Virginia Medeiros de Siqueira

Characterising filamentous fungal biofilm in drinking water distribution systems using microscopic and molecular techniques

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Universidade do Minho
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Tese de Doutoramento em Engenharia Química e Biológica

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Professor Doutor Nelson Lima

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There is an appointed time for everything. And there is a time for every event under heaven — A time to give birth, and a time to die; A time to plant, and a time to uproot what is planted. A time to kill, and a time to heal; A time to tear down, and a time to build up. A time to weep, and a time to laugh; A time to mourn, and a time to dance. A time to throw stones, and a time to gather stones; A time to embrace, and a time to shun embracing. A time to search, and a time to give up as lost; A time to keep, and a time to throw away. A time to tear apart, and a time to sew together; A time to be silent, and a time to speak. A time to love, and a time to hate; A time for war, and a time for peace.

King Solomon, Ecclesiastes 3:1-8 ("A Time for Everything")

To my family, I dedicate.

Resumo

A qualidade microbiológica de água de consumo tem sido uma das grandes preocupações de fornecedores governamentais e privados. Sistemas de distribuição de água são complexos ambientes onde algas, bactérias, fungos e protozoários cohabitam. Muitos destes microrganismos são patogenicos ao homem. Uma vez no sistema de distribuição, microorganimos podem aderir às superfícies das tubulações e formar biofilmes. Biofilmes são comunidades microbianas nas quais seus constituintes apresentem características diferenciadas, por exemplo, maior resistência a fatores adversos como desidratação, altas temperaturas, condições oligotroficas e ação de biocidas. Fungos são conhecidos por habitarem diversos ambientes aquáticos. Sua presença em sistemas de distribuição de água está associada a problemas de bloqueio de tubulações, produção de metabólitos e mudanças em propriedades organolépticas como odor e turbidez, e consequentemente, os fungos estão ligados à perda da qualidade da água. Além disso, por conta da sua habilidade em aderir a superfícies, os fungos são capazes de crescer como biofilmes em diferentes materiais, inclindo as paredes das tubulações. O presente trabalho visou o estudo de biofilmes de fungos filamentosos em sistemas de distribuição de água. Para isto, técnicas de microscopia de fluorescência e fluorescent in situ hybridization (FISH) foram utilizadas pretendendo-se detectar, monitorar e caracterizar biofilmes de fungos filamentosos em condições reais e laboratoriais. Corantes e sondas fluorescentes específicos foram aplicados, i.e. Calcofluor White R2R, DAPI, FUN-1, e as sondas EUK516 FUN1429. Microspheres Adhesion Assay, coloração com Cristal Violeta e MTT foram também utilizados. Adicionalmente, foi projetado um amostrador para formação de biofilmes in situ. Como resultados, foi observado que biofilmes de fungos filamentosos, quando comparados com sua forma planquitonica, possuem um comportamento diferenciado com relação à resistência contra agentes desinfectantes e a nívels de hidrophobicidade celular. Biofilmes de fungos filamentosos foram detectados em tubulações, amostradores e sistemas laboratoriais. Fungus filamentosos isolados de sistemas de água são capazes de formar biofilmes, apresentando caracteristicas morfológicas e fisiológicas diferenciadas. Em conclusão, fungos filamentosos são capazes de formar biofilmes sob diversas condições reais e laboratoriais. É provável que os fungos desempenhem um papel importante nas interações nos biofilmes em sistemas de água e, conseqüentemente, também na qualidade microbiológica da água. Assim, os fungos devem ser incluídos como membros consistentes dos biofilmes. Os resultados relatados neste trabalho representam novos conceitos na pesquisa micológica, e mais estudos na área podem levar a novas descobertas sobre a biologia dos fungos.

Abstract

Microbial quality of drinking water has been one of the greatest concerns of governmental and privative water suppliers. Water distribution systems are complex environments where algae, bacterial, fungi and protozoa cohabit. Many of these microorganisms are pathogenic to human and are associated with loss of water quality. Once within a water distribution system, microorganisms can attach to pipe surface and form biofilms. Biofilms are microbial communities wherein theirs constituents present differentiate features when compared with their planktonic form, e.g. increased resistance against adverse factors such as dryness, high temperatures, oligotrophic conditions and biocides. Fungi are known to habit diverse aquatic environments. Their presence in water systems is associated with pipe blockage, produce of metabolites involved in organoleptic changes such as odour and turbidity, and consequently are linked with loss of water quality. Moreover, because of their ability to adhere to surfaces, fungi are able to grown as biofilms on different surfaces, including pipe walls. The present work aimed to study filamentous fungal biofilms in water distribution systems. For this, epifluorescence microscopy and fluorescent *in situ* hybridization (FISH) were applied intending to detect, monitor and characterise filamentous fungal biofilms in real and laboratorial water systems. Specific fluorescent dyes and probes were used, i.e. Calcofluor White R2R, DAPI, FUN-1, EUK516 and FUN1429 probes. Microspheres Adhesion Assay, Cristal Violet staining and MTT assay were also applied. Moreover, a device was designed for sampling fungal biofilms *in situ*. As results, was observed that filamentous fungal biofilms have differentiated behaviour concerning resistance against disinfectant and levels of cell hydrophobicity when compared with their planktonic form. Filamentous fungal biofilms were detected in replaced pipes, sampler devices and laboratorial water system. Moreover, filamentous fungi recovered from water systems were capable to form biofilms with specific morphological and physiological features. In conclusion, filamentous fungi are able to form biofilms under diverse real and laboratorial conditions. Fungi are likely to play an important role in microbial interactions within water biofilms and consequently in microbial water quality. Thus, fungi may be included as a consistent member of biofilms in drinking water systems. The results reported in this work represent new concepts in mycological research, and further studies in the area may lead to new insights in fungal biology.

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1. General Overview

Access to potable water is a basic human right. Governmental and privative water agencies are constantly facing challenges to provide safe water free of hazards to human health, i.e. consistent with pre-established parameters, including the microbiological ones. A water distribution system is seen as a complex ecosystem in which diverse microorganisms cohabit and are influenced by biotic and abiotic factors. Many of these microorganisms, including bacteria, protozoa, virus and fungi are pathogenic to human and are capable to survive after water disinfection. Moreover, microorganisms can also enter in the water distribution system through external pathways, e.g. mechanic failures in pipes.

Once there, microorganisms can attach to pipe surface and form biofilms. Biofilms are microbial communities developed on surfaces in which microorganisms are embedded in an extracellular polymeric matrix produced by themselves. Generally, the constituents of a biofilm present differentiate features when compared with their planktonic form, e.g. increased resistance against adverse factors such as dryness, high temperatures, oligotrophic conditions and biocides. Biofilms formed in water distribution systems are seen as a human health threat since they can harbour pathogenic microorganisms.

Many studies report the presence of bacteria such as *Helicobacter pylori*, *Escherichia coli*, *Pseudomonas* and *Micobacterium* within water biofilms. Because of their relevance as human pathogens, a lot of attention has been put on them and *ex situ* and *in situ* techniques have been developed and applied in this field. Despite awareness that biofilms are microbial communities, less attention was paid on others microbial constituents. Mycological studies have always detected filamentous fungi in diverse water systems including water distribution network, tap water, bottled water and ultra-purified water, but so far few studies were focused on fungal biofilm detection.

Fungi are well-known by their ability to decompose organic matter and to produce metabolites used in industry, e.g. enzymes. Fungi are eukaryotes, spore-producing organisms with absorptive nutrition and that usually produce filamentous branched somatic structures, known as hyphae that have ability to adhere to surfaces. Their presence in water systems is associated with pipe blockage, produce of metabolites involved in organoleptic changes such as odour and turbidity, and consequently are linked with loss of water quality. Additionally, some fungal species recovered from water are human pathogen and/or potential mycotoxin producers. Little is known about the role played by fungi in water systems and how their interaction with other microorganism influences biofilm formation.

Recently published data report studies in laboratorial biofilms formed by pathogenic fungi such as *Aspergillus fumigatus*, *Candida* spp., and fungi of industrial interest (i.e. enzyme producers) such as *A.*

niger. Fungal biofilm formation has also been report in historical monuments, historic window glass, sediments in rivers and lakes and acid mine drainage microbial communities. However, studies in water distribution systems have so far produced scarce published data. Thus, new insights and implementation of new methods are needed in this research field.

Fluorescence microscopy has become an essential tool in biofilm studies. Fluorescent staining, including fluorescent *in situ* hybridization (FISH), has provided great benefits in this area. Its use allows *in situ* biofilm detection and further nondestructive analyses that give information of biofilms architecture, metabolic activity and microbial diversity. Together with sampler device implementation, laboratorial methods and specific fluorescent dyes (e.g. fluorochromes and fluorescent labeled probes) may add crucial information about filamentous fungal water biofilms.

2. Aims

The aims of the present work are:

1. The improvement and implementation of techniques to fungal water biofilm detection, viability and biomass quantification using fluorescent dyes in biofilms developed under real and laboratorial conditions;

2. Study fungal water biofilm architecture, physiology, disinfectant resistance and hydrophobicity;

3. The implementation of sampler devices in a water distribution system, fungal water biofilm detection under real conditions and *in situ* fungal identification using specific probes for Fluorescent *in situ* hybridization;

4. The implementation of a flow-chamber reactor to study laboratorial fungal water biofilm formation under monitored conditions;

5. Characterisation and identification of fungi recovered from water biofilms: biofilm kinetics formation and architecture.

3. Framework

This PhD thesis is composed by 8 chapters.

The **Chapter 1** is composed by literature review including relevant and actual references giving a wide overview of key topics in biofilms, biofilms in water systems, fungi and fungal biofilms in water systems and common applied techniques in this research area. The following chapters are composed by the results achieved with laboratorial experiments using laboratorial and real water biofilms and discussion with current literature.

The **Chapter 2** reports drawbacks faced by researchers to assess filamentous fungi biomass. Hence, a methodology using image analyses was described intending to measure fungal biomass using a proper convertor factor and converting biovolume into biomass. This technique allows very little fungal biomass estimation and fungal biofilm biomass measurement *in situ* using specific fluorescent dye.

The **Chapter 3** describes the effectiveness of sodium hypochlorite against spores and biofilms of *Penicillium expansum*. Fungal cell viability was assessed after treatment with disinfectant using FUN-1 and plate culture methods. Biofilms showed resistance against sodium hypochlorite and a higher resistance when compared with spores. FUN-1 is a rapid method to assess fungal cell viability when compared with plate culture method.

The **Chapter 4** reports the use of contact angles measurement (CAM) and microsphere adhesion assay (MAA) to assess fungal hydrophobicity of *Penicillium expansum* and *P. brevicompactum* grown as solid culture, liquid culture and biofilms. Solid culture of both species were classified as hydrophobic but liquid culture and water biofilms showed different levels of hydrophobicity when MAA was applied. CAM showed to be more useful to assess hydrophobicity on solid cultures, and MAA was more proficient to assess directly the cells surface hydrophobicity and was useful for characterise different zones of hydrophobicity within the biofilm which may be involved in fungal ecological functions.

The **Chapter 5** describes a flow-chamber reactor used to monitor fungal biofilms formation under monitored conditions. The model system enables simultaneous microscopy analysis of biofilms formed *in situ* and monitoring water parameters such as pH, free chlorine levels and temperature. Specific fluorescent dye was used for fungal biofilms detection. Fungal biofilm was observed mainly after 8 months on coupons. After 8 months of analyses the system was unset and the pipes and joints were removed and used for fungal isolation. The results indicate that time of exposure and free chlorine levels are important factors in fungal biofilm formation. Additionally, a total of 48 fungal isolates were recovered from biofilms on pipes and joints surfaces. *Aspergillus* spp., *Cladosporium* sp. and *Penicillium* spp. were the predominant fungi.

The **Chapter 6** characterises six filamentous fungal strains recovered from biofilms formed in a flow-chamber reactor. Cristal Violet stain and MTT assay together with image analyses were used to investigate the capability of biofilm formation and to characterise its morphology and physiology. Although each fungus presented a different pattern of biofilm development, spore adhesion, monolayer and EPS production were observed in all fungal species and resemble biofilm kinetics currently described in literature.

The **Chapter 7** describes a sampler device that can be inserted directly into pipes within water distribution systems (WDS), hence exposing biofilms to conditions experienced *in situ*. Calcofluor White M2R staining and fluorescent *in situ* hybridization with morphological analyses using epifluorescent microscopy were used for fungal biofilm analysis. DAPI was also applied for bacterial observation. Filamentous fungal in biofilms were detected predominantly after 6 months on coupons exposed to raw, decanted water and at the entrance of the water distribution system. Algae, yeast and bacteria were also observed representing a high biodiversity. The sampling device, morphological examination and specific fluorescent dyes provided an in situ approach for monitoring filamentous fungal biofilm formation within WDS.

Chapter 8 includes main conclusions and perspectives

4. Scientific output

Published papers in peer reviewed international journals

Siqueira VM, Oliveira HM, Santos C, Paterson R, Gusmão N, Lima (2011) Filamentous Fungi in drinking water, particularly in relation to biofilm formation. IJERPH - International Journal of Environmental Research and Public Health, (8): 456-469.

Accepted paper in peer reviewed international journals

Siqueira VM and Lima N (2011) Surface hydrophobicity of culture and water biofilm of *Penicillium* spp. Current Microbiology.

Submitted paper in peer reviewed international journals

Siqueira VM, Oliveira HMB, Santos C, Paterson RR, Gusmão NB, Lima N (2011) Biofilms from a Brazilian water distribution system are biodiverse and include filamentous fungi.

Chapter of book

Siqueira VM and Lima N. Efficacy of free chlorine against water biofilms and spores of *Penicillium brevicompactum*. In: Water contamination emergencies: monitoring, understanding and acting. Ulrich Borchers, K Clive Thompson (Org.). Cambridge: RSC Publishing, 2011, pp. 157-165.

Poster presentations in international conferences

Siqueira VM, Oliveira H M, Santos C, Paterson RR, Gusmão N, Lima N. Fungos nas águas de consumo: Qual a sua relevância? In: 26º Congresso Brasileiro de Microbiologia, Foz do Iguaçu, Paraná, Brasil, 2011.

Siqueira VM, Oliveira H M, Santos C, Paterson RR, Gusmão N, Lima N. Filamentous fungi in drinking water in relation to biofilm formation. In: FEMS 2011 - 4th Congress of European Microbiologists, Geneva, Switzerland, 2011.

Oliveira H, Siqueira V, Santos C, Lima N, Gusmão N. *In situ* detection of fungal biofilms in a water distribution system, Alto do Céu, Recife, Brazil. In: Biofilms in Nosocomial Fungal Infections Institut Pasteur, Paris, France, 2011.

Oliveira HM, Siqueira VM, Barbosa P, Santos C, Lima N, Gusmão N. Fungos em água de abastecimento. In: Congresso Brasileiro de Recursos Genéticos, Salvador. CBRG - Congresso Brasilleiro de Recursos Genéticos, 2010.

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Siqueira VM and Lima N. Fungal Biofilms. In: II Dia do aluno de pós-graduação da Uminho, Braga, Portugal, 2009.

Oliveira HM, Goncalves AB, Siqueira VM, Carneiro-Da-Cunha GM, Lima N. Detecção in situ de fungos filemntosos em tubos da rede de distribuição de água potável na área metropolitana de Recife. In: 1º International Workshop on Biotechnology, 3º Alfa-Valnatura Meeting, 3º Scientific Journey of LIKA, Recife, Brasil, 2008.

Chapter 1

Literature Review

1.1 Microbiology of drinking water

Water covers seven tenths of the Earth's surface and occupies an estimated total volume of 1.38 x 10^9 km^3. Most of this water occurs as oceans (96.1 % of global water); the remaining 3.9 % of water (including polar ice-caps) occurs mainly as polar ice and ground water. Non-polar surface freshwaters, including soil water, lakes, rivers and streams occupy approximately 0.0013 % of the global water (Sigee 2005). Only c.a. 2.6 % of planet´s water is available as potential drinking water (Szewzyk et al. 2000).

The European Union Council Directive 98/83/EC and the World Health Organization (WHO) state that drinking water is "free from any microorganisms and parasites and from any substances which, in numbers or concentrations, constitute a potential danger to human health". Although the access to safe drinking water is essential to health and is one of the basic human requirements, it is estimated that 1.1 billion people worldwide is deprived of it (WHO, 2000). Guideline limits for microbial and chemical parameters were created to achieve attainment of a comparable quality of drinking water in all countries belonging to the European Union (Council Directive 98/83/EC).

Water quality is one great concern for water consumers and many problems in water distribution systems are microbial in nature. Primary and opportunistic pathogens are able to survive water treatment and disinfection and, consequently, they can be found trough the water distribution system, either as planktonic or biofilms assemblages (Wrigth et al. 2004). In developing countries, where access to clean water and sanitation are not the rule, the problem takes a higher dimension. Differently, in Europe and US is common each house be supplied with clean and treated water, what does not mean an elimination of potential microbial waterborne diseases. Nowadays pathogens cause contamination and diseases outbreaks even with modern technologies and an efficient treatment (Szewzyk et al. 2000); examples of waterborne outbreaks in 19 European countries were listed (WHO 2001). In US, from 1970 until 2006, a total of 833 outbreaks associated with drinking water were reported, resulting in 577,991 cases of illness and 106 deaths (Craun et al. 2010). In Brazil, from 1999 until 2008, 343 waterborne outbreaks were notified with 10 089 cases of illness and 8 deaths. Interestingly, in 234 of 343 outbreaks in Brazil the ethologic agent was not identified (www.portal.saude.gov.br). Generally the most threatening microbial risks are associated with ingestion of water contaminated with human or animal feces. Cabral (2010) published a review in which a general characterization of the most important bacterial diseases transmitted through water is presented.

A distribution water system can be seen as a very complex environment in which diverse factors (e.g. pipe material, water flow, nutrients level, concentration of disinfectant, temperature and pH) may interfere in the water microbiology (Berry et al. 2006). The idea of a drinking water system as an ecosystem becomes even more complex if all the others habitats surrounding it are considered (Szewzyk et al. 2000). Storage, end point and plumbing also contribute for the microbiological content (Wingender and Flemming 2004).

Microorganisms in water distributions systems are not directly harmful to humans, however they may cause aesthetic (turbidity, odor), technical (biofouling) and health related (contamination with pathogenic microorganisms) problems (Monteil et al. 1999; Cabra and Pinto 2002; Flemming 2002; Paterson and Lima, 2005). Chlorine has been used as a popular disinfectant for controlling microbial growth in water but microorganisms may survive the disinfection process or may penetrate the water network following mechanical failures such as main breaks, faulty joints, vales or during network repairs and are able to attach to pipes and proliferate as biofilms (Momba et al. 2000). Biofilms are functional consortia of microorganisms (bacteria, fungi, algae, protozoa and viruses) and their formation on pipe walls are linked with microbial water problems such as increased resistance to disinfection and persistence of pathogens (Szewzyk et al. 2000). Water is an essential element for life, thus every effort should be made to achieve a drinking water quality as safe as possible.

1.2 Biofilm – definition and basic concepts

It is a wonderful time to work on microbial biofilms

(O'Toole and Ghannoum 2003)

In fact there has been an explosion of studies examining microbial biofilms in the last twenty years which have been accompanied by the development and improvement of the techniques that revolutionized our understanding of biofilms but, despite the several advantages brought about by the new techniques, a very simple question still remains: what is a biofilm?

The term biofilm is self-explanatory but even for biofilm researchers its definition remains controversial for many reasons. For example, *film* semantically implies a continuous and relatively thin layer but many biological structures regarded as biofilms are neither continuous nor thin (Lewandowski and Beyenal 2007). Moreover, research on biofilms has developed into interdisciplinary work and scientists

involved are from different research fields what leads to individual judgments regarding the professional area. Consequently, it can be said that there are almost as many definitions as there are scientists working in biofilms field. Facing the problematic definition, Wimpenny (2000) listed the types and descriptions of microbial systems that are related to biofilms but as a result of these diverse definitions, still left divergences in the application of the terminology.

Despite the difficulties in defining biofilm, and the diversity of pathways utilized to make a biofilm documented, the past decades has revealed common phenotypes conserved among biofilms. Thus, observing similarities among very different biofilms will likely teach us much. In this study, we use a simple and widely accepted definition which says that biofilms are microbial communities formed by microorganisms attached to a surface and enclosed in a matrix of extracellular polymeric substances (Costerson et al. 1995; Costerson 1999; Dolan 2002; Dolan and Costerson 2002; Stoodley et al. 2002; Harrison et al. 2005; Huq et al. 2008). As microbial communities, biofilms are assemblages of diverse species occupying the same, functional discrete, environment and have a complex level of organization with a distinctive structure, own activities and laws, which depend on the relationships between their constituents (Wimpenny 2000).

In general, for the development of a biofilm, the cell leaves its planktonic condition and attach to a surface and/or other cells within an exopolymeric matrix. In a biofilm, the structures of individual cells are not significantly altered, but the individuals become organized into a complex structure and display novel characteristics and phenotypes (Harding et al. 2009). The physical proximity of other cells promotes synergistic interactions and aid to microbial cells in numerous aspects of their life cycles. These benefits may include increased tolerance to chemical, biological and physical stresses, efficient capture of nutrients, enhanced cell to cell communication and colonization of host tissues (Lewis 2001; Mahmoud and O'Toole 2001). A typical bacterial biofilm development model can be described in five main stages: (1) adsorption, association or initial attachment of a single cell to a surface, (2) adhesion, (3) microcolony formation, (4) maturation and (5) dispersal (Figure 1.1).

The third stage of biofilm development in which cells form microcolonies is characterized by the production extracellular polymeric substances (EPS). EPS can represent 50-90% of the total organic matter of biofilms and are responsible for binding cells and other particulate materials together (cohesion) and to the surface (adhesion), i.e. providing the structural support for the biofilm maturation (Allison 2003). Polysaccharides are characteristic components of EPS but its chemistry is complex and in general also comprises proteins, nucleic acids, lipids, phospholipids and humic substances. Although polysaccharides have been well studied, the literature suggests a large variety, but uncharacterized, of components

produced by different species under different growth conditions (Sutherland 2001). Beyond mechanical stability, EPS protect biofilm against adverse conditions and biocides and also permit the development of microconsortia, concentration gradients, retention of extracellular enzymes, convective mass transport through channels, easy horizontal gene transfer, a matrix for exchange of signaling molecules and light transmission into the deeper layers of the biofilm structure (Flemming 2002).

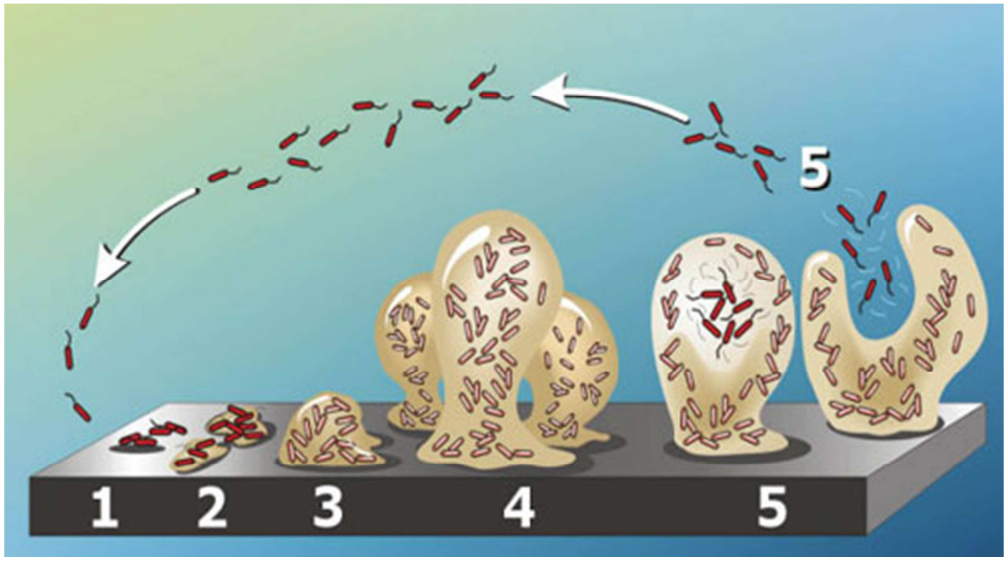

Figure 1.1 Illustration showing the development of bacterial biofilm as a five-stage process: (1) adsorption, association or initial attachment of a single cell to a surface, (2) adhesion, (3) microcolony formation, (4) maturation and (5) dispersal (Stoodley et al. 2002).

Biofilm formation at the interface between a solid substratum and a liquid is a common phenomenon in natural, medical and industrial environments. In water distribution systems it is estimated that 95% of microbial biomass is in biofilms (Momba et al. 2000) thus biofilms are considered a main reservoir of pathogens and a great threat to safe drinking water. The understanding of biofilms in water distribution systems will provide us important knowledge in microbial ecology of water distribution systems and insights to deal with problems related to them.

1.3 Biofilms in water distribution systems

The occurrence of bacteria in drinking water has been reported since the 30´s. Adams and Kingsbury (1937) showed that although bacteria were present in finished water, they seemed to come from nowhere because they could not be detected in the point of entry. Intriguingly these bacteria were multiplying in the distribution system and only later with the advance of scanning electron microscope

(SEM) complex communities of microorganisms were detected on pipe surfaces (Ridgway and Olson 1981; Schoenen and Scholer 1985). These findings make researchers realize that water disinfection was merely inactivating planktonic bacteria and that some bacteria were able to survive treatment process and were becoming adapted to the distribution system environment. Thus, monitoring for microbial water quality in the water distribution system may be difficult because significant microorganisms can be introduced after treatment. Additionally, growth of bacteria on pipe walls, i.e. biofilms, also can provide a shelter for microorganisms (EPA 1992).

Nowadays the focuses of microbial water quality studies still remain on monitoring planktonic microorganisms, even though it is long since known that in water systems the majority of microorganisms live together as biofilms (Costerson et al. 1987). In a water distribution system, biofilms are composed by microorganisms which survived water disinfection or entered into the distribution system through mechanic failures. These microorganisms provide a seed which multiply in the distribution system when the right conditions for growth are given. These conditions include factors such as the disinfectants used and the maintenance of a residual concentration in the system, the resistance of microorganisms to disinfectants, the nature and concentration of biodegradable compounds in the treated drinking water and the material of pipe used in the system (Momba et al. 2000). Others factors which influence biofilm formation and development are listed in Table 1.1.

Table 1.1 Surface, water phase and call variables in water biofilm formation and development (adapted from Dolan et al. 2002).

Surface	Texture or roughness, hydrophobicity, conditioning film
Water phase	Water flow/stagnation, pH, temperature, pressure, oxygen supply and demand Incident light, presence of residual disinfectants and nutrients availability
Cell	Cell surface hydrophobicity, extracellular appendages, extracellular polymeric substances, signaling molecules and surface-associated polysaccharides or proteins

Investigations in water systems biofilms have shown that these communities are usually composed by a heterogeneous microbial assemblage. The microbial composition of a biofilm is influenced many factors and may vary conforming the local. Generally, microbial characterization reveals the existence of a diverse and complex community wherein microorganisms interact and are influenced by the surrounding environment as well (Keevil et al. 1995; Wimpenny et al. 2000) (Figure 1.2). Although many studies aim to describe biofilms structure and composition, little is known about predominant species and primer and

secondary colonizers, and important features for biofilm succession remain not understood (Kerr et al. 2003).

In fact, most of microorganisms in a biofilm are not directly harmful for human but biofilms represent a threat for human health since they can act as supports for stickiness, accumulation and persistence of others microorganisms (Paris et al. 2009). Biofilms can also act as refuge for bacteria and protect them against disinfectants (Berry et al. 2006). Many studies have report biofilms harbouring potential pathogenic bacterial such as *Pseudomonas, Mycobacter, Campylobacter, Klebsiella, Aeromonas*, *Legionella* spp., *Helicobacter pylori* and *Salmonella typhimurium* (Park et al. 2001; Azevedo et al. 2006; Lehtola et al. 2007; Helmi et al. 2008).

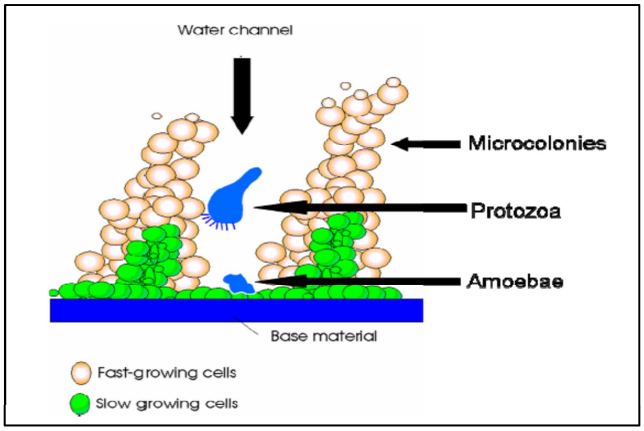

Figure 1.2 Structure and microbial composition of water biofilm (Keevil et al. 1995).

Biofilms in water distribution systems are also related with aesthetic problems, e.g. changes in taste, odour and colour of water. Filamentous bacteria such as *Actinomyces, Nocardia*, *Streptomyces* and *Arthrobacter* and certain filamentous fungi have been linked with taste and odour complains (Olson and Nagy 1994; Paterson et al. 2007). Biofouling, i.e. undesirable accumulation of biotic deposits on a surface, may cause corrosion in cast iron pipes and may be induced or enhanced by microbial activity (Flemming 2002). Therefore, for water companies and industries, biofouling can represent losses in billion extents.

Control strategies have been applied to prevent and eliminate unwanted biofilms. Water quality control strategies are mainly based on the prevention of biofilm formation by regular cleaning and disinfecting before microorganisms attach firmly to surfaces (Simões et al. 2006). The identification of materials that do not promote or can even suppress biofilm formation is also applied as a preventive strategy (Kerr et al. 2003). In food industry, the use of enzyme-based detergents as bio-cleaners is seen a viable option to overcome problems caused by biofilms (Augustin et al. 2004). The use of bacteriophages

as control strategy has also been studied (Curtin and Donlan 2006; Sillankorva et al. 2008), although relatively little information is available on the action of bacteriophages on biofilms and how biofilm composition and environmental factors influence bacteriophages actions (Sillankorva et al. 2004; Sutherland et al. 2004). More detailed information about biofilm control strategies can be accessed in a recent review given by Simões et al. 2010.

In water distribution systems, biofilms are composed by different microbial species functioning in a consortium, and the existence of multiple interspecies interactions may influence biofilm initial stages and subsequent development. The simple production of a metabolite, for example, can interfere in biofilm development. Moreover, mixed species biofilms may be thicker and more stable than single species biofilms, what further influences their susceptibility to biocides (Elvers et al. 2002).

1.4 Fungi and fungal biofilms in water distribution systems

1.4.1 Fungi

Fungi are a ubiquitous and diverse group of organisms belonging to the kingdom Fungi which was firstly considered as the fifth kingdom by Whittaker (1959). According to the most recent classification this kingdom comprises one subkingdom, seven phyla, ten subphyla, 35 classes, 12 subclasses, and 129 orders (Hibbett et al. 2007). It has been estimated that 1.5 million species exist worldwide and about only 120 000 species have been described to date (Kirk et al. 2001). Currently, novel fungi continue to be isolated, such as the high diverse group recently named "cryptomycota" (Jones et al. 2011).

Despite difficulties do define the limits of the group, mycologists have defined fungi as "*eukaryotic, spore-producing, achlorophyllous organisms with absorptive nutrition that generally reproduce both sexually and asexually and whose usually filamentous branched somatic structures, known as hyphae, typically are surrounded by cell walls*" (Alexopoulus et al. 1996). Based on their lifestyle, fungi are characterized by heterotrophic nutrition and cosmopolitan distribution (Kendrick 1992). As a matter of didactic and a practical approach to classification, fungi have been divided into groups based on their morphology, i.e. filamentous fungi (or moulds), yeasts and mushrooms (Figure 1.3).

As a diverse and dynamic group, fungi are involved in many activities that affect human both in a good or bad way. In general, a single most important role that fungi play is not specifically identified yet but fungi are the most important on Earth agent of decay (Alexopoulus et al. 1996) and play a predominant role in recycling organic matter in the environment. Fungi are often observed on decaying foodstuff on

which some fungi produce toxins (mycotoxins); many of them are plant and human pathogens. In addition, fungi are used to produce commercial products such as antibiotics (e.g., penicillin), organic acids (e.g. citric acid), industrial alcohol (e.g. biofuel) and enzymes (e.g. amylases). Moreover, fungi are also used in food industry for the production of a diverse range of important foodstuffs such as bread, beer, cheese, meats, and soy sauce (Paterson and Lima 2005).

Some fungi known as true aquatic fungi (e.g. *Chytridiomycota* and *Zygomycota*) are primarily adapted to aquatic environments and are known to produce spores (zoospores) morphologically adapted to disperse in running waters (Wurzbacher et al. 2010). Terrestrial fungi generally need a solid substrate for dispersal of their spores and are primarily adapted to environments such as soil, organic material and air, and anything in contact with air (Kirk et al. 2001). On the other hand, terrestrial fungi are often passively introduced into lakes, rivers and streams. Species of *Aspergillus* and *Penicillium,* well-known terrestrial fungi, are among the most common fungal isolates from freshwater and marine environments. Hence there may be no simple and exclusive division of fungi into aquatic and terrestrial types (Park 1972).

Water distribution systems are aquatic environments where fungi can enter, survive and proliferate, although water networks are considered an 'unnatural' habitat for them (Hageskal et al. 2009). Among the huge diversity of fungi already isolated from water distribution systems, opportunistic pathogens, e.g., *Cryptococcus* spp. and *Aspergillus fumigatus* must receive special attention, mainly in locals such as hospital where water may serve as a transmission route for fungal infections (Anaissie and Costa 2001). Moreover, because of absorptive nutrition mode, secretion of extracellular enzymes and apical hyphal growth, fungi are especially adapted for growth on surfaces (Jones 1994), therefore forming biofilms on pipe walls and contributing to loss of water quality.

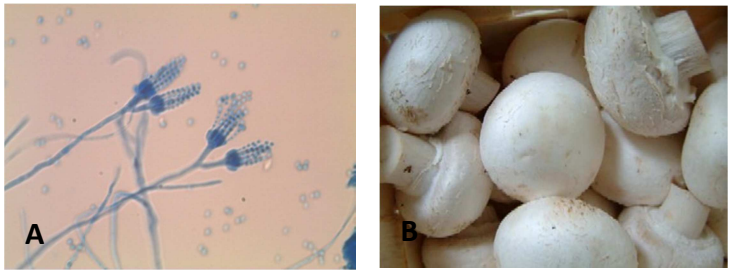

Figure 1.3 Fungal groups based on morphological characteristics: filamentous fungus (A) and mushroom (B).

1.4.2 Fungi in drinking water

A drinking water distribution system provides a habitat for microorganisms wherein, even after an efficient disinfection, bacteria, fungi, viruses, protozoa and algae may be present. Certain microorganisms

are well known water contaminants but bacteria are probably the most frequently studied group of microorganisms with respect to the quality of drinking water. The main reason for the infrequent discuss on fungi as pathogenic microorganisms in water lays on the fact that the consumption of fungal contaminated drinking water does not lead to acute disease (Hageskal et al. 2009). Although significance of fungi in drinking water remains underestimated, fungi play an important role in water quality since a water distribution system is a very complex environment in which abiotic and biotic factors may interfere in water microbiology. Moreover, fungi are able to produce compounds associated with organoleptic problems, i.e. unpleased odor and taste (Paterson and Lima 2005).

Despite fungi have not been directly associated with water contamination problems, in the last decade several studies have contributed to increase our knowledge on the occurrence of fungi in water systems (Table 1.2). In general, the results show that the recovery of fungi varies between 7.5–89 % positive samples, and that there is a considerable variation among the levels of fungi and in the samples. Moreover, fungi have been recovered from many types of water, e.g. raw water, treated water, heavily polluted water, distilled or ultra-pure water and bottled drinking water (Hageskal et al. 2009). The results of investigations show in common a wide diversity of fungi isolated and among them potentially pathogenic, allergenic, and toxigenic species. *Aspergillus fumigatus* is one of the most significant fungal pathogenic species causing infections in immunocompromised patients in hospitals and has been isolated from municipal water distribution system (Grabińska-Łoniewska et al. 2007) and hospitals (Warris et al. 2001; Hayette et al. 2010) indicating that water may disseminate harmful fungal species to private homes and hospitals (Kelley et al. 2003).

Since potentially pathogenic fungi have emerged as a leading cause of hospital-acquired infections (Anaissie et al. 2003), water in hemodialysis centers has also received special attention. At hemodialysis units, immunocompromised patients are in constantly contact with dialysis water which comes direct in contact with the bloodstream with the only interposition of a semipermeable artificial membrane (Pontoriero et al. 2005). Studies have shown that fungi are constantly isolated from water used for haemodialysis procedures, alerting the need of international standards monitoring not only for bacteria, but also for fungi (Arvanitidou et al. 2000; Pires-Gonçalves et al 2008).

In such a complex environment where bacteria, fungi, viruses and protozoa cohabit, fungal secondary metabolite can be produced as a response for these constant microbial interactions. *Penicillium* and *Aspergillus* are well known mycotoxin producers (Paterson and Lima 2005) and have been often isolated from drinking water (Table 1.2). For example, Paterson et al. (1997) reported aflatoxin produced by *Aspergillus flavus* in water from a cold water storage tank; Kelley et al. (2003) concluded that

mycotoxins and other metabolites can be produced by fungi in water; Criado et al. (2005) detected citrinin produced by *Penicillium citrinum* in mineral bottled water; Paterson et al. (2007) reported the production of zearalenone by *Fusarium graminearum.* Although mycotoxins produced in water may be extremely diluted and represent a minor concern, their concentrations may increase and may become a hazard to human health especially when water is stored in cisterns, reservoirs or even in bottles, for prolonged periods (Hageskal et al. 2009).

The diverse performances applied for analysis represent a critical point with respect to the study of fungi in water (Hageskal et al. 2009). Culture depending methods, that are time consuming and limited to specific culture media, are often used but yet there is no international standard method described, neither an acceptable or normal level of fungi established. So far the only exception is Swedish water regulation authority (Anon. 2003) that includes specifications on fungi. Membrane filter techniques are mostly employed (Table 1.2), but the wide range of applying volumes may result in different detection limits of fungi in the water. In addition, different culture media, temperatures and time of incubation will consequently result in recovery of different genera and species. Problems related to the quantification and identification of filamentous fungi make more difficult the comparisons between the different studies and may explain much of the variation in the results obtained (Hageskal et al. 2009).

Hageskal et al. (2009) published an extensively review with a complete overview about the study of fungi in drinking water and concluded that (1) fungi are relatively common in water distribution systems, (2) potentially pathogenic, allergenic and toxigenic species are isolated from water in high concentrations, (3) the main limitations of fungal water studies lie within the methodology, (4) the methods for analyzing fungi in drinking water should be standardized and (5) fungi may influence the water quality.

Table 1.2 Filamentous fungi recovered from diverse water supplies in different countries

Country, local, year	Time	Water analysed	Main isolation method	Most frequent fungal isolates	Authors
United Kingdom, UK, 1996	Autumn and Spring	Surface water and network	Membrane filtration, direct plating and Bating	*Aspergillus*, *Cladosporium*, *Epicoccum*, *Penicillium* and *Trichoderma*	Kinsey et al. 1999
Greece, Thessaloniki, 1998	Unique collect with 126 potable water samples	Tap water (hospital and community)	Membrane filtration	*Penicillium, Aspergillus* and *Acremonium*	Arvanitidou et al. 1999
Greece, haemodialysis units, 1998	Unique collect with 255 samples	Municipal water supplies of haemodialysis centres	Membrane filtration	*Penicillium* and *Aspergillus*	Arvanitidou et al. 2000
Norway, Oslo, 1999	6 months	National Hospital University of Oslo	Membrane filtration	*Aspergillus* spp., *Aspergillus fumigatus* and *Trichoderma* sp.	Warris et al. 2001
Germany, North Rhine-Westphalia, 1998/9	12 months	Drinking water	Pour-plating	*Acremonium*, *Exophiala*, *Penicillium* and *Phialophora*	Göttlich et al. 2002
Turkish, Istanbul, 2005	Unique collect with a total of 100 samples	Water system of a hospital	Membrane filtration	*Penicillium* spp., *Aspergillus* spp. and *Acremonium* spp.	Hapcioglu et al. 2005
Portugal, Braga, 2003/4	12 months	Tap water	Membrane filtration	*Penicillium* and *Acremonium*	Gonçalves et al. 2006
Brazil, Maringá, 2007	Unique collect with a total of 60 samples	Tap and bottled mineral water	Membrane filtration	Yeast and filamentous fungi	Yamaguchi et al. 2007
Norway, 14 networks, 2002/3	December, June and September	Drinking water (surface and groundwater)	Membrane filtration	*Penicillium, Trichoderma* and *Aspergillus*	Hageskal et al. 2007
Poland, Warsaw, 2007	2 years	Municipal water supply system	Membrane filtration	*Aspergillus nigerI, Aspergillus fumigatus*	Grabińska-Łoniewska et al. 2007
Brazil, São Paulo, 2008	3 months	Water system of a Hemodialysis centre	Membrane Filtration	*Candida parapsilosis*, *Fusarium* spp. and *Trichoderma* spp.	Pires-Gonçalves et al. 2008

Table 1.2 Continuation

Pakistan, Karachi, 2007	Unique collect with a total of 30 samples	Water and fruit juice	Direct plating	*Aspergillus niger* and *A. clavatus*	Nazim et al. 2008
Nigeria, Calabar, 2009	3 months	Borehole and sachet water	Membrane Filtration	*Aspergillus, Rizopus, Fusarium* and *Penicillium*	Okpako et al. 2009
Portugal, Lisbon, 2009	4 months	Surface water, spring water, and groundwater	Membrane Filtration	*Aspergillus*, *Cladosporium*, *Penicillium*	Pereira et al. 2009
Austrália, Queensland, 2007/8	18 months	Municipal water	Membrane Filtration	*Cladosporium*, *Penicillium*, *Aspergillus* and *Fusarium*	Sammon et al. 2010
Brazil, Recife, 2009/10	5 months	Water treatment station and tap water	Membrane Filtration	*Penicillium*, *Aspergillus* and *Phoma*	Oliveira 2010
Belgium, Liège, 2010	4 months	Tap water of the University Hospital of Liège	Membrane Filtration	*Aspergillus fumigatus* and *Fusarium* spp.	Hayette et al. 2010
Iran, Sari, 2011	1 year	4 universities hospitals	Membrane Filtration	*Aspergillus*, *Cladosporium*, *Penicillium*	Hedayati et al. 2011

1.4.3 Filamentous fungal biofilms

Bacterial and yeast biofilms have been greatly studied in the last twenty years (Chandra et al. 2001; Kumamoto and Vinces 2005; De Beer and Stoodley 2006; Walker and Marsh 2007; Shi and Zhu 2009). Consequently, there are well-defined models, criteria and phenotypes for characterizing bacterial and yeast biofilms. On the other hand, a lack of information about filamentous fungal (ff) biofilms still remains, tough ff are extremely adapted to grow on surfaces (Jones 1994).

Indeed, the term "biofilm" is rarely applied to ff but there have been several descriptions indicating that ff grow as biofilms in different medical, environmental and industrial settings (Anaissie et al 2003; Gutierrez-Correa and Villena 2003; Mowat et al. 2007; Mowat et al. 2008). Harding et al. (2009) proposed criteria for biofilm formation by ff which are grouped in (1) structural features such as complex aggregated growth, surface-associated growth of cells and secreted extracellular polymeric matrix and (2) altered gene expression resulting in phenotypic changes that include enhanced tolerance to antimicrobial compounds or biocides changes in enzyme or metabolite production and/or secretion physiological changes. The reports above mentioned demonstrate that the structural and phenotypic criteria can be fulfilled by some filamentous fungi.

Based on already published descriptions for ff and drawing from bacterial and yeast models, Hardling et al. (2009) proposed a preliminary model for ff biofilm formation (Figure 1.4) in which biofilm development follows six main steps including:

- Propagule adsorption: deposition of spores or other propagules such as hyphal fragments or sporangia;
- Active attachment to a surface: includes secretion of adhesive substances by germinating spores and active germlings;
- Microcolony formation: production of a polymeric extracellular matrix that allows the growing colony to adhere tenaciously to the substrate;
- Initial maturation: formation of compacted hyphal networks or mycelia and hypha–hypha adhesion and the formation of water channels via hydrophobic repulsion between hyphae or hyphal bundles;
- Maturation: it is characterized by the formation of reproductive structures;
- Dispersal or planktonic phase: involves spore dispersal or release, or the dispersal of biofilm fragments.

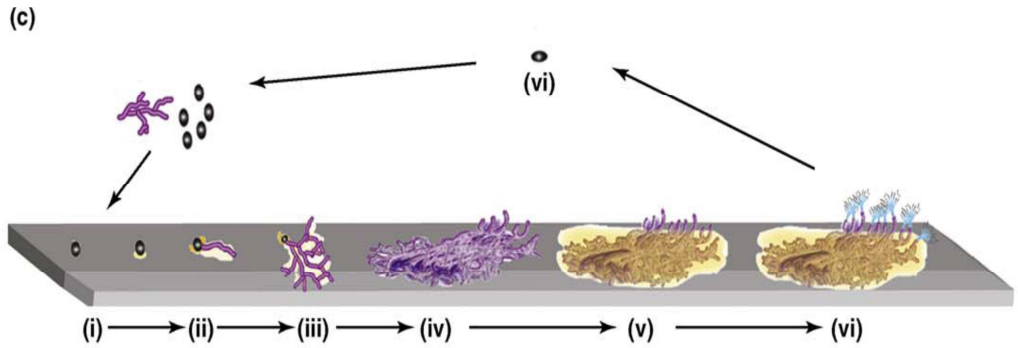

Figure 1.4 Ff biofilm formation model: (i) adsorption, (ii) active attachment, (iii) microcolony I (germling and/or monolayer), (iv) microcolony II (mycelial development, hyphal layering, hyphal bundling), (v) development of the mature biofilm, and (vi) dispersal or planktonic phase (Hardling et al. 2009).

An investigation in *Aspergillus niger* biofilms and pellets showed that these types of growth have two main structural differences: only biofilms exhibited surface heterogeneity and interstitial voids with well-defined channels. Other differences include the growth direction, i.e. biofilm growth was mainly vertical, and a specific biomass distribution as well (Villena et al. 2010). This same work reported that structural differences were associated with a differential physiological behavior regarding enzymatic production.

Compact hyphal balls of *Aspergillus fumigatus* were characterized as biofilms once they presented production of an extracellular polymeric matrix, differential gene expression, and differential sensitivity to antifungal drugs (Beauvais et al. 2007; Mowat et al. 2008). Increased resistance against biocides is one characteristic often described for biofilms, and several studies have been carried out to evaluate the *in vitro* susceptibility of pathogenic fungal biofilms (Jabra-Rizk et al. 2004; Bonaventura et al. 2006; Seidler et al. 2008).

In environmental studies, ff biofilm descriptions have been reported as well. For example, in historical monuments ff biofilms was described forming complex consortia with cyanobacteria and algae resulting in bioweathering of the substrata and thus causing biodeterioration (Grbić et al. 2010). An investigation in microbial communities on the surfaces and within the painting layers of mural paintings of a church showed that the main biofilm formers were microscopic fungi belonging to the genera *Acremonium, Aspergillus, Cladosporium* and *Fusarium* (Gorbushina et al. 2004). Müller et al. (2001) also described microbial colonization of the surface of historic glass panels aging from 30 to 600 years and found a heterogeneous colonization with ff as the dominant group. Phylogenetic analysis revealed that in acid mine drainages biofilms the majority of the sequences belonged to fungi (Baker et al. 2009). All these reports have in common natural ff biofilm growing in oligotrophic environments, showing high tolerance

against adverse factors (e.g. temperature and dryness), and intimate interaction with other microorganisms such as bacteria and algae.

Understandings of ff biofilm development, dynamics and interactions require further research for a better clarification about this naturally occurring growth form, thereby its impact and role in medical, industrial and environmental areas.

Table 1.3 Study of filamentous fungal biofilms in water systems.

Country	Material age	Origin	Main methods applied	Fungal components	Authors
Brazil	30-60 days	Cooling water system	SEM	Filamentous fungi	Lutterbach and França 1996
UK	-	Flowing water processing tanks	Modified Robbins Device; SEM	*Fusarium solani*, *Fusarium oxysporum* and bacteria	Elvers et al. 1998
USA	3 months	Municipal water distribution System	Light microscopic; SEM; direct inoculation	*Aspergillus* spp. and *Penicillium* spp.	Doggett 2000
Portugal	-	Potable water up-flow laboratorial reactor	FISH; Calcofluor staining	Eukarya and filamentous fungi	Gonçalves et al. 2006
France	7 – 10 months	Nancy's drinking water network	Flow chamber; optical microscopy	*Filamentous fungi biofilms*	Paris et al. 2009
Poland	160 days	Tap water	SEM	Filamentous fungi	Traczeweska and Sitarska 2009
Australia	7-26 months	Municipal Water Distribution System	Calcofluor, DAPI; SEM	Filamentous fungi	Sammon et al. 2011
Brazil	-	Municipal Water Distribution System	FISH; Calcofluor staining; FUN-1	Eukarya and filamentous fungi	Siqueira et al. 2011

1.4.4 Filamentous fungal biofilms in water systems

Although filamentous fungi have been commonly recovered from drinking water, and are often listed as integrant of microbial water biofilms (Kerr et al. 2003), ff biofilms in drinking water system have been disregarded, and the focus of most research have been put on bacterial biofilms, especially on those linked with water-related illness (Huq et al. 2008). Drinking water systems are undoubtedly complex environments wherein bacteria, fungi, protozoa, viruses and algae cohabit and interact. Each

microorganism plays its own roles and should not be underestimated, either as a potential threat to human health or as functional part of this unique ecological niche. Despite the undervalued participation of filamentous fungal biofilms in drinking water microbiology, a few reports have been published (Table 2.3). Based on these data, it would be reasonably to include filamentous fungi in biofilm illustration as an integrant of this microbial community (Figure 1.5).

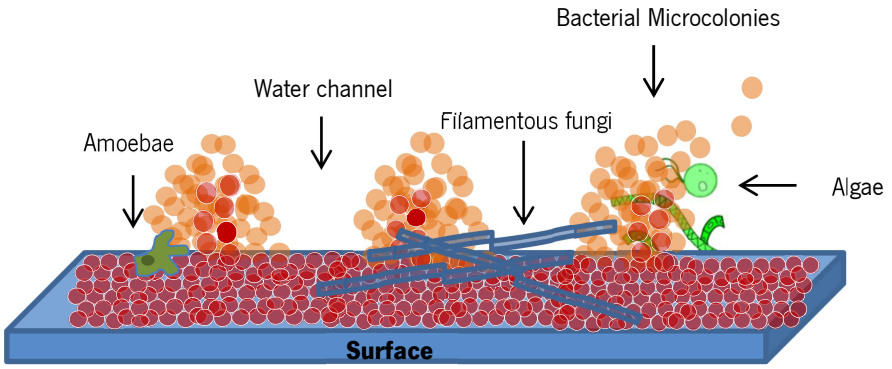

Figure 1.5 Microbial heterogeneity and architectural structure of water biofilm.

The study of biofilms in drinking water systems is prone to errors since the main drawbacks in this area are related to the variation in scientific methodology (Berry et al. 2004; Hageskal et al. 2009). Others features such as representativeness of samples, heterogeneity of environment and sort of techniques applied must be taken into consideration (Figure 1.6). For example, different source of water and variable time of exposure are commonly find in studies of ff biofilms in water systems (Table 1.3), and comparisons between results become difficult. Additionally, in water networks the collection of pipes is not easy since it would be necessary their removal; consequently *in situ* approaches are scarce. Pilot systems in laboratory are used instead.

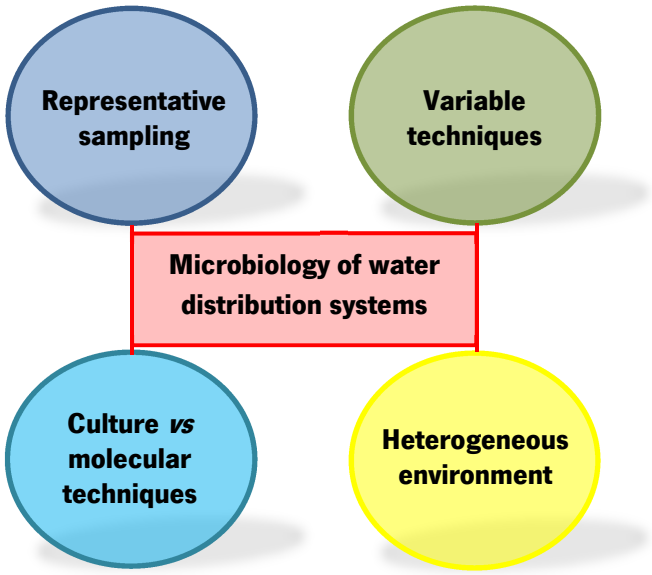

Figure 1.6 Drawbacks in microbial studies of water distribution systems.

Table 1.4 gives examples of common techniques and their main characteristics applied for detection of planktonic and biofilm in water distribution systems.

Table 1.4 Common techniques applied for studies in microbiology of water distribution systems.

	Techniques			
Characteristics	**Water samples**	**Pipe sections**	***In situ* sampler**	**Laboratorial devices**
Representativeness	Variable volumes; not representative when in small volumes; only cultivable microorganisms	Good representative of pipes material and water quality	Good representative of pipes material; needs a long exposure time	Represents only one point of the water network
Availability and cost	Inexpensive	Available only when are replaced	Cheap and always available; doesn't interfere in the water network	Relatively inexpensive
Degree of contamination	Unlikely to be contamined during transport and sample	Easily to be contamined during transport and sample	Unlikely to be contamined during transport and sample	Unlikely to be contamined during transport and sample
Physical challenge	Heavy when in large volumes	Easy to handle	Easy to handle	Easy to handle

1.5 Methods to study fungal biofilms

Research on biofilms is an interdisciplinary work in which researchers from different areas are involved. The field of interest will determine the specific approaches to be applied and may include microscopical, microbiological, molecular biological, (bio)-chemical and/or physical methods. The following scheme (Figure 1.7) summarizes research fields and techniques applied in biofilm studies. Is beyond our objectives give a detailed description of each technique, thus the following text describe only the techniques used in this work.

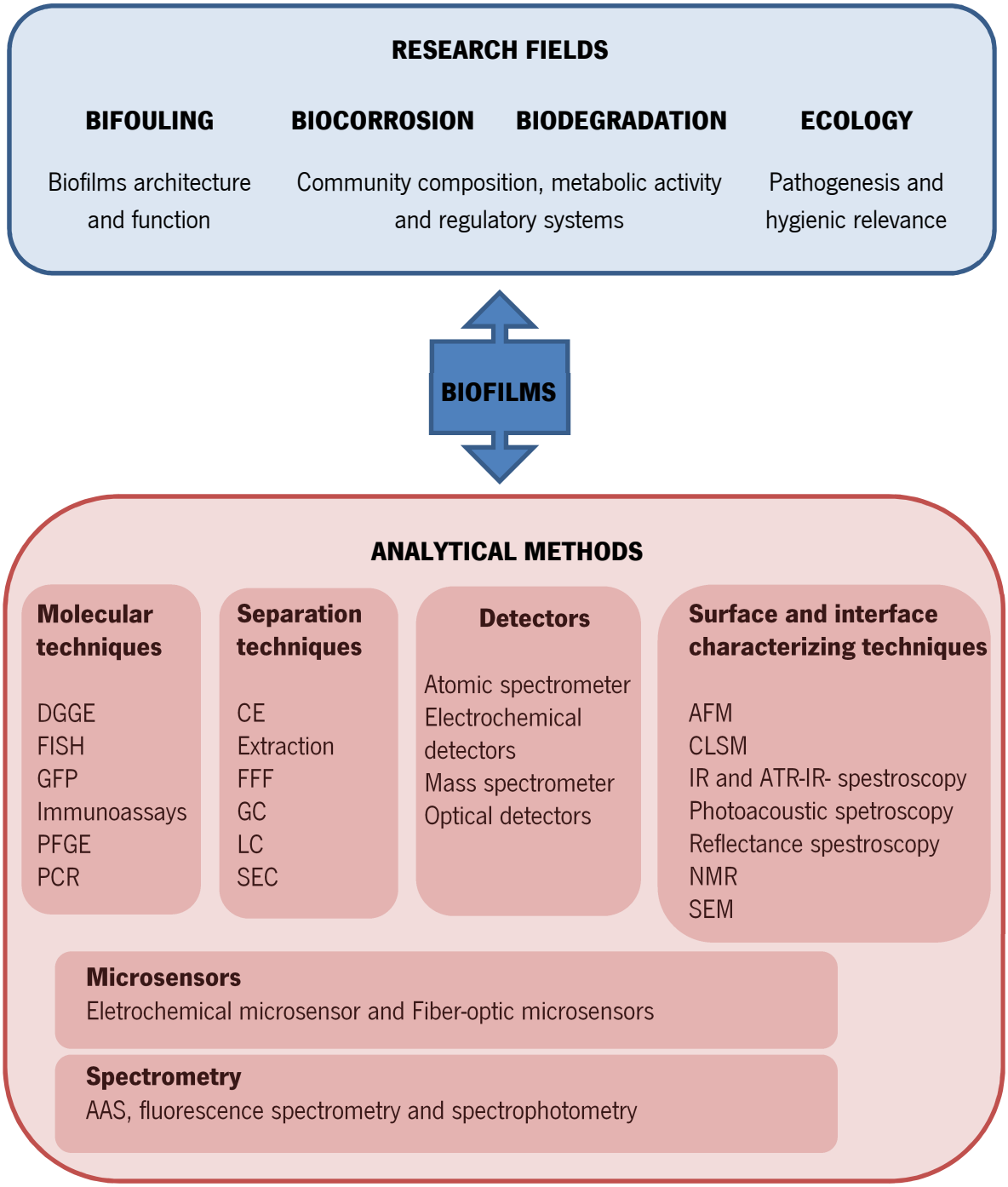

Figure 1.7 Research fields and analytical techniques applied in biofilm research (Denkhaus et al. 2007).

AAS atomic absorption spectrometry, AFM atomic force microscopy, ATR attenuated total reflectance, CE capillary electrophoresis, CLSM confocal laser scanning microscopy, DGGE denaturing gradient gel electrophoresis, FFF field-flow fractionation, FISH fluorescent in situ hybridization, GC gas electrophoresis, GE gel electrophoresis, GFP green fluorescent protein, IR infrared, LC liquid chromatography, NMR nuclear magnetic resonance, PCR polymerase reaction, PFGE pulsed filed gel electrophoresis, SEC size exclusion chromatography, SEM scanning electron microscopy, ST XM scanning transmission X-ray microscopy

1.5.1 Fluorescence microscopy and fluorochromes

Fluorescence is a property of some atoms and molecules to emit light at longer wavelengths after absorbing light of a particular and shorter wavelength. In a fluorescence microscope, the specimen is illuminated with light of short wavelength, e.g. ultraviolet light. Part of this light is absorbed by the specimen and re-emitted as fluorescence (Herman 1998). The key components of fluorescence microscopy include an excitation light source, wavelength selection devices (a set of well-balanced filter combinations), objectives and detectors.

Fluorescence microscopy has become an essential tool in biology science as it has attributes that are not readily available in other optical microscopy techniques. The use of different fluorochromes has made it possible to identify cells and submicroscopic cellular components with a high degree of specificity. The fluorescence microscope can reveal the presence of a single fluorescing molecule. In a sample, through the use of multiple staining, different probes can simultaneously identify several target molecules (Lichtman and Conchello 2005).

Fluorochromes (or fluorescent dyes) are molecules that are used by virtue of their fluorescent properties. Their efficiency as fluorescent molecules and their wavelength of absorption and emission depend on the more external electron orbitals in the molecule. When fluorochomes absorb light energy, alterations in the vibration, electronic and rotational states of the molecule can occur. This energy sometimes moves an electron into a different orbital, an “excited state” is set and fluorescent is emitted (Lichtman and Conchello 2005). Many organic molecules have intrinsic fluorescence (i.e. autofluorescence or primary fluorescence). Secondary fluorescence represents the emission produced after a molecule is combined with a primary fluorescent molecule (Altemüller and van Vliet-Lanoe 1990). Few autofluorescence molecules are useful for specific labeling in biological systems; synthetized compounds have been more often applied.

Fluorescent staining has provided great benefits for biofilm research. The use of fluorescent dies allows nondestructive analyses and gives information of several important characteristics of biofilms; architecture, metabolic activity and microbial diversity can be assessed in a same biofilm. The main characteristics and applications of the fluorescent dyes used in this work are listed in the following topics.

1.5.2 Calcofluor White M2R

Calcofluor White M2R (CW) (4,4’-bis[4-anilino-6-bis(2-ethyl)amino-s-triazin-2-ylamino]-2,2’-disulfonic acid) is a well-known fluorescent brightener and since the early 1940s has been used in the paper, textile,

and related industries as agents to whiten and to prevent "yellowing" of papers and fabrics (Zollinger 1991). CW binds to β(1-3) and β(1-4) polysaccharides, such as found in cellulose and chitin, and when excited with ultraviolet (UV) fluoresces with an intense bluish/white color (Harrington and Hageage 2003). Absorption spectra for aqueous solutions of CW show absorption over the range 300 to 412 nm, with an absorbance peak at 347 nm, i.e. the maximum excitation and fluorescence occurs with UV light.

CW is a symmetric molecule with two triazol rings and two primary alcohol functions on both sides of an ethylene bridge. The fluorophore shows a high affinity for chitin forming hydrogen bonds with free hydroxyl groups which stains fungal cell walls blue (Figure 1.8). As a selective staining, CW has been particularly useful in detection and morphological studies of fungi. Its use in microbiology was first reported by Darken (1961; 1962) as a dye useful for viewing cell walls of fungi and bacteria; in clinical mycology, its use was first described in the 80's (Hageage and Harrington 1981) and since that has found extensive use for the rapid detection of microorganisms.

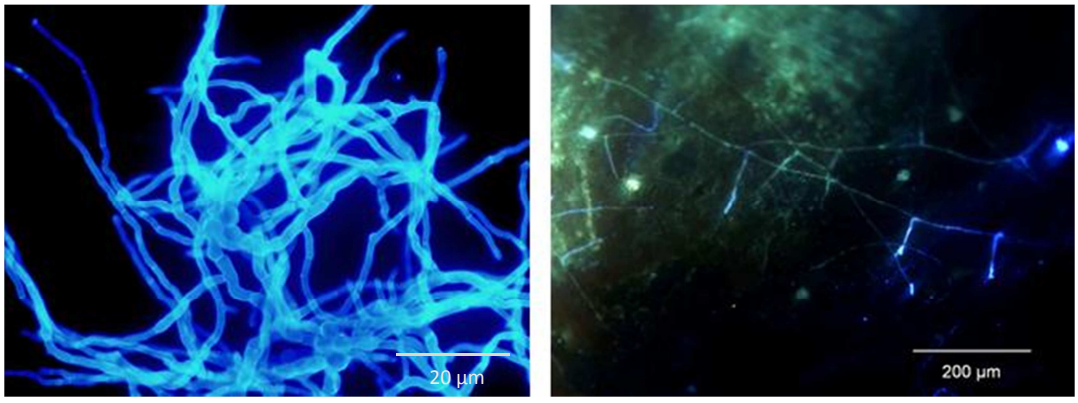

Figure 1.8 *Penicillium brevicompactum* culture (A) and biofilm on pipe surface (B) after CW staining

Specific chitin-binding of CW makes it a good stain to detect and quantify fungi (Ruchel et al. 2004) and has been successfully used for medicine in the detection of pathogenic fungi (Harrington and Hageage 2003; Henry-Stanley et al. 2004; Luther et al. 2005) and for environmental mycological studies (Nunan et al. 2001; Li et al. 2004; Baschien et al. 2008).

Biofilms are complex communities of microorganisms that develop on surfaces. Fungi are excellent colonizers of surfaces (Elvers et al. 2002) and form biofilms in diverse environments. CW was used to detect and characterize drinking water fungal biofilm (Gonçalves et al. 2006; Paris et al. 2009; Siqueira et al. 2011); *Candida* spp. biofilms on medical devises (Chandra et al. 2001; Kuhn et al. 2002; Ahariz and

Courtois 2010); and *Aspergillus fumigatus* biofilms (i.e., mycetoma) (Beauvais et al. 2007; Mowat et al. 2008).

CW has been also employed to visualize the surrounding of the biofilm cells (Stewart et al. 1995) and was used to detect biofilms through the visualization of the EPS (extracellular polymeric substances). Rezende et al. (2003) evaluated the use of CW to detect biofilms formed by foodborne pathogens in five different surfaces. These authors made use of CW´s characteristic to binds to polysaccharides containing β-D-glucans produced by the bacteria and concluded that CW can be used as a nonspecific test for detection of biofilm. Although CW was originally described for staining of β(1-3) and β(1-4) polysaccharides (Maeda and Ishida 1967), Thurnheer et al. (2004) showed that CW reacted with α(1-3) and α(1-6) polysaccharides presented in bacterial biofilms. Paramonova et al. (2007) applied CW to measure bacterial biofilm thickness; within a *Pseudomonas aeruginosa* biofilm, cells and EPS were distinguished after CW staining (Shih and Huang 2002) and interactions in a mixed bacterial biofilm were studied using CW as well (Cowan et al. 2000).

1.5.3 FUN-1

FUN-1 is a membrane-permeate fluorescent probe which stains nucleic acids in most cell types, producing diffuse green to green-yellow cytoplasmic staining in live or membrane-compromised dead cells. In metabolic active cells, FUN-1 (1 μM up to 50 μM) is metabolically converted into orange/red Cylindrical Intra Vacuolar Structures (CIVS) in less than an hour. The CIVS are approximately 0.5 - 0.7 μm in diameter and range from one to several μm in length. They are frequently observed moving freely in the intravacuolar space and have distinct orange-red fluorescence when excited by light from about 470 nm to 590 nm (Figure 1.9). Fluorescence labeling with FUN-1 stain provides spectral and morphological information not available with other single-dye fluorescence-based methods for viability determination in yeasts and fungi (Millard et al. 1997).

Most microbiological studies apply culture dependent methods which have as main disadvantage the several time-consuming steps and don´t reliably report on the metabolic capacity of slow-growing or no dividing cells. FUN-1 stain is nowadays a useful tool in mycological studies and has been often used to differentiate viable and non-viable fungal cells in antifungal susceptibility tests in conjunction with flow cytometry (Millard et al. 1997; Wenisch et al. 1997; Pina-Vaz et al. 2001) and fluorescent microscopy (Lass-Flörl et al. 2001; Gangwar et al. 2006; Chee et al. 2009; Pinto et al. 2009; Hua et al. 2011). FUN-1 has also been used extensively for examining survival in microbial communities, particularly in

environments from where culturing is difficult or the analysis *in situ* is important (Stan-Lotter et al. 2003; Olsson-Francis and Cockell 2010; Wurzbacher et al. 2010).

Biofilm formation is a common mechanism utilized by microorganisms to survive in different environments. In fact, experimental studies have shown that fungal pathogenic biofilms are less susceptible to antifungal drugs. FUN-1 has also been applied to assess the metabolic activity of the fungal cells after contact with antifungal drugs (Lass-Flörl et al. 2001; Pinto et al. 2009) and has been proved to be a fast and reliable method. In water biofilms, it was applied to detect viable cells within *Penicillium brevicompactum* biofilms after disinfection with sodium hypochlorite (Siqueira and Lima, 2010). Viability of fungal biofilms from water distribution system was detected *in situ* using FUN-1, without the need of culture dependent methods (Siqueira and Lima 2011).

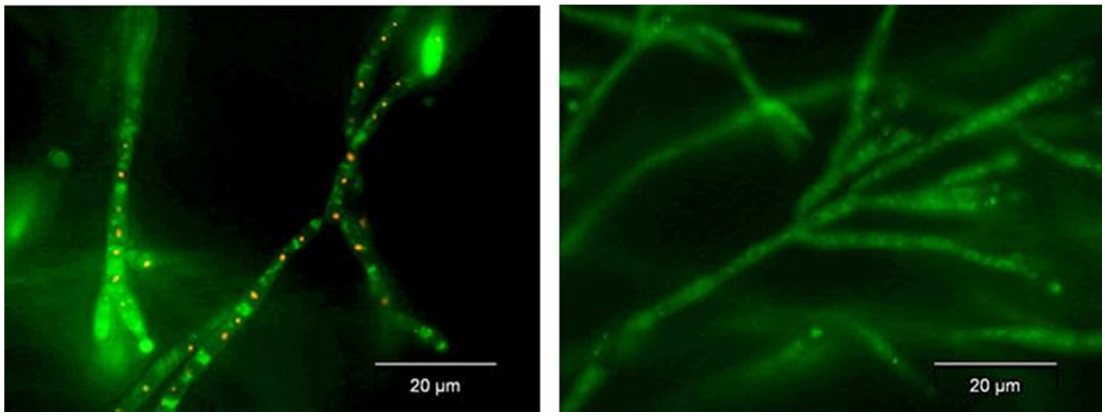

Figure 1.9 *Penicillium brevicompactum* after FUN-1 staining: metabolic active mycelia with red CIVS and no metabolic active mycelia with fluorescent green cytoplasm.

1.5.4 DAPI (4¹,6-diamidino-2-phenylindole,dihydrochloride)

The blue-fluorescent DAPI nucleic acid stain is a highly fluorescent cationic dye which specifically binds to adenine-thymine-rich DNA (A-T rich DNA) (Figure 1.10). DAPI is water soluble and solutions are stable for weeks if kept cold. The dye shows a very high stability in UV light and has an absorbance maximum at 340 nm and a fluorescence maximum at 488 nm.

DAPI has been commonly used for microscopy as a nuclear counterstain in multicolor fluorescent techniques and can also serve to fluorescently label cells for analysis in multicolour flow cytometry experiments. DAPI has been widely applied in water biofilms research (Kormas et al. 2010; Deines et al. 2010b).

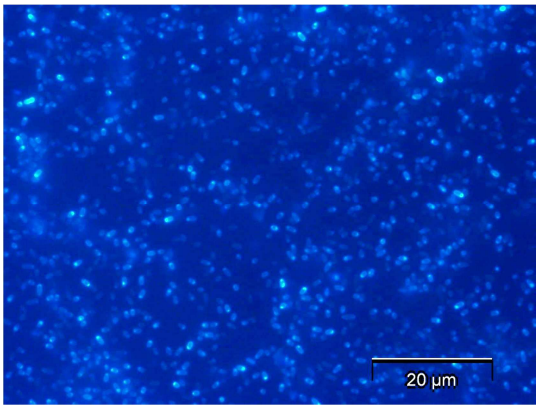

Figure 1.10 *Pseudomonas reactans* after DAPI staining.

1.5.5 Fluorescent *in situ* hybridization

The classical microbiology says that in order to study a microorganism it is necessary to isolate it from an original matrix or substratum. This isolation can be done only in an appropriate medium, from the plates showing separately grown colonies and only those microorganisms capable of growing, multiplying and forming visible colonies under that specific condition will be characterized. It is also known that any environmental change during the study can affect the whole composition of the microbial community, thus limiting the overview of the ecosystem considered. Additionally, the traditional methods used to assess the microbial communities are laborious, time-consuming and prone to statistical and methodological errors (Moter and Göbel 2000).

Although new culture media has been developed and new microorganisms are continuously isolated, only a small fraction of the existent microorganisms can be isolated, remaining a lack of knowledge which is most severe for complex multi-species microbial communities. In the last decades, the development of methods which avoid cultivation has become necessary. In this context, fluorescence *in situ* hybridization (FISH) is one of most used techniques.

FISH detects nucleic acid sequences by a fluorescently labeled probe that hybridizes specifically to its complementary target sequence within the intact cell (Figure 1.11). Giovannoni et al. (1988) was the first to use radioactively labeled rRNA-directed oligonucleotide probes for the microscopic detection of bacteria. DeLong et al. (1989) first used fluorescently labeled oligonucleotides for the detection of single microbial cells. When compared to the radioactive probes, fluorescent probes are safer, offer better resolution and do not need additional detection steps. Moreover, fluorescent probes can be labeled with

dyes of different emission wavelength thus enabling detection of several target sequences within a single hybridization step.

FISH with rRNA-targeted oligonucleotide probes has been developed for the *in situ* identification of individual microbial cells and is now a well-established technique (Amann et al. 2001). rRNAs are the main target molecules for FISH for reasons such as (1) they can be found in all living organisms; (2) they are relatively stable and occur in high copy numbers; (3) and they include both variable and highly conserved sequence domains (Amann et al. 1995). Hundreds rRNA-targeted oligonucleotide probes for FISH have been described, together with a large online database providing an encompassing overview of over 700 published probes and their characteristics (Loy et al. 2003).

The choice of probes for FISH must consider specificity, sensitivity and ease of sample penetration. A typical oligonucleotide probe is between 15 and 30 nucleotides long and covalently linked at the 5′-end to a single fluorescent dye molecule. Different fluorescent dyes and their characteristics are listed in Table 1.5. Direct fluorescent labeling is most commonly used and is also the fastest, cheapest and easiest way because it does not require any further detection steps after hybridization (Moter and Göbel 2000).

FISH procedure typically includes the following steps: (i) fixation of the specimen; (ii) preparation of the sample, possibly including specific pretreatment steps; (iii) hybridization with the respective probes for detecting the respective target sequences; (iv) washing steps to remove unbound probes; (v) mounting, visualization and documentation of results (Figure 1.11).

Table 1.5 Characteristic of common fluorochromes applied for FISH.

Fluorochrome	**Colour**	**Max. excitation λ (nm)**	**Max. emission λ (nm)**
Alexa488	Green	493	517
AMCA	Blue	399	446
CY3	Red	552	565
CY5	Red	649	670
CY7	Violet	743	767
DAPI	Blue	350	456
Fluorescein	Green	494	523
Rodamine	Red	555	580
TAMRA	Red	543	575
Texas red	Red	590	615
TRITC	Red-orange	550	580

Over the last decade, sensitivity and speed have made FISH a powerful tool for phylogenetic, ecologic, diagnostic, and environmental studies (Kempf et al. 2000; Thurnheer et al. 2004; O'Sullivan et al. 2007; Bishop 2010; Wurzbacher et al. 2010). FISH not only provides insight into microbial community

structure, but can also be used in combination with confocal laser scanning microscopy (CLSM) for accurate reconstruction of the spatial arrangement of microbial communities in their habitat (Amann et al. 1990).

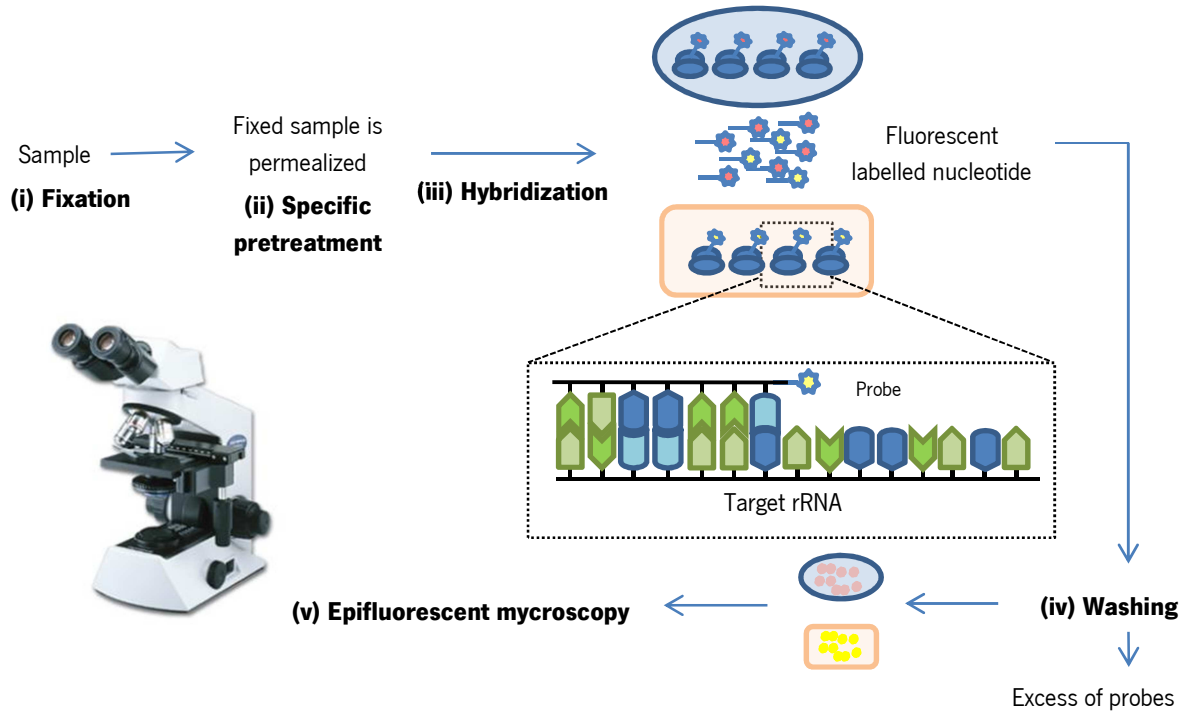

Figure 1.11 Flow diagram with main steps to perform FISH

In food industry, in order to monitor sanitation practices, FISH is becoming an important tool to identify specific microorganisms in mixed communities without the need for isolation in pure cultures. This technique allowed a rapid and accurate enumeration of *Pseudomonas* in dairy plants and the accurate validation of pasteurization treatment and the prediction of shelf life of pasteurized milk (Gunasekera et al. 2003). Another example in the food industry is the use of FISH with group- and species-specific oligonucleotide probes which provided an insight to the microbial composition of Gruyere cheese surface (Kolloffel et al. 1999). FISH is also useful in wine production (Blasco et al. 2003; Stender et al. 2001) and environmental water (Ootsubo et al. 2003)

Diverse microorganisms form complex microbial communities and commonly attach to solid surfaces as biofilms in natural environments and in engineered systems. Characteristics such as architecture, physiological status and genetic diversity of the cells contained within biofilms are of great interest for clinical, industrial and environmental microbiology (Thurnheer et al. 2004).

The application of molecular techniques has enabled *in situ* monitoring of microbial biofilm communities and has provided information about phylogenetic affiliations, spatial distribution, functions and activities (Aoi 2002). FISH have contributed to the clarification of the *in situ* microbial community structure in various types of biofilm communities such as glass windows surfaces (Müller et al. 2001), acid mine drainage (Baker et al. 2004) bioreactor systems (Aoi 2002; Schmidt et al. 2000), water pipe lines surfaces (Gonçalves et al. 2006; Bragança et al. 2007; Siqueira et al. 2011), and *in vitro* mixed biofilms (Thurnheer et al. 2004).

Although FISH has gained great acceptance in the scientific community and the undoubted advantages of its use, this technique has technical and conceptual problems which must be taken into consideration. Methodological factors, e.g. the type of fluorochrome, formamide and sodium chlorine concentrations, hybridization temperature and environmental factors, e.g. ecosystem type, dominant phylogenetic group and microbial physiological state, are linked to FISH performance (Bouvier and Del Giorgio 2003). Furthermore, autofluorescence of microorganisms themselves, especially fungi, can lead to false positive results (Moter and Göbel 2000).

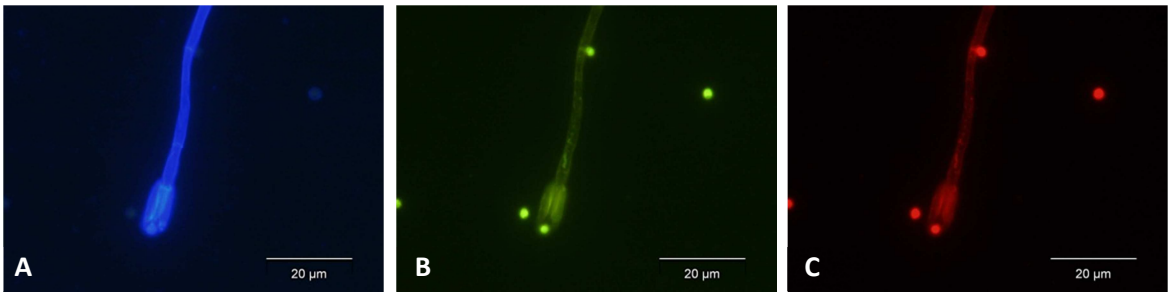

Figure 1.12 *Penicilliium brevicompactum* after CW staining (A) and FISH (B – FUN-1429 probe and C - EUK516 probe).

1.5.6 Samplers device

The development of microbial biofilms has been the subject of many studies; researchers aim to elucidate a large number of undesirable effects concomitant with the emergence of biofilms. Laboratorial devices are developed for investigations of different aspects of biofilms, allowing biofilm formation under controlled conditions of physical (flow velocity, shear stress, temperature, properties of the substratum etc.), chemical (composition and amount of nutrients, organic and inorganic particles, ions, etc.), and biological (composition of the microbial community – single or mixed – type of microorganism) parameters (Denkhaus et al. 2007).

There are many examples of samples devices (Table 1.6) which usually share a common idea of a hollow structure holding removable coupons where the biofilm can grow on, and a system fed with a continuous liquid flow as well (Figure 1.13). However, investigations in drinking water biofilms face a major problem to study biofilm within the distribution system: the lack of suitable experimental systems that both represent conditions within real pipe networks and enable the effects of abiotic factors to be explored in a controlled environment. Moreover, *in situ* approaches in replaced pipes are not suitable for systematic studies since their removable cannot follow a well-defined schedule. Apart from difficulties, an ideal device would fulfill demands such as mimic the real conditions of the water network and yet be straight forward to insert and handle, be convenient for transportation and storage, maintain the integrity of biofilms and allow *in situ* analyses of the biofilms. In this context, samplers (Figure 1.14) in which biofilms could be detected have been designed (Sammon et al. 2011; Siqueira et al. 2011).

Table 1.6 Comparison of samplings devices for studying drinking water biofilms (Adapted from Deines et al. 2010a).

Sampling device	**Benefits**	**Limitations**	**References**
Rotating disc reactor (RDR)	Coupons of different materials can be applied; Constant conditions possible; Microscopic imaging possible	Controlling shear difficult; Coupons engineered flat;	Murga et al. 2001 Möhle et al. 2007
CDC biofilm reactor (CBR)	Microscopic imaging possible; Coupons of different materials can be applied; Consistent biofilm samples and growth conditions; Surface treatments and antimicrobial agents can easily be tested	Controlling shear difficult; Coupons engineered flat	Goeres et al. 2005
Biofilm annular reactor (BAR)	Coupons or slides of different materials can be applied; Liquid/surface shear similar to pipe flow shear; Variable treatments can be easily applied	Biofilm cells have to be detached from slides for microscopic analysis when used without coupons; Coupons engineered flat	Batté et al. 2003a Batté et al. 2003b
Propella reactor	Reactors are made from water distribution pipe material; Water flow velocity can be controlled by the propeller	Coupons fabricated from stainless steel or cast iron; Coupons engineered flat; Non-destructive microscopic analysis of biofilms not possible	Parent et al. 1996 Appenzeller et al. 2001
Robbins device	Direct staining of biofilm bacteria for microscopy possible	Use of glass, cast iron, or stainless steel slides; Turbulent flow around the mounted slides affects biofilm development	Manz et al. 1993 Kalmbach et al. 1997
Modified Robbins device (MRD)	Coupons at the end of the pegs are flush and can be connected to pipelines to study in situ biofilm formation	Flow chambers are square and not round; Coupons engineered flat;	McCoy et al. 1981 Kharazmi et al. 1999 Millar et al. 2001

Table 1.6 Continuation

Prévost coupon	Suitable for studying biofilm formation *in situ* in pipelines	Coupons cannot be used for microscopic analysis; Coupon material iron; Surface flat/curved?	LeChevallier et al. 1998 Prévost et al. 1998
Bioprobe monitor	Coupons of different materials can be applied; Allows studying *in situ* biofilm development in a pipe system; Coupon surface flush with pipe wall	Coupons engineered flat/curved?	LeChevallier et al. 1998
Pipe sliding coupon holder	Easily installed within a pilot-scale system; Coupons can be fixed to slides for microscopic analysis	Turbulent flow around coupons affects biofilm development; Coupons are engineered flat; No continuous sampling possible	Chang et al. 2003
Biofilm sampler	Can be used *in situ* in large distribution systems; Holders can easily be taken out and coupons be processed in the lab; Biofilm cell loss minimized	Coupons engineered flat; Coupon material PVC	Juhna et al. 2007
PWG coupon	Can be used *in situ* in pilot-scale WDS and WDS; Coupon surface flush with curved pipe wall; In situ analysis of both biofilm structure and community possible using the same coupon; Coupons can be out of most pipe materials	Coupons can be made out of cast iron but cannot recreate the vast variety and complexity of "old" non-lined cast iron pipes, and cement linings which would have insufficient strength	Deines et al. 2010a
Biofilm pipe sampler	Can be used *in situ* in WDS; Easy insertion, handling and removal of each sampler; The sampler can be filled with water, preserving the integrity of biofilms	Coupons engineered flat;	Siqueira et al. 2011
Biofilm sampler	Coupons of different materials can be applied; Holders can easily be taken out and coupons be processed in the lab;	Coupons engineered flat;	Sammon et al. 2010

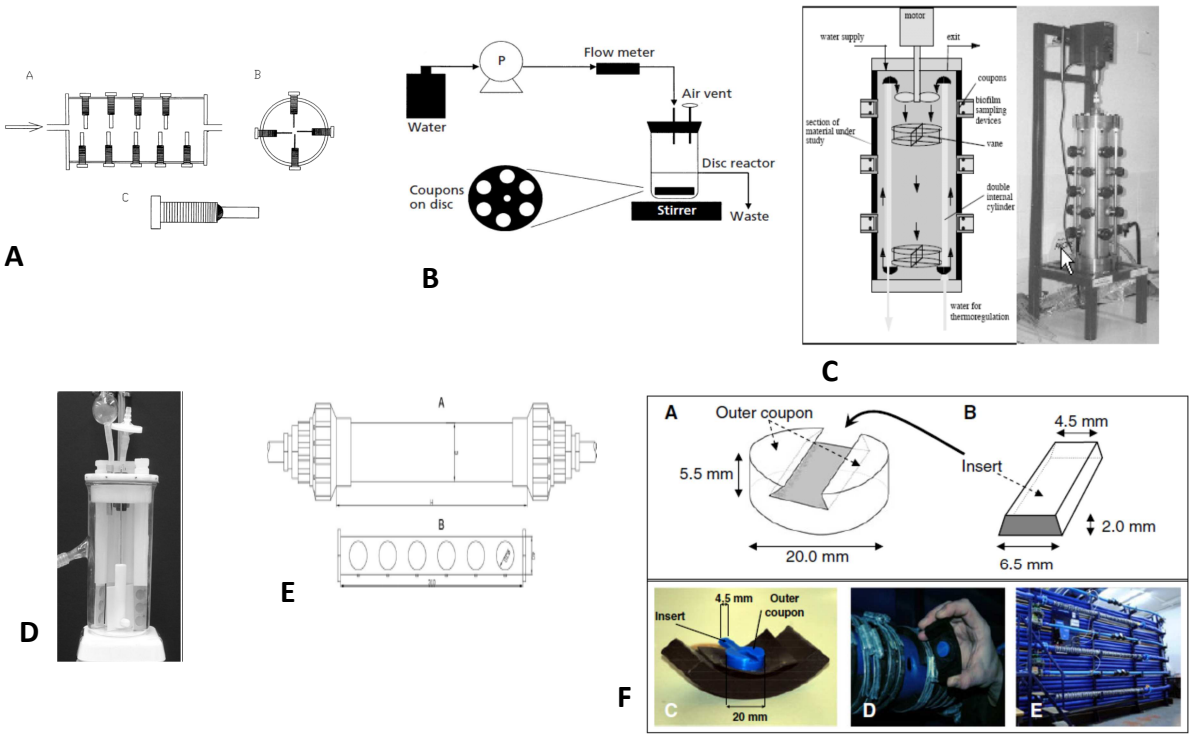

Figure 1.13 Examples of samples devices used for drinking water biofilms studies in laboratorial and/or pilot scale. A - Robins Device (Manz et al. 1997), B - Rotating disc reactor (Murga et al. 2001), C - Propella™ Bioreactor (Simões et al. 2008), D - CDC biofilm reactor (Goeres et al. 2005), E - Biofilm sampler (Juhna et al. 2007) and F - Pennine Water Group coupon (Deines et al. 2010).

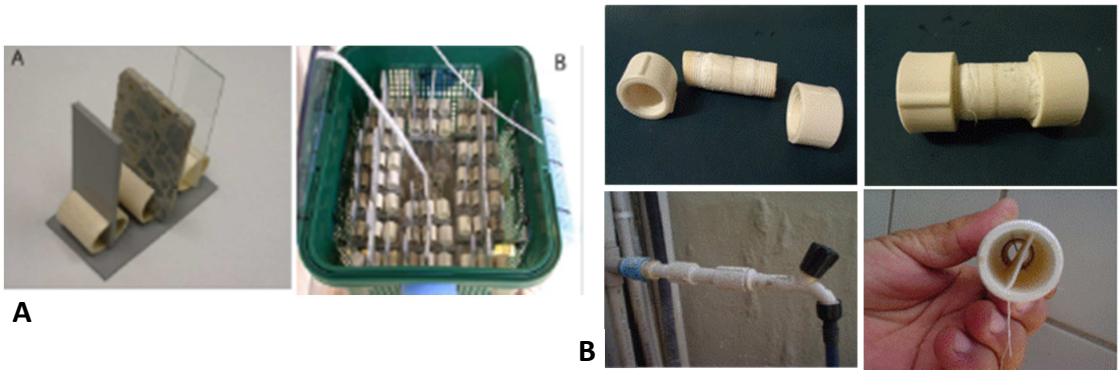

Figure 1.14 Examples of samples devices used for drinking water biofilms studies *in situ* in water distribution systems. A - Coupons and apparatus for biofilm formation (Sammon et al. 2011) and B - Sampler pipe device (Siqueira et al. 2011).

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Chapter 2

Fungal biomass quantification by image analyses

2.1 Introduction

The study of filamentous fungi in drinking water has received great attention in the last years (Göttlich et al. 2002; Gonçalves et al. 2006; Yamaguchi et al. 2007; Pereira et al. 2009; Oliveira 2010; Sammon et al. 2010). Researchers in this area have to deal with drawbacks associated to fungal quantification, mainly because of the variety of applied methodologies and their limitations, and the lack of international standardised methods as well (Hageskal et al. 2009). Nonetheless, quantification of fungi in water is mostly assessed by counting total fungal colonies on agar plates, referred to as colony-forming units (CFU) per volume of water sample investigated (Mara and Horan 2006). Filamentous fungi have a diverse morphology that changes according to environmental growth conditions (Figure 2.1), and produce branched hyphal structures which are constituted by many cells. Thus, after grow on agar plates, one colony may not represent one single cell, consequently a precise number of fungi is never provide. Additionally, not all fungi are able to grow under laboratorial conditions and they can be also outcompeted on culture plates if overgrowth of bacteria occurs, or be inhibited if the antibacterial compounds used are also effective against them (International Mycological Institute 1996).

Other methods such as analyses of ergosterol (Ruzicka et al. 2000; Joergensen 2000; Montgomery et al. 2000; Kelley & Paterson 2003) and dry weight (Reeslev and Kjøller 1995) have been also applied in fungal biomass quantification. The development of computational image processing as a toll for the study of fungal culture systems came about to quantify morphological variation in mycelial structures in industrial fermentations (Cox et al. 1998).

Approaches to biofilm analysis include microscopic, microbiological, molecular biological, (bio)-chemical and physical methods. Analytical questions with regard to biofilms can be divided into information about the components, the architecture and the processes occurring within biofilms (Denkhaus et al. 2007). Biovolume is defined as the product between the cell volume and the total number of cells; on the assumption that the microorganism is a sphere, ellipsoid or cylinder its volume can be estimated by measuring its radius, diameter or length. This value can be converted into biomass using a proper conversion factor (Kell et al. 1990; Madrid and Felice 2005). Several methods have been used for the estimation of microorganism biovolume, including electronic sizing, flow cytometry and different microscopic techniques such as scanning

electronic microscopy, confocal laser scanning microscopy, normal light microscopy and epifluorescence microscopic (Bölter et al. 2002).

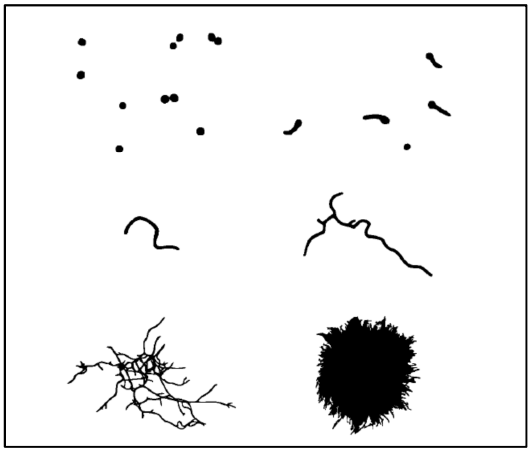

Figure 2.1 Different morphological forms of filamentous microbes: growth commences from approximately spherical spores (typically < 10 µm in diameter), which, over time, produce simple branched hyphal structures (hyphal diameter is generally < 10 µm). These can, in turn, develop into complex, composite architectures termed mycelia, while the agglomeration of biomass in submerged culture can result in the formation of dense, approximately spherical configurations termed "pellets", which may be up to several millimetres in diameter (Barry 2010).

The use of microscopy techniques associated with image analyses has become a valuable tool for research in many areas (Daniel et al. 1995; Tucker et al. 1992; Solé et al. 2008; Bölter et al. 2002). The fluorescent stains and epifluorescence microscopy had already found great acceptance in Ecology, Medical and Bioengineer research. Image analyses are widely used to quantify fungal hyphae growing in batch cultures (Thomas and Packer 1990) and on soil (Bolton et al. 1991; Morgan et al. 1991). When compared with techniques such as plate counts and colony forming units the microscopic methods are less prone to subjectivity and need less labour. The short time of analysis, a simple measurement procedure and the low operation cost makes fluorescent microscopy perhaps the most promising technique is this field.

The relationship between structure and activity of biofilms and the factors which shape their physical form are crucial information for use or control of biofilms in various environments. An increasing understanding of the morphology and kinetic formation of laboratorial biofilms allow us to examine the patterns of development and interaction for further understanding of ecological traits. Within this context, biomass of microorganism is a basic requirement for understanding how different factors interfere in the physical characteristics, development and maturation of biofilms (Stoodley et al. 1999). The aim of this work was to describe an optical

method for determining the fungal biomass in laboratorial filamentous fungal water biofilm developed under laboratorial conditions. Calcofluor White M2R was used as staining in conjunction with epifluorescent microscopy.

2.2 Material and Methods

2.2.1. Microorganism

Penicillium brevicompactum (MUM 05.17) supplied by Micoteca da Universidade do Minho (MUM, Braga, Portugal), was chosen as an example, as it is the most commonly ff isolated from tap water (Gonçalves et al. 2006). *P. brevicompactum* maintained on Malt Extract Agar (MEA: malt extract 20 g, peptone 5 g, agar 20 g, distilled water 1 l) plates was used throughout the study. Spores were washed from 5-day cultures with distilled water, counted in a Neubauer chamber and diluted to give 10^5 spores/ml. This suspension was used as inoculum.

2.2.2. Biofilm formation

The biofilm formation was made in 6-well dishes with PVC (polyvinyl chloride), PP (polypropylene) and PE (polyethylene) coupons (1cm x 1cm) previously sterilized, and water added with 0.1% of glucose under room temperature (25±2 °C) and 120 rpm. The biofilm growth was confirmed when small points of mycelium were observed on the surface of the coupon and were analysed with 24, 48 and 72 h of growth.

2.2.3. Staining and Image analysis

Calcofluor White M2R (25 μM) was used as staining for the visualization of fungal cells walls. Following incubation in the dark for 15 min in room temperature, the coupons were microscopically observed. An Olympus BX51 epifluorescence microscope using UV light equipped with 40x/0.30 and 10x/0.65 objectives was used. The images were acquired with a colour camera Zeiss AxioCam HRc using the software CellB®. The excitation wavelength for Calcofluor was 346 nm and the signal acquired was blue. In each image all the hyphal length stained with Calcofluor was semi-automatically calculate by click and drag operation of the computer mouse in an enlarged image on the screen. The software CellB® generated a database with all the calculated values which were processed by WordExecel (Microsoft®) where the final biovolume and biomass were calculated.

2.2.4. Biovolume

The fungal biomass (in gram), derived from the biovolume, was calculated as "Biomass = density x π r^2 L", where r was the radius of fungal hyphae (in centimetre), d = 1.09 g cm^{-3} was considered to be the density of fungal hyphae (Schnürer 1993) and L was the length of fungal hyphae (in centimetre). The diameters of 20 intersection points of fungal hyphae were chosen for measurements and calculated its average.

2.3 Results

Calcofluor M2R stains the cell walls of fungal hyphae and allowed the detection of living plus empty fungal filaments attached to coupons. The length of fungal hyphae in samples of water biofilms was easily measured, and the method may be applied to evaluate filamentous fungal biofilms development.

P. brevicompactum MUM 05.17 was able to form biofilms on PVC, PP and PE coupons under the conditions presented here. PVC coupons showed autofluorescence which may interfere in image analyses, thus this material is no recommended for studies that use fluorescent microscopy. The steps followed to assess fungal biofilm biomass are described in Figure 2.2.

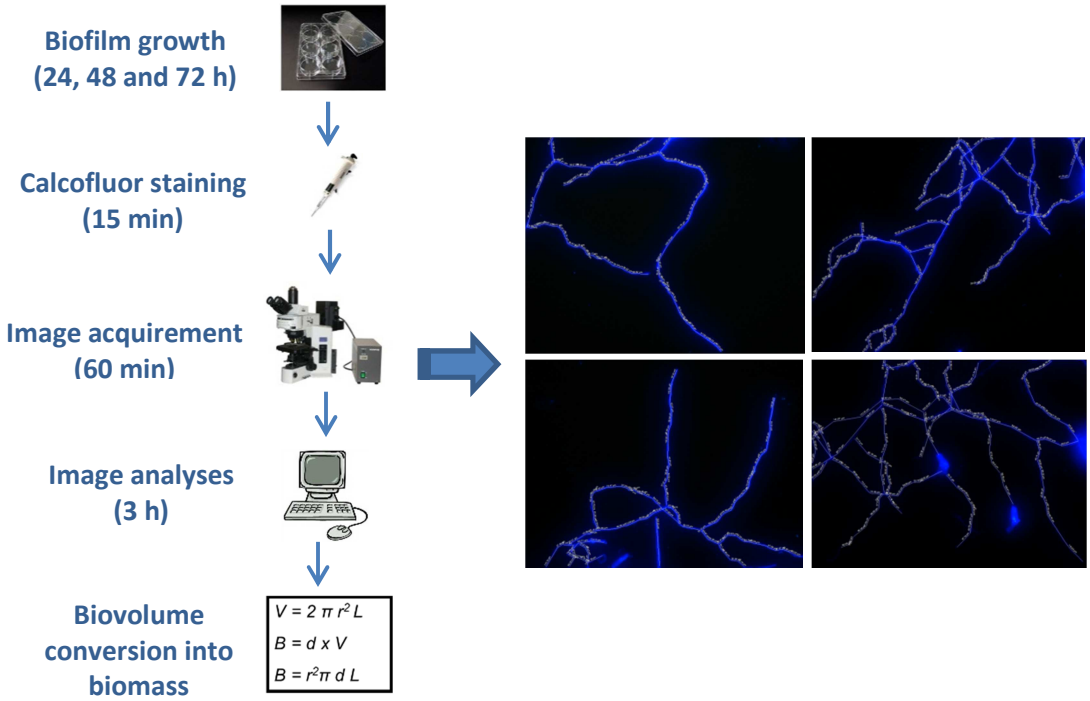

Figure 2.2 Scheme showing the steps to assess biomass of filamentous fungal biofilms.

The analyses were made after 24, 48 and 72 h of growth on PVC, PP and PE and different structural features and biomass values were found among the different aged biofilms on the different surfaces. This method allowed biomass measurement up to 72 h of growth, since the limit of the technique is generated by a superimposition of mycelia, when the biofilm can be characterized as a "clump" (Figure 2.4). After biofilms growth, c.a. four hours were needed to do all experiment, since the staining until the conversion of biovolume into biomass values. From each coupon an average of 20 images were generated.

Three coupons of each material were analysed and biomass was calculated every 24 h (Figure 2.5). Initial adherence and conidial germination were observed in the first 24 h on all coupons but a different rate of growth was observed after 48 h of development (Figure 2.3). Biofilms on PVC surface were greater at 48 h and 72 h reaching 53.0 ng and 107.5 ng respectively, i.e. two times more biomass when compared with biofilms on PP surface which had 48.4 ng at 72 h.

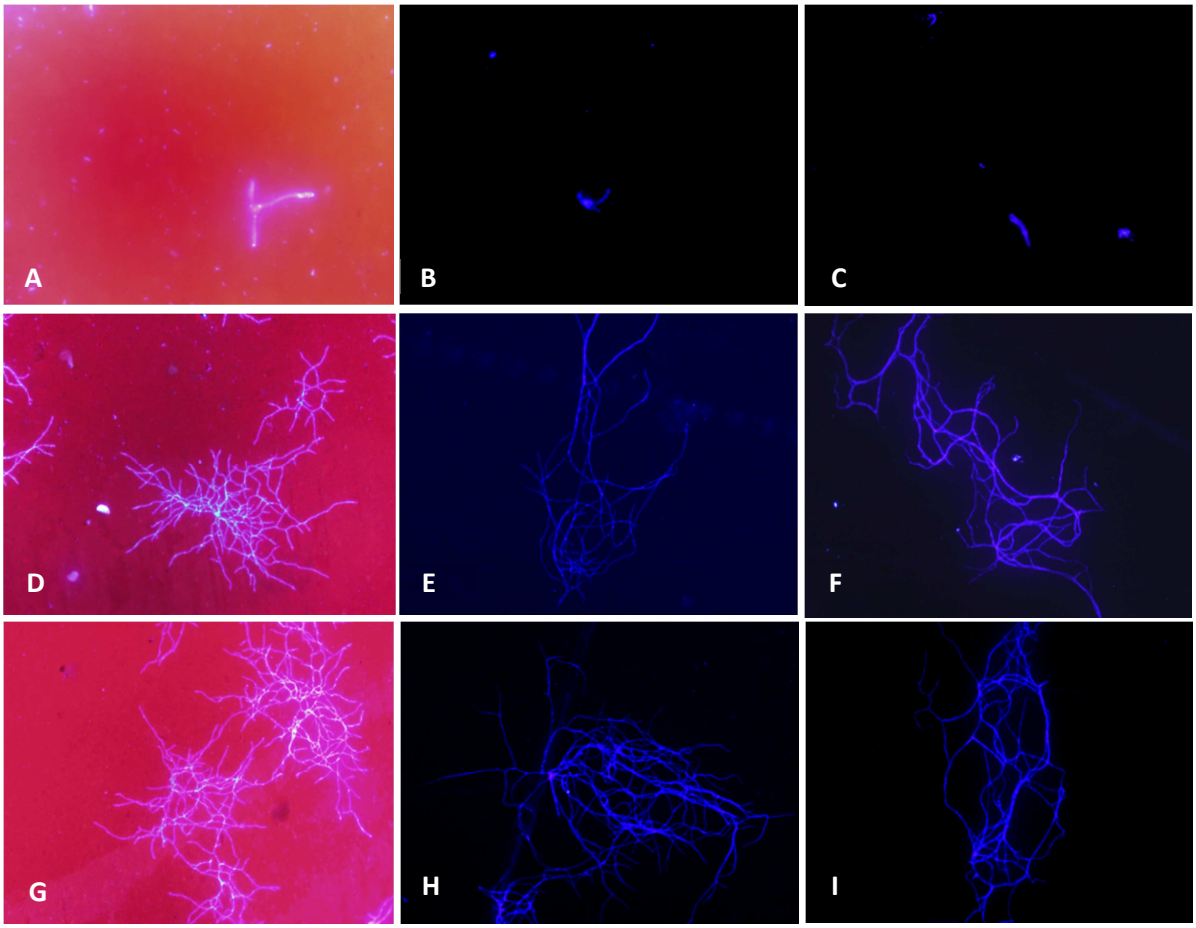

Figure 2.3 Biofilm of *P. brevicompactum* grown on PVC/PP/PE 24 h (A, B and C); PVC/PP/PE 48 h (D, E and F); PVC/PP/PE 72 h (G, H and I).

A difference in biomass distribution was also evident among biofilms on the three materials. When compared with PP and PE, biofilms on PVC surfaces showed a higher mycelial density with a more organized structure (Figure 2.3 G).

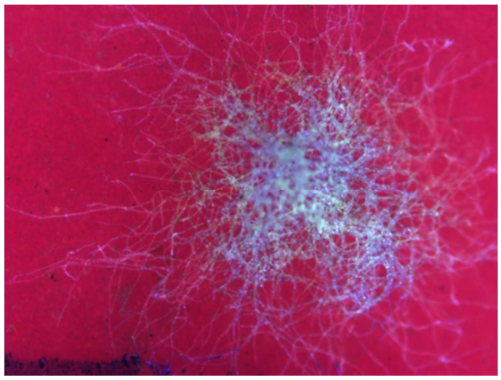

Figure 2.4 Superimposition of mycelia in *P. brevicompactum* biofilm on PVC after 96 h of growth.

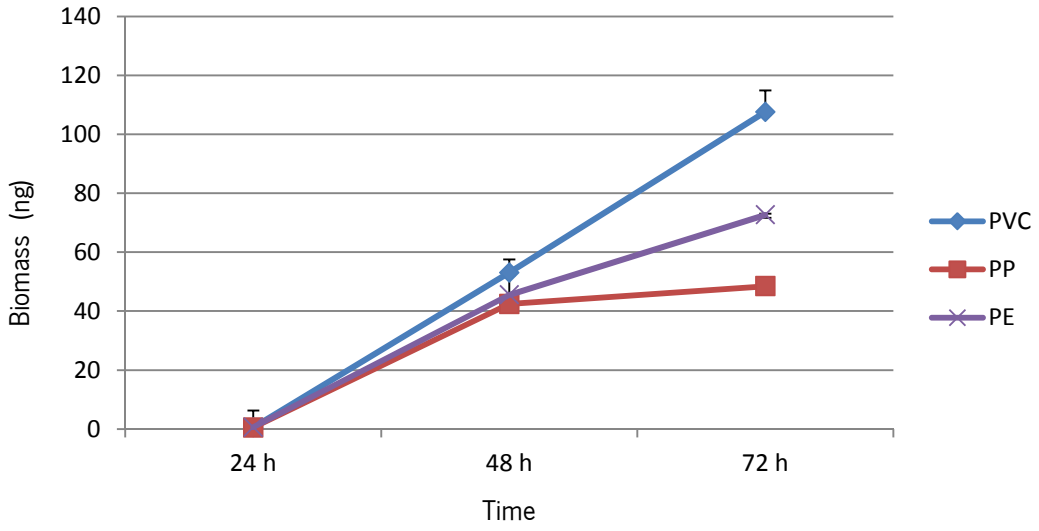

Figure 2.5 Biofilm biomass values of *P. brevicompactum* biofilms grown on different surfaces.

2.4 Discussion

Among others methods proposed to determine fungal biomass, hyphal length measurements, conversion to hyphal biovolumes, and further conversion to biomass constitute a traditional method for fungal biomass measurement in aquatic environments, plant litter systems (Gessner and Schwoerbel 1991), batch cultures (Thomas and Packer 1990) and soil (Morgan et al. 1991). The development and improvement of this method came about to reduce sample

analyses time and observer bias. Adams and Thomas (1988) firstly described a semi-automatic image processing system to derive measurements of fungal filaments and reported that this method has greater reproducibility between operators and especially between laboratories. In the last two decade, with the emergence of computer, digital cameras and digital image programs, the problems related to subjective and prone analyses to errors were overcome (Barry 2010).

Görs et al. (2007) applied ergosterol as biomarker to assess biomass of fungal biofilms on in- and outdoor artificial surfaces. Ergosterol is a main component of fungal membranes but its content depends on various factors, such as culture age, growth rate, carbon and nutrient availability, temperature and oxygen (Charcosset and Chauvet 2001). Moreover, ergosterol is also found in cell membrane of some microalgae and protozoa. Thus, fungal biomass may be underestimated by the ergosterol method. Zhao et al. (2005) emphasized that studies relying on a single biomarker such as ergosterol is unwise if the objective is to monitor microbial communities. In water biofilms, the use of CFU per volume of water was applied to estimate fungal biomass (Sammon et al. 2011). Generally, the sampling method needs steps such as scraping and swabbing, and then there are possible effects on the integrity of a fungal cell. Additionally, fungi have a variety of structural morphology, including mycelial clumps, single hyphal elements, hyphal fragments, and spore aggregations, and a precise CFU number per volume of sample is difficult to be estimated.

The use of fluorescent microscopy and specific fluorescent dyes in association with image analyses gives additional information besides biomass. Our results showed that using image analyses it was possible to estimated fungal biomass and correlate it with biofilm morphology on different material. Surface material is commonly reported as one of factor that influence bacterial biofilm (Zhou et al. 2009; Yu et al. 2010) and fungal biofilm (Sammon et al. 2011; Siqueira et al. 2011) formation. In this study, fungal biofilms showed different rate of development and thus different biomass when PVC, PP and PE coupons were used as surface for biofilm grow. PVC coupons showed to be the best material for fungal biofilm development, though it has high autoflurescence and may not be suitable for fluorescent microscopic studies.

CW stains all fungal cell walls, i.e. it does not discriminate live from dead cells. On the other hand, CW can be applied together with others fluorescent dyes which give information about the metabolic state of cells in a same sample (Ingham and Klein 1984). Moreover, the use of fluorescent dyes allows *in situ* analyses, and fungal biomass can be measured and correlated with others parameters in natural habitats. CW has been successfully used for fungal

quantification in diverse habitats such as fresh water lakes (Rasconi et al. 2009), seawater and marine sediments (Gutiérrez et al. 2010), within decomposing leaves (Daniel et al. 1995) and soils (Ananyeva et al. 2008).

Variation in convertor factor values is reported as an interferential factor in filamentous fungal biomass measurement using hyphal biovolume. Hyphal diameter and fungal density can interfere in the final biovolume calculation. Fungal grow within solid culture media has commonly used as reference method to calculate hyphal diameter (Gessner and Newell 2002), and consequently leads to errors if these same values are applied for samples on natural substrates (Müller et al. 2000; Pollack et al. 2008). Some studies use pre-established values available in published data, but since hyphae thickness is affected by several conditions (Daniel et al. 1995), the calculation of an average value for hyphal diameter under the ongoing experimental conditions was taken as the best solution for this problem during this study.

Although it is accurate and reliable method, biomass quantification by image analyses is based on laborious and tedious procedure, especially if it is necessary to analyze a large number of images in which high densities of cells are present. On the other hand, is a nondestructive method in which, if specific fluorescent dyes are used (i.e. fluorescent labeled probes and Fluorescent *in situ* hybridization), total and individual fungal biomass can be assessed together with microbial diversity and structural and physiological features. In conclusion, image analysis may be an appropriate method to measure the fungal biofilm biomass and the use of specific fluorescent dyes is useful especially within heterogeneous environments.

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Chapter 3

Efficacy of free chlorine against water biofilms and spores of *Penicillium brevicompactum*

3.1 Introduction

Biofilm is a complex community of microbes (bacteria, protozoa, filamentous fungi, yeasts and other microorganisms), organic and inorganic material accumulated amidst a microbially produced organic polymeric matrix attached to a surface (O'Toole et al. 2000).

The structural and phenotypic changes associated with the development of a mature biofilm aid microbial cells in numerous aspects of their life cycles. One frequently measurable change in the phenotype of cells in a biofilm, when compared to their planktonic counterparts, is significantly increased tolerance to chemical, biological or physical stresses (Chandra et al. 2001; Harrison et al. 2005). Other benefits may include tenacious attachment to surfaces, colonization of host tissues, expression or enhancement of virulence traits, efficient capture of nutrients and enhanced cell-to-cell communication (Harding et al. 2009).

Both bacterial and yeast biofilm have been widely studied but less attention have been given for filamentous fungi (ff) biofilms (O'Toole et al. 1999; Huq et al. 2008). Fungi are especially adapted for growth on surfaces, as evidenced by their absorptive nutrition mode, their secretion of extracellular enzymes to digest complex molecules, and apical hyphal growth (Elvers et al. 2001; Villena et al. 2009). Despite the fact that the term 'biofilm' is rarely applied to ff, there are several descriptions indicative of biofilm formation in different medical, environmental and industrial settings (Dogget 2000; Beauvais et al. 2007; Paterson et al. 2006; Mowat et al. 2008; 2009).

The occurrence of biofilms can be a source of taste, odour and visual appearance problems resulting in poor drink water quality (Hageskal et al. 2009). Moreover, fungi in potable water distribution systems may have direct effects on human health (allergenic or toxigenic species), contribute to the occurrence of nosocomial infections in immune-compromised individuals and contaminate foodstuffs during processing or preparation (Elvers et al. 2001; Anaissie et al. 2008). Microbially-induced corrosion, loss of indicator organism utility and the persistence contamination in water can be problems related with the development of biofilms (US EPA 1984; Geldreich 1996). Water systems worldwide have been shown to be colonized with pathogenic filamentous fungi (Hageskal et al. 2009). Although in recent years studies of fungi in

drinking water has received attention (Göttlich et al. 2002; Hageskal et al. 2006; Gonçalves et al. 2006; Ribeiro et al. 2006; Yamaguchi et al. 2007; Pereira et al. 2009), detailed researches of ff biofilms on water are rare.

Water for human consumption is often disinfected before goes to the distribution system to ensure that potential microbial pathogens are inactivated. Chlorine, chloramines or chlorine dioxide are most often used because they are very effective disinfectants (US EPA 1984). In contrast, biofilms can protect microbes from disinfectants and allow microbes injured by environmental stress and disinfectants to recover and grow. Moreover, biofilms react with chemical disinfectants reducing their availability for inactivating pathogens in the water (Berger et al. 2000). As a result, biofilm can be considered one of the reasons for persistent microbial contamination of the water. Conventional water treatment (coagulation/flocculation, filtration, and chlorination) can be effective in removing microfungal contaminants from water but a possible re-contamination can occur if supplementary chlorination of all water service reservoirs is not routinely carried out (Sammon et al. 2010).

The objective of this work was analyse the susceptibility of *Penicillium brevicompactum* biofilms and its single spores against free chlorine, the most common disinfectant used routinely in water treatment.

3.2 Material and Methods

3.2.1 Spores

Penicillium brevicompactum MUM 05.17 supplied by Micoteca da Universidade do Minho (MUM, Braga, Portugal) was chosen as a model as it is the most commonly filamentous fungi isolated from Portuguese tap water (Gonçalves et al. 2006). Spores were collected from a 7-day pure culture in malt extract agar (MEA: malt extract 20 g, peptone 5 g, agar 20 g, distilled water 1 l) at 25 °C by adding 2 ml of distilled water to plate. The spore suspension was re-suspended and vortexed for 1 min before quantification using a Neubauer counter chamber. The suspensions were standardized by dilution with water to a final concentration of 10^5 spores/ml.

3.2.2 Biofilms

A spore suspension of 10^5 spores/ml was also used to perform biofilms. The biofilms were grown in 6-well plates at room temperature and 120 rpm. The spore suspension was added to each well which contained 5 ml of glucose solution (0.1 %). Then the PVC (polyvinyl chloride), PP

(polypropylene) and PE (polyethylene) coupons (1 cm x 1 cm), previously autoclaved at 121 °C during 15 min, were placed into the wells with the reverse face touching the well bottom and staying all under the water. After a time period 48, 72 and 96 h of incubation the biofilm on the coupons were used for free chlorine susceptibility test.

3.2.3 Concentrations of Free Chlorine

Sodium hypochlorite solutions were prepared with bleach and distilled and deionised water and adjusted to pH 7.0 ± 0.1 using HCl. For free chlorine determination, in the absence of iodide ion, free chlorine reacted instantly with DPD (N,N-diethyl-p-phenylenediamine) to produce a red colour, which was measured immediately with a colorimeter (Ion specific meters, Hanna Instruments, HI 93701, light emitting diode @ 555nm, range 0.00 to 2.5 mg/l, resolution 0.01 mg/l). The survival of *P. brevicompactum* spores and biofilms were determined after exposure to 0.015, 0.3, 0.6, 0.125, 0.25, 0.5 and 1 (v/v) of sodium hypochlorite, i.e., 0.02, 0.05, 0.25, 1.83, 1.98, 2.13 and 2.38 mg/l of free chlorine, respectively, during 15 minutes. A 10% sodium hypochlorite solution was used as negative control. This dilution reflects the concentration currently used for surface sterilization.

3.2.4 Treatment with Free Chlorine

Spores. Pellets of 10^5 spores were re-suspended in 1 ml of free chlorine solution (Table 1), mixed by inversion to ensure full contact and then incubated at room temperature (25±1 °C) for the required exposure time (15 min). The samples were mixed by inversion at least twice during incubation period, centrifuged and at the end the supernatants were discarded. Pellets were immediately washed with abundant distilled water and then centrifuged. This process was repeated three times. Finally, the resulting pellets were re-suspended in 1 ml distilled water. Positive controls were treated in similar way, with the exception that distilled water replaced the free chlorine solutions. In this study, free chlorine Minimum Inhibitory Concentration (MIC) was the lowest concentration at which at least 90 % of spores were inactivated after contact with the disinfectant solution (value expressed as a percentage of the colonies formed by positive control). For each concentration test and control, three replicates were made.

Biofilms. The PVC, PE and PP coupons were washed with distilled water to remove the non-adherent cells. Each coupon was transferred to another 6-well plate with different free chlorine

solutions (0.02 mg/l, 1.57 mg/l and 2.38 mg/l) and submerged. After 15 min the coupons were taken off and washed three times with distilled water to remove the disinfectant. All biofilms submitted to the different free chlorine concentrations and grown on different surfaces were done in triplicate. Both spores and biofilms were exposed to free chlorine solutions using the same conditions.

3.2.5 Spores Viability test

Culture test. Spores viability was determined by their germination capability. The samples 1 ml volume of the spore suspension was plated using a pour plating method. To facilitate the determination of the number of viable spores, three 10-fold dilutions were prepared from each sample. A 1 ml volume of the spore suspension from the last dilution (i.e., maximum of 10^2 spores) was added to a sterile disposable Petri dish; 10 ml PDA (46±2 °C) were then added and the mixture was gently swirled to evenly distribute the spores. The germination capability was confirmed after 72 h of incubation at 25 °C by the visible grown of colonies.

FUN-1 staining. *P. brevicompactum* spores without treatment were used to establish the method. The resulted images were used as standard for further comparison with treated spores. From each free chlorine concentration tested, one spore suspension was chosen for FUN-1 staining (Molecular Probes, The Netherlands). FUN-1 stains the dead cells with a diffuse yellow-green fluorescence and the metabolically active cells with red Cylindrical Intra-Vacuolar Structures (CIVS). For FUN-1 staining, 15 µL of the spore suspension plus 15 µL of FUN-1 solution were added on a glass slide, homogenised, following incubation in the dark at 30 °C during 30 min and observed under an Olympus BX51 epifluorescent microscope using UV light equipped with 40x/0.30 and 10x/0.65 objectives and a filter set (EX 450-490 nm, EM 520 nm). The images were acquired with a colour camera Zeiss AxioCam HRc using the software CellB®. Storage and handling of reagents were performed as recommended by the supplier.

3.2.5 Biofilm Viability test

Culture test. The biofilms recover was determined by plating the coupons on potato dextrose agar (PDA) plates. Before plating, the coupons were washed with distilled water and put on the culture media with the biofilm touching the culture medium surface. The development of colonies was observed until 72 h of plating at 25 °C.

FUN-1 staining. From each free chlorine concentration tested, one coupon was chosen for FUN-1 staining. Negative and positive controls were also submitted for *in situ* viability test with FUN-1 staining (methodology already described in section 3.2.5).

3.3 Results

The results after treatment with different free chlorine concentrations against spores and biofilms are shown in Table 3.2 and 3.3, respectively.

Table 3.1 Sodium hypochlorite concentrations and the corresponding nominal concentrations of free chlorine. All disinfectant data refer to soluble free chlorine concentrations.

Sodium hypochlorite concentration (% v/v)	**Free chlorine nominal concentration (mg/l)**
1	2.38
0.5	2.13
0.25	1.98
0.125	1.83
0.6	0.25
0.3	0.05
0.015	0.02

Table 3.2 Survival of *P. brevicompactum* biofilms determined by germination capability in solid culture medium after contact with different concentrations of free chlorine.

	Biofilms								
	PVC			**PE**			**PP**		
Free chlorine (mg/l)	Biofilm age								
	48h	72h	96h	48h	72h	96h	48h	72h	96h
0.07	+	+	+	+	+	+	+	+	+
1.57	+	-	+	-	+	+	+	+	-
2.38	+	-	+	-	-	+	-	-	-
Positive control	+	+	+	+	+	+	+	+	+
Negative control	-	-	-	-	-	-	-	-	-

Visible grow (+); non-visible grow (-)

3.3.1 Germination capability

Spores. Low concentrations of free chlorine (0.02 and 0.05 mg/l) had no effect on the inactivation of *P. brevicompactum* spores. By the other hand, high concentrations (1.98, 2.13 and 2.38 mg/l) reduced to zero the number of viable spores. The number of fungal colonies

observed on dilution plates derived from each treatment were enumerated 3 days post inoculation. No new colonies were observed 5 days post inoculation, thereby confirming that slow developing spores were not overlooked in the earlier counts. A free chlorine 1.83 mg/l concentration can be considered the Minimum Inhibitory Concentration. Negative control inactivated all spores. Positive control gave an average of 2.5 x 10^2 spores recovered per millilitre.

Table 3.3 Survival of *P. brevicompactum* spores determined by germination capability in solid culture medium after contact with different concentrations of free chlorine. *each value is the mean of three independent assays (each assay with three replicates). Values expressed as a percentage of the colonies formed by positive control.

Free chlorine (mg/l)	**Spores recovered* (%)**
2.38	0
2.13	0
1.98	0
1.83	8.2
0.25	23.7
0.05	98.4
0.02	99.5

Biofilms. The lowest free chlorine concentration did not show any significant activity after 15 min exposures. For 0.07 mg/l of free chlorine, biofilms with 48, 72 and 96 h aged showed similar results with development of visible colonies after 48h of incubation. For the intermediary free chlorine solution (i.e., 1.57 mg/l), most of biofilms were resistant and showed visible colonies until 72 h of inoculation. For the highest free chlorine concentration (i.e., 2.38 mg/l), age and material coupons interfered in the biofilm resistance; 96 h aged biofilms on PVC and PE were resistant, whereas none 48 h and 72 h aged nor biofilms on PP presented colonies grown until 72 h of incubation.

The effects of free chlorine solutions in the deactivation of biofilms seem to be more related with the delay of the development of visible grown than with the inactivation itself. For example, for the 48 h aged biofilms on PVC, all free chlorine solutions tested did not inactivated the cells but the recovery time was different for each solution.

3.3.2 FUN-1 staining

Spores. After 30 min of incubation with FUN-1 viable spores from a 7-day pure culture were detected by conversion of FUN-1 dye into bright orange-red CIVS (Figure 3.1 A). The positive results allowed the comparison with treated spores. No evidence of autofluorescence was recovered in unstained spores. Effectiveness of free chlorine solutions against biofilms could be analysed by the FUN-1 staining under the conditions presented in this work.

For highest free chlorine concentration, the spores were inactivated and didn't form CVIS. The visualization of only diffuse green to green-yellow cytoplasmic staining indicates a membrane-compromised dead cell (Figure 3.2 B). After exposure to the lowest free chlorine concentration viable spores shown, in addition to diffuse green to green-yellow cytoplasmic staining, CIVS which had distinct orange-red fluorescence. For the intermediary free chlorine concentration both viable and non-viable spores were detected (Figure 3.2 A). The results could be correlated qualitatively with conventional plating and was less time consuming.

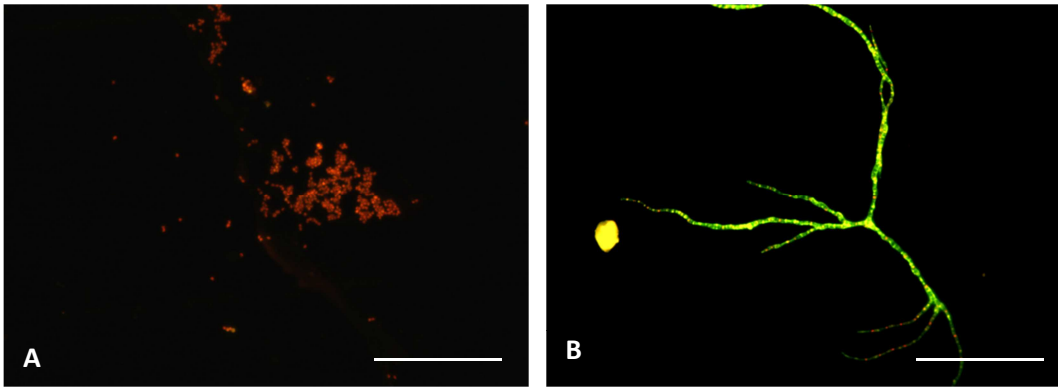

Figure 3.1 FUN-1 staining: the visualization of only diffuse green to green-yellow cytoplasmic staining indicates a membrane-compromised dead cell; in metabolically active cells CIVS, wich have distintic orange-red fluorescence, are produced. Spores and biofilm of *P. brevicompactum* after FUN-1 staining. Positive controls of spores (A) and biofilm (B). Scale bar = 50 µm.

Biofilms. Viable biofilms were detected after 30 min of incubation with FUN-1 by conversion of FUN-1 dye into bright orange-red CIVS (Figure 3.1 B). The results allowed the comparison with treated biofilms. We investigated whether FUN-1 can be used to detect viability after exposure to free chlorine solution. In general, the intensity of fluorescent signals was lower in the treated biofilms when compared with non-treated biofilms. Nevertheless, the results obtained with treated biofilms after FUN-1 staining were conclusive under the conditions presented in this work.

Effectiveness of free chlorine solutions against biofilms could be analysed by the FUN-1 staining under the conditions presented in this work (Figure 3.2 C-D). Although the results did not allow quantitative analyses, the qualitative results were conclusive. *P. brevicompactum* biofilms took up and converted the FUN-1 dye into bright orange-red CIVS. Free chlorine susceptibility test compared with conventional plating is more rapidly determined by viability analysis with FUN-1. The results suggest that analysis of biofilm viability with a fluorescent probe provides rapid and reproducible detection of cell inactivation. FUN-1 staining needs 30 min of incubation, whereas cultivation needs at least 48h of incubation. Instead of the better development of biofilms on PVC coupons, its autofluorescence affected fluorescent signals and the image analyses making this material not the most recommended. By the other hand, PP and PE coupons did not show this problem and are recommended for laboratorial biofilm development and fluorescence analyses.

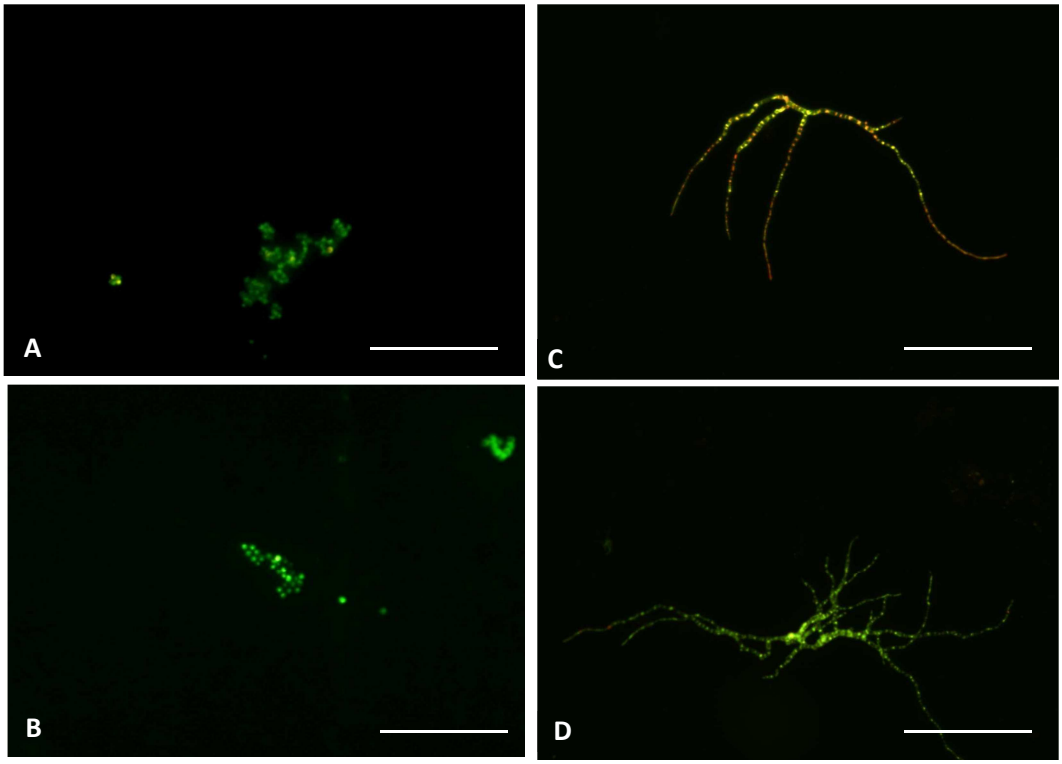

Figure 3.2 Spores and biofilm of *P. brevicompactum* after FUN-1 staining. Spores after contact with 1.83 mg/l (A) and 1.98 mg/l of free chlorine (B). Biofilms after contact with 1.83 mg/l (C) and 2.38 mg/l (D) of free chlorine.

3.4 Discussion

Many studies about filamentous fungi from water have been published in the last years but there is still a lack of information about filamentous fungi biofilms from water distribution system. Moreover, there are also few studies examining the effects of free chlorine on filamentous fungi biofilms from water, thence the results presented in this work are discussed mainly with studies published about bacterial biofilms. In this study, the relationship between the attachment of filamentous fungi to surfaces and disinfection with hypochlorite solution was analysed with *P. brevicompactum* biofilms. Biofilms on different surfaces and different aged were tested with crescent free chlorine solutions. Free spores were also tested. Pour plating method and FUN-1 staining were used for viability tests.

The results indicate that attachment of spores to surfaces and the development of a biofilm provide features for fungi to survive disinfection. Even in a 10^5 spores/ml suspension, spores were vulnerable to the lowest free chlorine concentration, whereas biofilms were resistant to the highest concentration. Attachment to a surface alters the way a disinfectant interacts with a microorganism and its efficacy may also be unsatisfactory against pathogens within flocs or particles, which protect them from disinfectant action (LeChevallier et al. 1988; WHO 2008). The free chlorine acts within the cell membrane inactivating microorganisms indicating that resistance against chlorine is linked with structural features provided by biofilms. The negatively charged exopolysaccharides are also efficient in protecting cells from positively charged biocides by restricting their permeation through binding. Additionally, a small portion of cells (persisters) could survive the common causes of cell death by the induction of quiescence in certain biofilm pockets. Such quiescent cells are noted for their resistance to biocides (Schwartz et al. 2003). In fact, microorganisms growing attached to surfaces often display a distinct phenotype that provides resistance to biocides (Srinivasan et al. 1995; Cochran et al. 2000; Morató et al. 2003).

There are previous studies showing that the characteristics of the pipe material can influence the formation of bacterial biofilms and the survival of pathogens in drinking water (Schwartz et al. 2003; Niquette et al. 2000; Norton et al. 2004; Lehtola et al. 2005). Biofilms 96 h aged on PVC surface were more resistant when compared with same aged biofilm on PP and PE surfaces. According to LeChevallier et al. (1990) increase disinfection efficiency is not based solely on disinfection concentration, i.e., two times more disinfectant concentration does not result in twice inactivation. Biofilms on PVC showed a different structural composition with an organized mycelial development and a mature structure. Biofilms on PP and PE surfaces showed

cells sparsely distributed and were less resistant against chlorine inactivation suggesting that more than cellular density the biofilm architecture can difficult the transport of the disinfectant to the biofilm interface increasing the chlorine demand for cell inactivation. Lehtola et al. (2004) compared biofilm formation on cooper and PE surface and found that biofilms had a different rate of development and a different microbial community structure but that after one year microbial numbers in biofilms in water were similar in both materials.

Often household plumbing is constructed of plastic or copper, in some certain cases of stainless steel. Into household plumbing there are increases in the temperature and the content of chlorine decreases and consequently, microbial numbers increase in the water distributed throughout the buildings (Zacheus and Martikainen 1995, 1997). In this study, for filamentous fungi biofilm, PVC was the best surface for its development. In contrast, Yu et al. (2010) indicate some plastic materials, such as PVC, for drinking water distribution pipes, due to its low biofilm formation potential and little microbial diversity in biofilm.

In a research with filamentous fungi, Ramírez-toro et al. (2002) showed that organisms were able to colonize glass slides even in the presence of chlorine concentrations higher than those normally found in distribution systems. These authors also found that older biofilms are more resistance to chlorine and that attachment allowed survivals 2 to 10 times higher than planktonic cells. The highest concentration of chlorine used in this work was greater than the concentration advised by the World Health Organization (between 0.2 and 0.5 mg/l) (WHO 2008). The antimicrobial activity of chlorine depends on the amount of hypochlorous acid which, in turn, depends on the pH of water, the amount of organic matter in the water and on the temperature of water. However, excessive treatment, i.e. hyperchlorination, has several known and potential negative effects on product sensory quality, in environment and human health. Moreover, disinfection with free chlorine can also be affected by pipe surface and biofilm age (LeChevallier et al. 1988).

The efficiency of disinfection is important to reduce microorganisms in water and to avoid contamination. According to Council Directive 98/83/EC, a 0.5 mg/l concentration of free chlorine, after an exposure of 30 min, guarantees a satisfactory disinfection. The already published researches about biofilms disinfection showed that this free chlorine concentration is not effective against bacterial biofilms. In an overview, the water distribution network is under many different changes such as pH, nutrients, pipes material, temperature that influence in chlorine effectiveness what make disinfection a complex step of water treatment.

The study of biofilms has increased in the last years and the development of new methodologies has a great importance. Biofilms from water distribution system are known as a resource of microorganisms and recontamination with a consequently reduction in water quality. FUN-1 staining associated with fluorescence microscopy is a non-destructive analytical technique which in association with others fluorescent dyes provides metabolic and morphological analyses.

FUN-1 has been widely applied for antifungal susceptibility tests. Balajee and Marr (2002) reported a flow cytometric assay relied on conidial metabolism of the viability dye FUN-1 with spores of *Aspergillus* sp. Susceptibility of *Candida* sp. clinical isolates was also investigated with FUN-1 staining and the authors suggested it as an alternative and rapid method emphasize that the use of fluorescent viability assays can indicate the presence of a viable but not cultivable spores state (Pina-Vaz et al. 2001; Vanhee et al. 2008). De Vos et. al. (2006) used fluorescent dyes to detect fungi in hospital waters, dialysis fluids and endoscopic rinse. The results of these authors shown that in general, fluorescent labelling techniques detected more fungi in water than plate methods.

In this study, FUN-1 staining did not allow a quantitative analyse since a biofilm, as its own definition already explains, is not compose of free cells but the qualitative analyses were conclusive and the results corresponded with conventional plating results. The results already published are most about detection of yeast and spores viability and the application of FUN-1 dye for filamentous fungi biofilm are few or were not published yet.

In conclusion, we presented a simple and reproducible methodology for the study of the effectiveness of free chlorine against filamentous fungi biofilms from water. For this, we applied conventional plating and FUN-1 staining and we have shown that FUN-1 is efficient and offered rapid and reliable results for laboratorial biofilms and more studies are necessary to apply the methods in real biofilms. Furthermore, this is the first report about FUN-1 staining for susceptibility and viability analyse of a filamentous fungi biofilms from water. Finally, *P. brevicompactum* biofilms were capable to survive after exposure to a high free chlorine concentration whereas free spores were susceptible.

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Chapter 4

Surface hydrophobicity of culture and water biofilm of *Penicillium* spp.

4.1 Introduction

Hydrophobicity is related to many factors of fungal life and is crucial for fungal survival and adaptation (Wösten 2001). Filamentous fungi are known to produce hydrophobins which are small proteins localized on the outer surface of their cell walls (Wessels 1997). The hydrophobins form an amphipathic membrane whose hydrophobic side is exposed to the exterior whilst the hydrophilic surface is bound to the cell wall polysaccharides and confer water repellent properties (Whiteford and Spanu 2002). Hydrophobicity seems to function in cases of symbiosis between fungi and plants (ectomycorrhizae) or algae and/or cyanobacteria (lichens) and mediate attachment of hyphae to hydrophobic surfaces (Linder et al. 2005).

Hydrophobic interactions are of major importance in the firm adhesion of diverse microorganisms to water-solid interfaces (Donlan and Costerton 2002). Although the hydrophobic effect has been considered to be nonspecific, it is known that a large number of bacterial and fungal pathogens depend on hydrophobic interactions for successful colonization of a host (Doyle 2000); fungal-bacterial biofilms can be mediated by hydrophobic and electrostatic interactions wherein the fungal cell acts as a surface for bacteria to be attached on (Morales and Hogan 2010). The relation between hydrophobicity and fungal-bacterial interactions has been described in soil (Ritz and Young 2004; de Boer et al. 2005) and medical studies like in oral tissues, oral prostheses, implanted medical devices and urinary tract infections (Doyle 2000; Howard and Douglas 2002; Morales and Hogan 2010).

Although some studies about hydrophobicity in filamentous fungi (ff) have been recently published (Chau et al. 2009; 2010), ff have been largely excluded from hydrophobicity measurements. Characteristics such as dimorphic growth, variety of size and shape of hyphae and spores, and a complex cell wall make difficult the use of the same methods already applied for bacteria to ff (Hazen 1990). Moreover, the filamentous nature of fungal mycelial mats is difficult to handle, easily damaged and its surface may vary depending on the growth conditions (Doyle and Rosenberg 1990). Methods such as microbial adhesion to hydrocarbons (MATH),

hydrophobic interaction chromatography (HIC) and contact angle measurements (CAM) are widely used for cell surface hydrofobicity determination (Doyle and Rosenberg 1990; Doyle 2000). Contact angles (CA) represent a measure of a surface interaction with a liquid and have been commonly used to quantify the cell surface hydrophobicity of bacteria and yeasts (Henriques et al. 2004; Paramonova et al. 2009; Mazumder et al. 2010; Epstein et al. 2011). In contrast, Hazen (1990) suggested microsphere adhesion assay (MAA) as the best method for measuring hydrophobicity of ff. Recently, Chau et al. (2009) presented a new method to evaluate mycelial hydrophobicity based on CAM method.

Hyphal hydrophobicity influence bacterial adhesion to fungal cell and influence fungal-bacterial biofilm interactions (Perotto and Bonfante 1997; de Boer et al. 2001; Li and Palecek 2008). As matter of consequence, the characterisation of hyphal hydrophobicity is important for understanding its function and role in fungal-bacterial biofilms like the ones have been found in water distribution system (Siqueira et al. 2011).

In this study, CAM and MAA were applied to assess hydrophobicity of *Penicillium brevicompactum* and *Penicillium expansum* to compare hydrophobicity between mycelium mats from solid culture, liquid culture and fungal water biofilms and determine if MAA is an effective approach to characterise the hydrophobicity on fungal surfaces developed under these conditions.

4.2 Material and Methods

4.2.1 Microorganisms

Penicillium brevicompactum (MUM 05.17) and *Penicillium expansum* (MUM 00.02) supplied by Micoteca da Universidade do Minho fungal culture collection (MUM, Braga, Portugal) were chosen based on their high occurrence in Portuguese tap water distribution system (Gonçalves et al. 2006). None of them were previously characterised as hydrophobic or hydrophilic. The fungi were maintained on Malt Extract Agar plates (MEA: malt extract 20 g, peptone 5 g, agar 20 g, distilled water 1 l) at 25 °C.

4.2.2 Formation of mycelial mats on solid culture

This methodology was followed as described by Chau et al. (2009). Briefly, pre-cleaned microscope slides (7.6 cm x 2.5 cm) were sterilized by flaming after dipping them in 96 % ethanol solution. Then, the slides were transferred aseptically to sterilized Petri dishes.

Approximately 1 ml of the MEA or Water Agar Glucose medium (WGA: agar 20 g, glucose 10 g, distilled water 1 l) was spread uniformly on the slide using a micropipette. The slide medium was then allowed to harden. Under aseptic conditions, 5 µl of fungal spore suspension from a 7 day growing culture was inoculated on the centre of the slide medium and incubated at 25 °C for 7 and 21 days. Three replicates of each fungus grown in each slide medium were used for CAM.

4.2.3 Formation of mycelial mats in liquid culture

A suspension of 10^5 spores/ml was used to perform fungal liquid culture. The suspension was added to 2 ml Eppendorf tubes previously sterilized containing 1.5 ml of Water Glucose medium (WG: WGA without agar) or Malt Extract Broth (MEB: MEA without agar). Afterwards, the Eppendorf tubes were vortex and then were agitated gently at 23±2 °C for 3 days. Three replicates of each fungus grown in each liquid culture medium were used for MAA.

4.2.4 Formation of submerged water fungal biofilms

The biofilms were formed in 6-well plates containing 5 ml of WG or MEB medium and polypropylene coupons (1 cm x 1 cm), previously autoclaved at 121 °C during 15 min, placed into the wells with the reverse face touching the well bottom and completely submerged under the liquid medium. To form fungal biofilms, a suspension of 10^5 spores/ml was inoculated and incubated at 23±2 °C temperature and 120 rpm during 7 days. Three replicates of each fungal biofilm formed in each culture medium were used for CAM and MAA analyses.

4.2.5 CAM

Viewing and acquisition of CA images were performed by the sessile drop technique using a CAM apparatus (model OCA 15 Plus; DataPhysics Instruments GmbH, Germany). All the measurements were performed at room temperature using water droplets (3 µl). Contact angles measurements to be consistent and stable required a steady fungal growth and the procedures were also made in way not change the fungal colony properties. The contact angles were measured at the edge, middle and centre points of mycelial mats which representing the ages of the fungal colony: from the youngest to the oldest zones, respectively. Contact angle measurement >90° is interpreted as hydrophobic property of the surface.

4.2.6 MAA

MAA was used to test the surface hydrophobicity of mycelia mats grown in liquid cultures and fungal biofilms as described by Beauvais et al. (2007). Latex beads of sulfate-modified polystyrene with red fluorescence and an average diameter of 0.5 µm (L9777, Sigma; EX 575 nm/ EM 610 nm) were used. These beads have a low negative charge density with >90 % of their surfaces available for hydrophobic interactions (Hazen and Hazen 1987). Mycelial mats from liquid cultures and biofilms were washed with 0.1 M KNO_3 pH 6.5 and mixed with a 10^9 microspheres/ml suspension. The mixture was vortexed for 30 s and extensively washed with the KNO_3 solution. The adhesion of the microspheres was observed under an Olympus BX51 epifluorescent microscope using UV light (EX 470 nm, EM 505 nm).

Calcofluor White R2 (CW) which stains fungal cell walls was used as counterstain to improve morphological analysis of mycelia. The excitation/emission wavelength for CW was 346/433 nm and the signal acquired was blue. The images were acquired with a Zeiss AxioCam HRc colour camera using the software CellB®.

4.3 Results

The CAM were applied to edge, middle and centre zones of mycelial mats on solid culture (Figure 4.1) and in randomly points of biofilms (Figure 4.2). The contact angles values are summarised in Table 4.1. At 7 and 21 days the mycelial mats in both solid media did not allow apply the CA method at the centre zones. The high hydrophobicity observed did not consent the water drop to be released from the tip of the needle (Figure 4.1A). The presence of high number of spores in this oldest zone could be the cause of the very high hydrophobicity observed because after contact with the colony surface the water drop was covered by spores (Figure 4.1B). For this same reason, CA were not determined at middle zone of 21 days aged cultures in MEA. In contrast, *P. expansum* and *P. brevicompactum* for 7 days solid cultures in MEA in edge and middle zones (Figure 4.1C and D) and for 21 days in edge zone showed hydrophobic surface properties (Table 4.1). In WGA, both fungi showed hydrophobic features when the CA were measured at 21 days as well at 7 day for the middle zone of the colony. However, at the edges of 7 days aged colonies CA were <90° and these zones were classified as hydrophilic. Notwithstanding this, the whole 7 and 21 days aged colonies of *P. expansum* and *P. brevicompactum* were classified as hydrophobic.

In contrast with solid cultures, biofilms of P*. expansum* (Figure 4.2A) and *P. brevicompactum* (Figure 4.2B) formed in MEB with 7 days showed hydrophobicity (CA

91.3±2.0°) and hydrophilicity (CA 67.0±3.0°), respectively. It was impossible to measure contact angles of biofilms in WG medium because there was not enough fungal growth to cover an area on the coupon where the drop could be placed on.

Mycelial mats or fungal biofilms *P. brevicompactum* and *P. expansum* did not show different morphologies between them. In contrast and as expected, morphological differences were observed when mycelial mats and fungal biofilms were compared. In liquid media, the mycelial mats have grown typically as pellets (i.e., highly entangled masses of hyphae) ranging from 1.5 up to 3 mm of diameter (Figures 4.3A-C and 4.5A-C). On the other side, biofilms have grown covering the coupon surface heterogeneously and showing specific characteristics such as a low rate of growth, interstitial voids and vertical direction growth (Figures 4.4A-C and 4.6A-C). Fungi grown in liquid cultures were classified as hydrophilic since a little amount of microspheres was observed on hyphae (Figures 4.3D-F and 4.5D-F). In biofilms, a high number of microspheres could be seen attached to hyphae, characterizing them as hydrophobic (Figures 4.4D-F and 4.6D-F) but in the same microscopic fields were possible to observe hyphae totally uncovered thus classifying them as hydrophilic (Figures 4.4I and 4.6I). This feature differs between biofilms and mycelial mats from liquid cultures: while within the biofilms the microspheres showed preferential attachment on some hyphae (Figures 4.4G-I and 4.6G-I) in liquid culture the distribution seems to be randomly (Figures 4.3G-I and 4.5G-I).

Table 4.1 Contact angles (θw) values of mycelial mats on solid cultures.

Fungus	**Colony zone**	**Solid culture**			
		MEA (θ_w)		**WGA** (θ_w)	
		7 days	**21 days**	**7 days**	**21 days**
P. expansum	Centre	ND	ND	ND	ND
	Middle	129.4±1.7°	ND	112.5±1.5°	110.4±1.0°
	Edge	112.6±1.3°	103.7±1.0°	66.3±1.9°	102.6±1.0°
P. brevicompactum	Centre	ND	ND	ND	ND
	Middle	119.4±1.5°	ND	96.3±2.0°	98.3±1.2°
	Edge	118.2±2.3°	110.2±1.3°	83.7±1.0°	99.5±1.5°

ND = not determined; The values are the mean ± standard deviation of three replicates.

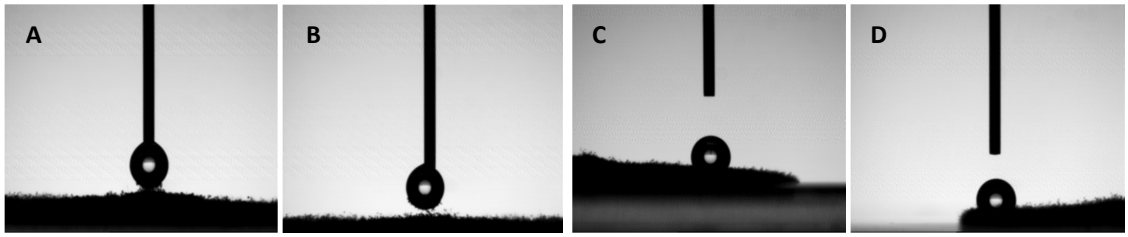

Figure 4.1 Images of water drop on the surface of three zones of the fungal colony of *P. expansum* developed in 7 days in MEA solid culture. A: Centre zone; B: Centre zone with fungal spores surrounding the water drop; C: Middle zone; D: Edge zone.

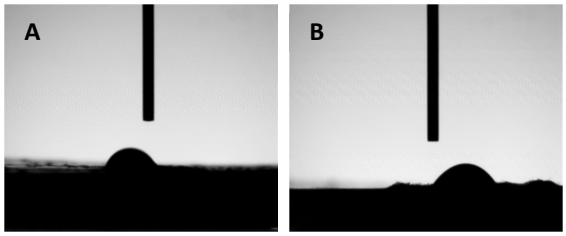

Figure 4.2 Images of water drop on biofilm surface developed in 7 days in MEB liquid culture. A: *P. expansum*; B: *P. brevicompactum*.

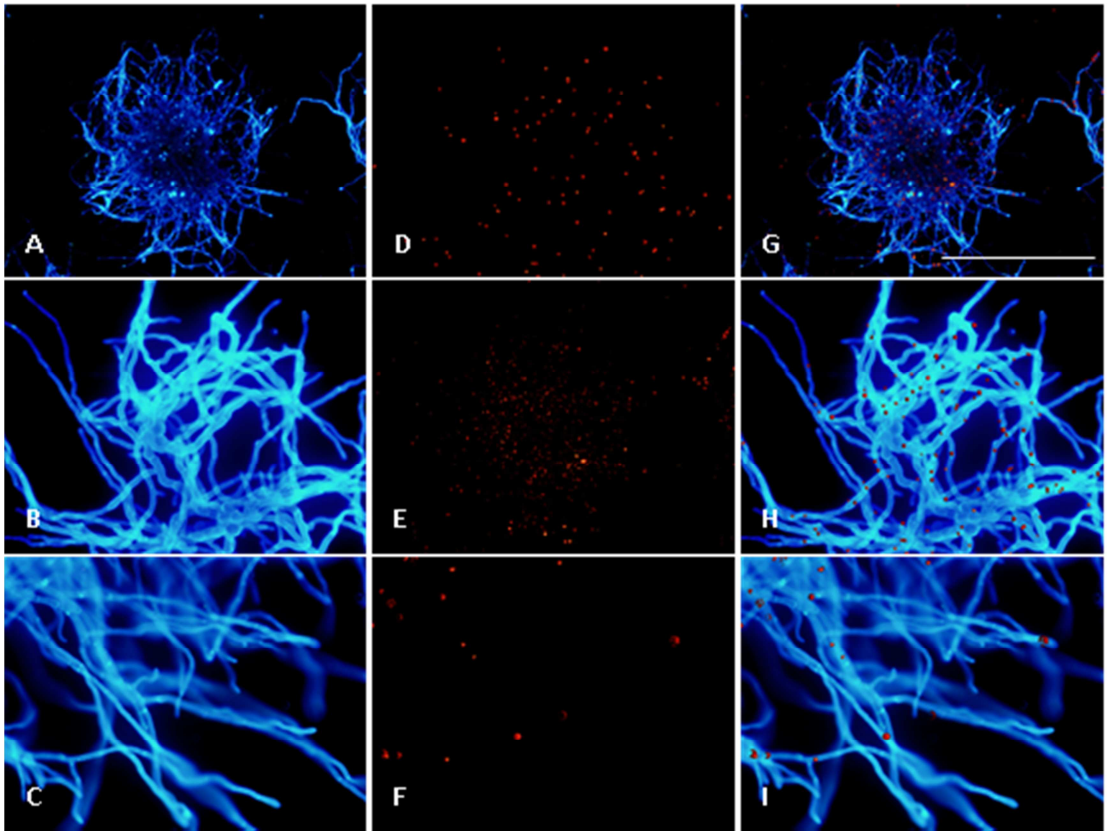

Figure 4.3 *P. expansum* mycelial mats in MEB. A-C: Calcofluor White R2 staining; D-F: Microsphere adhesion; G-I: Superimposed images. Scale bar = 200 µm.

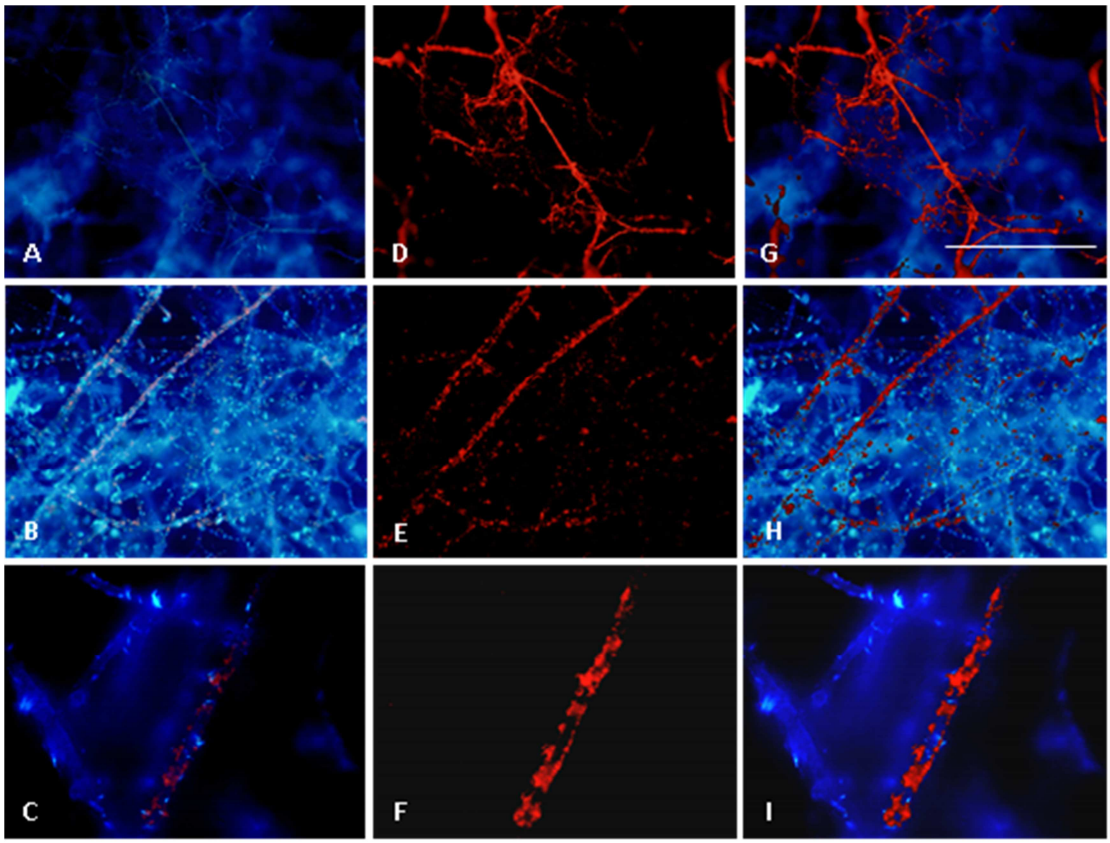

Figure 4.4 *P. expansum* biofilm in MEB. A-C: Calcofluor White R2 staining; D-F: Microsphere adhesion; G-I: Superimposed images. Scale bar = 200 µm.

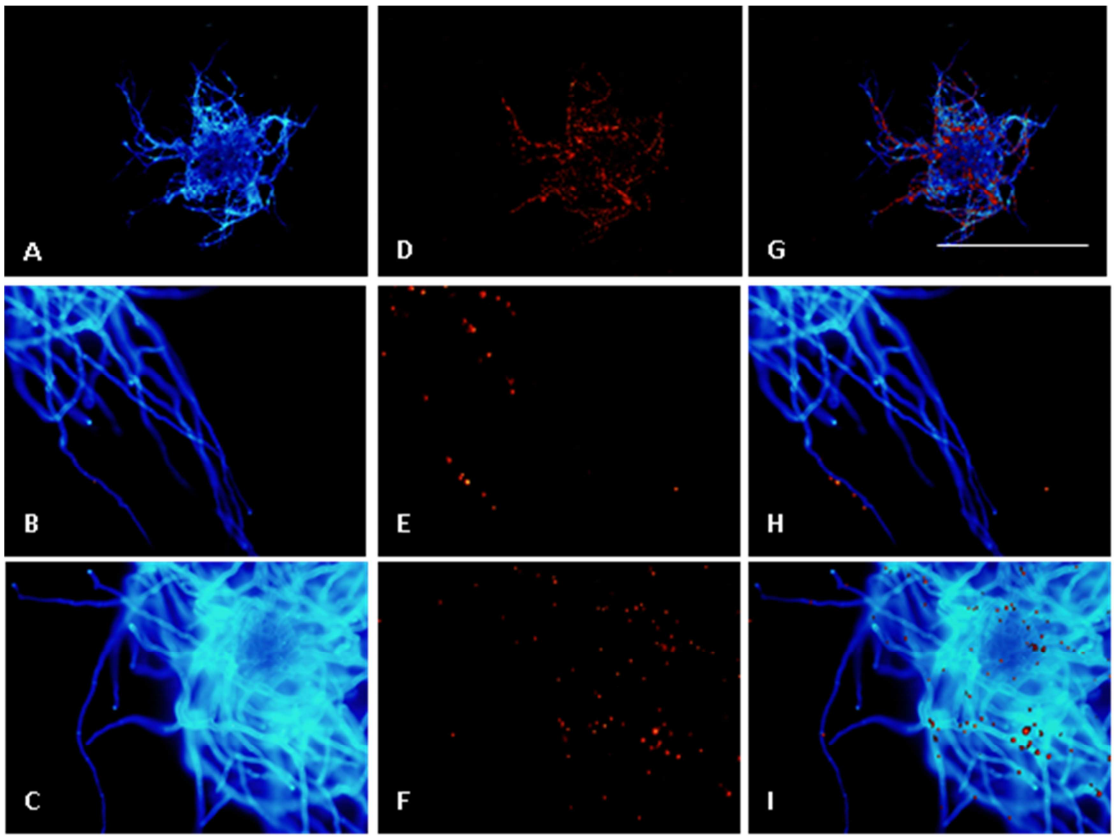

Figure 4.5 *P. brevicompactum* mycelial mats in MEB. A-C: Calcofluor White R2 staining; D-F: Microsphere adhesion; G-I: Superimposed images. Scale bar = 200 µm.

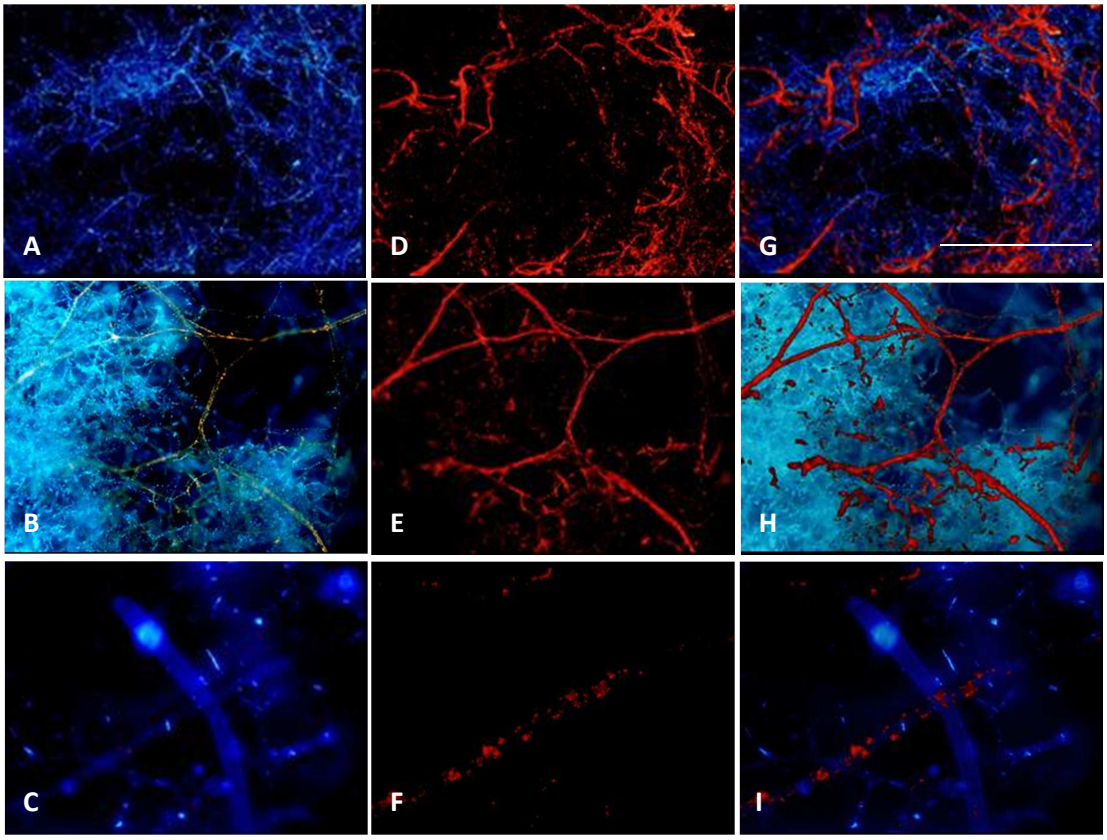

Figure 4.6 *P. brevicompactum* biofilm in MEB. A-C: Calcofluor White R2 staining; D-F: Microsphere adhesion; G-I: Superimposed images. Scale bar = 200 µm.

4.4 Discussion

Our results showed that *P. expansum* and *P. brevicompactum* colonies have high hydrophobicity in 7 and 21 days old cultures in MEA. These findings are in line with Chau et al. (2009) which using CAM also reported high hydrophobicity for *Penicillium auranteogriseum* in a 10 day culture on Potato Dextrose Agar. Others deuteromycetes such as *Cladosporium* sp. (Smits et al. 2003) and *Cladosporium cladosporioides*, *C. minourae* and *Alternaria* sp. (Chau et al. 2009) were also characterised as hydrophobic using CAM. Additionally, fungal spores of *P. oxalicum* and *P. expansum* were also characterised as hydrophobic using the phase distribution test and the retention on polystyrene assay, respectively (Pascual et al. 2000; Amiri et al. 2005).

The different hydrophobicity levels found in *P. expansum* and *P. brevicompactum* 7 and 21 days old in WGA can be due the intense colonization at the zone of inoculation (Busscher et al. 1984) which reflect a change in growth state from the point of inoculation to the edge of the colony (Wessels et al. 1991), and/or the presence of sporulation and the amount of spores produced (Smits et al. 2003; Cavalcante et al. 2008). Spores of Penicillium are well known to be very hydrophobic (Amiri et al. 2005) and colonies in solid cultures of most of this taxon, which

had radial growth, is easily visible the gradate increase of sporulation from the point of inoculation up to the edge of the colony. This features may be the main reason for high contact angles values obtained and, consequently, high hydrophobicity. Furthermore, in this study the sporulation was higher in colonies grown in MEA when compared with those grown in WGA. The levels of nutrients can interfere in the hydrophobicity because the higher the nutrients are the higher the fungal sporulation is, or the higher the nutrients are the higher the proteins excreted by the fungus are (Smits et al. 2003; Chau et al. 2009). Chau et al. (2009) suggested a new Chronos-amphiphilic class which covers fungi with shifting hydrophobicity over time and space.

It is know that environmental conditions such as temperature, nutrients source and humidity can affect the hydrophobicity (Smits et al. 2003). The hydrophobicity can also change with the form of growth, e.g. if the microorganism is grown on solid or in liquid media or, if it grows attached to a surface as biofilms. The interference of the form of growth is detected when we compare the results between solid culture and biofilms of the same fungus. While the colonies showed in general high CA values, biofilms showed <90° contact angles. Hazen (1990) says that the fungal cell layer formed on a surface must be uniform in depth to get a reliable contact angle, but uniformity may not be achievable with ff cells. Nevertheless, the limitations of the technique can be questioned since CA is extremely affected by surface heterogeneity, a characteristic linked with ff biofilms (Villena et al. 2009).

In aqueous environments, hydrofobins produced by ff are known to be involved in the mechanism of fungal hyphae to lower the surface tension and grow into the air; once escaped, these hyphae may further differentiate to simple or elaborate spore-bearing aerial structure which are covered with a hydrophobic film (Wösten and Wessels 1997; Wösten and Willey 2000). The greater interest among the results presented in this study, is that MAA was able to assess how different growth (i.e., biofilms) influences the levels of fungal cell surface hydrophobicity. Within biofilms, the hyphae projected out of the denser hyphae layer and exposed to the outer inner of the biofilm, were those which presented higher hydrophobicity. This observation is in line with the model for the formation of fungal aerial structures, which postulate that hyphae are cover by hydrophobin film with its hydrophobic side exposed to the air (Wösten et al. 1994), but with a particular feature: the hydrophobic hyphae are still in contact with the liquid medium. Nevertheless, they still seem to be projected out of the biofilm core to create a differentiated mycelial zone which can be associated with further interactions in aquatic environments.

Villena et al. (2009) described biofilms of *Aspergillus niger* and reported a vertical growth direction, well-structured channels, a specific biomass density distribution and differential physiological behaviour (i.e. enzymatic production) as important characteristics of biofilms. The depth and the presence of channels between groups of hyphae were also reported in *Aspergillus fumigatus* biofilms and are related with the vertically polarized growth (Beauvais et al. 2007). Thus, morphological and physiological behavior of ff biofilms can be correlated with different hydrophobicity levels. Moreover, structural and physiological functions within ff biofilms seem to be correlated with specific hyphae, corroborating the concept of biofilms as communities with complex levels of organization and cell activity (Wimpenny et al. 2000).

Hydrophobicity also influences the cell adherence to a wide variety of surfaces and thus in the biofilm formation as well (Chandra et al. 2005; Pompilio et al. 2008; Norouzi et al. 2010) and can render microorganisms more pathogenic (Beauvais et al. 2007; Kuntiya et al. 2005). Hydrophobicity can also affect microbial interaction in different environments. Fungi and bacteria often share habitats, whether they live together as free-living organisms in natural ecosystems or as intimate partners in a symbiotic relationship (Valdivia and Heitman 2007). The hyphosphere, i.e. the surface and direct surroundings of fungal hyphae, can be considered the hotspot of microbial interactions. In aquatic habitats hyphosphere-associated bacteria seem to have the potential to affect the fungal performance significantly (Baschien et al. 2009). Although many studies have described bacteria attached to fungal hyphae (de Boer et al, 2001, 2004; Hogan et al. 2007; Hoffman and Arnold 2010), the mechanisms for this attachment and in particular the role of fungal surface characteristics are greatly unknown.

Biofilms in water distribution system have been widely investigated in the last years (Huq et al. 2008; Simões et al. 2010; Wingender and Flemming 2011). Mixed-species bacterial biofilms are usually described (Watson et al. 2004; Braganra et al. 2007) but a lack of information about fungal-bacterial interaction within biofilms still remains, though fungi and fungal biofilms are also relevant to water quality (Paterson and Lima 2005; Hageskal et al. 2009; Sammon et al. 2011; Siqueira et al. 2011). Biofilms are complex communities wherein interspecies and intraspecies interactions occur and influence initial stages of biofilm formation and its subsequent development and permanence (Elvers et al. 2002). Future work should aim to find out if hydrophobicity is related to attachment of bacteria to fungal hyphae and how it interferes in fungal-bacterial interaction, specifically within water biofilms.

In conclusion, the results of this work showed a relevant difference when CAM and MAA are applied. In this study, CAM showed to be more useful to assess hydrophobicity on solid cultures, i.e. the results represent the hydrophobicity of the whole colony. By the other hand, MAA was more reliable to assess directly the cells surface hydrophobicity and was useful for characterise different zones of hydrophobicity within the biofilm. Moreover, MAA is a reproducible and simple technique and it can be used to assess patterns of hydrophobicity and provide better understandings about filamentous fungal biofilm architecture.

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Chapter 5

Monitoring filamentous fungal biofilm formation in a laboratorial flow chamber reactor by image analyses

5.1 Introduction

Governmental agencies and water companies are constantly concerned about water quality; there is an evident preoccupation to provide water free of hazards to human health. In this sense, the study of microbial biofilms in water distribution systems (WDS) has received great attention, since biofilms are widely considered a source of microbial contamination (Szewzyk et al. 2000; Huq et al. 2008). WDS are very complex environments in which diverse factors may interfere in water microbiology and biofilm formation as well (Berry et al. 2006). Abiotic factors such as temperature, disinfectant residuals and type (Gagnon et al. 2005), organic matter (Norton and LeChevallier 2000), nutrient concentrations (Chu et al. 2005), substratum (Zhou et al. 2009), and hydraulic characteristics (Lehtola et al. 2006; Manuel et al. 2007) influence biofilm formation in WDS. The idea of a WDS as an ecosystem becomes even more complex if all the others habitats surrounding it are considered (Szewzyk et al. 2000).

Nowadays, the different techniques applied in this field are incredible numerous and include microscopic, microbiological, molecular biological, (bio)-chemical and physical approaches (O'Toole et al. 1999; Flemming et al. 2000). Additionally, researchers have been developing *in vitro* systems for growing and testing microbial biofilms; they are known as biofilm reactors. A biofilm reactor is defined as a group of compartments and their components determining biofilm structure and activity and it is basically composed of three main compartments: (1) the surface where the microorganisms are attached, (2) the biofilm and (3) the solution of nutrients. Batch systems, flow-cells, modified Robbins device and annular reactor can be listed as the most common biofilm reactors used in laboratorial experiments (Goeres et al. 2005).

These systems provide conditions for biofilm development and, even though they do not completely reflect the conditions in real pipe networks, they are very useful for biofilm characterization in laboratorial experiments. For example, for the study of filamentous fungal water biofilms the implementation of biofilm reactor in laboratorial studies is quite important,

since this is a very new area in water quality research that requires evaluation and optimization of new techniques. Hence, a biofilm reactor was developed by our group and was applied for laboratorial experiments and was performed together with specific fluorescent dye for filamentous fungal biofilm detection. Filamentous fungi isolation was also performed to assess fungal diversity in biofilms within the biofilm reactor.

5.2 Material and methods

5.2.1 Model system

In order to produce biofilm samples a flow chamber (Figure 5.1) was constructed to be operated under monitored conditions. Selected properties are shown in Table 5.1.

Table 5.1 Flow chamber conditions.

Flow chamber characteristics	**Closed flow**	**Open flow**
PVC coupon area (inner surface)	2.52 cm^2	2.52 cm^2
Number of cells	4	4
Number of coupons	4	4
Tank volume	2.15 l	-
Flow chamber volume	45 ml	45 ml
Total volume (with pipes)	2.3 l	-
Reynolds number*	2513 (transitional turbulence conditions)	≤ 2100 (laminar conditions)

* At the cells modules

The model system consisted of two acrylic flow chambers, silicone pipes, a circulation pump, an acrylic tank and connectors. The cells were mounted with flanges at each end, were joined by screws, and tightened by O-rings. The system was set up in two different systems named open flow and closed flow. The open flow was continuously fed with tap water from the municipal distribution network of Braga, Portugal. The closed flow was set up in continuation with the open flow and fed with the same tap water, but the water was kept in a recirculation system. Biofilm samples were collected by removing the cell. Disinfection of all system was carried out with sodium hypochlorine solution (10%) followed by washing with distilled and autoclaved water.

No additional disinfectant was allowed to enter into the system during all experiment. The model system was operated under constant conditions during a total of 8 months; analyses were performed at each 2 months. During this period, free chlorine levels (Ion specific meters, Hanna Instruments, HI 93701, light emitting diode @ 555nm, range 0.00 to 2.5 mg/l, resolution 0.01 mg/l), pH (pH Meter 526, WTW, Multical®) and temperature of the water were also monitored.

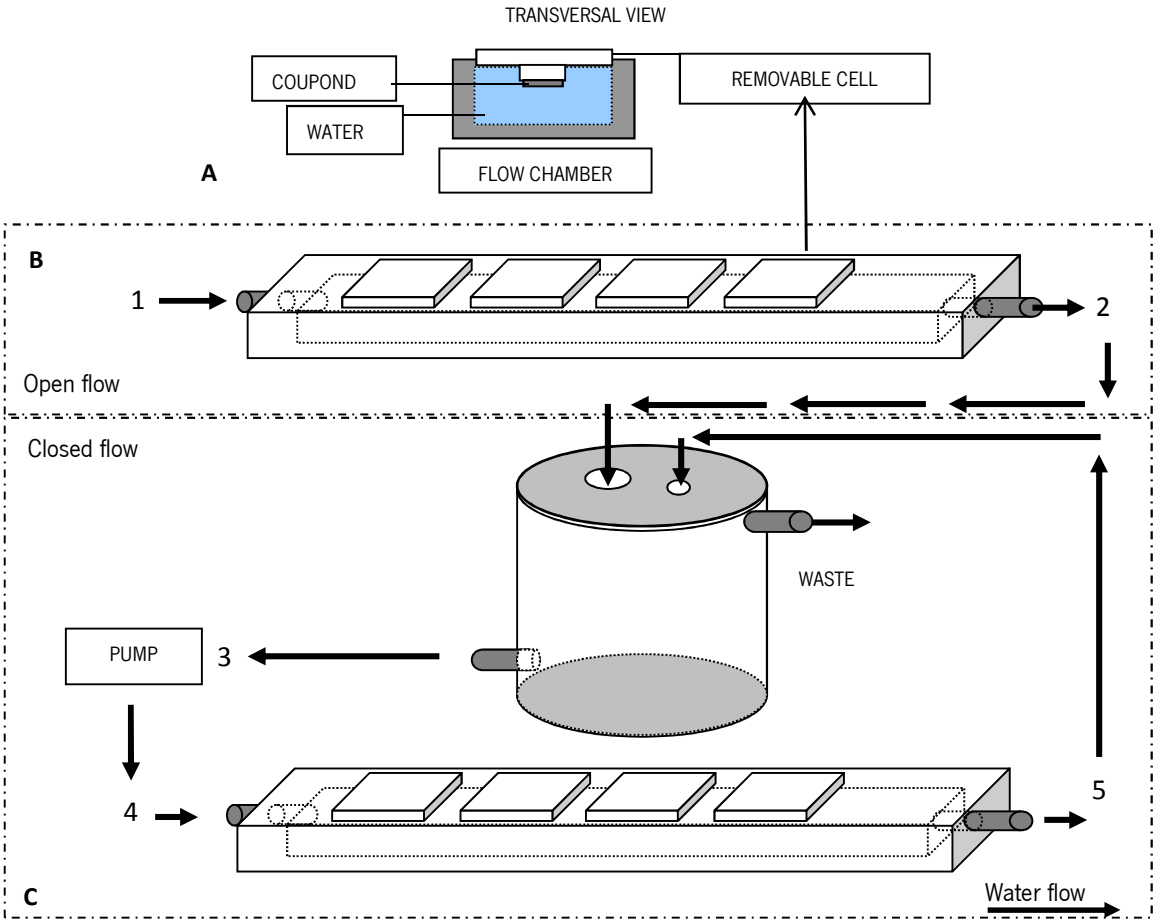


Figure 5.1 General overview of model system: **A** – Transversal view of acrylic flow chamber with a removable cell; **B** – Acrylic flow chamber of open flow with four cells and **C** – Tank, pump and acrylic flow chamber of closed flow with four cells. Arrows represent water flow inside silicone pipes.

5.2.2 Fluorescent staining and microscopy

Calcofluor White M2R (CW) (25 µM) was used as staining for fungal detection. Following incubation in the dark for 15 min in room temperature, the coupons were microscopically observed. The excitation wave length for CW was 346 nm and the signal acquired was blue. An

Olympus BX51 epifluorescence microscope using UV light equipped with 100x/1.3, 40x/0.30 and 10x/0.65 objectives was used. The images were acquired with a colour camera Zeiss AxioCam HRc using the software CellB®.

5.2.3 Biomass quantification

The fungal biomass (in gram), derived from the biovolume, was calculated as "Biomass = density x π r^2 L", where r was the radius of fungal hyphae (in centimetre), d = 1.09 g cm^{-3} was considered to be the density of fungal hyphae (Schnürer 1993) and L was the length of fungal hyphae (in centimetre).

5.2.4 Fungal isolation

After 8 months of analyses the system was unset and the pipes and joints were removed and used for fungal isolation. For this, three samples of five chosen points named (1) inlet open flow, (2) inlet tank, (3) inlet pump, (4) inlet close flow and (5) outlet closed flow were used (Figure 5.1). The inner surface of pipes and joints were scraped using sterile blades; the scrape samples were placed into tubes containing 1 ml of saline solution (0.85 % NaCl), and homogenised using a vortex. One hundred millilitres of each sample was spread onto Petri dishes containing the culture media Malt Extract Agar plates (MEA: malt extract 20 g, peptone 5 g, agar 20 g, distilled water 1000 ml) added with chloramphenicol (50 mg) (Biokar Diagnostics, France) and Neopeptone Glucose Rose Bengal Aureomycin plates (NGRBA: neopeptone 5 g, glucose 10g, chlortetracycline solution (1.0 g/100 ml) 14 ml, rose Bengal solution (1.0 g/150 ml) 3.5 ml, agar 20 g, distilled water 1000 ml). Culture plates were incubated at 25 °C during 7 days and colonies were counted and identified to genus level.

5.3 Results

A total of 8 coupons (4 from open flow and 4 from the close flow) were analysed at each 2 months during a total of 8 months. Filamentous fungi were detected in all samples since the first analyses, but a higher number of filaments stained with CW and a more diverse morphology were observed mostly in the last month (Figure 5.2 – 5.5). PVC coupons showed a very high autofluorescence. Although the autofluorescence decreased along time, this sort of material may not be used for fluorescent microscopy because the autofluorescence can lead to a false negative

detection. Since CW has blue signal, it was possible to detect the stained structures and the experiment was carried out until 8 months.

From the fourth month of analyses, diverse algae attach to coupons were also observed (Figure 5.3D; Figure 5.4G and H; Figure 5.5G and H). These algae resemble the genera *Chlorella* (Figure 5.4H and 5.5G), *Staurastrum* (Figure 5.3D and 5.5H) and *Tetraedron* (Figure 5.4G) which are commonly find in freshwater environments (Sigee 2005). They were observed mainly attached to coupons exposed to the closed flow.

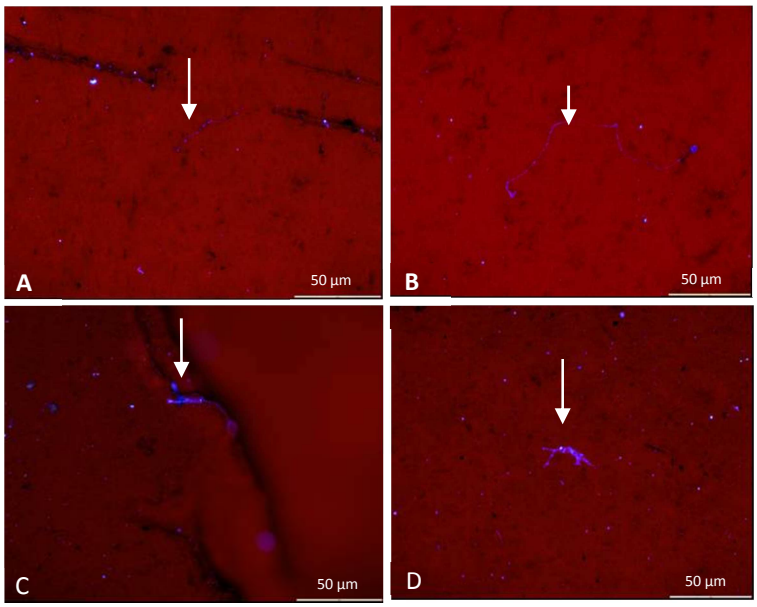


Figure 5.2 Filamentous fungi (arrows) detection on coupon surface after 2 months of exposure and CW staining. A and B - open flow; C and D - closed flow.

Fungal biomass was calculated by conversion of biovulome into biomass using a proper conversion factor, i.e. the density of fungal hyphae (Johan Schnürer 1993). Biomass values are listed in table 5.2 and represent all detected filaments attach to coupon. The closed system showed a higher attached biomass, i.e. 3.49 mg/cm^2 after 8 months.

Table 5.2 Time course of fungal biomass on coupon surfaces over 8 months in open and closed flow.

Local	**Fungal biomass (mg/cm^2)**			
	2 months	4 months	6 months	8 months
Open flow	1.86	1.23	2.3	2.89
Closed flow	2.18	2.26	2.93	3.49

Table 5.3 Fungal colony-forming units (CFU) recovered from scraped pipes and joint of model system after 8 months.

Local	**Culture media**		**Total per local**
	MEA	NGRBA	
Inlet open flow	4	0	4
Inlet tank	6	5	11
Inlet pump	7	1	8
Inlet closed flow	9	6	15
Outlet closed flow	7	3	10
Total	33	15	48

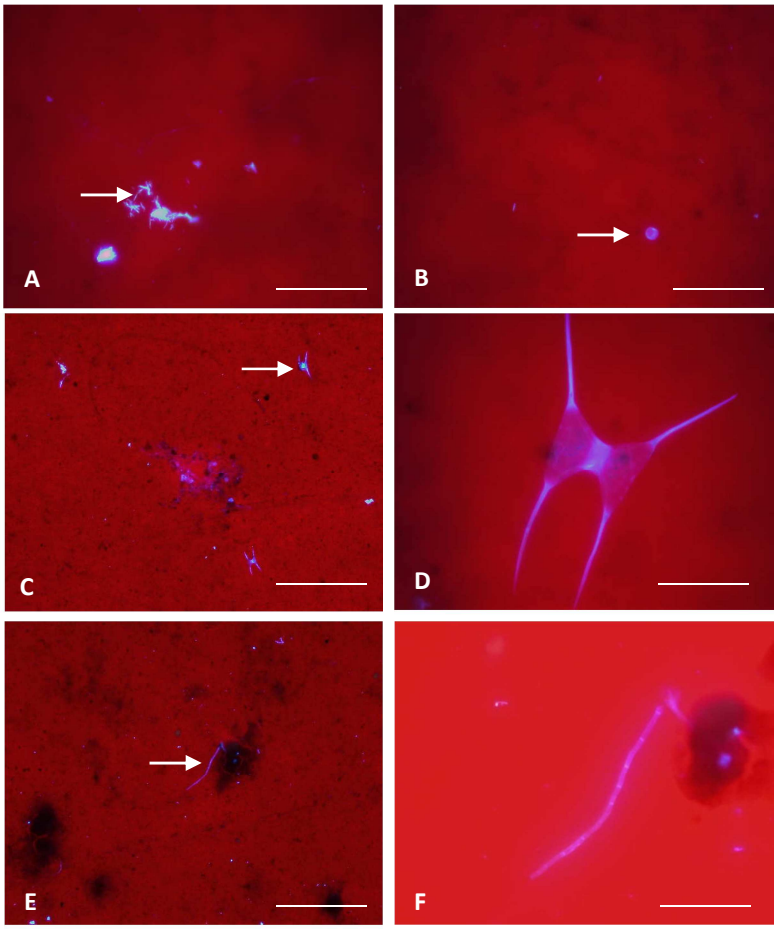


Figure 5.3 Filamentous fungi and algae (arrows) detected on coupon surface after 4 months of exposure and CW staining. Open flow: A and B. Closed flow C – G. Scale bar = 20 µm.

Free chlorine, pH and water temperature were also assessed during the experiment (Table 5.4). It wasn't detected a high variance in these parameters along the 8 months of experiment, but a considerable change was verified in free chlorine levels when tap water (open flow) and tank water (closed flow) were compared, i.e. tank water showed very low free chlorine levels. Free chlorine was not detectable at tank water in 6th and 8th months. No significant changes in water temperature and pH were observed during the experiment (Table 5.4).

Table 5.4 Free chlorine, pH and temperature during 8 months of experiment (values are the average of measurements during the respective period).

	Time			
	2 months	4 months	6 months	8 months
Free chlorine (mg/L)				
Tap water	0.66	0.66	0.45	0.7
Tank	0.05	0.04	-	-
pH				
Tap water	6.9	7.2	6.8	7.0
Tank	6.5	6.7	6.2	6.4
Temperature (°C)				
Tap water	16	16	15	16
Tank	18	20	19	19

- No detectable

A total of 48 isolates were recovered from the different chosen points in the model systems (Table 5.3). *Aspergillus* spp., *Cladosporium* sp. and *Penicillium* spp. were the predominant fungi. A higher number of isolates was recovered at the local named inlet closed flow but a higher diversity was detected at the local named inlet pump (Table 5.5).

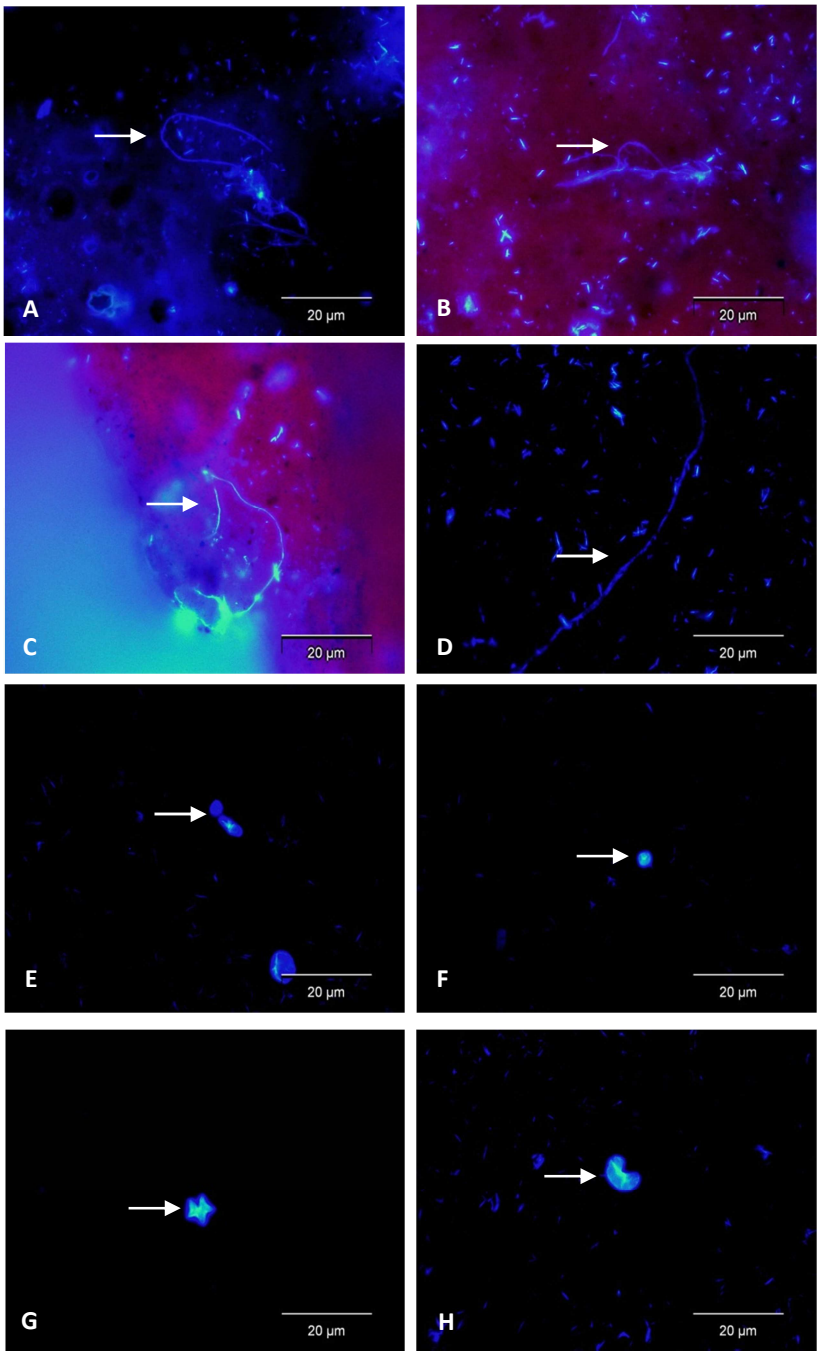


Figure 5.4 Filamentous fungi and algae (arrows) detection on coupon surface after 6 months of exposure and CW staining. A and B - open flow; C, H - closed flow.

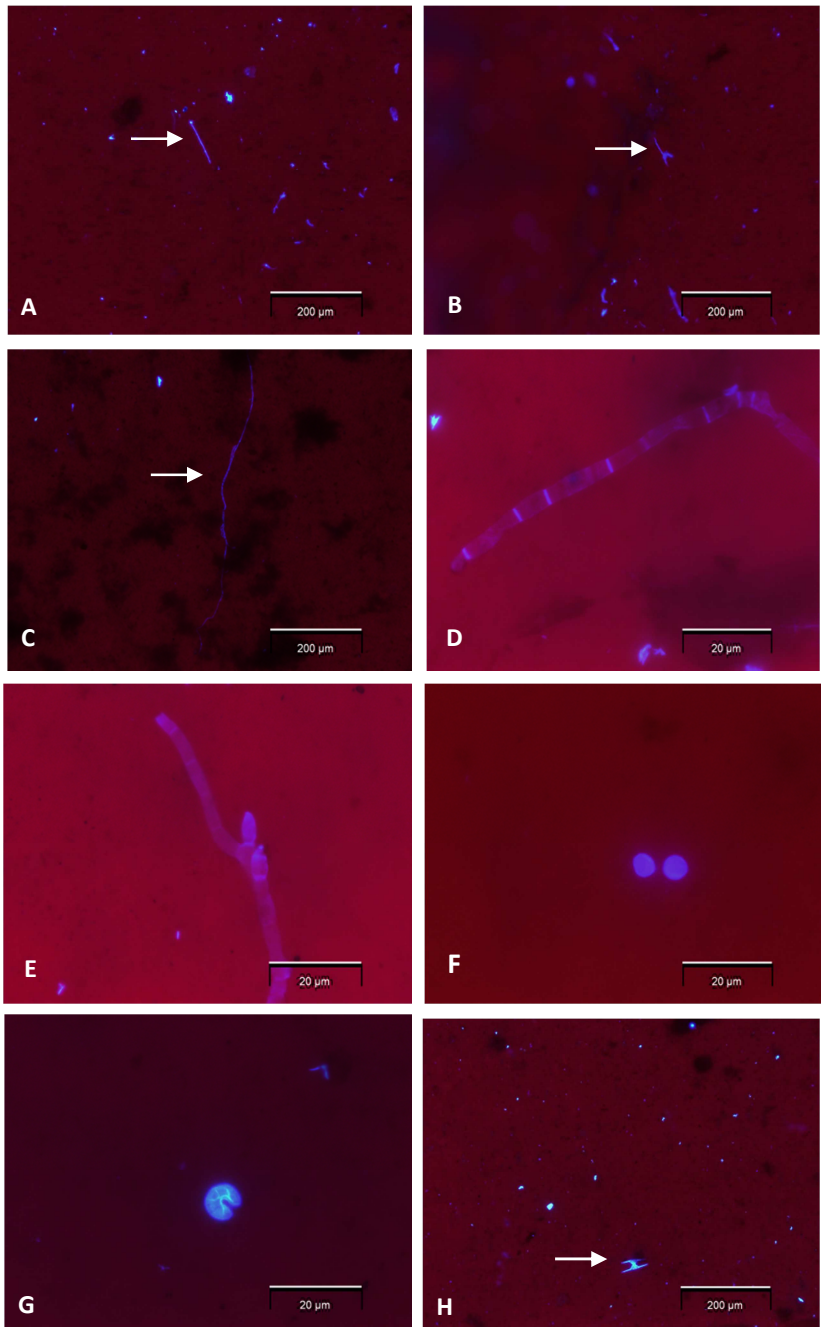
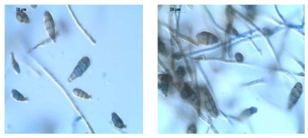
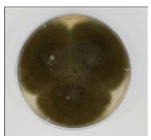
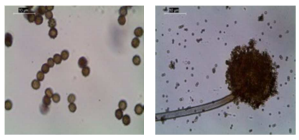
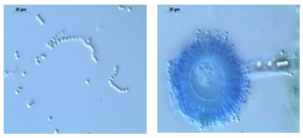
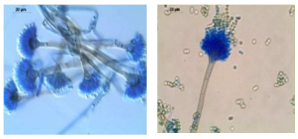
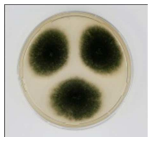
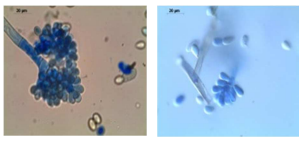
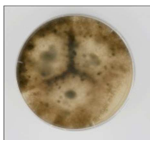
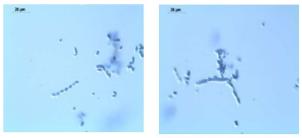
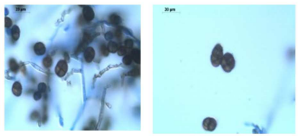
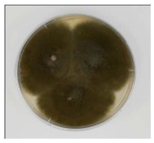
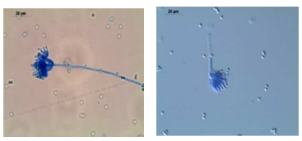
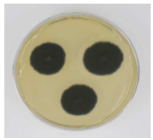
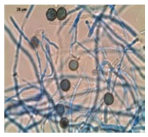
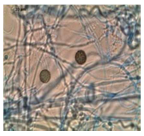
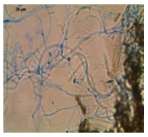
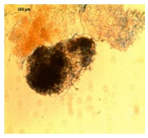
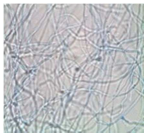


Figure 5.5 Filamentous Fungi and algae (arrows) detection on coupon surface after 6 months of exposure and CW staining. A and B - open flow; C, H - close flow.

Table 5.5 Fungi recovered from pipes and joints of flow-chamber.

Fungi	Local	Number per local	Total	Microscopy	Macroscopy
Alternaria sp.	Inlet closed flow	1	1		
Aspergillus section Nigri	Inlet closed flow/Inlet open flow/Outlet closed flow	4/1/5	10		
spergillus section Flavi	Inlet tank/Inlet pump/Inlet closed flow/Outlet closed flow	3/3/2/3	11		
Aspergillus spp.	Inlet tank/Inlet closed flow	3/1	4		
Botrytis sp.	Inlet closed flow	1	1		
Cladosporium sp.	Inlet closed flow/Outlet closed flow	2/2	4		
Epicocum sp.	Inlet tank/Inlet pump	2/1	3		
Penicillium spp.	Inlet open flow/Inlet tank/Inlet pump/Outlet closed flow	3/1/3/2	9		

Periconia sp.	Inlet closed flow	1	1			
Coelomycete	Inlet pump	1	1			
No sporulation	Inlet tank/Inlet closed flow/Outlet closed flow	1/2/1	3			

5.4 Discussion

Despite the existence of various studies about filamentous fungi in water systems (Hageskal et al. 2009) fungal biofilms has only recently received some attention (Siqueira et al. 2011; Sammon et al. 2011). As a new area of research, a lack of specific and standardized methodologies still remains a key challenge. In this study, a flow chamber reactor was designed to operate under monitored conditions to study filamentous fungal biofilm formation.

The main drawback along the experiments was the autofluorescence of PVC coupons, but as CW has blue signal, it was decided to carry out the experiment. Here, PVC coupons were chosen because it is the same material used in most water distribution systems. The choice of coupons is a decisive step in biofilm studies since coupon material can interfere in biofilm formation and in the final analyses as well. Different coupons are applied for biofilm studies; glass, steel, and plastic are the most common (Murga et al. 2001; Simões et al. 2006; Pamp et al. 2009; Deines et al. 2010). CW staining was a rapid and easily manipulated fluorescent dye for fungi detection. Despite its capability to stain others microorganisms and EPS (Gallo et al. 1989; Biegal et al. 2002; Rasconi et al. 2009; Rezende et al. 2003), fungi could be easily differentiated by morphological characteristics such as thick septate hyphae. Filamentous fungi were detected in all time of analyses and showed a crescent attachment along time.

Time of exposure seems to be a very important factor for filamentous fungal biofilm formation (Nagy and Olson 1985). In another study of our group in which coupons were exposed

directly to water network, it was necessary at least 6 months to detect filamentous fungi attached to coupons (Siqueira et al. 2011). Similar results were reported by Sammon et al. (2011) that verified filamentous fungi attached to coupon surface after 7 months. Nonetheless, in real replaced pipes, which had been exposed to water network for years, were detected mature filamentous fungal biofilm straightly attached to pipe surface (Sammon et al. 2011; Siqueira et al. 2011). The formation of a previous biofilm was also reported as an important factor for fungal biofilm development. Garny et al. (2009) reported a complete change in water biofilm structure from a bacterial single cells biofilm to a strong fungal network, mainly after detachment events. These same authors pointed out that in the end of six weeks biofilms were dominated by filamentous growth and hypothesised that bacterial detachment favoured fungal growth within the remaining base biofilm.

The *in vitro* ability of microorganisms to produce biofilms is usually tested under optimised laboratorial conditions such as temperature, nutritional source and pH. Despite real conditions are more variable and biotic and abiotic factors may influence all the system, in laboratorial studies some parameters can be monitored and add more information about their influence in biofilm formation.

More filamentous fungi were detected on coupon surface exposed to tank water, i.e. in the closed flow which presented low levels of free chlorine and where the water flow was characterised as transitional (Reynolds number = 2513). Studies in bacterial biofilms show that stable and higher water flow rates produce thinner and more cohesive layers less prone to release bacteria into the bulk water, and thus limiting biofilm growth (Percival et al. 1999; Cloete et al. 2003). However, in a WDS the water flow changes from laminar to turbulent and stagnant (no-flow) which occurs in reservoirs or where the water consumption is low, e.g. in buildings (Manuel et al. 2007). In this study, free chlorine levels decreased substantially when tap water and tank water were compared. The higher number of filaments detected on coupons exposed to transitional flow, i.e. closed flow, may be due to flow water velocity in association with low levels of free chlorine.

Chlorine and chloramines are common disinfectants used in drinking water to inactivate microorganisms (WHO 2008), but a disinfectant residue does not protect the distribution system from recontamination with polluted water, microorganisms attached to particles or mechanical failures (Schwartz et al. 2003). Moreover, re-chlorination along a water distribution network is necessary in order to keep disinfectant levels enough to act against microorganisms. This is also

an important feature for water in household plumbing and for water kept in reservoirs in which chlorine levels may be influenced by temperature, light exposure and pipe material (Niquette et al. 2000). Additionally, it has also been reported that microbial biofilms are capable to decrease chlorine levels by itself (Berger et al. 2000). In agreement with others authors (Codony et al. 2005; Gagnon et al. 2005), was observed that the presence of higher levels of chlorine in tap water did not prevent microbial attachment to coupon surface. Moreover, fungal spores are known to show high resistance against disinfectants (Okull et al. 2006).

Cellular forms apparently belonging to the group of microalgae were also detected. These microalgae are characterised by a porous morphology what probably facilitates the trapping of smaller cells such as bacteria and fungal spores (Lutterbach and França 1996). Furthermore, microalgae produce organic compounds which can be used as nutrients by bacteria (Haak and McFeters 1982) establishing a partnership between microorganisms and contributing for the formation of multispecies biofilms. The ability of fungi to form associations with algae was recently reported in studies involving biofilms in historic monuments (Crispim et al. 2004; Grbić et al. 2010). Interactions between different microorganisms play an important role in the development and maintenance of biofilms (Parsek and Greenberg 2005). In water distribution systems new insights are still needed to understand the established microbial relations between fungi and others microorganisms.

In mycological studies of drinking water there is a number of fungal species frequently isolated, e.g. species belonging to the genera *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium* and *Penicillium* (Hageskal et al. 2009). *Aspergillus*, *Cladosporium* and *Penicillium* were the predominant fungi in this study. This finding is in agreement with previous studies where few species are predominant among all isolates (Göttlich et al. 2002; Gonçalves et al. 2006). Nonetheless, the precise species composition observed in different studies varies considerably, i.e. specific environmental characteristics of the individual distribution systems may influence considerably the microbial communities. Moreover, most of techniques are directed to study planktonic fungi, and fungal biofilms remains neglected. Sammon et al. (2011) observed a variance in number and diversity when fungi were recovered from water and from pipe surfaces and related these found to drawbacks such as disturbance of biofilms when the sampling method (i.e. scratching) is applied and the variable fungal morphology. Thus, direct visualisation may represent a reliable alternative to study fungal biofilms.

In biofilm studies, microbial quantification is estimated by the standard plate count of colony forming unity (CFU) (Ivnitsky et al. 2007; Meier et al. 2008; Wong et al. 2010), one of the best known and oldest microbiological techniques (Harris and Kell 1985). Despite its wide use, several criticisms have been made of it, mainly because it necessarily involves cell reproduction, i.e. only cells which are capable of reproducing themselves under the given conditions are counted (Berlutti et al. 2003). In mycological studies, researchers have to overcome an additional drawback, i.e. the multicellular characteristic of filamentous fungi. Thus, methods non-culture dependent such as fluorescent staining and direct measurements can add important information. In this sense, fungal biomass was also measured using the biovolume concept for filamentous microorganisms (Madrid and Felice 2005), i.e. in the coupons, fungal quantification was assessed *in situ* by image analyses and further calculation of biovolume and its conversion into biomass (in grams).

The use of direct observation has advantages such as the assessment of biomass per area, and its structural distribution and morphology. Stahl et al. (1995) say that the most significant source of error in the direct microscopic method of estimating fungal biomass in soil is observer subjectivity which can be minimized with extensive observer training. In this study, the fungal biomass measured showed very low values (10^{-8} g) which would not be detected by others techniques such as ergosterol that have a limited detection (Zhao et al. 2005). Although it may apparently represent a small number, it must be taken in count that coupon has 2.52 cm^2 of area, a very small area if compared to huge pipe surface area in water distribution systems.

In conclusion, the use of a flow chamber reactor and specific fluorescent dye allowed the detection of filamentous fungal biofilm. Moreover, the systems provided information in fungal adherence and grow under monitored conditions and fungal biofilm formation could be related with parameters such as free chlorine levels.

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Chapter 6

Biofilm kinetics of filamentous fungi recovered from water biofilms

6.1 Introduction

Filamentous fungal have been frequently isolated from aquatic environments such as rivers, streams, lakes and sea (Wurzbacher et al. 2010). Water distribution systems (WDS) are nowadays seen as complex aquatic environments in which high diverse microorganisms cohabit (Szewzyk et al. 2000). Regardless of their importance for human health, little is known about their microbial ecology. Since WDS are large, complex highly interconnected and dynamic, with variable hydraulics, input sources and behavior (Deines et al. 2010), quality water supply has become a great challenge for governmental agencies and water industry. The focuses of microbial water quality studies still remain on monitoring planktonic microorganisms, despite scientists' awareness that in water systems the majority of microorganisms live together as biofilms (Costerton et al. 1987).

Research in filamentous fungi biofilm in water distribution systems has only recently received attention. Most of previous mycological studies are focused on pathogenic fungi, e.g. *Candida* spp. (Chandra et al. 2001; Douglas 2003; Williams et al. 2011) and *Aspergillus fumigatus* (Mowat et al. 2008; Bruns et al. 2010; Müller et al. 2011). Since increased resistance against antimicrobials is a very well-known and clinically relevant biofilm feature, these studies have especially established suitable methods for antimicrobial biofilm susceptibility assay (Kuhn et al. 2002; Harriott and Noverr 2009; Silva et al. 2010). Recently, Ramage et al. (2011) reported the importance of fungal biofilm phenotype concept in medical and industrial mycological research. These authors described schematically *Aspergillus* biofilms development (Figure 6.1) and discussed morphological, physiological and molecular features related to both fungal virulence and enzymatic production.

On the other hand, others investigations have reported the detection of filamentous fungal within biofilms in different habitats such as rocks (Gorbushina 2007), fuel tanks (Srivastava et al. 2006) historic monuments (Gorbushina et al. 2004; Grbić et al. 2010; Lan et al. 2010) and acid mine drainage (Baker et al. 2009). Nonetheless, the improvement and standardization of suitable methods for laboratorial studies of filamentous fungi biofilms are still needed.

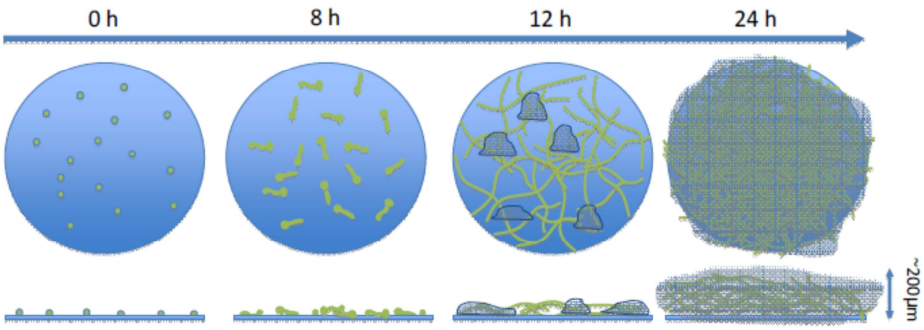

Figure 6.1 *Aspergillus fumigatus* biofilm development: initial adhesion of conidia, germling formation, a monolayer of intertwined hyphae and mature 3-D filamentous biomass encased within EPS (Adapted from Ramage et al. 2011).

The objective of this work is to investigate the capability of biofilm formation and characterise morphologically and physiologically the biofilms of six fungal species isolated from biofilms in a water system.

6.2 Material and Methods

6.2.1 Fungal isolates

Aspergillus sp. 1 (section Nigri), *Aspergillus* sp. 2 (section Flavi), *Alternaria* sp., *Botrytis* sp., *Cladosporium* sp. and *Penicillium* sp., were used in this study. These strains were recovered from biofilms developed in a flow chamber reactor set up at Mycological Laboratory of the Biological Engineering Centre, University of Minho, Braga, Portugal, during a previous study. The fungi were maintained in MEA (MEA: malt extract 20 g, peptone 5 g, agar 20 g, distilled water 1 l) at 25 °C.

6.2.2 Biofilm formation

Spores of each strain were harvested from 7 days aged pure culture in MEA by adding 2 ml of saline solution (0.85 %) to plate. The spore suspension was re-suspended and vortexed before quantification. The suspensions were standardized by dilution with saline solution (0.85 %) to a final concentration of 10^5 spores/ml and using a Neubauer counter chamber.

For biofilm kinetics, 100 µl of spore suspension and 100 µl of culture media MEBroth (MEB: malt extract 20 g, peptone 5 g, distilled water 1 l), R2A Broth (proteose peptone 0.5 g;

casamino acids 0.5 g; yeast extract 0.5 g; Dextrose 0.5 g; soluble starch 0.5 g; dipotassium phosphate 0.3 g, magnesium sulphate $7H_2O$ 0.05 g; sodium pyruvate 0.3 g; distilied water 1 l; pH 7.2) and sterilised tap water were added per well into 96-well, flat-bottomed polystyrene microtiter plates. Media-only blanks were also set up. The plates were incubated at 30 °C for 24 h and analyses were made at 4, 8, 12 and 24h.

6.2.3 Biofilm kinetics

Biofilm quantification

Biofilm biomass was assessed using a protocol described by Mowat et al. 2007. Briefly, at each time interval (i.e. 4, 8, 12 and 24 h), the spent culture medium was removed from each well and the adherent cells were washed with phosphate-buffered saline (PBS; 0.1 M, pH 7.2). These were air-dried and 200 µl of 0.5% (w/v) crystal violet solution was added for 30 min. The solution was then removed until excess stain was removed. The biofilms were distained by adding 200 µl 95 % ethanol to each well. The ethanol was gently pipetted to completely solubilize the crystal violet for 1 min, the ethanol was transferred to a clean 96-well microtitre plate and the OD_{570} was read using a microtiter plate reader (Model Synergy HT; BIO-TEK). The OD values are proportional to the quantity of biofilm biomass, which comprises hyphae and extracellular polymeric material (the greater the quantity of biological material, the higher the level of staining and absorbance).

MTT assay

MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) is a yellow soluble tetrazolium salt that is converted into a insoluble purple crystal by metabolically active cells. MTT (Sigma) solution was aseptically prepared by dissolving the MTT powder at a concentration of 5 mg/ml in sterile PBS at room temperature and stored at 4 °C in a dark, screw-cap container. At each time point, 0.2 mL of MTT solution were added to each well and the 96-well plates were incubated at 37 °C during 3 h. After this period, the supernatant was then discarded and 0.2 ml dimethyl sulfoxide (DMSO) was added to each well to solubilize the MTT, which had been cleaved into an insoluble purple formazan through the metabolism of the live cells. Biofilm development was assayed by loading 0.2 ml of the solubilized MTT into a flat-bottom, 96-well polyvinyl chloride microtitre plate, and absorbance measured at OD_{570} nm using a microtiter plate reader (Model Synergy HT; BIO-TEK).

6.3 Results

Biofilm formation by *Aspergillus* sp. 1, *Aspergillus* sp. 2, *Botrytis* sp., *Alternaria* sp., *Cladosporium* sp. and *Penicillium* sp. grown in different culture media over 24 h was characterised, as revealed by both the colorimetric MTT assay and Cristal Violet (CV) biomass estimation. Figure 6.2 shows the mean value of absorbance level for CV staining (total biofilm), and Figure 6.3 shows the mean value of absorbance level for MTT staining (viable cells). Additionally, image analyses were used to assess morphological characteristics (Figures 6.4 – 6.9).

A direct relation was observed between biomass (CV), and biofilm development (Figures 6.2 and 6.4 – 6.9). On the other hand, it was not observed a direct relation between biomass (CV) and viable cells (MTT) (Figure 6.2 and 6.3). The time when adherence of spores, monolayer development and EPS production were observed for each fungus biofilm are described in Table 6.1.

Table 6.1 Time of detection of spore adherence, monolayer and EPS production in fungal biofilms grown in different culture media.

Fungus	**Water**			**R2A**			**MEB**		
	Ad	Mn	EPS	Ad	Mn	EPS	Ad	Mn	EPS
Aspergillus sp. 1	-	-	-	4 h	8 h	24 h	< 4 h	4 h	8 h
Aspergillus sp. 2	-	-	-	4 h	8 h	24 h	< 4 h	4 h	8 h
Alternaria sp.	4 h	8 h	24 h	4 h	8 h	24 h	< 4 h	4 h	8 h
Botrytis sp.	-	24 h	-	4 h	8 h	24 h	< 4 h	4 h	8 h
Cladosporium sp.	-	-	-	8 h	24 h	*	4 h	8 h	12 h
Penicillium sp.	24 h	-	-	8 h	24 h	*	< 4 h	4 h	8 h

Ad: Adherence; Mn: Monolayer; EPS: extracellular polymeric substances

- no biofilm formation; * not detected

In water, biofilms showed a higher cell activity in the first 4 h of analyses (MTT), and lower along time (Figure 6.3A). In adverse conditions, spores may enter in a dormancy status with lower cells metabolic activity.

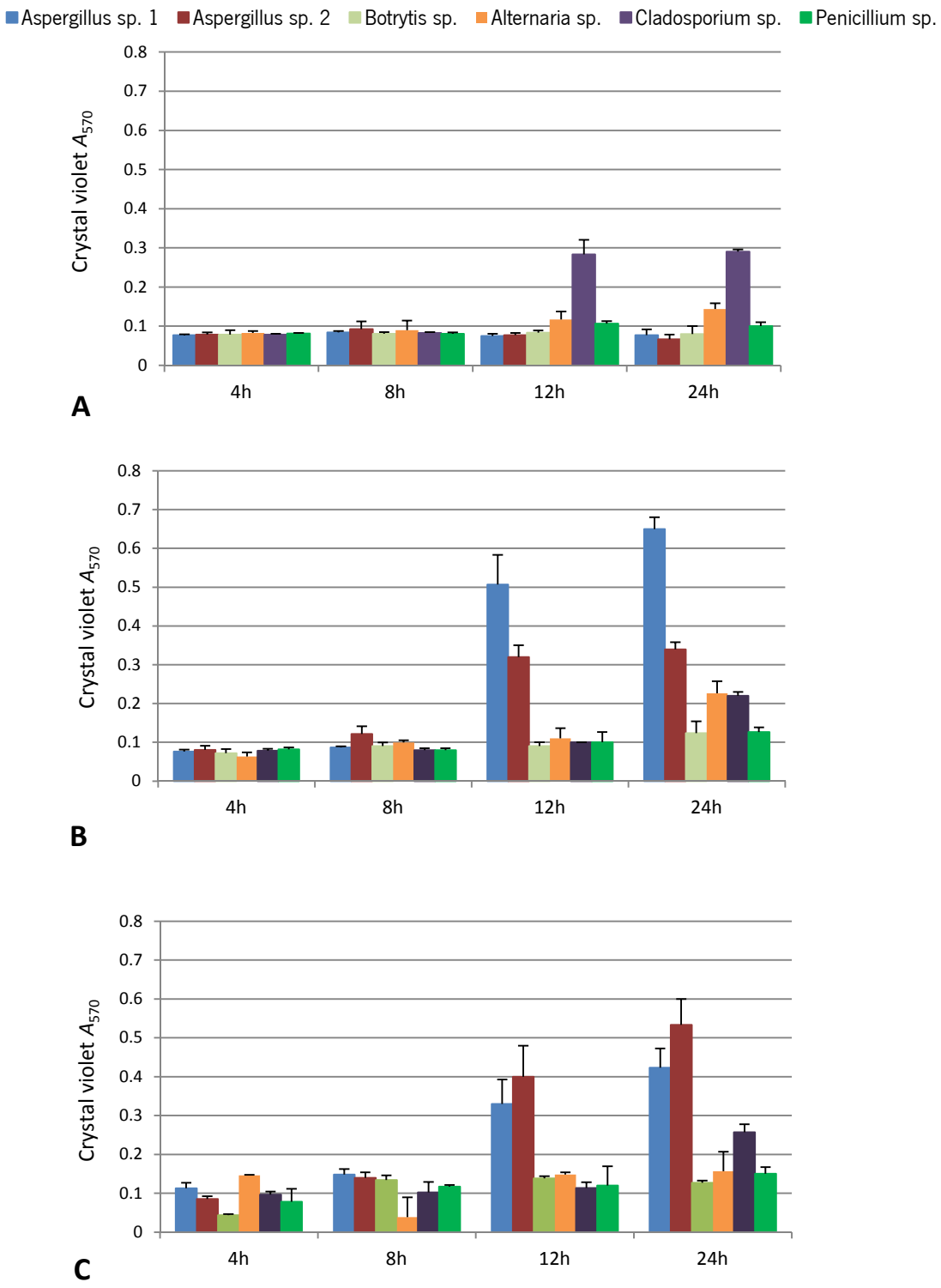

Figure 6.2 Total biomass (CV) in filamentous fungal biofilm grown in different culture media: A – water, B – R2A and C – MEB. The means ± standard deviations for at least three replicates are illustrated.

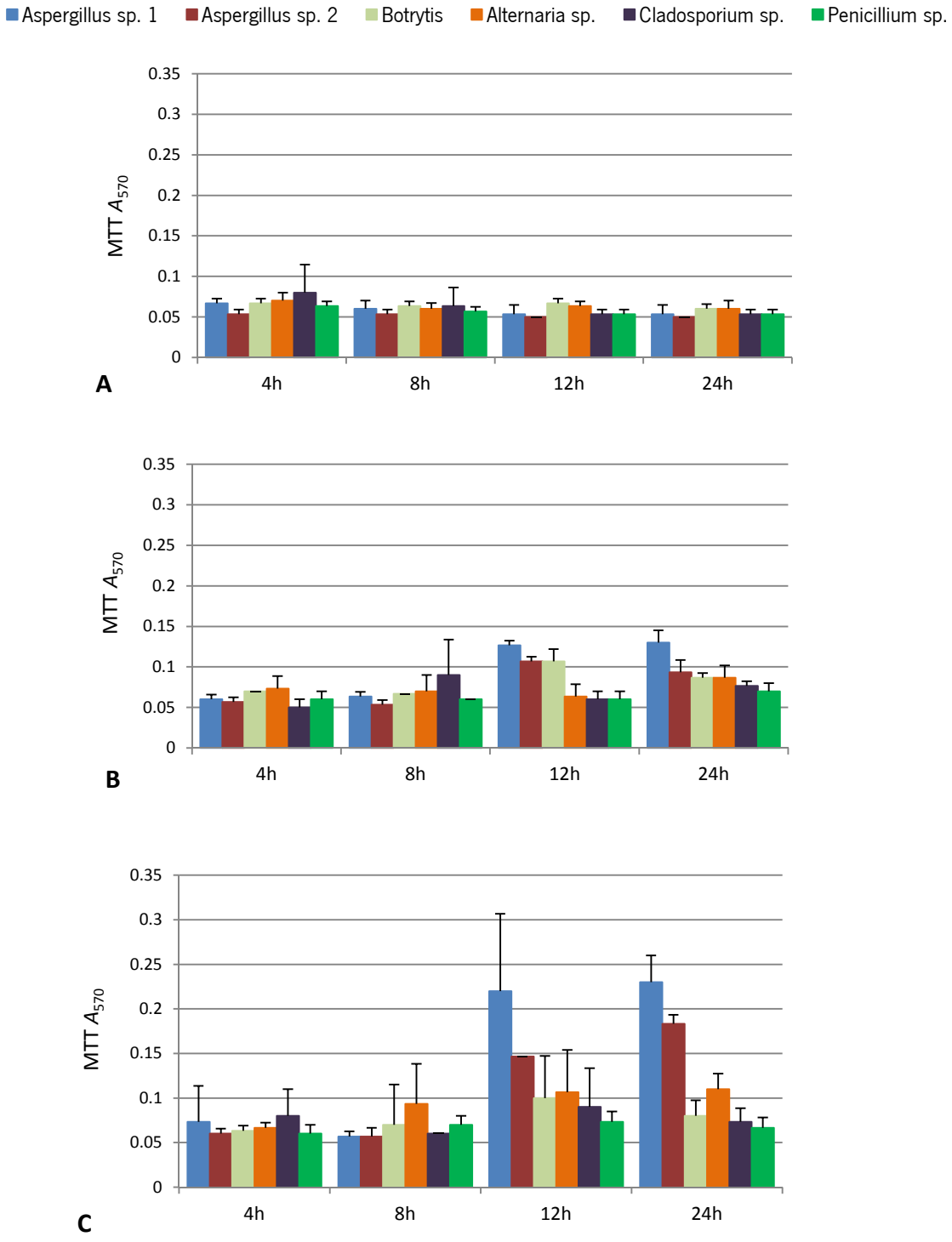

Figure 6.3 Total viable cells (MTT) in filamentous fungal biofilm grown in different culture media: A – water, B – R2A and C – MEB. The means ± standard deviations for at least three replicates are illustrated.

Different pattern of biofilm development were observed between the culture media and fungi. *Alternaria* sp. and *Botrytis* sp. were the only fungi that formed biofilm in water and

Penicillium sp. showed spore adherence after 24 h under this same condition (Figure 6.4 and 6.5). In R2A, biofilms were formed mainly after 12 h and EPS production was observed in both *Aspergillus* sp., *Alternaria* sp. and *Botrytis* sp. biofilms at 24 h (Figure 6.6 and 6.7).

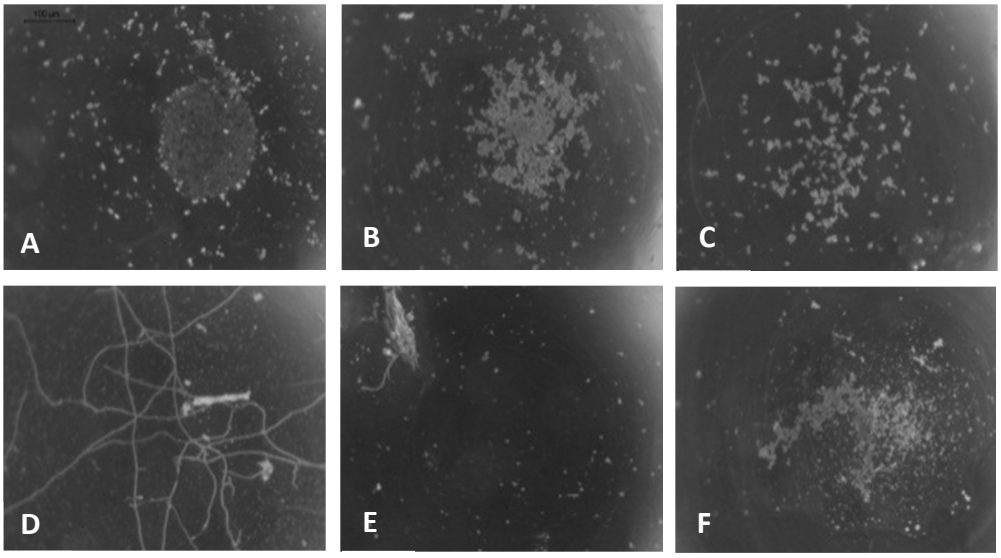

Figure 6.4 *Aspergillus* sp. 1 (A), *Aspergillus* sp. 2 (B), *Botrytis* sp. (C), *Alternaria* sp. (D), *Cladosporium* sp. (E) and *Penicillium* sp. (F) after 8 h in water.

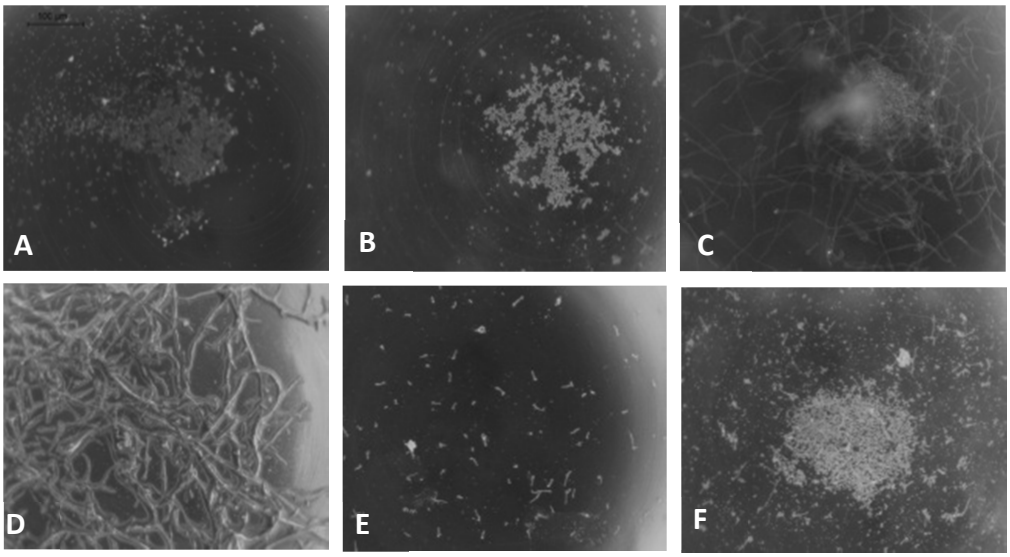

Figure 6.5 *Aspergillus* sp. 1 (A), *Aspergillus* sp. 2 (B), *Botrytis* sp. (C), *Alternaria* sp. (D), *Cladosporium* sp. (E) and *Penicillium* sp. (F) after 24 h in water.

In MEB, after 8 h, the fungi formed mature biofilm (Figures 6.8 and 6.9), with the exception of *Cladosporium* sp. that showed mature biofilm in MEB after 12 h. MEB showed to be the best culture medium for biofilm formation, i.e. in which biofilm grew faster and showed a very well structured shape.

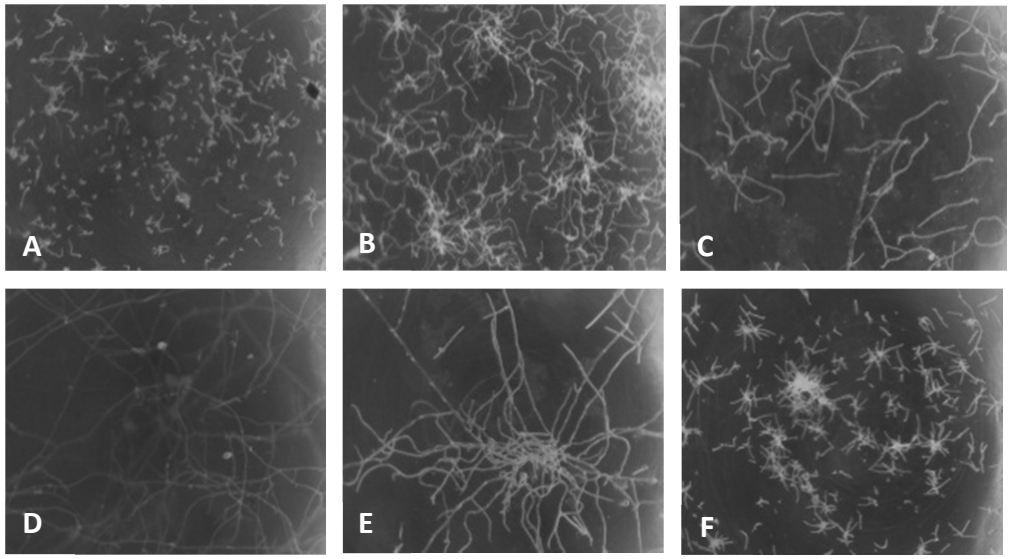

Figure 6.6 *Aspergillus* sp. 1 (A), *Aspergillus* sp. 2 (B), *Botrytis* sp. (C), *Alternaria* sp. (D), *Cladosporium* sp. (E) and *Penicillium* sp. (F) after 8 h in R2A Broth.

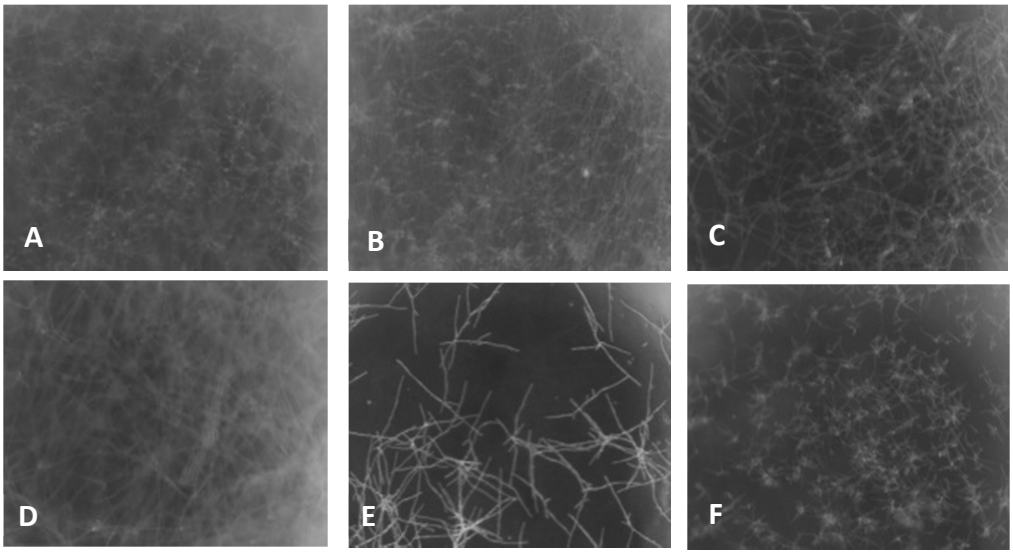

Figure 6.7 *Aspergillus* sp. 1 (A), *Aspergillus* sp. 2 (B), *Botrytis* sp. (C), *Alternaria* sp. (D), *Cladosporium* sp. (E) and *Penicillium* sp. (F) after 24 h in R2A Broth.

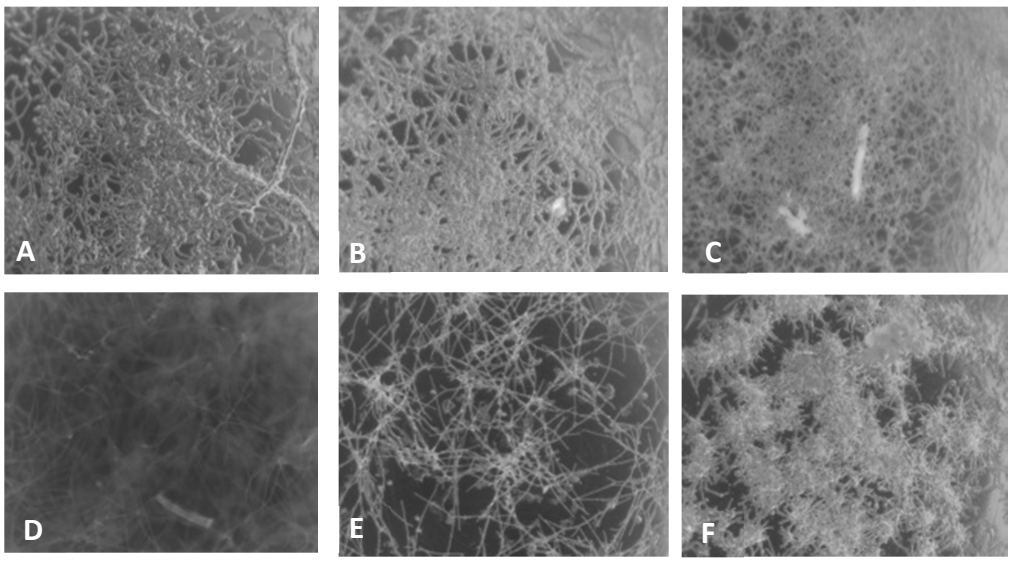

Figure 6.8 *Aspergillus* sp. 1 (A), *Aspergillus* sp. 2 (B), *Botrytis* sp. (C), *Alternaria* sp. (D), *Cladosporium* sp. (E) and *Penicillium* sp. (F) after 8 h in MEB.

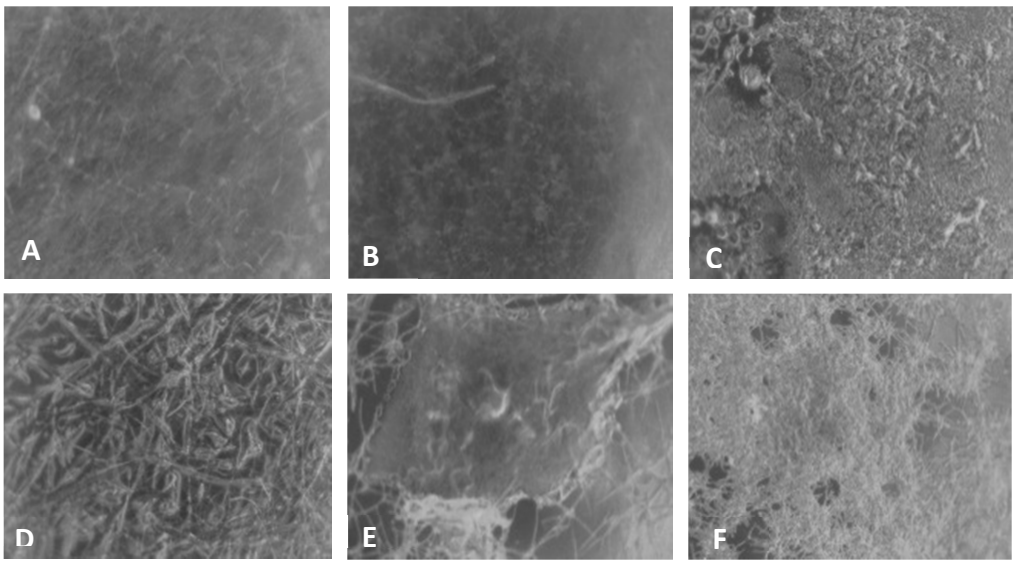

Figure 6.9 *Aspergillus* sp. 1 (A), *Aspergillus* sp. 2 (B), *Botrytis* sp. (C), *Alternaria* sp. (D), *Cladosporium* sp. (E) and *Penicillium* sp. (F) after 24 h in MEB.

6.4 Discussion

The biofilm formation pattern observed in this work resembled the kinetics of biofilm formation of *Aspergillus fumigatus* recently proposed by Ramage et al. (2011) (Figure 6.1).

Previously, Harding et al. (2009) proposed a model for filamentous fungal biofilms which includes five stages: (i) propagule adsorption, (ii) active attachment to a surface, (iii) microcolony formation I, (iv) microcolony formation II, (v) maturation or reproductive development and (vi) dispersal or planktonic phase. In this work, was not observed reproductive development, e.g.

production of sporangia and spores over 24 h of growth. However, the dispersal phase can be represented by hyphal fragments which also act as dispersal propagules.

Despite this model has been based on bacterial and yeast models, it is important to highlight that unique features related to fungal biology distinguish bacterial from fungal biofilm formation: fungi commonly have more than one planktonic form (i.e. sexual and asexual spores, sporangia and hyphal fragments), these dispersive forms are not unicellular and often float in water and air as well, and the development of specialized reproductive tissues to produce dispersive forms (Harding et al. 2009). Moreover, hyphae growth is characterised by two or three dimensions which is achieved by branching. Branch formation provides a greater regulation growth while bacterial unicellular growth is characterised by colonies which are formed by cells pilling and pushing each other apart (Prosser 1983). Thus, studies in filamentous fungal biofilm must take in consideration these specific fungal features which leads to a specific way of how biofilm analyses should be carried on.

Typical filamentous fungal biofilm morphology is described as a complex three dimensional structure with cells usually enclosed within an extracellular matrix consisting of polymeric substances (EPS) (Stoodley et al. 2002). Mainly after 12 h of biofilm growth, was observed a layer of substances surrounding fungal hyphae, i.e. EPS. EPS can represent 50-90% of the total organic matter of biofilms and are responsible for binding cells and other particulate materials together (cohesion) and to the surface (adhesion), i.e. providing the structural support for the biofilm maturation (Allison 2003).

In previous studies, the spore density in the initial spore suspension was verified as a key factor in the development of biofilms by filamentous fungi (Mowat et al. 2007). Singh et al. (2011) found that for *Rhizopus oryzae, Lichtheimia corymbifera,* and *Rhizomucor pusillus* the initial inoculum play a key role in germination of the adhered spores as well as structural integrity of the biofilms formed, a phenomenon also described in studies in *Aspergillus niger* (Villena & Gutierrez-Correa, 2006) and *A. fumigatus* biofilms (Ramage et al. 2011).

Bonaventura et al. (2006) selected an inoculum size of 10^5 CFU/ml, an adhesion time of 1 h, and a biofilm formation time of 72 h as optimal experimental conditions for growing *Trichosporon asahii* biofilm on polystyrene surfaces. Our findings showed that time for spore adherence and further biofilm development varied for each fungus under different culture conditions. Nonetheless, spore characteristics such as size and surface proprieties may also influence biofilm kinetics.

Airborne fungal spores have a hydrophobic surface which aids dispersal, prevents desiccation and may provide a barrier to the entry of toxicants (Laseter et al. 1968). Many studies report that fungal spore hydrophobicity influences their capacity to adhere to biological surfaces, and that spore hydrophobicity is significantly influenced by culture conditions (Singh et al. 2004). Fungal cultures in culture media which have high nutrient content are likely to produce more hydrophobic spores; the opposite is found when culture media with low nutrient content are used (Holder et al. 2007). As spore hydrophobicity may interfere in spore adhesion and consequently in biofilm formation, attention must be paid on previous culture conditions. Nonetheless, surface hydrophobicity of substrate may also interfere in spore adhesion. Thus, optimal condition for filamentous fungal biofilm characterisation may significantly vary between different fungal species.

The colorimetric method using the dye MTT has been cogitated as an alternative to traditional methods for in vitro susceptibility testing of fungi (Meletiadis et al. 2000) and has been applied in fungal biofilms (Jahn et al. 1996; Krom et al. 2007). In this work, MTT values did not correlated with biofilm biomass assessed by CV, mainly in older biofilms, i.e. 24 h aged. Older biofilms showed a higher EPS production, which may interfere in the assessment of MTT to cell. Freimoser et al. (1999) demonstrated that MTT method serve as a measure for cell densities of the entomopathogenic fungi *Neozygites parvispora* and *Entomophthora thripidum* and emphasized that for this two fungi the incubation period had to be longer (i.e. 16 h) because the cell wall might act as an additional barrier for the uptake of MTT. Thus, MTT may not be the best method to evaluate cell viability in mature biofilms.

In conclusion, the results present here show that the filamentous fungi studied in this work are able to form biofilms under the applied conditions. Although each fungus presented a different pattern of biofilm development, spore adhesion, monolayer and EPS production were observed in all fungal species over 24 h of analyses. Moreover, characteristics of spores and culture conditions may play an important role in filamentous fungal biofilm kinetics and must be taken into consideration for further studies in this area.

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Chapter 7

Filamentous fungal biofilm detection and sampler implementation in a water distribution system, *Alto do Céu*, Recife, Brazil

7.1 Introduction

Aquatic environments are vast and biodiverse. Microorganisms occupy particular niches in rivers, streams, lakes and sea. Filamentous fungi have been known for centuries in such environments. For example, in the (a) the Middle Ages aquatic fungi were recognized as parasites of fish, (b) mid-nineteenth century these organisms were observed on several algae and substrates and were referred to as phycomycetes possessing rhizoids and zoospores, and (c) 1940's filamentous fungi were described in running water and were referred to as Ingoldian fungi (Wurzbacher et al. 2010). Indeed, novel fungi continue to be isolated from aquatic environments in breakthrough discoveries relating to interfaces between major life forms, such as the "cryptomycota" (Jones et al. 2011).

Biofilms are functionally-organised microbial communities grown on a surface amidst a matrix of exopolysaccharide (EPS) 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 harbor pathogenic microorganisms (Huq et al. 2008): hence biofilms are correlated with reduced microbial water quality. 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 architecture and microbial composition (Momba et al. 2000). Virus, protozoa, fungi and algae may be incorporated in drinking water biofilms (Momba et al. 2000; Gonçalves et al. 2006; Helmi et al. 2008; Traczeweska and Sitarska 2009; Villanueva et al. 2010) although, generally, bacteria are the dominant component. It is relevant to point out that Taylor et al. (2001) identified 307 fungal species recognised as emerging pathogens and biofilms contained one or more of these species (WHO 2003). Nevertheless, it remains the exception to find valid reports of filamentous fungi in biofilms.

Biofilm filamentous fungi in WDS have been recorded in few studies (Nagy and Olson 1985; Doggett 2000; Kelley et al. 2003; Sammon et al. 2011; Siqueira et al. 2011). However,

researchers have been hampered by non-standard methodology, difficulties in quantification of filamentous fungal, and a lack of mycological expertise compared to that for bacteria. Screening aquatic environments for filamentous fungi using molecular biology is not so developed as for bacteria and conventional cultural techniques suffer drawbacks. For example, it is difficult to determine whether a conidium, conidiophore, or hyphal fragment represents a single fungus (Gonçalves et al. 2006). The use of *ex situ* techniques (e.g. swabbing or scraping) may exclude non-culturable microorganisms and are unsuitable for biofilm structural analysis. Specific dyes and fluorescent microcopy may be useful to overcome some of these problems. However, new methods are required to understand the role played by filamentous fungal biofilms in microbial water quality, as factors influencing filamentous fungal biofilm formation and interactions with other microorganisms remains unknown.

In this study, we describe a device for sampling fungal biofilms which is composed of hollow pipes containing flat coupons. The samplers were developed for use in "real" situations in water treatment stations and WDS. Replaced pipe samples were also studied. Specific fluorescent dyes for fungal detection were employed. This work was carried out in parallel with other investigations which recovered planktonic filamentous fungi along the same WDS and is reported elsewhere (Oliveira 2010).

7.2 Materials and Methods

7.2.1 Water Supply System

The *Alto do Céu* WDS has been operating since 1958 and produces approximately 10% of the total volume distributed in the metropolitan region of Recife, Pernambuco, Brazil. The water treatment plant (WTP) of *Alto do Céu* is designed to treat 1000 l/s but it operates at a 20% overload occasionally. The raw water comes from three pumping stations and is treated by flocculation, sedimentation, filtration, and disinfection (*ca*. 5 ppm chlorine) before leaving the plant. The water is pumped to two storage reservoirs with capacities of 5000 m^3 and 20000 m^3. Only the 20000 m^3 reservoir was used for the present study. The water supply at the metropolitan region of Recife is intermittent and follows a schedule published online by COMPESA (www.compesa.com.br), thus the network is subjected to variable pressure and water flows.

7.2.2 Sampler devices and replaced pipes

Samplers consisted of PVC hollow pipes (1.5 cm diameter x 7-10 cm length). Threads were cut in the ends of each sampler to enable multiple samplers to be attached or a cap to be connected to close the device after removal (Figure 7.1). Coupons of PVC or acetate were inserted inside the pipe intended as surfaces for adhesion of microorganisms and biofilm formation.

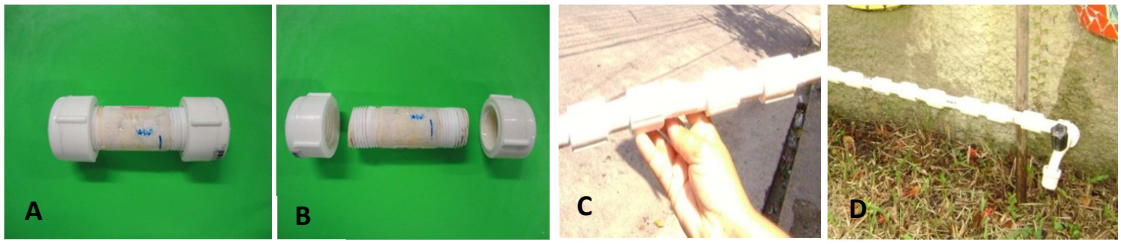

Figure 7.1 Samplers device consisted of hollow PVC pipes and round screwed caps (A and B); Samplers device screwed and set up in place (C and D).

The samplers were installed at different sampling points: (a) the WTP (raw water, decanted water and 20000 m^3 reservoir) and (b) the entrance, middle and exit of the distribution network. The samplers were removed for analyses monthly for 6 months, with a final sample at 12 months. The samplers were refilled with onsite water covered with round screwed caps and sent to the laboratory under refrigeration for further analyses after removal from the network.

A total of 10 replaced pipes samples were randomly collect during technical maintenance procedures in the water distribution network. At the lab, these pipes were cut into small pieces (c.a. 1 cm^2) and storage in PBS solution at 4 °C until analyses. The steps followed for *in situ* analyses are described in Figure 7.2.

7.2.3 *In situ* detection

Calcofluor White M2R (CW) (Molecular Probes Europe, Leiden, the Netherlands) allows the visualization of fungal and other cell walls (e.g. algae) because of its high affinity for β(1-3) and β(1-4) polysaccharides found in cellulose, carboxylated polysaccharides and chitin. CW (25 μM) 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 10x/0.65, 40x/0.30 and 100x/1.3 objectives. 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 (4¹,6-diamidino-2-phenylindole,dihydrochloride) (100 nM/ml) was

added directly to the coupons for 30 min and 25 °C to visualise bacteria. The excitation wavelength for DAPI was 340 nm and the signal was blue.

Morphological characteristics such as (a) presence and regularity of septa, or septa like structures, (b) diameter of filaments, (c) size and shape of cells and (d) shape of sporophores were also assessed to assist in differentiating filamentous fungi, yeast, algae and bacteria.

7.2.4 FUN-1 staining

FUN-1 stains the dead cells with a diffuse yellow-green fluorescence and the metabolically active cells with red Cylindrical Intra-Vacuolar Structures (CIVS). For FUN-1 staining (Molecular Probes, The Netherlands), 15 µl of FUN-1 solution were added on samples, homogenised, following incubation in the dark at 30 °C during 30 min and observed under an Olympus BX51 epifluorescent microscope using UV light as already described.

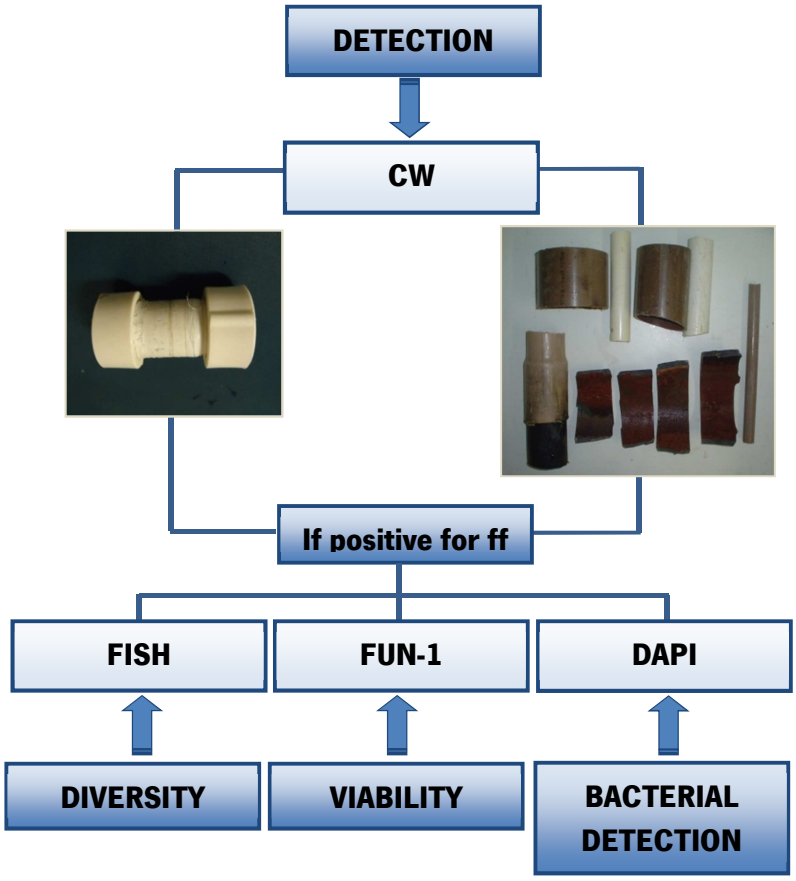

Figure 7.2 Stepwise approach used to study in situ filamentous fungal water biofilms using fluorescent staining techniques.

7.2.5 Fluorescent *in situ* hybridization (FISH)

The protocol for FISH was adapted from Nuovo (1997). Briefly, the 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 NaCl 5M, 40 µl Tris 1M, 300 µl formamide, 130 µl Milli-Q water, 4 µl SDS 10%, into 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 rest of the HB was poured into a Petri dish containing a paper tissue. The samples were hybridized for at least 3 hours at 46 °C in the Petri dish already saturated with HB. After this period, the samples were rinsed with preheated (water bath; 48 °C) wash buffer (WB) (1 ml Tris 1M, 3180 µl NaCl 5M, 50 µl SDS 10%; 49 ml Milli-Q water, in a Falcon tube) and incubated for 20 min. After this period, the samples were gently rinse 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) labelled with the red Cy3 at the 5' terminal was used. For fungi, the FUN1429 probe specific for a wide range of Eumycota (5'-GTGATGTACTCGCTGGCC-3´, MWG Biotech, Ebersberg, Germany) labelled with Oregon-Green at the 5' terminal for FISH was employed (Baschien et al. 2008). The samples were visualized using the Olympus BX51 epifluorescent microscope as above.

7.3 Results

7.3.1 Sampler device

Fungal-like structures on PVC coupon surfaces were observed after 3 months of exposure at the entrance to the WDS after staining with CW (Figure 7.3). None of the others samples at the different points of collection supported recognizable fungal-like structures after 5 months and no samples placed inside the reservoir showed these after 12 months of exposure.

Fungal-like structures were observed in the coupons at different points of the water network after 6 months of exposure (Figure 7.4). Filamentous structures were detected mainly in the samples collected from the beginning of the WDS (Figure 7.4A and B). At the middle and end of the WDS only dispersed fragments were observed (Figure 7.4C-F; red arrows). Once again these fungal-like structures appeared to be on the coupon surface. Highly fluorescing CW surrounding the filaments was observed (Figure 7.4A and B; green arrow).

The coupons exposed to raw and decanted water demonstrated a higher colonization of hyphae, and reproductive structures such as spores (Figures 7.5 - 7.7). Enlargement are provided in Figures 8.5A' and 8.5B', where septate hyphae and germinating spore can be observed.

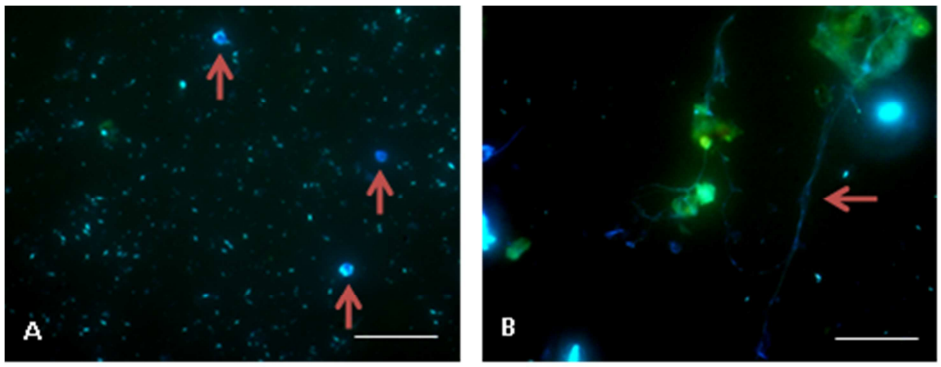

Figure 7.3 PVC coupons after 3 months of exposure and stained with CW. Yeast-like (A) and filamentous fungal-like (B) structures (red arrows). Scale bar = 50 µm.

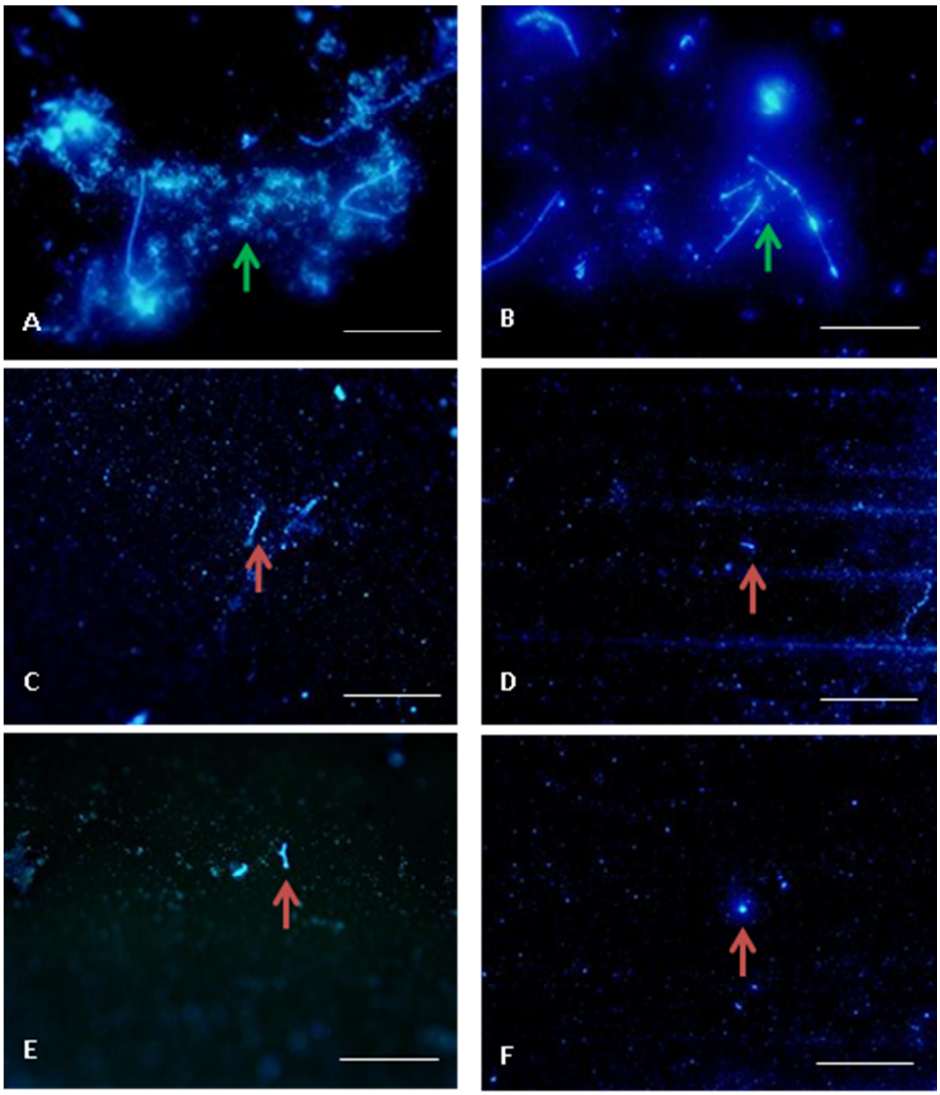

Figure 7.4 Acetate (A, C and E) and PVC (B, D and F) after 7 months of exposure at the beginning (A, B), middle (C, D) and end (E, F) of the WDN. Scale bar = 200 µm.

Figure 7.6 demonstrates a sporing structure which resembles the condiophores produced by some *Alternaria* species. An enlargement is provided in Figure 8.6A'. Filaments without septa were also observed and representing non-septate fungi, or algae which are also stained by CW. Figure 7.7 shows a spiral shaped structure which resembles microalga. In particular, a microalgae that resembled *Spirulina platensis,* was observed (Figure 7.7). Bacterial cocci were detected on fungal hyphae (Figure 7.8).

Another demonstration of filamentous fungi is presented in Figure 7.9. Structures stained with CW and EUK516 also stained with FUN1429 which is specific for the subphylum of fungi, the *Pezizomycotina*.

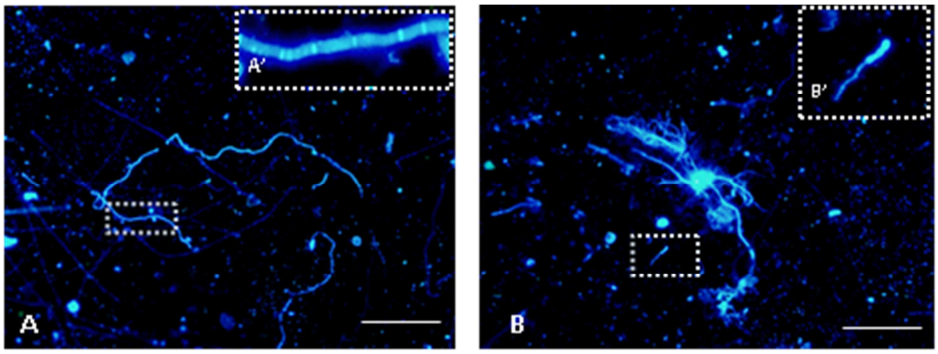

Figure 7.5 Acetate coupon after 12 months of exposure to raw water and stained with CW. Filamentous fungi septate hyphae (detail A') and germinating spore (detail B'). Scale bar = 200 µm.

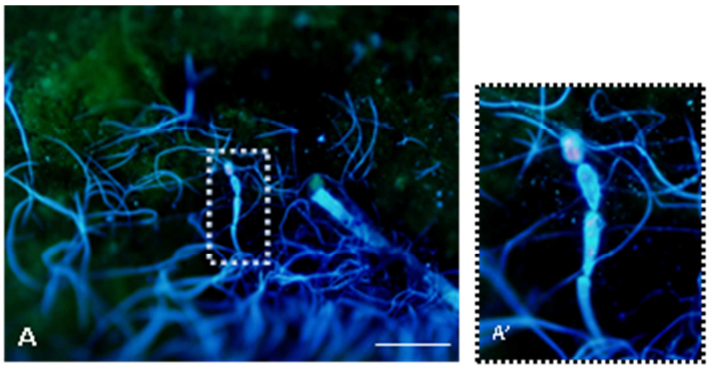

Figure 7.6 Acetate coupon after 12 months of exposure to decanted water and stained with CW. Fungal-like reproductive structure (detail A'). Scale bar = 50 µm.

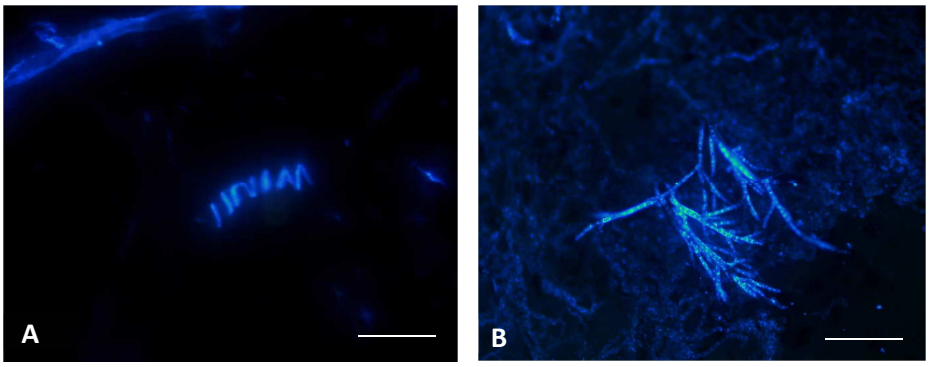

Figure 7.7 Acetate coupon after 12 months of exposure to raw water and stained with CW. Algae-like structures (A and B). Scale bar = 20 µm (A) and 200 µm (B).

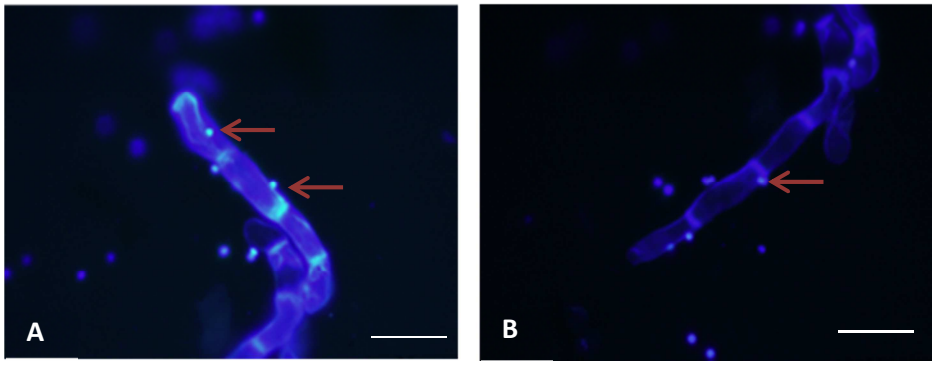

Figure 7.8 Visualization of bacteria (red arrows) nearby and on filamentous fungi hyphae after DAPI and CW staining. Scale bar = 20 µm.

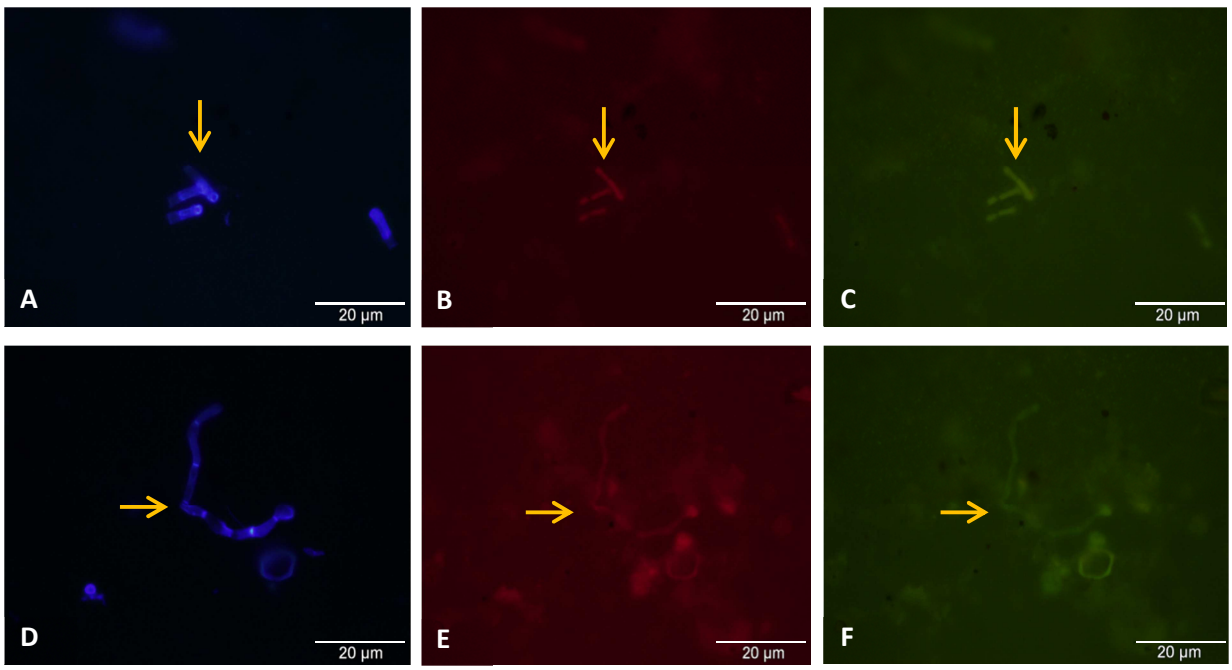

Figure 7.9 Detection of filamentous fungal after CW staining (A and D), and analysis with FISH with EUK516 (B and E) and FUN1429 (C and F) probes. Acetate (A – C) and PVC coupons (D – F).

7.3.2 Replaced pipes

After CW staining filamentous fungal biofilms were detected in 6 of 10 replaced pipes samples (Figure 7.10). Well-developed hyphae tightly adhered to pipe surface and presumptive reproductive structures were also observed (Figure 7.10; yellow narrows).

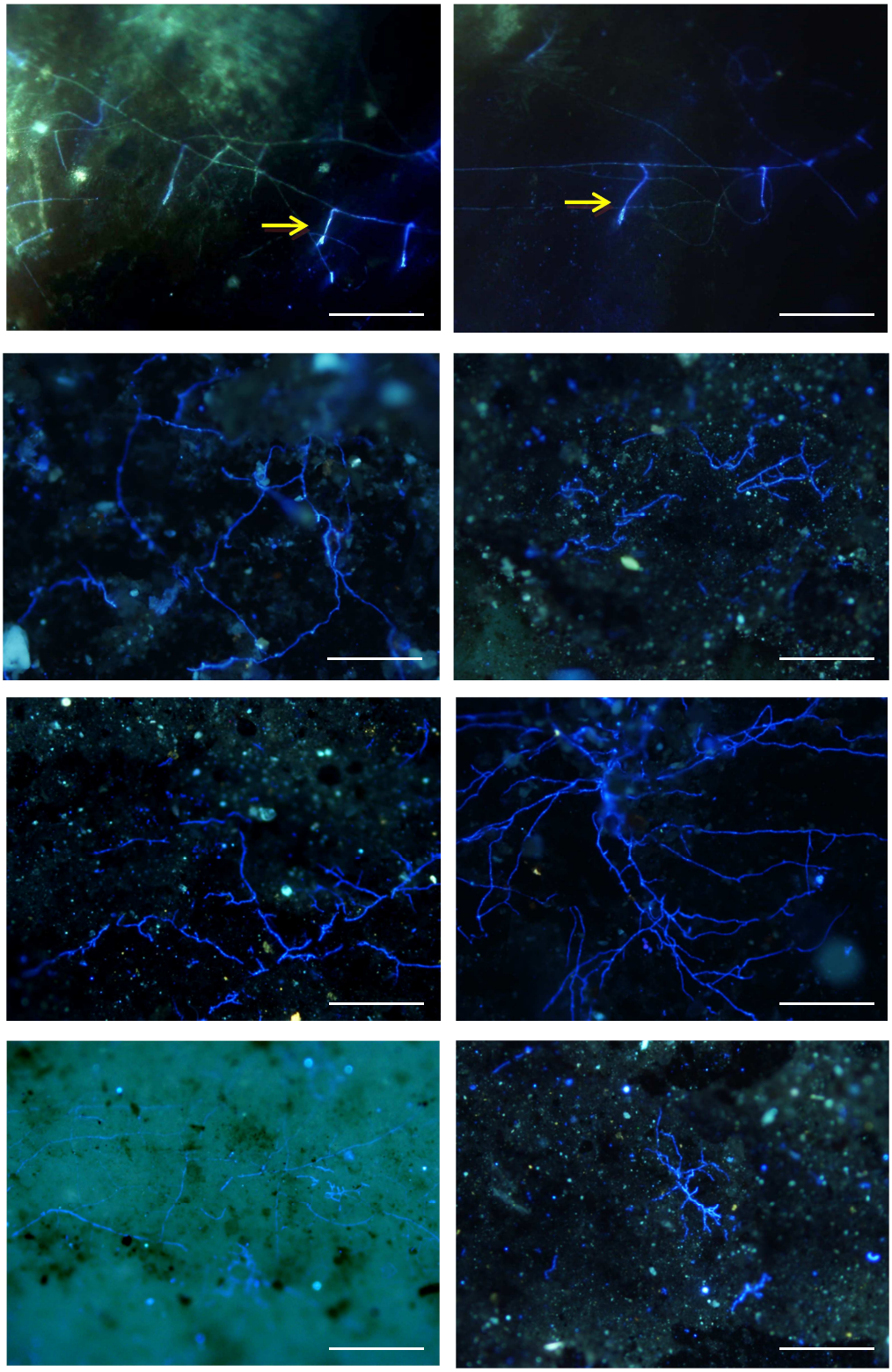

Figure 7.10 Filamentous fungal biofilms detected after CW staining in replaced pipe samples. Scale bar = 200 μm.

Since the real pipes demonstrated filamentous fungi within the biofilm, the stepwise approach, as defined in Figure 7.2, was continued. The FUN-1 staining for viability showed red CIVS inside of the fungal vacuoles (Figure 7.11) demonstrating that the fungi were viable in the biofilm. The CIVS are ATP-dependent which is correlated with fungal viability.

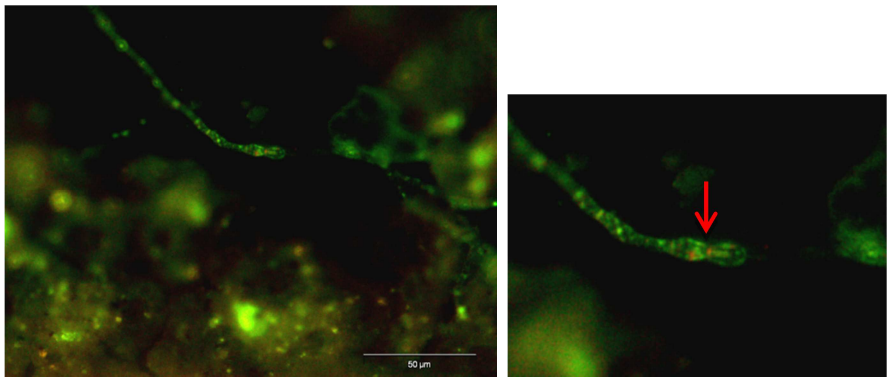

Figure 7.11 Visualization of red CIVS (arrow) and green diffuse hypha after FUN-1 staining biofilm in real replaced pipe samples.

7.4 Discussion

In this present work, filamentous fungi in biofilms were detected throughout a WDS using sampling devices especially developed for the purpose. Fungus-like structures on coupon surfaces were observed after 3 months of exposure; after 12 months filaments and microbial heterogeneity were detected. A fungus resembling *Alternaria* was observed as were unidentified member of the *Pezizomycotina*.

The contents of the samplers were subjected to different conditions as experience in the WDS (e.g. levels of disinfectant, water flow, nutrient availability, pH and temperature). Bacteria, yeasts and algae were also attached to coupon surface representing a diverse microbial community. The organisms were not removed after a washing procedure and may be initiating the first step of biofilm development in which the deposition of spores or other fungal propagules occurs (Harding et al. 2009), although the microorganisms were not tightly adhered to the coupon surfaces.

Sammon et al. (2011) did not detected fungal biofilm growing on PVC, glass or concrete coupons, though diverse fungi were 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). Filamentous fungi were also detected 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 (a) exposed to water flow and oligotrophic conditions for years, and (b) 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 and highly absorbent. The presence of melanin may increase its stability and resistance to adverse conditions (Nosanchuk and Casadevall 2003), thus fungi are often found in anthropogenicly disturbed areas (e.g. industrial and municipal waste water) and in ultraoligotrophic environments such as water distillation apparatus (Wainwright 2005).

The most usual control strategy taken against biofilm accumulation is to maintain a definite concentration of disinfectant residuals. Levels of chlorine varied from 0 up to 4.6 mg/l, decreasing towards the end of the network (Oliveira 2010). 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 is reduced if the biofilm is already present (Lewis 2001; Schwartz et al. 2003; Zhou et al. 2009). For this reason, it is important to ensure sufficient disinfectant along the entire water network in order to control microorganisms in bulk water and biofilm development on the pipe surfaces. Unexpectedly, we detected more fungi attached to coupon surface at the entrance to the water network, i.e. immediately after water treatment, and fewer at the exit of the water network (Figure 8.4); this result may reflect the necessity of a long exposure time for fungal biofilm development and not the effectiveness of chlorine against fungi. Nonetheless, fungi are able to withstand high levels of chlorination (Doggett 2000) and are more resistant when located in biofilms than when they are planktonic conidia (Siqueira and Lima, 2011). Additionally, the chlorine depletion measured at the end of the water network may favour

the development of microbial communities (Codony et al. 2005), and consequently loss of water quality.

Based on morphological characteristics, the presence of algae was also verified during the experiments. *Spirulina platensis* (Figure 7.7) is a blue-green alga found abundantly worldwide in pollution-free, fresh-water environments (Kumar et al. 2011) and was observed on coupon surface. In general, it can be difficult to distinguish between filamentous algae and fungi in circumstances described herein. Some algae can form filaments made from individual cells which gives the appearance of septa although they are often very regularly spaced. Filamentous bacteria have much smaller diameters when compared to fungi what is useful for differentiating between the two organisms. Villanueva et al. (2010) studied river biofilms composed by algae, bacteria and ciliates, and found that biofilm formation was faster at higher temperatures, while nutrient availability influenced the mature biofilm more. The temperature at the water treatment station varied from 23.5 °C up to 25 °C (Oliveira 2010) and may also influence the levels of algae attached to coupons surface.

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, although fungi may have an important role in biofilms that is often overlooked due to the complexity of the investigations. It is well known that fungi produce antibiotics and mycotoxins that kill bacteria although the extent that this occurs in Nature is unclear. In this study we observed bacteria surrounding fungal hyphae (Figure 7.8). 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 do not represent a mature biofilm. However, their importance in forming biofilms must not be underestimated. The relation between fungi and bacteria is unclear and would need different approaches to clarify what kind of interaction occurs. Nonetheless, these current findings highlight the concept of natural biofilms as diverse microbial communities.

Fungi are well-known producers of biologically active secondary metabolites (e.g. antibacterial penicillin, patulin). The role played by these compounds in natural environments is greatly not understood but some evidences indicate that they can act as chemical defenders when fungi interact with others microorganisms or as quorum sensing molecules (Rasmussen et al. 2005). Many of the fungi recovered from drinking water are able to produce mycotoxins (human toxic secondary metabolite) in water (Kelley et al. 2003; Criado et al. 2005; Paterson et al. 2007). Although the amount of mycotoxin in a water distribution system may be very dilute, their concentrations may increase and become a hazard to human health especially when water is stored in cisterns, reservoirs or even in bottles for prolonged periods (Hageskal et al. 2009). Certainly, the presence of *Fusarium* mycotoxins in freshwater systems is becoming recognised as a potential health hazard even at low concentrations (Bucheli et al. 2008; Gromadzka et al. 2009). It is interesting that an *Alternaria* may have been detected in the present study as representatives of these are well-known mycotoxin producers. However, data is unavailable concerning the effects of mycotoxins in microbial interactions within filamentous fungal water biofilms.

FISH has been widely applied as a method for rapid and specific identification of individual microbial cells within their natural environments and have overcome some drawbacks of cultivation dependant methods (Amman et al. 2001; Bottari et al. 2006). However, FISH should not be used as the sole tool to characterize (freshwater) fungal community (Baschien et al. 2008) and morphology must be determined. It is particularly important that conidiophores, or other fungal sporing structures are searched for in biofilms as this gives direct evidence of the presence particular fungal taxa as described herein. Moreover, through the use of species-specific probes it is possible to identify fungal species (Baschien et al. 2008) and potentially mycotoxin producers or opportunistic pathogenic may be able to be detected in the future. 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 with a degree of taxonomic detail.

The use of samplers which allowed analyses of coupons *in situ* is a useful innovation when studying biofilms. CW was a rapid and efficient stain to detect fungi and filamentous fungi which could be differentiated by well-known morphologies. FISH allowed the detection of specific group of eukaryotic microorganism and fungi, and hence confirming their presence. Fungi are

likely to play an important role in microbial interactions within water biofilms and consequently in microbial water quality.

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Chapter 8

Conclusions and Perspectives

8.1 Main conclusions

The choice and application of suitable methods is a main step for studies in filamentous fungal (ff) biofilm. Microscopic and molecular techniques, i.e. fluorescent microscopy together with specific fluorescent dyes (Calcofluor White R2R, DAPI, FUN-1) and probes (EUK516 and FUN1429), are appropriated methods which provide information about biofilm biomass, morphology, structure, physiology and composition. Additionally, the presented sampler device is appropriated for biofilm formation in situ, i.e. exposed under real conditions.

Diverse ff habit drinking water systems and are capable to adhere to surface and form biofilms. Time of exposure and disinfectant level are major factors which influence ff biofilm formation. In both real and laboratorial conditions, ff were capable to survive and form biofilms in the presence of chlorine, disinfectant commonly used in drinking water treatment stations. Thus, ff biofilms represent a refuge for fungi and consequently a source for contaminations in drinking water systems.

Laboratorial ff biofilms are more resistant against disinfection when compared with their planktonic form. Using FUN-1 staining, metabolically active cells were detected after biofilm exposure to high concentrations of free chlorine. Moreover, the use of FUN-1 allows the detection of metabolic active cells within ff biofilm in replaced pipes. FUN-1 is a rapid and reliable technique to access cell viability within fungal biofilms.

Ff water biofilms have different hydrophobicity levels when compared with solid and liquid fungal cultures. Microspheres adhesion assay is a reproducible and simple technique that can be used to directly assess patterns of cell surface hydrophobicity. Using this technique, ff showed different zones of hydrophobicity within the biofilm, a specific feature which can be associated with microbial interactions in aquatic environments.

Ff recovered from water systems are diverse and capable to form biofilms under specific laboratorial condition and exhibit patterns of development which resemble those recently described in literature, i.e. spore adherence, monolayer and EPS production. Nonetheless, among the fungal isolates studied, each one has a different pattern of behaviour which may influence in further implementation of techniques in this area.

Biofilms formed under real conditions, i.e. on pipes and samplers surfaces exposed along a drinking water distribution network, are composed by fungi, algae and bacteria. Although the relationship between the microbial components within biofilms is not yet clarified, these biofilms are complex microbial communities indeed. Ff biofilms are present in replaced pipes collected from a water distribution network. Thus, the development and maintenance of the biofilms in such pipes reflect a high capability of adaptation and resistance to water flow, oligotrophic conditions, and diverse abiotic factors such as temperature, pH and residual disinfectant.

8.2 Perspectives

The results of this work have highlighted several aspects of filamentous fungal biofilms in drinking water systems. Nonetheless, the information provided here is not sufficient to answer all questions which came about along these years of research.

Drinking water systems are very complex environments in which several factors influence their microbial composition. Variation in these factors may lead to very different approaches and new insights in this area. Tanking in count that water systems are everywhere, e.g. houses, hospitals, restaurants, industries, and in a wider view the water systems of cities, the possibilities of further studies using samples from different local are outstanding.

Many of ff such as Penicillium spp. and Aspergillus spp. recovered from water systems are known as mycotoxin producers. As mixed microbial communities biofilms are composed by diverse microorganisms which are constantly in interaction. Further studies are needed to clarify how mycotoxins influence these interactions. In this sense, it is necessity further implementation of methodologies to studies mixed species biofilms, with special focus on bacteria-fungi interactions.

The use of specific probes for Eukaryotes and Fungi provide important information about biofilm composition and, together with Calcofluor and morphological analyses, prove the presence of ff biofilms in the water systems. On the other hand, further studies for the design of more specific fluorescent probes, e.g. for mycotoxigenic and pathogenic fungal species such as Penicillium brevicompactum and Aspergillus fumigatus, will surely add estimated value for research in microbiology of water systems of food industries and hospitals.