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Summary

This study reports the presence of filamentous fungi (ff) in drinking water including biofilms. Ff are not studied sufficiently in drinking water. Ff were highest in winter and had an indirect relation with other microorganisms. Pathogenic fungi were not observed at the mesophilic temperatures used. *Penicillium expansum* and *P. brevicompactum* were observed which may affect biofilms by mycotoxin production. FISH and calcofluor methods indicated presumptive ff in biofilms in water distribution systems.

Introduction

Fungi in drinking water have been recognised for decades. Growth has been described as "oligotrophic" in such dilute systems (Kelley et al. 2003). The number of studies of filamentous fungi (ff) in water is increasing slowly from an unsatisfactorily low level (Gonçalves et al. 2006a). Some of the problems associated with ff growth may be (a) unsightly appearance, (b) blocked pipes in distribution systems, (c) odours, (d) pigments, (e) source of potentially-pathogenic and allergy-causing fungi, and (f) mycotoxin production (Paterson and Lima 2005). Furthermore, the possibility of drinking water being a target of criminal acts cannot be ignored in this security conscious age (Paterson 2006). Biofilm formation by bacteria in water systems is undesirable and the problems of contamination are compounded in these multi-microbial systems. Ff involvement in biofilms has been demonstrated only in rather specific circumstances. The detection of ff by conventional methods in biofilms is complex, indirect and time consuming. To overcome these problems a combination of two fluorescent techniques for presumptive direct detection

was tested in the present work: (a) Fluorescence In Situ Hybridization (FISH), and (b) staining with Calcofluor white M2R fluorescent dye.

Material and Methods

Full details of how fungi were isolated from tap water are provided in Gonçalves et al. (2006a). Briefly, fungi were filtered from tap water and incubated on the various media described. The resulting colonies were counted and the fungi identified from standard morphological characters.

FISH and Calcofluor techniques: details are provided in Gonçalves et al. (2006b). The oligonucleotide probe EUK516, 5'-ACCAGACTTGCCCTCC-3', was labelled with the dye Cy3. The real samples, i.e., coupons, were treated similarly. First the PVC-C or cast iron (CI) coupons were dehydrated for 10 min in 90% (v/v) ethanol. Then, 500 μL of the hybribisation solution with 5 ng/μL probe was applied to the samples which were incubated for 3 hours at 46 ºC. The hybribisation solution was removed by incubating the samples in 10 mL of washing solution for 20 min at 48 ºC. The samples were rinsed with distilled water. As negative controls, samples of pure culture were treated as mentioned above but without addition of the probe.

The presence of filamentous structures in the biofilms was analysed *in situ* with Calcofluor White M2R stain. To the water biofilms in the coupons, about 300 mL of the dye solution was applied to the surface of the coupons which were incubated in the dark at room temperature for 30 min. After the staining procedure, the samples were washed with 3 mL of sterile distilled water for microscopy.

After FISH and Calcofluor staining, the microscope slide with pure culture and the coupons with biofilm samples were observed under an epifluorescent microscope. The excitation wavelength for the Calcofluor stain was 346 nm and the signal acquired is blue, while for Cy3 the excitation wavelength was 543 nm and the signal acquired is red. Image processing was carried out with the Zeiss software package. Virtual images, resulting from the overlaping of the two images obtained for each stain, blue or red, were created using the Paint Shop Pro 7 image processing programme.

Results

There appeared to be an increase in ff during the winter months of 2003/4 which corresponded to a low level of bacteria and yeast (Fig. 1left). This increase was at a similar time to a large increase in odour-producing genus *Acremonium* (Fig. 1rigth). *Penicillium* strains were isolated frequently including *P. brevicompactum* which was isolated at a low level throughout the sampling period. Unidentified penicillia and *P. expansum* were isolated at a high level in the spring of 2003 and 2004 respectively. FISH demonstrated eukaryotic microorganisms after 5 hours while the Calcofluor method revealed chitinous or cellulosic filamentous structures in less than one hour which may represent ff (Fig. 2).

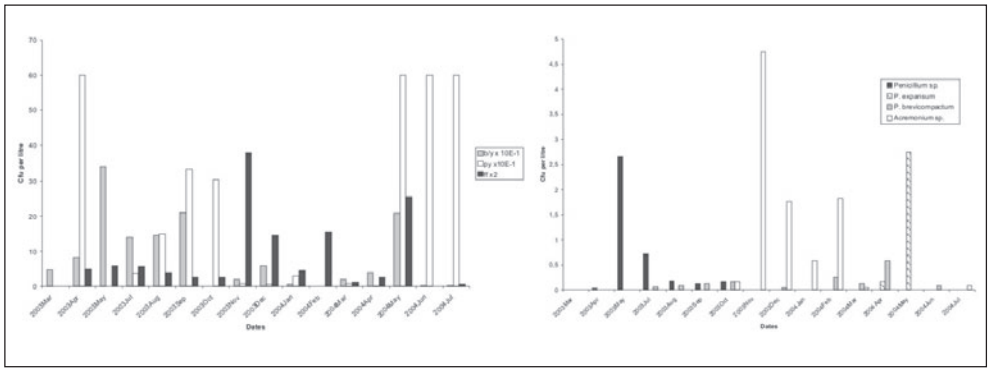

Figure 1. left) Cfu of bacteria/yeast (b/y) on TWA, CMA/2, and OSM; presumptive yeast (py) on NGRBA; and filamentous fungi (ff) from NGRBA, and right) Distribution of the key taxa throughout the sampling period. TWA = tap water agar; CMA/2 = corn meal agar half-strength; OSM = oomycete selective medium; NGRBA = neopeptone glucose rose Bengal aureomycin.

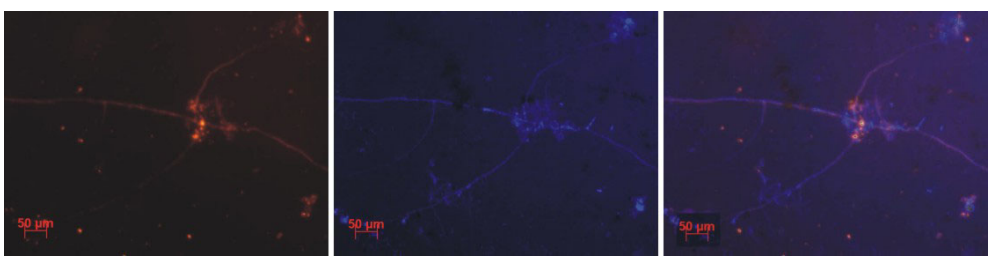

Figure 2. Mycological examination of PVC-C coupon. left) Detection of fungal mycelia using EUK516 probe, middle) Detection of fungal mycelia by Calcofluor staining and, right) Overlap of the two images using an image processing programme.

Conclusions

Fungi were isolated readily from the Portuguese tap water and a seasonal effect was observed with ff being more common in winter. Potentially pathogenic *Aspergillus fumigatus* strains were not isolated although higher incubation temperatures need to be employed for optimal isolation of this species. Other pathogenic fungi were not apparent. However, potentially mycotoxigenic *P. expansum* and *P. brevicompactum* strains were common. These may be more important ecologically in terms of the interactions with other microorganisms than from a direct health risk from toxin production, although subsequent contamination of food may be an issue. FISH and Calcofluor staining provide rapid and direct presumptive information on the involvement of ff in biofilms which form in water.

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