NRC Research Press

ARTICLE

Biofilms from a Brazilian water distribution system include filamentous fungi

V.M. Siqueira, H.M.B. Oliveira, C. Santos, R.R.M. Paterson, N.B. Gusmão, and N. Lima

Abstract: Filamentous fungi in drinking water can block water pipes, can cause organoleptic biodeterioration, and are a source of pathogens. There are increasing reports of the involvement of the organisms in biofilms. This present study describes a sampling device that can be inserted directly into pipes within water distribution systems, allowing biofilm formation in situ. Calcofluor White M2R staining and fluorescent in situ hybridization with morphological analyses using epifluorescent microscopy were used to analyse biofilms for filamentous fungi, permitting direct observation of the fungi. DAPI (4′,6-diamidino-2-phenylindole) was applied to detect bacteria. Filamentous fungi were detected in biofilms after 6 months on coupons exposed to raw water, decanted water and at the entrance of the water distribution system. Algae, yeast, and bacteria were also observed. The role of filamentous fungi requires further investigations.

Key words: filamentous fungi, biofilm, water distribution system, fluorescent in situ hybridization (FISH), in situ detection.

Résumé : Les champignons filamenteux de l'eau potable peuvent bloquer les conduites d'eau, produire une détérioration biologique organoleptique et ils constituent une source d'organismes pathogènes. Il y a de plus en plus de données qui démontrent une implication de ces organismes dans les biofilms. L'étude présente décrit un dispositif d'échantillonnage qui peut être inséré directement dans les conduites de systèmes de distribution d'eau permettant la formation de biofilm in situ. La coloration au Calcofluor White M2R et l'hybridation fluorescente in situ ont été utilisées parallèlement à des analyses morphologiques par microscopie en épifluorescence afin d'analyser les biofilms de champignons filamenteux, permettant une observation directe des champignons. Le DAPI (4′,6-diamidino-2-phenylindole) a été appliqué afin de détecter les bactéries. Après 6 mois, des champignons filamenteux ont été détectés dans les biofilms présents sur des échantillons exposés à l'eau brute décantée ainsi qu'à l'entrée des conduites du système de distribution d'eau. Des algues, des levures et des bactéries ont aussi été observées. Le rôle des champignons filamenteux nécessite d'être examiné plus à fond. [Traduit par la Rédaction]

Mots-clés : champignons filamenteux, biofilm, système de distribution d'eau, FISH, détection in situ.

Introduction

Aquatic environments are vast and biodiverse. Microorganisms occupy niches in rivers, streams, lakes, and the sea, from which filamentous fungi are well known. In (*i*) the Middle Ages, aquatic fungi were recognized as fish parasites; (*ii*) the mid-nineteenth century, phycomycetes were observed on water-associated algae and substrates; and (*iii*) the 1940s, "Ingoldian fungi" were isolated in running waters and described (Wurzbacher et al. 2010). Indeed, novel fungi continue to be discovered from aquatic environments in groundbreaking discoveries such as those described for the "cryptomycota" (Jones et al. 2011).

Drinking water distribution systems (WDS) represent the system of reservoirs, pipes, and treatment facilities employed to transport water to the public. They are environments in which filamentous fungi can survive and proliferate (Hageskal et al. 2009). Filamentous fungi in drinking water can block water pipes, can cause organoleptic biodeterioration, and are a source of pathogens.

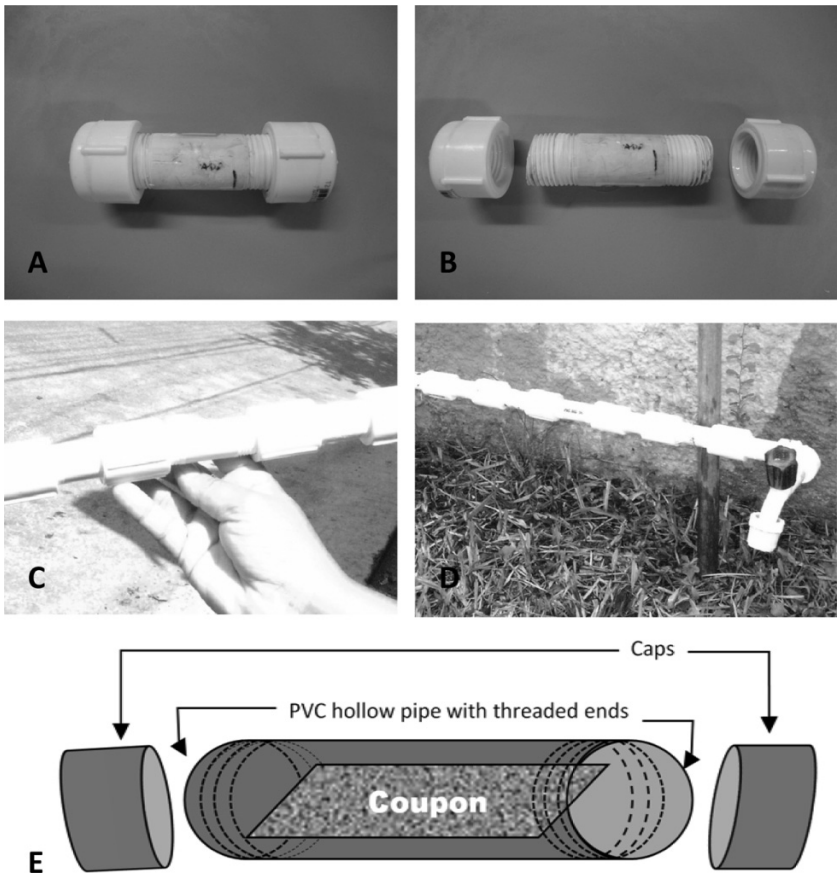
Most microorganisms in WDS are attached to surfaces within biofilms, although some are detected as planktonic cells (Flemming et al. 2002; Johnson 2007), and planktonic filamentous fungi were detected in a WDS by Oliveira (2010). Biofilms are functionally organised microbial communities growing amidst a matrix of exopolysaccharides produced by the inhabiting microorganisms. They are a microbial survival mechanism providing protection from toxic compounds, desiccation, thermal stress, nutrient depletion, and predation (Flemming et al. 2002). A human health threat is present, since they may harbour pathogenic microorganisms (Huq et al. 2008); hence, biofilms are correlated with reduced microbial water quality. Biofilm development in WDS is influenced by biotic and abiotic factors (e.g., levels of disinfectants, pipe material, temperature, water flow, and microbial interactions), which influence the architecture and microbial composition of biofilms (Momba et al. 2000). Viruses, protozoa, fungi, and algae may be incorporated into drinking water biofilms (Momba et al. 2000; Gonçalves et al. 2006; Helmi et al. 2008; Traczewska and Sitarska 2009; Villanueva et al. 2010), although, generally, bacteria are the dominant component. Taylor et al. (2001) identified 307 fungal species as emerging pathogens, and biofilms have been reported to contain some of these (WHO 2003). Nevertheless, reports of filamentous fungi in biofilms remain few and somewhat inconclusive (Doggett 2000; Kelley et al. 2003; Sammon et al. 2011; Siqueira et al. 2011).

Research of filamentous fungi has been hampered by nonstandard methodology, difficulties in quantification, and a lack of mycological expertise compared with that of bacteria. Screening aquatic environments for filamentous fungi using molecular biology is not so developed as are the screening methods for bacteria, and conventional cultural techniques also suffer drawbacks.

Received 30 August 2012. Revision received 14 December 2012. Accepted 14 December 2012.

V.M. Siqueira, C. Santos, R.R.M. Paterson, and N. Lima. Institute for Biotechnology and Bioengineering (IBB), Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal.
H.M.B. Oliveira and N.B. Gusmão. Department of Antibiotics, Federal University of Pernambuco, Av. Prof. Morais Rego, 1235, 50670-901, Recife, Pernambuco, Brazil.
Corresponding author: N. Lima (e-mail: nelson@ie.uminho.pt).

Fig. 1. Sampler device consisting of hollow polyvinylchloride pipes and round screw caps (A and B). Sampler device in place (C and D). Diagram to show the position of the coupon fixed to the pipe (E).



For example, it is difficult to state with authority whether a conidium, conidiophore, or hyphal fragment, etc., represent a single fungus (Gonçalves et al. 2006). The use of ex situ techniques (e.g., swabbing or scraping) may exclude unculturable filamentous fungi and destroy the biofilm if structural analysis is required. Specific dyes and fluorescent microscopy may overcome some of these problems. However, new methods are required to understand more fully the role played by filamentous fungal biofilms in microbial water quality, as the factors influencing these biofilms remain unknown.

WDS biofilms were sampled in the present study and the microbes determined, with a particular emphasis on filamentous fungi, by employing specific dyes.

Materials and methods

Water distribution system

The Alto do Céu, Recife, Pernambuco, Brazil, WDS has been operating since 1958 and produces approximately 10% of the total volume of water distributed to the metropolitan region. The water treatment plant that supplies the WDS is designed to treat 1 m^3 water/s but operates at a 20% overload occasionally. The raw water is sourced from 3 pumping stations and is treated by flocculation, sedimentation, decantation, filtration, and disinfection (ca. 5 mg/L chlorine) before leaving the plant. The water is pumped to 2 storage reservoirs with capacities of 5000 m^3 and 20 000 m^3, although only the 20 000 m^3 storage reservoir was used for the present study. The water supply in Recife is intermittent and follows a schedule published online by COMPESA (www.compesa.com.br), thus the WDS is subjected to variable water pressure and flows.

Sampling device

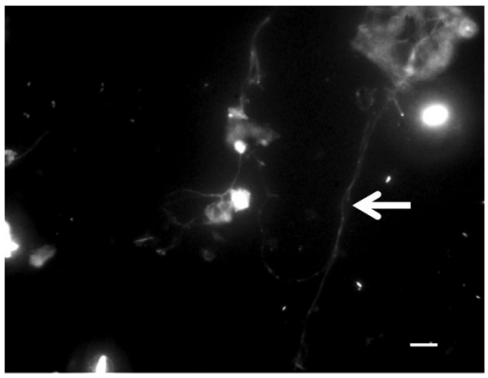
Samplers consisted of polyvinylchloride (PVC) hollow pipes (1.5 cm diameter by 7–10 cm length). Threads were cut in the ends of each sampler to enable attachment of multiple samplers or connection of caps to enclose the device after removal from the WDS (Fig. 1). Polyethylene or acetate coupons were inserted inside the pipe to permit biofilm formation (Fig. 1E). The coupons were made from readily obtained materials such as ice cream box caps (0.53 mm thick) and transparent stationary sheets (0.54 mm thick) as a source of polyethylene and acetate, respectively. However, the polyethylene coupons were abandoned due to their intense autofluorescence, whereas the acetate coupons had only a weak autofluorescence. In addition, the pipes collected from the samplers were cut into small pieces (about 2 cm × 2 cm), and the internal surfaces were used as PVC coupons; the same analytical procedures were followed as for the other devices.

The samplers were installed in raw water; in decanted water; in the 20 000 m^3 storage reservoir; and at the beginning, middle, and end of the WDS. Two samplers were removed every month for 6 months for analysis, with a final sample after 12 months. The samplers were refilled with WDS water, sealed with caps, and sent to the laboratory under refrigeration for analysis. Each coupon was cut into 3 for staining.

In situ detection

Calcofluor White M2R (CW) (4,4′-bis[4-anilino-6-bis(2-ethyl) amino-*s*-triazin-2-ylamino]-2,2′-disulfonic acid, Molecular Probes Europe, Leiden, the Netherlands) allows the visualization of the cell walls of fungi and other organisms (e.g., algae) because of its affinity for β(1–3) and β(1–4) polysaccharides in cellulose, carboxylated poly-

Fig. 2. Polyvinylchloride coupons after 3 months of exposure to the water distribution system and after staining with Calcofluor White M2R. Filamentous fungus-like structures (arrows). Scale bar = 20 μm.



saccharides, and chitin. CW (25 μmol/L) was added to each sample following incubation in the dark for 15 min at room temperature. These samples were observed under UV light using an Olympus BX51 epifluorescent microscope equipped with 10×/0.65, 40×/0.30, and 100×/1.3 objective lenses. The images were acquired with a Zeiss AxioCam HRc camera with software CellB. The excitation wavelength for CW was 346 nm and the signal was blue. DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride) (100 nmol/L) was added directly to the coupons for 30 min at 25 °C to assist morphological observations of the bacteria (size and shape). The excitation wavelength for DAPI was 340 nm and the signal was blue. Although the CW and DAPI signals are blue they are distinctive in colour intensity and brightness under microscopic observation.

The following morphological characters were assessed to differentiate between filamentous fungi, yeast, algae, and bacteria: (*i*) septa or septa-like structures, (*ii*) diameters of filaments, (*iii*) size and shape of cells, and (*iv*) shape of sporophores.

Fluorescent in situ hybridization

The protocol for fluorescent in situ hybridization (FISH) was adapted from Nuovo (1997), and samples were dried for 10 min at 46 °C; dehydrated in 70%, 80%, and 96% (*v*/*v*) ethanol for 10 min each; and air dried. Hybridization buffer (HB) (360 μL of 5 mol/L NaCl, 40 μL of 1 mol/L Tris, 300 μL of formamide, 130 μL of Milli-Q water, 4 μL of 10% SDS, in a 2 mL Eppendorf tube) was pipetted onto the whole surface of each sample. Each probe (4 μL) was added and gently homogenized with the HB. The remainder of the HB was poured into a Petri dish containing a paper tissue. The samples were hybridized for at least 3 h at 46 °C in the Petri dish saturated with HB. After this period, the samples were rinsed with preheated (water bath; 48 °C) wash buffer (1 mL of 1 mol/L Tris, 3180 μL of 5 mol/L NaCl, 50 μL of 10% SDS; 49 mL of Milli-Q water, in a Falcon tube) and incubated for 20 min. The samples were gently rinsed with ultrapure water and dried with compressed air.

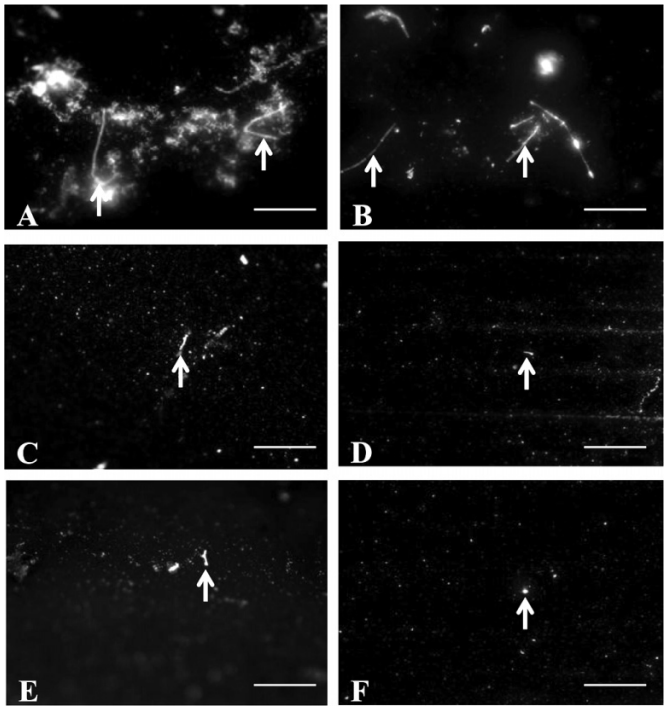
For eukaryotes, the universal rRNA probe specific for Eukarya, EUK516 (5′-ACCAGACTTGCCCTCC-3′, MWG Biotech, Ebersberg, Germany) (Baschien et al. 2008) labelled with the red Cy3 at the 5′ terminal, was used. For filamentous fungi, the FUN1429 probe (5′-GTGATGTACTCGCTGGCC-3′, MWG Biotech, Ebersberg, Germany) (Baschien et al. 2001), specific for the subphylum Pezizomycotina of Ascomycota, was used and was labelled with Oregon-Green at the 5′ terminal for FISH (Baschien et al. 2008). The samples were visualized using an Olympus BX51 epifluorescent microscope as described above.

Results and discussion

In situ detection

Filamentous fungi in biofilms were detected throughout the WDS. After 3 months of exposure within the WDS, structures that resembled filamentous fungi were observed (Fig. 2) as a filamentous mat stained by CW. After 6 months (Fig. 3) and 12 months (Figs. 4–7), filaments and additional microbial heterogeneity were detected. None of the other samples supported fungal structures after 5 months, and samples inside the storage reservoir did not demonstrate fungi. The internal surfaces of the PVC coupons from the sampler pipes demonstrated biofilm formation more clearly than the acetate coupons.

Fig. 3. Filamentous structures stained with Calcofluor White M2R (arrows). Acetate (A, C, and E) and polyvinylchloride (B, D, and F) coupons after 6 months of exposure to water at the beginning (A, B), middle (C, D), and end (E, F) of the water distribution system. Scale bar = 200 μm.



Filamentous fungal structures were observed in the acetate and PVC coupons at different points along the WDS after 6 months of exposure (Fig. 3). Filamentous structures were detected mainly in the samples collected from the beginning of the WDS (Figs. 3A and 3B), and at the middle and end of the WDS, only dispersed fragments were observed (Figs. 3C–3F; arrows). These fungal-like structures were observed on the coupon surfaces, and highly fluorescing CW staining surrounding the filaments was observed (Figs. 3A and 3B; arrow).

The coupons exposed to raw and decanted water demonstrated a higher colonization of hyphae and reproductive structures such as conidia (Figs. 4–6) than coupons from the other WDS sampling points. Enlarged images demonstrate septate hyphae and germinating conidia (Figs. 4A′ and 4B′).

Figure 5 demonstrates a structure that resembles a condiophore produced by some *Alternaria* species. An enlargement is provided in Fig. 5A′, where the conidia are produced in an acropetal chain; however, typical divisions in vertical and horizontal directions were not observed. Nonseptate filaments were also observed and represent fungi or algae and these were also stained with CW. Figure 6 shows a spiral-shaped structure, which resembled the microalga *Arthrospira* sp. (Fig. 6), and coccoid bacteria were detected on the surface of fungal hyphae (Fig. 7).

Another demonstration of filamentous fungi is presented in Fig. 8. Structures stained with CW and EUK516 and also stained

Fig. 4. Acetate coupon after 12 months of exposure to raw water and staining with Calcofluor White M2R. Filamentous fungi septate hyphae (detail A′) and germinating spore (detail B′). Scale bar = 200 μm.

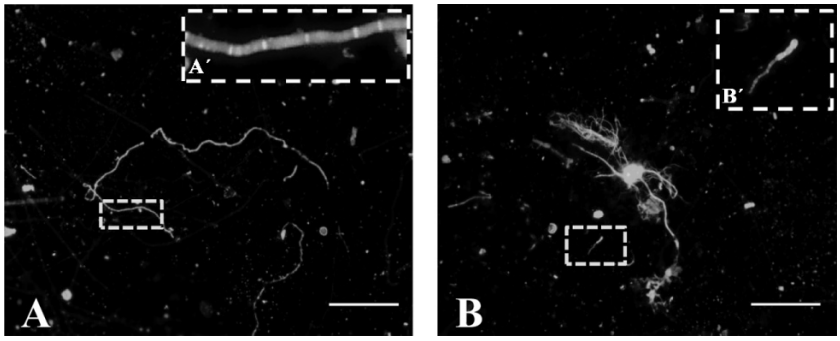


Fig. 5. Acetate coupon after 12 months of exposure to decanted water and staining with Calcofluor White M2R. Fungal-like reproductive structure (detail A′). Scale bar = 50 μm.

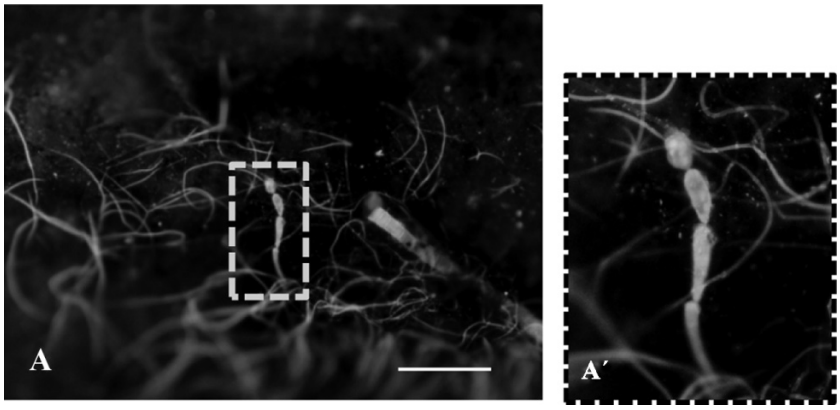


Fig. 6. Acetate coupon after 12 months of exposure to raw water and staining with Calcofluor White M2R. Algae-like structures (A and B). Scale bar = 20 μm (A) and 200 μm (B).

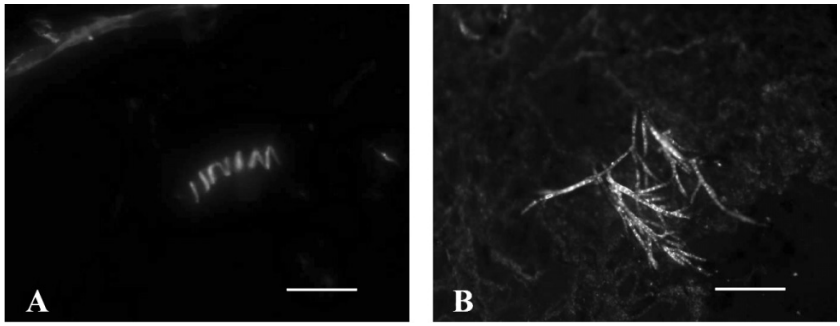
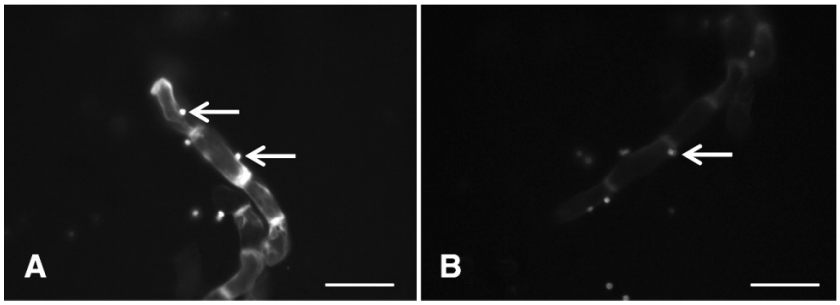


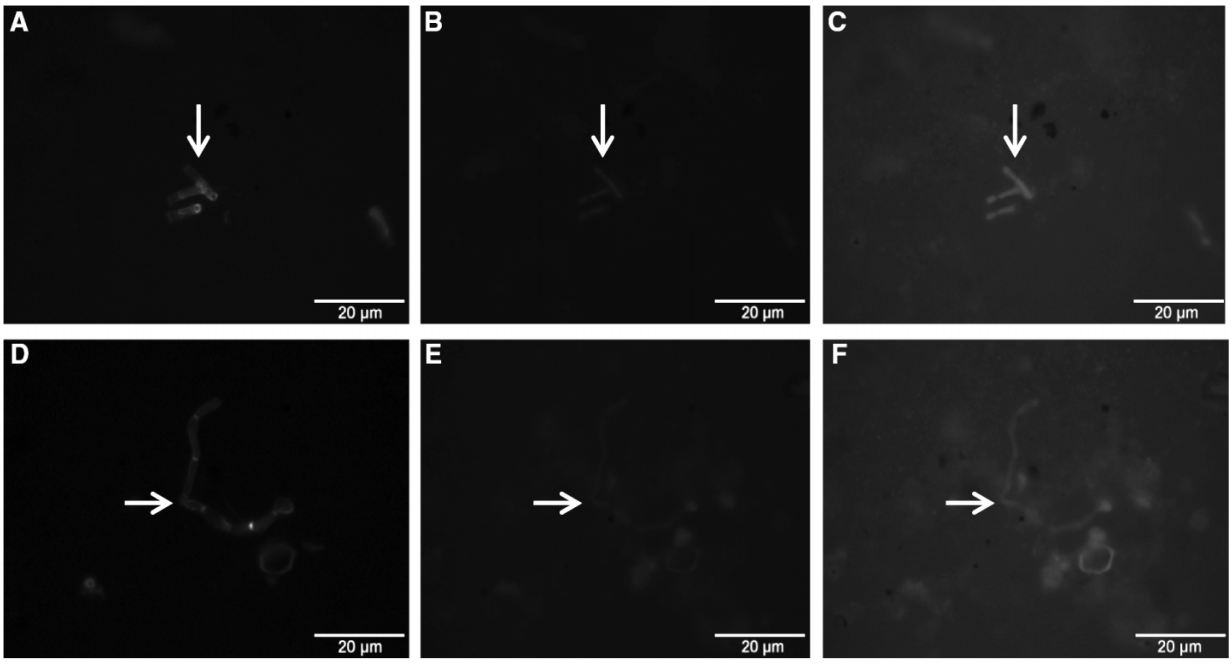
Fig. 7. Bacteria (arrows) nearby and on filamentous fungi hyphae after DAPI (A) and Calcofluor White M2R (B) staining. Scale bar = 20 μm.



with FUN1429, which is specific for the subphylum of fungi, the Pezizomycotina. This finding provided confirmation of the fungal nature of these structures. In contrast, not all filamentous structures stained by the EUK516 probe were visualized using the other 2 staining methods, indicating the eukaryotic organisms on the coupons were different from the subphylum Pezizomycotina fungi.

Sammon et al. (2011) did not detect filamentous fungal biofilms on PVC, glass, or concrete coupons, though diverse fungi were

Fig. 8. Detection of filamentous fungi after Calcofluor White M2R staining (A and D), and analysis with fluorescent in situ hybridization using probes EUK516 (B and E) and FUN1429 (C and F). Acetate (A–C) and polyvinylchloride coupons (D–F).



recovered from the same coupons after scraping. On the other hand, these authors observed hyphae on pipe sections, pipe dead ends, and sediments collected from the same water network to where the coupons were placed. The findings support the view that fungal biofilms in treated water develop slowly and are controlled predominately by the environmental conditions. Pipe material, pipe age, speed of water flow, and presence of deposits also affect biofilm development in treated water (Nagy and Olson 1985; Niquette et al. 2000; Zacheus et al. 2001; Martiny et al. 2003). Siqueira et al. (2011) detected filamentous fungi on replaced pipes from the same water network as described in the present results, confirming that filamentous fungi are able to grow as biofilms on pipe surfaces. These findings corroborate Doggett (2000) and Sammon et al. (2011) who also detected filamentous fungal biofilms in replaced pipes. Nagy and Olson (1985) emphasize that older pipes support a more diverse community composed of diverse microorganisms, including filamentous fungi.

The development and maintenance of the biofilms in such pipes reflect a high capability of adaptation and resistance, as they had been (*i*) exposed to water flow and oligotrophic conditions for years, and (*ii*) influenced by diverse abiotic factors, such as temperature, pH, and residual disinfectant. The hyphal cell wall consists of multiple layers of polysaccharides, which render it very stable. In addition, the presence of melanin in some fungi may increase stability and resistance to adverse conditions (Nosanchuk and Casadevall 2003), thus fungi are often found in anthropogenically disturbed areas (e.g., industrial and municipal waste water) and in ultra-oligotrophic environments, such as water distillation apparatus (Wainwright 2005).

The most usual control strategy taken against biofilm accumulation is the use of disinfectants. In Oliveira (2010), levels of chlorine varied from 0 to 4.6 mg/L, decreasing towards the end of the network. The activity of chlorine is affected by many factors, such as temperature, pH, and organic matter (Kerr et al. 2003). Chlorine is efficient at controlling biofilm formation, but this efficiency is reduced if the biofilm has already formed (Lewis 2001; Schwartz et al. 2003; Zhou et al. 2009). Hence, sufficient disinfectant is required along the entire WDS to control microorganisms in bulk water and biofilm development on the pipe surfaces. Unexpectedly, we detected more fungi attached to the coupon surfaces at the entrance to the water network, i.e., immediately after water treatment, and fewer at the exit of the water network (Fig. 3). This result may reflect the necessity of a long exposure time for fungal biofilm development and not the effectiveness of chlorine, which needs time to be homogeneously dissolved in water as free chlorine. In addition, we can hypothesize that the higher fungal bioload that comes from the water treatment plant will be dispersed through the WDS, which will be responsible for the observed gradient of fungal biofilms. Nonetheless, fungi are able to withstand high levels of chlorination (Doggett 2000) and are more resistant when located in biofilms (Siqueira and Lima 2011).

There are various ways in which microorganisms interact within biofilms (Burmølle et al. 2006; Christensen et al. 2002; Nielsen et al. 2000; Tait and Sutherland 2002), but little is known about fungal–bacterial interactions. In this study we observed bacteria surrounding fungal hyphae (Fig. 7). Fungal hyphae may play functions similar to those of bacterial biofilm extracellular polymers, such as retarding desiccation, providing sites for adhesion of other microorganisms, and serving as a source of support and nutrition (Jones 1994). The filamentous nature of the fungi may assist in maintaining the structure of biofilms. Paris et al. (2009) studied the distribution and persistence of allochthonous particles inoculated into biofilms composed by bacteria and filamentous fungi and verified that adherence occurred almost exclusively on the biofilms and not directly on the uncolonized walls.

Filamentous fungi were detected on coupon surfaces in the present work, but this does not represent a mature biofilm. However, their importance in forming biofilms must not be underestimated (Harding et al. 2009). These current findings highlight the possibility of natural biofilms containing filamentous fungi.

FISH should not be used as the sole tool to characterize a (freshwater) fungal community (Baschien et al. 2008); morphology must also be determined. It is particularly important that conidiophores, or other fungal sporing structures, are searched for in biofilms, as this gives direct evidence of particular fungal taxa as described herein. It is evident that more work is required on the contribution of filamentous fungi to water biofilms, although the

present paper is the first that describes direct observation of fungi in detail.

Conclusion

The samplers described herein allowed analyses of coupons in situ and are useful when studying biofilms. CW was a rapid and efficient stain to detect filamentous fungi, which could be differentiated by morphology. FISH allowed the detection of specific groups of eukaryotic microorganism and fungi. Fungi are likely to play an important role in microbial interactions within water biofilms and, consequently, in microbial water quality. Finally, more work is required to determine the role of filamentous fungi in biofilms.

Acknowledgements

The authors acknowledge Companhia Pernambucana de Saneamento (COMPESA) for its support in making the work by H.M.B. Oliveira possible. V.M. de Siqueira is supported by the grant SFRH/BD/43719/2008 from Fundação para a Ciência e Tecnologia (FCT), Portugal.

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