

## 22. Fluorescent techniques including FISH for *in situ* fungal detection in water biofilms

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Filamentous fungi are a ubiquitous and diverse group of eukaryotic organisms and may contribute, along with bacteria, yeasts, protozoa and viruses, to the formation of biofilms in water distribution systems. However, fungal involvement in biofilms has only been demonstrated recently. It is a demand to understanding ecological roles of the fungi in biofilms and which is their importance on this peculiar niche. Furthermore, these fungi may be responsible for the production of tastes, odours and mycotoxins in drinking water making their early detection important.

The detection of filamentous fungi by conventional methods is complex, indirect and time consuming. To overcome these problems a combination of two fluorescent techniques for direct detection was tested: (a) Fluorescence *In situ* Hybridization (FISH) employing the eukaryotic universal rRNA probe EUK516 (5'ACCAGACTTGCCCTCC3') or the eumycotic probe FUN1429 (5'GTGATGTACTCGCTGGCC3') both from MWG Biotech, Ebersberg (Germany) and labeled with the red Cy3 at the 5'-terminal, followed by (b) staining with Calcofluor White (CW).

CW is a symmetric molecule with two triazol rings and two primary alcohol functions on both sides of an ethylene bridge. CW is a fluorescent probe capable of making hydrogen bonds with  $\beta$ -(1 $\rightarrow$ 4) and  $\beta$ -(1 $\rightarrow$ 3) polysaccharides. The fluorophore shows a high affinity for chitin forming hydrogen bonds with free hydroxyl groups which stains fungal cell walls blue.

After FISH and CW staining, the microscope slide with pure culture (*Penicillium brevicompactum* as control) and the coupons with biofilm samples were observed under an Axioskop epifluorescent microscope (Carl Zeiss, Germany) using UV light equipped with 40X/0.30 and 10X/0.65 objectives. A BX61 Olympus vertical FLUOVIEW1000 confocal laser scanning microscope (CLSM) for *in situ* analysis was also used. CLSM combines the advantages of digital fluorescence and the possibility to detect optical sections of biofilm samples with enhanced vertical and axial resolution and quality. The excitation wavelength for the CW stain was 346 nm, while for Cy3 the excitation wavelength was 543 nm and the signal acquired is red.

FISH demonstrated eukaryotic microorganisms after approximately 5 hours while the CW method revealed chitinous filamentous structures in less than one hour. When the two methods were combined, additional resolution was obtained from the images of filamentous walls (blue) with intact protoplasm (red). In conclusion, FISH and CW staining provide rapid, direct and unambiguous information on the involvement of filamentous fungi in biofilms which form in water.

To expand our knowledge about fungal biofilms the FUN-1 (Molecular Probes, The Netherlands) was applied to study fungal viability and metabolic activity. FUN1 is a membrane permeant nucleic acid binding dye that initially stains both live and dead cells bright yellow-green fluorescence (530 nm) when excited by 488 nm. However, after appropriate incubation the dye is converted intracellularly by metabolically active cells into red Cylindrical Intra-Vacuolar Structures (CIVS). The results demonstrate that the fungi in water biofilms are metabolic actives which indicate that they participate on the biofilm dynamics and architecture.