filtration	*Aspergillus, Fusarium, Penicillium and Trichoderma*	[45]
Portugal (Lisbon), 2006-08	Surface, spring and groundwater for the production of drinking water	Membrane filtration, spread plate and pour plate	*Aspergillus, Cladosporium* and *Penicillium*	[46,47]
Australia (Rockhampton), 2007-08	Municipal water supply system	Membrane filtration	*Aspergillus, Cladosporium, Fusarium* and *Penicillium*	[48]
Australia (Rockhampton), 2007-10	Municipal water supply system	Glass, PVC and concrete coupons (Biofilm)	*Aspergillus, Cladosporium* and *Penicillium*	[15]
Brazil (São Paulo), 2007-08	Water distribution system of paediatric haematopoietic stem cell units	Membrane filtration	*Aspergillus Cladosporium, Penicillium* and *Purpureocillium*	[49]
Portugal (Centre), 2008-12	Untreated water (groundwater, spring and surface water)	Membrane filtration	*Penicillium* and *Trichoderma*	[50]
Italy (Marche region), 2010-11	Water treatment and distribution system of haemodialysis units	Membrane filtration	*Alternaria, Cladosporium* and *Tricophyton*	[51]
Brazil (São Paulo), 2011-12	Tap water from wells (groundwater)	Membrane filtration	*Acremonium, Aspergillus, Fusarium* and *Penicillium*	[52]
China (Xiamen), 2011-12	Drinking water (surface and tap water)	Membrane filtration	*Aspergillus, Fusarium, Penicillium, Phialophora* and *Trichoderma*	[17]
United States of America (Pittsburgh, PA), 2011-12	Hospital hot water system	Membrane filtration	*Aspergillus, Cladosporium, Penicillium, Peniophora* and *Rhodosporidium*	[53]
Brazil (Recife), 2013−14 and 2015	Water distribution system (groundwater)	Membrane filtration	*Aspergillus, Fusarium, Penicillium* and *Trichoderma*	[54]
United States of America (Anonymous), 2014, 2016	Drinking water treatment plant	Membrane filtration	*Alatospora, Aspergillus, Penicillium, Taphrina* and *Tuber*	[55]
China (Guangdong Province), 2015	Water supply reservoirs, drinking water treatment plants	Direct plating	*Aspergillus* and *Cladosporium*	[18]
Colombia, 2015	Drinking water network (surface water)	Water-surface interface scraping (Biofilm)	*Paecilomyces, Paraconiothyrium* and *Penicillium*	[56]
United Kingdom (Southwest region), 2014-15	Surface and groundwater chlorinated networks (bulk water and biofilms)	Membrane filtration	*Aspergillus, Basiodobolus, Cladosporium, Plectosphaerella,* and *Rhexocercosporidium*	[7,57]
Poland (Lodz), 2016	Recreational surface water	Centrifugation	*Alternaria, Aspergillus* and *Penicillium*	[58]
Spain (Valencia), 2019-20	Drinking water network	Membrane filtration	*Aspergillus* and *Cladosporium*	[59]

the lack of a uniform approach for detection or isolation of fungi. The most usual isolation pathways for analysing fungi in drinking water are based on water filtration followed by either conventional culturing methods or molecular approaches. Membrane filtration techniques are the most usually employed (Table 1) with varying volumes of water. Other techniques performed include direct plating with low volumes of water as well as centrifugation [18,31,35,58].

Since there is no standardisation approach in the isolation of fungi, the culture medium used tends to vary among researchers. Among the most commonly reported media are Sabouraud dextrose agar (SDA), Sabouraud glucose agar (SGA), malt extract agar (MEA), cornmeal agar (half-strength) (CMA/2), Czapek Dox agar (CZ), Dichloran 18% glycerol agar (DG18), Dichloran Rose Bengal Chloramphenicol agar (DRBC), Neopeptone glucose Rose Bengal aureomycin (NGRBA) and potato dextrose agar (PDA) [41,43,64]. Some of these media have higher nutrition content than others, thus being more selective towards some fungal species. Hence, an extensive culturomics approach, applying different designed media, should be used in order to increase the probability of obtaining the highest coverage representation of the fungi present in water. As pure fungal cultures are obtained, the

identification of fungi is usually performed through morphological identification keys, which could lead to misidentification at the species level. For instance, if the fungi do not sporulate, it cannot be identified morphologically. To improve this step, molecular approaches such as PCR and sequencing were employed. The recommended genetic marker for basic fungal identification is the internal transcribed spacer (ITS) region which has already been used in the majority of studies [3,39,47,65,66]. However, there are limitations in separating all fungal species when using only this genetic barcode, with high relevance for *Aspergillus* and *Penicillium* genera. Hence, secondary markers such as the 18S ribosomal small subunit, β-tubulin or the translation elongation factor 1-alpha (TEF-1α) should be added in complementation [39,57,67].

Biofilm sampling from DWDS has evolved with time. The most usual method of sampling has been performed by scrapping or swabbing water related surfaces [32,33,35,41,56,68]. Swabs were then directly plated onto solid media or previously suspended in a sterile saline solution before being plated. Other methods for studying biofilms *in situ* include cutting out pipes, which could then be placed onto medium for culturing, or the use of sampling devices inserted into the pipe (Fig. 1) [3,26,57,64].

Pipe cut-out is, however, considered as a destructive sampling method and has other disadvantages, such as being expensive and labour-intensive. It is also difficult to repeat the experiment since pipe cut-outs have to be replaced after sampling [64,69]. The use of devices inserted into pipes is, therefore, the currently preferred sampling method. These devices are usually coupons of different materials such as glass, polyvinyl chloride (PVC), polyethylene, acetate, cast-iron or concrete, to encompass the high variability of materials present in DWDS [3,7,15,26]. The majority of older DW networks were made of iron-based materials. More recently, polymeric materials such as PVC have been preferred because they are easier to handle. Different materials have different surface physicochemical properties that influence biofilm formation, including microbial diversity [70–72]. Shortly, physical adsorption is generally a reversible process in which one monolayer is formed and involves nonspecific bonds (London and van der Waals forces). In contrast, chemical adsorption involves specific chemical bonds (electrostatic, covalent and hydrogen bonds), dipole interactions and hydrophobic interactions [73]. The devices can be organised repeatedly within an operational DWDS, allowing biofilms to grow *in situ* without removing the original pipe. For the isolation and identification of fungi from biofilms, the methodology was usually culture-dependent, the same as for fungi in bulk water. Nevertheless, these methods have several limitations such as temperature and incubation time which will culminate in the recovery of different fungal genera and species [74]. Consequently, the use of culture-independent methods has gained vital relevance to complement culture-dependent methods, or to detect and directly quantify fungal DNA in water. Among these methods are, for instance, Real Time Quantitative PCR (qPCR) and Denaturing Gradient Gel Electrophoresis (DGGE) [17,75]. A growing number of studies have also been using metagenomic approaches for the detection of fungi in bulk water or biofilm samples [7,57,68,75]. Different next generation sequencing (NGS) techniques (e.g. Ilumina and Nanopore) have been applied. The use of these techniques has allowed to understand and enhance not only the knowledge of fungal presence in water (can detect less abundant microorganisms) but also the microbial ecology of these ecosystems. This information has emphasised the need to understand and develop new indicators with the potential to be used to protect and promote water quality and safety [7]. For this reason, Hull et al. [76] highlighted the need to initiate and conduct a large-scale coordinated drinking water microbiome project.

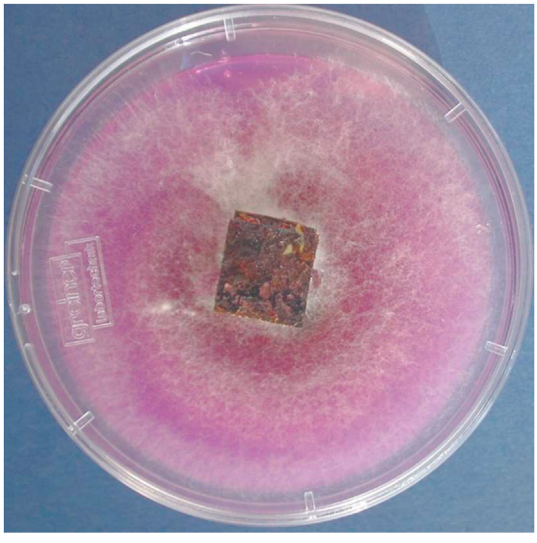

Fig. 1. Example of a cast-iron coupon (~15 × 15 mm) cut out from a water distribution system pipe in direct contact with NGRBA medium, showing the growth of a filamentous fungus. Source from the authors.

5. Fungal-bacterial interactions in biofilms

DWDS biofilms are complex communities with a high number of co-inhabiting microorganisms. This diversity leads to a variety of complex relationships involving inter- and intra-species interactions [3,7,57]. Although intra-species interactions may play an important role in the coexistence of some microbiomes, biotic interactions between distantly related organisms across the kingdoms of life also regulate the composition of these communities [77]. In many microbiomes, bacteria can coexist with different eukaryotic microorganisms, including fungi [78]. These interactions encompass, of course, biofilms (Fig. 2). As the microorganisms are closely embedded in an extracellular matrix, inter-kingdom biofilms containing bacteria and filamentous fungi can be considered a closer level of fungal-bacterial interaction, but this aspect is still poorly understood [78]. Different factors may affect these inter-kingdom interactions, including the presence of quorum sensing (QS) molecules. QS is a mechanism employed by microbial species to coordinate community behaviour. It relies on the production, release and detection of small signalling molecules, which in turn modify gene expression [79]. There are several types of QS signals: Gram-negative bacteria utilize *N*-acyl-homoserine lactones (AHLs)-type signals, whereas Gram-positive species use short oligopeptide signals. These QS systems are already well understood at the molecular level and reviewed elsewhere [80,81]. QS controls and regulates different bacterial population density-dependent processes, including biofilm formation, stress resistance, production of toxins and secondary metabolites and pathogenicity [82,83]. In contrast, eukaryotes, and in the scope of this review, filamentous fungi, have the ability to interfere with bacterial communication by producing molecular signals that interact with bacterial QS. These compounds are called quorum sensing inhibitors (QSI). They can mimic the structure or function of autoinducers, act as antagonists to the QS molecules as well as interfere with the stability and function of the regulator protein or the autoinducer synthase and hydrolysate signalling molecules [84]. As filamentous fungi do not have an active immune system, they must rely instead on chemical defence mechanisms. For this reason, they have been studied regarding their QSI potential, in particular the genus *Penicillium*. Patulin and penicillic acid were identified as being biologically

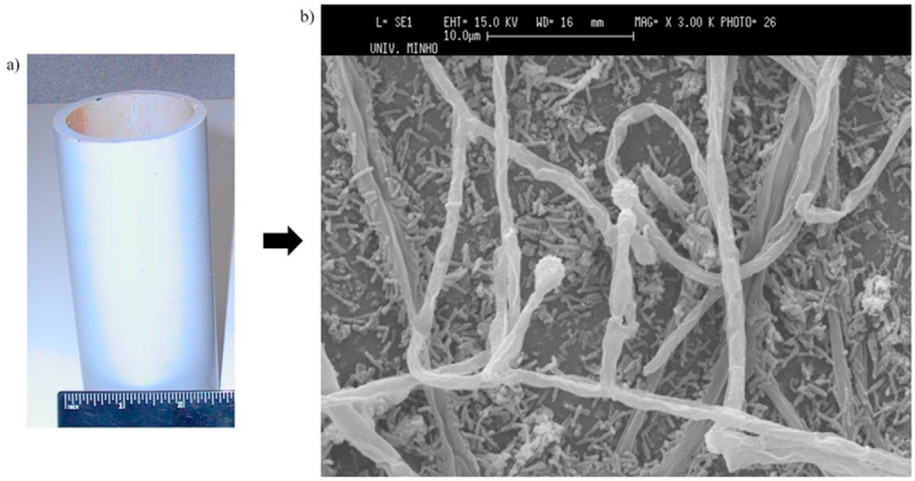

Fig. 2. (a) PVC-C tube from a water distribution system and (b) scanning electron micrograph of a PVC-C cut out coupon after one week in direct contact with NGRBA medium to promote the fungi growth only, showing in the basal layer a close relationship between filamentous fungi and bacteria. Source from the authors.

active QSI compounds [79]. Through DNA micro-array-based transcriptomics, the same authors showed that these QSI compounds down-regulated QS-regulated genes in *Pseudomonas aeruginosa* by 45% and 60%, respectively, indicating their specificity towards QS-regulated gene expression [79].

Different studies have found different relationships between fungi and bacteria. In mixed biofilms, both organisms can exist as dual complexes or fungi may offer biotic support for the establishment of a bacterial biofilm [85]. However, it has also been shown that fungi often colonize pre-established bacterial biofilms, and due to their different ecological requirements, it has been suggested that it can lead to a positive relationship between these microorganisms [28]. Reports of negative relationships may be observed due to culturing processes, where both fungi and bacteria are in direct competition for resources [41]. This variety in findings could, in sum, be a consequence from several factors, such as the difference in the composition of isolated species from the water systems, differences in methodologies or different biological mechanisms at play [6]. For this reason, a need arises for further research to explore the different correlations between fungi and bacteria and what are the factors influencing these interactions.

To demonstrate the heterogeneity in findings, in a single study, performed on bacterial-fungal biofilms in flowing water photoprocessing tanks using a model community, it was difficult to determine which interactions were present. Some species showed increased growth rate in mixed cultures while others showed a reduction, however, all species were present in a lower number than in single cultures, which was considered to be a result of limitation and competition for the nutrients available [86]. Douterelo et al. [57] reported a positive coexistence between the bacteria *Pseudomonas* and the fungi *Basidiobolus* in *in situ* biofilms. This could be due to ability of the fungi to produce extracellular enzymes that allow them to degrade high molecular weight compounds, releasing secondary metabolites that can potentially be used, in this case, by *Pseudomonas* [57]. In another study, a correlation was observed between the relative abundance of certain bacterial taxa such as Proteobacteria and Basidiomycota [3]. The same study also confirmed the presence of *Acremonium* and *Neocomospora* from early stages of biofilm formation to a more developed biofilm, forming essential communities with bacteria. Fungal contribution, in particular *Ascomycota*, is very important to the microbial ecology of real DWDS [7]. One of the roles of filamentous fungi in drinking water biofilms has consequently been associated with providing building blocks and/or biotic support through their hyphae for the establishment and colonization of surfaces by bacteria [7]. A recent study performed by Del Olmo et al. [59] detected a core microbial community throughout the network of a DWDS with microorganisms like *Pseudomonas*, *Aspergillus* or *Alternaria* being abundant in underlying and more consolidated material layers. This study revealed a diverse community of fungi which demonstrated a strong contribution to biofilm formation in DWDS, supporting concepts of mutual beneficial fungal-bacterial interactions. In addition, fungal-bacterial communities were found to be highly correlated, with bacteria being more diverse, whilst fungi showing more dominance and stability [59]. Pipe material has also been shown to influence microbial community composition, particularly bacteria [72]. Microbial communities from cast-iron pipes revealed to be more stable than communities from non-ferrous pipe materials [72].

To understand and try to clarify fungal-bacterial interactions in biofilms, several studies have recently been done under controlled laboratory conditions. These studies usually involve the interaction between one fungal and one bacterial species, as the effects observed will result directly from their interactions. In *Penicillium expansum* inter-kingdom biofilm formation with *Acinetobacter calcoaceticus*, it was observed that intertwined fungal hyphae increased the cell number of this bacterium, revealing a possible protective role of the fungi towards bacteria. Despite this protective effect, it was observed that when inoculated at the same time, bacteria could inhibit filamentous fungal spore germination and its development into a biofilm in the first 24 h of interaction [87]. Similar results were obtained in other studies with different microorganisms. For instance, in a 24 h co-culture study between the bacteria *P. aeruginosa* and the fungus *A. fumigatus*, direct contact between these two microorganisms as well as between the fungi and bacterial supernatant, resulted in an inhibition of fungal biofilm formation [88]. In another 24 h co-culture study, this bacterium was also able to inhibit spore germination of the fungus *Rhizopus microsporus* [89]. Fungal spore germination and hyphal development of several *Aspergillus* species were inhibited when in co-culture with the bacterium *Klebsiella pneumoniae* [90]. Additionally, the bacterium *Staphylococcus aureus* strongly inhibited fungal conidiation, filamentation and consequently biofilm formation of *A. fumigatus* by direct cell–cell contact [91]. This same bacterium was also able to negatively influence the filamentation and biofilm formation of the fungus *Fusarium falciforme* [92]. Most

of these studies were performed within 24 h of interaction, however, with medium renewal and removal of planktonic cells, after that time, an increase in fungal viability could be observed, which could lead to its germination and development into a biofilm [87]. Nogueira et al. [90] also showed that *Aspergillus* species remain viable after interaction with bacterial cells. This is particularly noteworthy because if the possibility of biofilm formation is provided for the fungi, or if a pre-established fungal biofilm is present, then, an advantage may also be given to opportunistic bacteria to replicate and proliferate in inter-kingdom biofilms inside DWDS. Flemming et al. [4] suggested that it is possible that drinking water biofilms can also help to inhibit the propagation of invading pathogens, thus safeguarding water quality. Understanding community dynamics, including the presence of fungi in DWDS can be the key to sustaining a beneficial and ultimately safe microbiome [59]. A practical example of this statement was observed in a study performed by Lahaye et al. [93] where the treatment of a DWDS of a pig farm using essential oils lead to the evolution of a positive bacterial biofilm. While the initial biofilms were essentially composed of fungi with hyphae being prominent, after treatment with essential oils, a decay of fungal population was observed to the benefit of new bacterial populations. This inversion of the biofilm lead to an improvement of the pig herd's health without addition of antibiotics by allowing a positive biofilm to colonize the water network while also having associated positive economic effects [93]. Several new approaches are also being undertaken to help the removal of pathogenic microorganisms from DWDS. For instance, direct potable reuse requires extensive advanced treatment of wastewater, which often involves combinations of ozonation, granular activated carbon filtration, microfiltration, reverse osmosis and an UV advanced oxidation process [94]. The granular activated carbon filter used in a study performed by Miller et al. [95] harboured an active and consistent microbial community over time. From a treatment process perspective, these filters used in a last stage can act as an additional barrier for organic pollutants while not increasing concentrations of opportunistic pathogens relative to conventional water systems. A diverse microbial community should lead to increased biological stability, capable of outcompeting opportunistic pathogens [95]. Because advance treatment decreases bacterial presence and diversity, treatments such as granular activated carbon filters should be used to increase finished water biostability [95].

6. Concluding remarks

Filamentous fungi are present in all environments, and water distribution systems are no exception. Several studies have demonstrated the presence of these microorganisms, including concerning pathogenic, toxigenic or/and allergenic species. The present work provides an updated overview of the occurrence of filamentous fungi in DWDS from the past two decades. Emphasis was given to biofilm formation along with the interaction with bacteria in fungal-bacterial biofilms. Several gaps and drawbacks were detected from the start and some recommendations arise from these needs.

Detection and/or isolation of filamentous fungi in DWDS should follow a uniform approach. Different methodologies, including culturomics and culture-independent methods, should be used to complement one another. Looking at community analysis, NGS techniques have a fundamental role and are also of great importance to analyse interactions between microorganisms. Even with some advances in the study of fungal-bacterial interactions, much more work is needed to fully elucidate these interactions. With differences in the methodology being used and the microorganisms being studied, there is a complex variety of observable relationships. For this reason, there is a necessity to further investigate the different interactions between fungi and bacteria in DWDS and what factors are affecting such associations.

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Conflict of interest

No conflict of interest was reported by the authors.

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