Universidade do Minho
Escola de Engenharia

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Water Biofilm Modulation by Fungi

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Water Biofilm Modulation by Fungi

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Trabalho efetuado sob a orientação do
Professor Doutor Nelson Lima

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Abstract

Water is indispensable to life and a safe and accessible supply must be available to all. The presence of microorganisms is a threat to this commitment. Biofilms are the main reservoir of microorganisms inside water distribution systems and they are extremely ecologically diverse. Biofilm formation in drinking water has mainly been studied regarding bacteria, however, filamentous fungi and bacteria can coexist inside these systems forming inter-kingdom biofilms. The ecology of a biofilm is a complex function of different factors, including the presence of microbial metabolites excreted by its inhabitants. The present work aimed to study the ability of filamentous fungi to interact and influence bacteria whilst forming inter-kingdom biofilms. For this purpose, different methods to analyse biofilms were applied, including total biomass, metabolic activity, bacterial colony forming units and epifluorescence microscopy. The study on the effect of quorum sensing molecules and fungal secondary metabolites on inter-kingdom biofilm formation and development was also done. In addition, an RT-qPCR method to quantify a gene involved in the patulin biosynthetic pathway was performed and the expression levels were compared with the patulin production in inter-kingdom biofilms. The results revealed fungal stage development is important in the first 24 h of biofilm formation. Inter-kingdom biofilm formation is microorganism dependent and inter-kingdom biofilms may provide an advantage to the opportunistic bacterium *Acinetobacter calcoaceticus* to replicate and proliferate. *Methylobacterium oryzae* biofilm formation and development was more susceptible to the presence of exogenous molecules than *A. calcoaceticus* biofilms. The effect of *M. oryzae* on *Penicillium expansum* biofilms is dependent on the time of interaction. More mature *P. expansum* biofilms appear to be more resistant to the inhibitory effect that *M. oryzae* causes towards *idh* gene expression and patulin production. *M. oryzae* affects patulin production by acting at the transcriptional level of the *idh* gene. In conclusion, a possible protection role of filamentous fungi towards opportunistic bacteria inside complex biofilms is reported. Moreover, quorum sensing and quenching molecules play a vital role in inter-kingdom communication and/or microbial assembly processes that influence biofilm formation between filamentous fungi and bacteria.

Keywords: Bacteria; biofilms; drinking water; fungi; fungal-bacterial interactions.

Modulação de biofilmes das águas por fungos

Resumo

A água é indispensável à vida e um fornecimento seguro deve estar disponível para todos. A presença de microrganismos é uma ameaça a este compromisso. Os biofilmes são a principal fonte de microrganismos em sistemas de distribuição de água e são ecologicamente muito ricos. A formação de biofilmes em sistemas de água de consumo tem sido estudada principalmente em relação às bactérias, contudo, nestes sistemas, fungos filamentosos e bactérias interagem formando biofilmes mistos. A ecologia de um biofilme é extremamente complexa e é suscetível a diferentes fatores como a presença de metabolitos microbianos excretados pelos seus habitantes. O trabalho aqui apresentado teve como objetivo o estudo da capacidade de fungos filamentosos interagirem e influenciarem bactérias enquanto formam biofilmes mistos. Com este propósito, diferentes métodos de análise de biofilmes foram usados, incluindo biomassa total, atividade metabólica, unidades formadoras de colónias bacterianas, e microscopia de epifluorescência. Foi também realizado o estudo do efeito de moléculas de quórum sensing e metabolitos secundários produzidos por fungos na formação e desenvolvimento de biofilmes mistos. Adicionalmente, foi usado um método de RT-qPCR para quantificar um gene relacionado com a via biossintética da patulina e os valores de expressão relativa foram comparados com a produção de patulina em biofilmes mistos. Os resultados revelaram que o estado de desenvolvimento do fungo filamentoso aquando da inoculação é importante nas primeiras 24 h de formação de biofilme. A formação de biofilmes mistos é dependente dos microrganismos presentes e pode conferir uma vantagem a bactérias oportunistas como a *Acinetobacter calcoaceticus* para se replicar e proliferar. A formação e desenvolvimento de biofilmes da *Methylobacterium oryzae* demonstrou ser mais suscetível à presença de moléculas exógenas do que os biofilmes da *A. calcoaceticus*. O efeito da *M. oryzae* em biofilmes do *Penicillium expansum* é dependente do momento de interação. Biofilmes fúngicos mais maduros parecem ser mais resistentes ao efeito inibitório que a *M. oryzae* causa na expressão do gene *idh* e na produção de patulina. Esta bactéria afeta a produção de patulina atuando ao nível transcricional do gene *idh*. Em conclusão, é aqui reportado um possível efeito protetor que o fungo filamentoso confere a bactérias oportunistas em biofilmes mistos. Além disso, a presença de moléculas de quórum sensing e de metabolitos secundários desempenham um papel fundamental por parte dos diferentes microrganismos em biofilmes mistos, que resultam na regulação de processos que levam à sua formação e estabelecimento.

Palavras-chave: Água de consumo; bactérias; biofilmes; fungos; interações fungo-bacterianas.

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General Introduction

1. General overview

Water is indispensable to life. Therefore, every effort should be done to achieve drinking water as safe as possible. The United Nations Sustainable Development Goal 6 (SDG 6) reinforces that "while substantial progress has been made in increasing access to clean drinking water and sanitation, billions of people – mostly in rural areas still lack these basic services. Worldwide, one in three people do not have access to safe drinking water, two out of five people do not have a basic hand-washing facility with soap and water". Clean water is still a luxury for poor regions. For this reason, water companies have the main goal of delivering microbiological safe water to the consumers, adequate in quantity and delivery pressure and satisfactory in terms of taste, odour, and appearance. This objective can be questioned when microorganisms are present in excess as their growth may affect the organoleptic properties of the water. These microorganisms can be found either as in planktonic forms inhabiting bulk water or as biofilms growing on pipes surfaces. Biofilms can be considered the main source of microorganisms in drinking water distributions systems and are responsible for serious effects, such as changes in the taste, turbidity, colour and odour of the water, corrosion of metallic pipes, disinfectant demand, potential accumulation and dispersion of pathogens and production of toxins. Primary and opportunistic pathogens are found in drinking water distribution systems because they can survive water disinfection. Special protection is provided to microorganisms embedded in biofilms such as sharing of nutrients and metabolic products and increased resistance to environmental stresses, such as hydrodynamic shear forces and disinfection.

While the term "biofilm" has been rarely applied to filamentous fungi, there has been indication that they grow as biofilms in diverse environmental, medical, and industrial. This is due to their high ability for growing on surfaces, as shown by their absorptive nutrition mode, secretion of extracellular enzymes to digest complex molecules, and apical hyphal growth

Under natural conditions, true monospecies biofilms are rare, and consequently, they are usually considered as complex communities. The ecology of a biofilm is a complex function of prevailing growth conditions, hydrodynamic shear forces and presence of microbial metabolites and molecules, such as cell-cell signalling communication molecules, excreted by its inhabitants. This diversity leads to a variety of complex interactions between the microorganisms that are present. Findings into the microbial ecology

of DWDS have shown that resistance of microorganisms to disinfectants, particularly chlorine, is affected by this microbial diversity. This information, has, however, been mainly obtained from studies with bacteria. Studies regarding fungi, in particular filamentous fungi, have been gaining attention due to their ability to develop biofilms, however, their interaction with bacteria in inter-kingdom biofilms is still poorly understood.

Quorum sensing (QS) is a mechanism employed by microbial species to coordinate community behaviour. It allows perception of population density by the production, release, and detection of small signalling communication molecules which in turn modify bacterial gene expression. In Gram-negative bacteria, the primary signalling molecules involved in this mechanism are *N*-acyl homoserine lactones. QS controls and regulates different bacterial population density-dependent processes, including, biofilm formation, stress resistance, production of toxins and secondary metabolites and pathogenicity. In contrast, eukaryotes have the ability to interfere with bacterial communication by producing molecular signals that interact with bacterial QS. These compounds are called quorum sensing inhibitors (QSI). As filamentous fungi coexist with bacteria and do not have active immune systems, they must rely instead on chemical defence mechanisms. Fungal secondary metabolites (mycotoxins) are now being considered as important players in ecological settings allowing the fungus to secure its environmental niche by providing protection against other microbes. The understanding of the mechanisms of microbial growth in drinking water distribution systems like the microbial ecology, specific mechanisms of adhesion, intra and interspecies interactions and the production of signalling molecules and other metabolites, will continue to provide needed insights to help resolve public health concerns associated with the biofilm formation on these systems.

2. Objectives

The objectives of the present work are:

1. To clarify, under laboratory conditions, the role of filamentous fungi in water biofilms through characterization of inter-kingdom biofilm formation with bacteria found in drinking water distribution systems, including the influence of fungal stage development.

2. To evaluate the ability of the fungi to produce quorum sensing inhibitors.

3. To study the effects of signalling molecules and fungal secondary metabolites on inter-kingdom biofilm formation between filamentous fungi and bacteria.

4. To investigate the effect of Gram-negative bacteria on the expression levels of mycotoxin-related genes and mycotoxin production on filamentous fungi biofilms.

3. Thesis layout

This PhD Thesis is divided in five chapters:

Chapter 1 is composed by a literature review including relevant and up to date references overviewing different key topics related to biofilms in drinking water distribution systems. These include biofilms in drinking water, filamentous fungal biofilm formation, the occurrence of filamentous fungi in drinking water distribution systems and the interaction between bacteria and filamentous fungi inside biofilms. The following chapters are composed by the results achieved with the practical experiments using laboratorial biofilms and their respective discussion with current literature.

Chapter 2 assesses and characterizes the ability of filamentous fungi and bacteria to form inter-kingdom biofilms. Biofilms were analysed in terms of total biomass, metabolic activity, bacterial colony forming units and morphology by epifluorescence microscopy. The quantitative methods revealed that biofilm mass increased over time for both single and inter-kingdom biofilms while specific metabolic activity decreased, in general, along the time points evaluated. Microscopic data visually confirmed the biofilm mass increase over time. Fungal stage development is important in the first 24 h of biofilm formation. Inter-kingdom biofilm formation is microorganism dependent and inter-kingdom biofilms may provide an advantage to the opportunistic bacterium *Acinetobacter calcoaceticus* to replicate and proliferate when compared with *Methylobacterium oryzae*.

Chapter 3 reports the effect of patulin, and *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL) on inter-kingdom biofilm formation between a filamentous fungus and bacteria isolated from drinking water. The filamentous fungus *Penicillium expansum* and the bacteria *A. calcoaceticus* and *M. oryzae* were used as model species. *M. oryzae* biofilm formation and development was more susceptible to the presence of the molecules than *A. calcoaceticus* biofilms. Patulin reduced *M. oryzae* biofilm growth while 3-oxo-C_{12}-HSL caused an increase after 48 hours. The presence of *P. expansum* had a detrimental effect

towards *M. oryzae* cell numbers, while an advantageous effect was observed regarding *A. calcoaceticus.* The overall results reveal that quorum sensing and quenching molecules have a significant effect in inter-kingdom biofilm formation and especially on bacterial numbers.

Chapter 4 reports the effect of the Gram-negative bacteria *M. oryzae* on the *idh* gene expression levels and patulin production of *P. expansum* mature biofilms. For this purpose, an RT-qPCR method to quantify *idh* mRNA levels was done. In addition, the *idh* expression levels were compared with the patulin production. The results obtained reveal that the effect of the bacterium on pre-established *P. expansum* biofilms is dependent on the time of interaction. More mature *P. expansum* biofilms appear to be more resistant to the inhibitory effect that *M. oryzae* causes towards *idh* gene expression and patulin production. This is a result of the increased concentration of patulin and reciprocates an inhibitory effect of *P. expansum* towards *M. oryzae*. A trend was observed between the *idh* expression and patulin production values. The results indicate that *M. oryzae* affects patulin production by acting at the transcriptional level of the *idh* gene.

Chapter 5 includes general conclusions and perspectives.

4. Scientific output

Part of the results presented in this thesis has been published elsewhere.

4.1 Published papers in peer reviewed international journals

Afonso TB, Simões LC, Lima N. 2021. Occurrence of filamentous fungi in drinking water: their role on fungal-bacterial biofilm formation. Research in Microbiology (In Press).

Afonso TB, Simões LC, Lima N. 2020. Effect of quorum sensing and quenching molecules on inter-kingdom biofilm formation by *Penicillium expansum* and bacteria. Biofouling, 36: 965-976.

Afonso TB, Simões LC, Lima N. 2019. *In vitro* assessment of inter-kingdom biofilm formation by bacteria and filamentous fungi isolated from a drinking water distribution system. Biofouling 35: 1041-1054.

4.2 Poster presentations in international conferences

Afonso TB, Simões LC, Lima N. 2019. *In vitro* effect of cell-cell signalling molecules on interkingdom biofilms between bacteria and filamentous fungi isolated from a DWDS. In: Congress of Microbiology and Biotechnology, Universidade de Coimbra.

Afonso TB, Simões LC, Lima N. 2017. Inter-kingdom biofilm formation between bacteria and filamentous fungi isolated from a drinking water distribution system. In: Congress of Microbiology and Biotechnology, Escola Superior de Biotecnologia, Universidade Católica, Porto.

Simões, LC, Afonso, T, Paterson, R. Lima, N. 2017. The role of filamentous fungi in the inter-kingdom complex association of water biofilms. In: Books of abstracts of CEB annual meeting 2017 (Ed: E.C. Ferreira) Universidade do Minho, Centro de Engenharia Biológica, p. 27.

Chapter 1

Literature Review

Part of the contents presented in this chapter were published on:

Afonso TB, Simões LC, Lima N. 2021. Occurrence of filamentous fungi in drinking water: their role on fungal-bacterial biofilm formation. Research in Microbiology (In Press).

1.1 Drinking water microbiology

Water is indispensable to life. It is the most common and abundant chemical compound on earth, essential for all socio-economic development and the healthy maintenance of ecosystems (Simões and Simões, 2013). It covers seven tenths of the earth's surface occupying an estimated total volume of 1.38×10^9 km^3. However, freshwater, and consequently, the water available as potential drinking water represents only approximately 2.6 % of global water (Szewzyk et al., 2000). For this reason, every effort should be done to achieve drinking water as safe as possible. The definition proposed by the United Nations (UN) for water security, and the centrality of water to achieving a larger sense of security, sustainability, development and human well-being is "the capacity of a population to safeguard sustainable access to adequate quantities of acceptable quality water for sustaining livelihoods, human well-being, and socio-economic development, for ensuring protection against water-borne pollution and water-related disasters, and for preserving ecosystems in a climate of peace and political stability" (Figure 1.1) (UN-Water, 2013). The UN Sustainable Development Goal 6 (SDG 6) reinforces that "while substantial progress has been made in increasing access to clean drinking water and sanitation, billions of people – mostly in rural areas still lack these basic services. Worldwide, one in three people do not have access to safe drinking water, two out of five people do not have a basic hand-washing facility with soap and water" (UN, 2020). This becomes even more dramatic in times of SARS-Cov-2 where access to clean water and handwashing with soap are crucial to controlling the individual and communitarian pandemic disease of COVID-19. Clean water is still a luxury for poor regions. For this reason, water companies have the main goal of delivering microbiological safe water to the consumers, adequate in quantity and delivery pressure and satisfactory in terms of taste, odour and appearance (Simões and Simões, 2013). This objective can be questioned when microorganisms are present in excess. Primary and opportunistic pathogens can also be found in water distribution systems because they can survive water treatment and disinfection. These pathogens can be transmitted to the population if untreated or inadequately treated water is consumed and may cause waterborne diseases (Simões and Simões, 2013). Several diseases are associated with waterborne outbreaks such as cholera, dysentery, encephalitis, hepatitis, legionellosis, leptospirosis, meningitis, poliomyelitis, pulmonary illness, salmonellosis and typhoid fever, with gastroenteritis being the most notorious disease associated with waterborne outbreaks (WHO, 2011).

A water distribution system is a very rich environment under the influence of various factors that can modulate water microbiology (e.g. temperature, pipe material, nutrients, pH, water flow and disinfectant concentration) (Berry et al., 2006). It can also be perceived as extremely complex systems

wherein bacteria, fungi (filamentous and yeast), protozoa, viruses and algae interact (Berry et al., 2006; Hageskal et al., 2009). Considering that each of the microorganisms present plays a role in these environments, their significance should not be underestimated as a potential threat to human health and well-being (Paterson and Lima, 2005; Siqueira et al., 2011).

The excess presence of microorganisms in water distribution systems may not be directly harmful to humans by causing disease, but they may cause aesthetic problems as their growth affects the organoleptic properties of the water (Flemming et al., 2002; Paterson and Lima, 2005). These microorganisms can be found either as planktonic forms inhabiting bulk water or as biofilms growing on pipes surfaces (Wright et al., 2004).

Figure 1.1 Infographic of the United Nations regarding the concept of water security (UN-Water, 2013).

1.2 Biofilm: definition and concepts

Biofilms are a functional consortium of microorganisms and their formation and development on pipe walls are linked with a variety of microbial water problems. In these biofilms, pathogenic microorganisms are protected from stress conditions such as chlorine, shear stress and temperature, which allows them to remain viable (Szewzyk et al., 2000).

Several attempts have been made towards a definition of biofilm. Views from different research fields often lead to individual definitions regarding the professional area. Facing this problem, Wimpenny (2000) listed the types and descriptions of microbial systems that are related to biofilms. Nevertheless, as a result of these different definitions, a gap in the application of the terminology was still observed. Even with the difficulties in defining biofilm, Donlan and Costerton (2002), after considering not only the readily observable characteristics but also other physiological features such as altered growth rate and the fact that biofilm microorganisms transcribe genes that their planktonic counterpart does not, attempted a new definition. So, according to these authors a biofilm can be defined as a "microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances (EPS) that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription" (Donlan and Costerton, 2002). This physical proximity of the cells leads to synergistic interactions between microorganisms and provides a variety of beneficial traits regarding their phenotypes. These alterations include increased tolerance to chemical, biological and physical stresses, efficient capture of nutrients, stronger attachment to surfaces, enhanced cell to cell communication and survival through severe conditions (Harding et al., 2009).

A typical bacterial biofilm development can be described as a five-stage process: (1) adsorption, association or initial attachment of cells to a surface – physico-chemical interaction of microorganisms with a surface, (2) adhesion – presence of appendages or EPS, cell clustering, (3) microcolony formation – cell multiplication, cell-cell aggregation, recruitment of planktonic single cells and increased EPS production leading to the formation of multi-layered aggregates, (4) biofilm maturation – biofilm reaches maximum thickness and surface coverage, (5) dispersal – dispersion of single cells from the biofilm (Figure 1.2) (Stoodley et al., 2002).

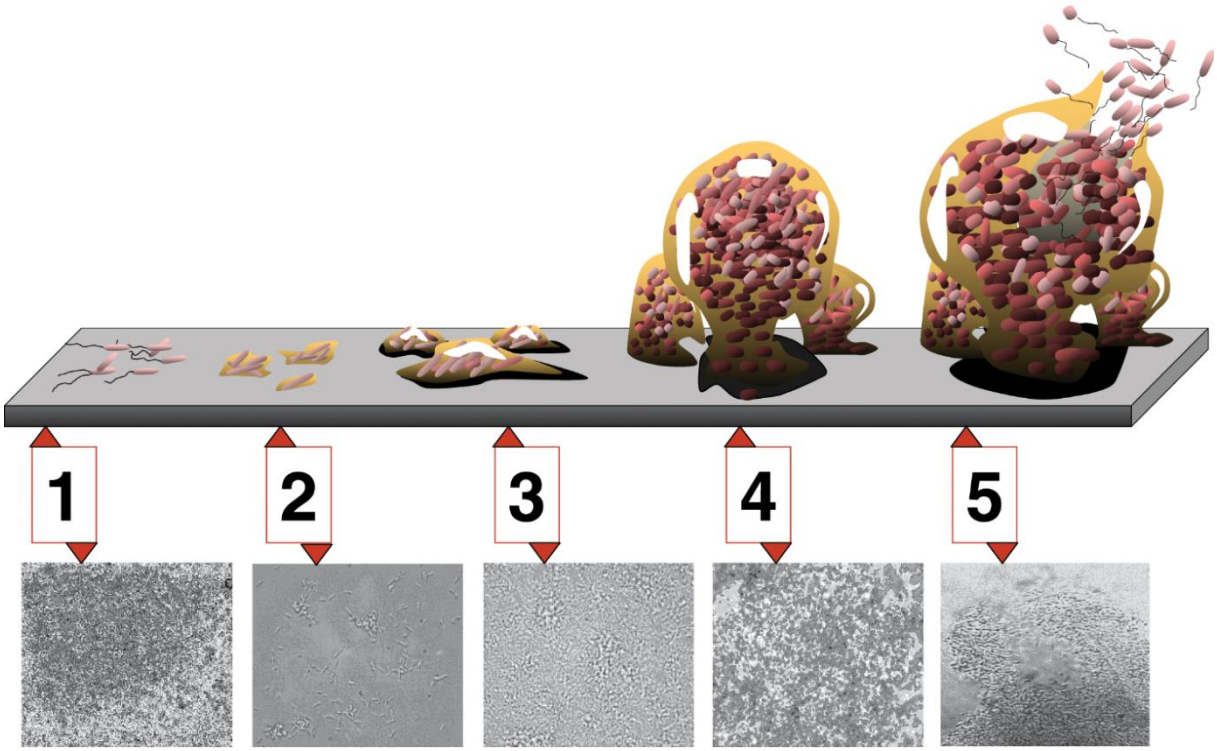


Figure 1.2 Development of a bacterial biofilm as a five-stage process: (1) initial attachment of cells to the surface, (2) adhesion, (3) microcolony formation, (4) biofilm maturation and (5) dispersion of single cells from the biofilm (Monroe, 2007).

EPS production in the third stage of biofilm formation is essential for the development of a mature biofilm. EPS can represent most of the total organic matter of biofilms and are responsible for binding cells in the matrix while providing support for the maturation of the biofilm. In addition to this mechanical aid, EPS also serve as protection against adverse conditions, retention of extracellular enzymes and a matrix for the exchange of signaling molecules that will ultimately influence community behavior (Flemming et al., 2002).

Biofilms are the main source of microorganisms in water distribution systems. It is estimated that 95% of the total microbial mass is attached to pipe walls, while only 5% is in the planktonic phase (Momba et al., 2000). For this reason, microbial growth in biofilms is extremely relevant and they are considered a major threat to safe drinking water.

1.3 Biofilms in drinking water distribution systems

Many problems in DWDS are microbial in nature, including biofilm growth, microbially mediated corrosion, and the occurrence and persistence of pathogens. The presence of biofilms themselves in

DWDS can have serious effects, including changes in the turbidity, taste, odour and colour of the water, an increase in cell numbers in the bulk phase, biocorrosion, disinfectant demand, increased habitat for potentially pathogenic microorganisms and production of toxins (Flemming et al., 2002; Paterson and Lima, 2005; Li et al., 2015; Zhou et al., 2017). A DWDS provides a great habitat for microorganisms, which are sustained by organic and inorganic nutrients present on the pipes and in the surrounding water (Sonigo et al., 2011). Understanding the microbial ecology of water distributions systems is essential to provide needed insights to help resolve public health concerns associated with biofilm formation in these environments.

In DWDS, under natural conditions, true monospecies biofilms are rare, and they are usually perceived as complex systems where different microorganisms interact (Douterelo et al., 2018a). The microorganisms that can be found in these systems are bacteria, fungi (filamentous and yeast), protozoa, viruses and algae (Berry et al., 2006; Hageskal et al., 2009). Several of these microorganisms are pathogens and able to cause waterborne diseases (Table 1.1).

Table 1.1 Pathogens associated to waterborne diseases and infections (Ashbolt, 2004; WHO, 2006; Karanis et al., 2007; Sonigo et al., 2011; Babič et al., 2017).

Bacteria	Protozoa	Virus	Fungi
Aeromonas spp.	*Acanthamoeba castellani*	Adenovirus	*Alternaria alternata*
Burkholderia pseudomallei	*Balantidium coli*	Astrovirus	*Aspergillus* spp.
Campylobacter jejuni	*Cryptosporidium parvum*	Calicivirus	*Candida* spp.
Escherichia coli	*Cyclospora cayetanensis*	Coxsackie virus	*Cladosporium* spp.
Helicobacter pylori	*Entamoeba histolytica*	Echovirus	*Exophiala* spp.
Legionella pneumophila	*Giardia lamblia*	Enterovirus	*Fusarium* spp.
Leptospira spp.	*Naegleria fowleri*	Hepatitis A virus	*Mucor* spp.
Mycobacterium spp.	*Toxoplasma gondii*	Hepatitis E virus	*Penicillium* spp.
Pseudomonas aeruginosa		Norovirus	*Trichoderma* spp.
Salmonella spp.		Poliovirus	
Shigella spp.		Rotavirus	
Toxic *cyanobacteria*			
Vibrio cholera			
Yersinia enterocolitica			

The diversity in the microbial community leads to a variety of complex relationships involving intraspecies and interspecies interactions (Douterelo et al., 2016). A conventional representation of a drinking water biofilm is presented in Figure 1.3.

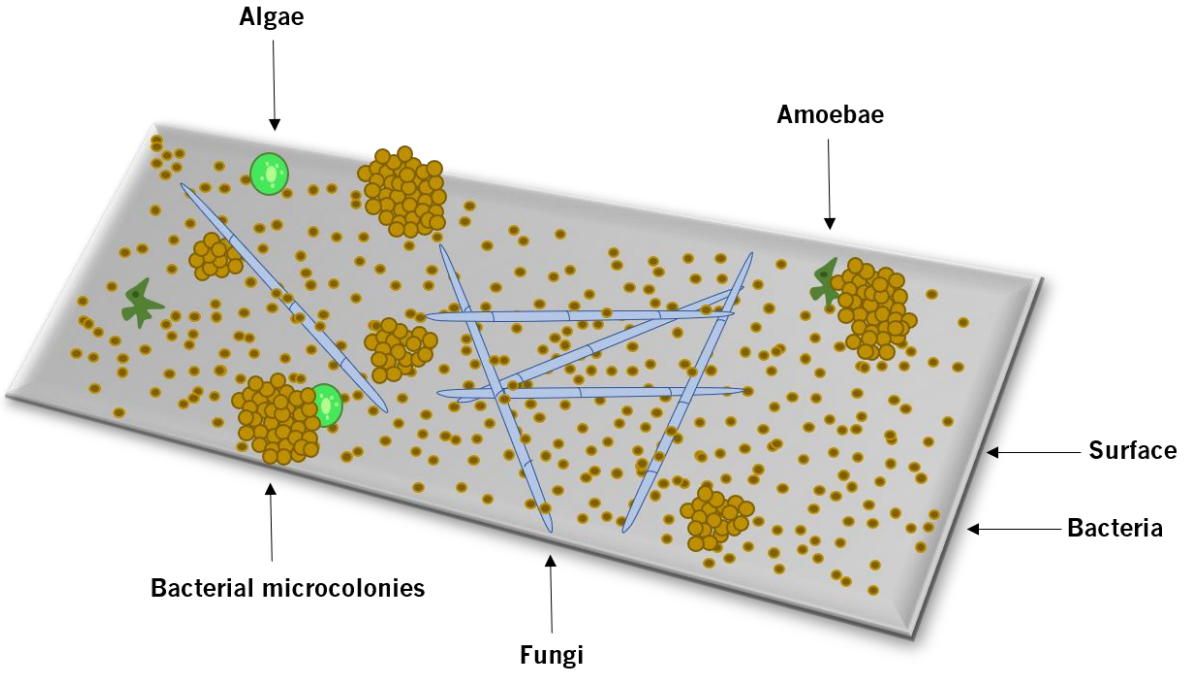


Figure 1.3 Microbial heterogeneity and architectural structure of water biofilms. Based on Siqueira (2011).

Interactions among bacterial species may have a profound influence on the initial stages of biofilm formation and consequent development (Stoodley et al., 2002). The ecology of a biofilm is a complex function of prevailing growth conditions, hydrodynamic shear forces, presence of microbial metabolites and molecules, such as cell-cell signalling communication molecules, excreted by its inhabitants (Bryers and Ratner, 2004). This diversity leads to a plethora of complex interactions between the microorganisms that are present. Findings into the microbial ecology of DWDS have shown that resistance of microorganisms to disinfectants, particularly chlorine, is affected by this microbial diversity (Simões et al., 2010). This information, has, however, been mainly obtained from studies with bacteria. In DWDS, Gram-negative bacteria are predominant over Gram-positive with *Pseudomonas* being the most abundant genera of found in water systems. Other predominant Gram-negative genera detected in these systems include *Acinetobacter, Aeromonas, Burkholderia, Enterobacter, Klebsiella, Methylobacterium, Sphingomonas* and *Xanthomonas* (Berry et al., 2006). Despite the possible presence of pathogenic microorganisms in DWDS, most of the microorganisms present in these systems are not directly harmful for human health. However, the dominant non-pathogenic microorganisms should not be neglected, since they play a major role in biofilm formation (Paris et al., 2009).

Studies regarding fungi, in particular filamentous fungi, have been gaining attention due to their biofilm formation ability, however, their interaction with bacteria in fungal-bacterial biofilms is still poorly understood.

1.4 Fungi and filamentous fungi biofilms

1.4.1 Fungi

Fungi are a diverse and abundant group of organisms belonging to the kingdom *Eumycota* (Viegas et al., 2016). The most recent classification of this kingdom comprises seven recognised phyla: *Basidiomycota*, *Ascomycota*, *Glomeromycota*, *Microsporidia*, *Blastocladiomycota*, *Neocallimastigomycota* and *Chytridiomycota* (Hibbett et al., 2007). Fungal classification is, however, very dynamic, as shown by the recently proposed phyla *Cryptomycota* (Jones et al., 2011). As a practical approach to the classification of fungi, division regarding their cellular organisation has been made. They range from microscopic single-cell species (yeasts) to species with massive mycelia. This latter large group of organisms can be characterised by hyphal growth supporting macroscopic sexual reproductive structures (e.g. truffles, mushrooms) and microscopic sexual or asexual reproductive structures, known as moulds or filamentous fungi (Lima and Santos, 2017). Fungi are ubiquitous and some of them, belonging to the phyla *Chytridiomycota,* are particularly adapted to aquatic environments. These fungi are known for producing zoospores morphologically apt to propagate in running waters. Filamentous fungi from other phyla in *Eumycota* are, however, mostly adapted to the terrestrial environment, such as soil and anything in interface with air, as they generally need a solid substrate for spore dispersal (Kirk et al., 2001).

1.4.2 Filamentous fungi biofilm formation

Although DWDS are not considered natural habitats for these filamentous fungi, they can often be introduced into these environments from different pathways, such as physical openings in storage facilities, treatment breakthroughs, leaking joints and adapters, cracks in pipelines and/or during maintenance or mains installation. Sammon et al. (2011) demonstrated that airborne spores can be an important external source of filamentous fungi propagules in a DWDS. Once inside these systems, fungi can survive the oligotrophic conditions by scavenging nutrients from the substrate which they colonize or the water in which they are inhabiting. Consequently, their presence may then cause additional problems to the water quality (e.g. unpleasant appearance with flocs and earthy pungent odours, the presence of pigments, pipe blockage, a source of potentially pathogenic and allergy-causing fungi and the presence of mycotoxins) (Al-gabr et al., 2014; Bai et al., 2017; G. Hageskal et al., 2007; Oliveira et al., 2018; Paterson and Lima, 2005; Sonigo et al., 2011). To maximize nutrient uptake, filamentous fungi will form hyphal mats. Due to their absorptive nutrition mode, secretion of extracellular enzymes that digest

complex molecules and apical hyphal growth, filamentous fungi have a high ability to grow on surfaces, thus forming biofilms (Jones, 1994). Fungal survivability and proliferation in DWDS are believed to be related to the ability to form biofilms (Simões et al., 2015).

Harding et al. (2009) proposed a six-step pioneer model for filamentous fungal biofilm formation (Figure 1.4) based on models for bacteria and yeasts: 1) propagule adsorption – deposition of spores or other propagules such as hyphal fragments or sporangia (physical contact of the organism with a surface); 2) active attachment to a surface – secretion of adhesive substances by germination spores and active germlings; 3) microcolony formation I – apical elongation and hyphal branching, hyphae exploration of the substratum, ramification across surfaces as a monolayer (production of a polymeric extracellular matrix that allows the colony to adhere to the substrate); 4) microcolony formation II (or initial maturation) – formation of compacted hyphal networks or mycelia and hypha-hypha adhesion, layering and the formation of water channels via hydrophobic repulsion between hyphae or hyphal bundles; 5) maturation – formation of fruiting bodies, sporogenous cells, sclerotia and other survival structures; 6) dispersal – spore dispersal or release, or the dispersal of biofilm fragments (detached cells act as new propagules to re-initiate the cycle).

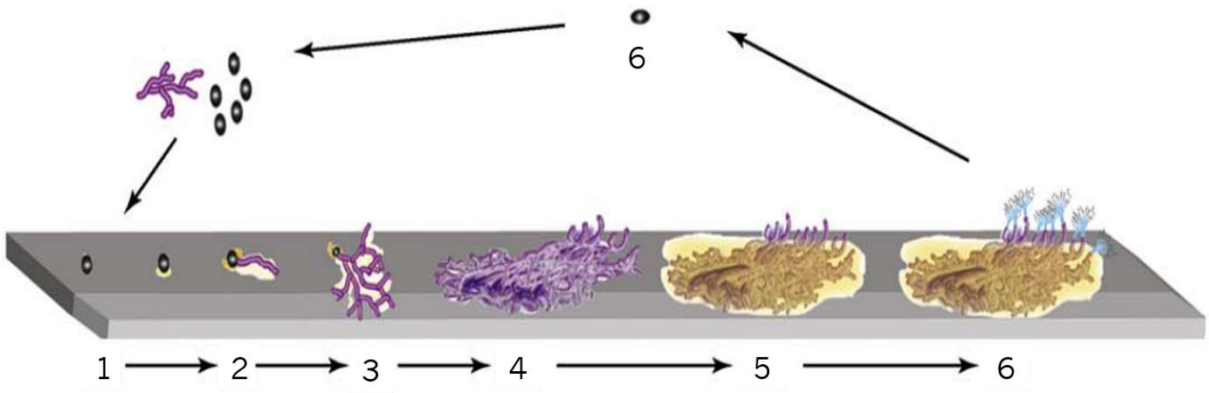


Figure 1.4 Filamentous fungi biofilm model. (1) Propagule adsorption; (2) Active attachment to a surface; (3) Microcolony formation; (4) Initial maturation; (5) Maturation or reproductive development; (6) Dispersal or planktonic phase (Harding et al., 2009).

Recently, Fernandes et al. (2019) updated this model by adding an initial step – surface conditioning – where the surface hydrophobicity and charge of spores and the substratum play key roles in the adhesion process (Figure 1.5). This initial physical contact can result in reversible adhesion, followed by irreversible adhesion with the secretion of adhesive substances by germinated spores and active germlings (Harding et al., 2009). Spore germination will ensue if suitable environmental conditions are met (Fernandes et al., 2019). After germlings start to form, they secrete hydrophobins that mediate adhesion and hyphae-

substratum interaction (Zampieri et al., 2010). Subsequently, hyphal differentiation produces a complex hyphae net (mycelium) that grows in all directions enclosed within a polymeric extracellular matrix, where quorum sensing (QS) molecules, similarly to bacterial biofilms, are present (Blankenship and Mitchell, 2006). The maturation stage for DWDS biofilms should mainly occur in reservoirs due to the requirement of a stable air-water interface for aerial growth and subsequent spore formation and air dispersion (Viegas et al., 2016). Finally, the dispersion stage occurs through the release of spores or different propagules in response to environmental stresses or biological stimuli (Harding et al., 2009). In drinking water, propagules can be dispersed by water flow, which can then establish new biofilms, further spreading the presence of filamentous fungi in drinking water (Fernandes et al., 2019; Sammon et al., 2011; Siqueira et al., 2013).

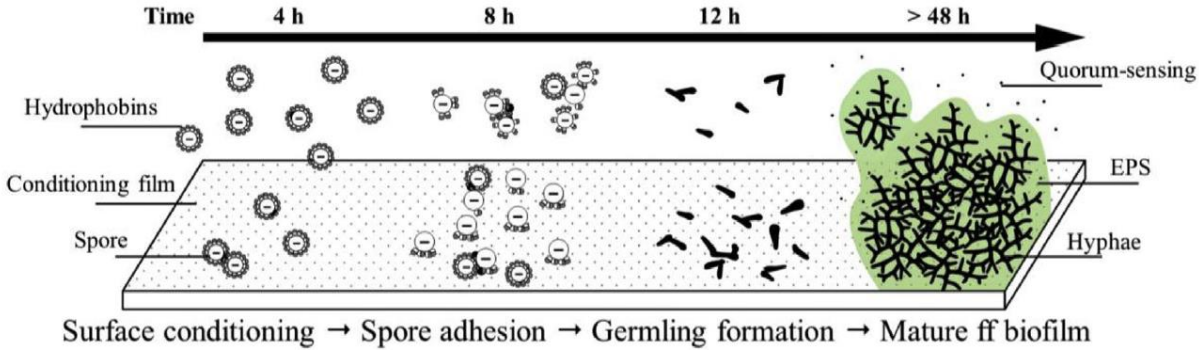


Figure 1.5 Updated stages of filamentous fungi biofilm formation (Fernandes et al. 2019).

1.5 Occurrence of filamentous fungi in drinking water distribution systems

Since the 70s, several works have reported the presence of filamentous fungi in DWDS worldwide. Table 1.2 lists and focuses only on the most relevant reports from the last two decades. A wide diversity of filamentous fungi has been isolated/detected from drinking water. The most frequent recovered species belong to the genera *Aspergillus, Cladosporium and Penicillium*, follow by and (Table 1.2). This might be related to their ability to secrete a pigment called melanin, which confers protection to spores against a variety of stresses, providing these microorganisms with a competitive advantage and greater resistance to water treatment (Sonigo et al., 2011). In addition, due to the hydrophobicity property of the spores from these genera, further protection is offered against water disinfection as spores tend to aggregate between each other and other particles (Sonigo et al., 2011).

Table 1.2 Most relevant filamentous fungi surveys in drinking water from the last two decades.

Location / Date	Water source	Isolation method	Most frequent fungal genera	References
United Kingdom, 1996	Surface water and network	Membrane filtration, centrifugation, direct plating	*Aspergillus, Cladosporium, Epicoccum, Penicillium* and *Trichoderma*	Kinsey et al., 1999
United States of America (Springfield, MO), 1997	Municipal water supply system	Pipe coupons (Biofilm)	*Aspergillus* and *Penicillium*	Doggett, 2000
Greece (Thessaloniki), 1998	Hospital and community tap water	Membrane filtration	*Acremonium, Aspergillus* and *Penicillium*	Arvanitidou et al., 1999
(Haemodialysis units)	Municipal water supplies of haemodialysis centres	Membrane filtration	*Aspergillus* and *Penicillium*	Arvanitidou et al., 2000
Germany (North Rhine-Westphalia), 1998-99	Drinking water	Pour-plating	*Acremonium, Exophiala, Penicillium* and *Phialophora*	Göttlich et al., 2002
Norway (Oslo), 1998-99	Hospital tap and shower water	Membrane filtration and swabs from water-related surfaces	*Aspergillus, Cladosporium, Paecilomyces* and *Trichoderma*	Warris et al., 2001
United States of America (Little Rock, AR), 1997-2000	Water distribution system of a Hospital	Membrane filtration, swab applicators	*Alternaria, Aspergillus, Paecilomyces* and *Penicillium*	Anaissie et al., 2002, 2003
(Houston, TX) 2000	Water distribution system of a Hospital	Membrane filtration, centrifugation, swab applicators	*Fusarium*	Anaissie et al., 2001
Greece (Thessaloniki, Athens and Heraklion), 2000	Hospital tap water	Membrane filtration	*Acremonium, Fusarium, Paecilomyces* and *Penicillium*	Panagopoulou et al., 2002
Poland (Warsaw), 2000-02	Municipal water supply system	Membrane filtration	*Aspergillus, Cladosporium* and *Fusarium*	Grabińska-Łoniewska et al., 2007
Norway, 2002-03	Drinking water (surface and groundwater)	Membrane filtration	*Aspergillus, Penicillium* and *Trichoderma*	(Hageskal et al., 2006, 2007, 2008)
Saudi Arabia (Riyadh region), 2003	Drinking water (60% desalinized water and 40% well water)	Pour-plating	*Aspergillus* and *Penicillium*	Nasser, 2004
Turkey (Istanbul), 2003	Water distribution system of a Hospital	Membrane filtration	*Acremonium, Aspergillus* and *Penicillium*	Hapcioglu et al., 2005

Portugal (Braga), 2003-04	Tap water	Membrane filtration and swabbing	*Acremonium* and *Penicillium*	Gonçalves et al., 2006
Belgium (Liège), 2005-06	Water distribution system of a Hospital	Membrane filtration	*Aspergillus, Fusarium, Paecilomyces* and *Penicillium*	Hayette et al., 2010
Austria, 2006	Drinking water and groundwater	Membrane filtration and plating	*Cladosporium,* Basidiomycetes, and *Penicillium,*	Kanzler et al., 2008
Brazil (São Paulo), 2006	Water distribution system of a haemodialysis unit	Membrane filtration	*Aspergillus, Cladosporium, Fusarium* and *Trichoderma*	Varo et al., 2007
	Water distribution system of a haemodialysis centre	Membrane filtration	*Aspergillus, Fusarium, Penicillium and Trichoderma*	Pires-Gonçalves et al., 2008
Portugal (Lisbon), 2006-08	Surface, spring and groundwater for the production of drinking water	Membrane filtration, spread plate and pour plate	*Aspergillus, Cladosporium* and *Penicillium*	Pereira et al., 2009, 2010
Australia (Rockhampton), 2007-08	Municipal water supply system	Membrane filtration	*Aspergillus, Cladosporium, Fusarium* and *Penicillium*	Sammon et al., 2010
Australia (Rockhampton), 2007-10	Municipal water supply system	Glass, PVC and concrete coupons (Biofilm)	*Aspergillus, Cladosporium* and *Penicillium*	Sammon et al., 2011
Brazil (São Paulo), 2007-08	Water distribution system of paediatric haematopoietic stem cell units	Membrane filtration	*Aspergillus, Cladosporium, Penicillium* and *Purpureocillium*	Mesquita-Rocha et al., 2013
Portugal (Centre), 2008-12	Untreated water (groundwater, spring and surface water)	Membrane filtration	*Penicillium* and *Trichoderma*	Oliveira et al., 2013
Italy (Marche region), 2010-11	Water treatment and distribution system of haemodialysis units	Membrane filtration	*Alternaria, Cladosporium* and *Tricophyton*	Schiavano et al., 2014
Brazil (São Paulo), 2011-12	Tap water from wells (groundwater)	Membrane filtration	*Acremonium, Aspergillus, Fusarium* and *Penicillium*	Arroyo et al., 2019
Brazil (São Paulo), 2012	Water distribution system of a Hospital	Membrane filtration	*Aspergillus, Cladosporium* and *Penicillium*	Arroyo et al., 2020
China (Xiamen), 2011-12	Drinking water (surface and tap water)	Membrane filtration	*Aspergillus, Fusarium, Penicillium, Phialophora* and *Trichoderma*	Al-gabr et al., 2014
United States of America	Hospital hot water system	Membrane filtration	*Aspergillus, Cladosporium Penicillium,*	Ma et al., 2015

(Pittsburgh, PA), 2011-12			*Peniophora* and *Rhodosporidium*	
Brazil (Recife), 2013-14 and 2015	Water distribution system (groundwater)	Membrane filtration	*Aspergillus, Fusarium, Penicillium* and *Trichoderma*	Oliveira et al., 2016
United States of America (Anonymous), 2014, 2016	Drinking water treatment plant	Membrane filtration	*Alatospora, Aspergillus, Penicillium, Taphrina* and *Tuber*	Ma et al., 2017
China (Guangdong Province), 2015	Water supply reservoirs, drinking water treatment plants	Direct plating	*Aspergillus* and *Cladosporium*	Bai et al., 2017
Colombia, 2015	Drinking water network (surface water)	Water-surface interface scraping (Biofilm)	*Paecilomyces, Paraconiothyrium* and *Penicillium*	Hurtado-McCormick et al., 2016
United Kingdom (South-west region), 2014-15	Surface and groundwater chlorinated networks (bulk water and biofilms)	Membrane filtration	*Aspergillus, Basiodobolus, Cladosporium, Plectosphaerella* and *Rhexocercosporidium*	Douterelo et al., 2016, 2018a
Poland (Lodz), 2016	Recreational surface water	Centrifugation	*Alternaria, Aspergillus* and *Penicillium*	Góralska et al., 2020
South Africa (Johannesburg), 2016-17	Drinking water network	Membrane filtration	*Aspergillus, Penicillium* and *Trichoderma*	Mhlongo et al., 2020
Spain (Valencia), 2019-20	Drinking water network	Membrane filtration	*Aspergillus* and *Cladosporium*	Del Olmo et al., 2021

Among the isolated filamentous fungi, potentially pathogenic, allergenic and toxigenic species have also been found. This is particularly concerning since several reports on the presence of these fungi have been obtained from hospital water systems (Arvanitidou et al., 1999, 2000; Ma et al., 2015; Mesquita-Rocha et al., 2013; Pires-Gonçalves et al., 2008; Schiavano et al., 2014; Varo et al., 2007; Warris et al., 2001). In some cases, the presence of pathogenic species (e.g. *Aspergillus fumigatus; Fusarium solani*) in drinking water, has led to the hypothesis of hospital water systems serving as transmission routes for fungal infections. These results indicate that hospital water contains high fungal diversity, including potential pathogens. Many of the fungal species found in drinking water have also allergenic potential (Paterson and Lima, 2005). Fungal species from the main genera recovered from drinking water have also been investigated towards their implication with asthma and other respiratory problems, regarding indoor environments (Denning et al., 2006). Some of these health adverse effects may arise not only from the fungi itself but also from the production of secondary metabolites and volatile organic compounds (VOC's). Several species from both *Penicillium* and *Aspergillus* genera are known mycotoxin producers. Mycotoxins cause a variety of health problems and are known to be carcinogenic

and capable of impairing the immune system in both humans and animals (Babič et al., 2018). Of all the different mycotoxins than can be produced, aflatoxins (*Aspergillus* spp.) and zearalenone (*Fusarium* spp.) are some of the most relevant and have been detected in drinking water (Paterson and Lima, 2005; Russell and Paterson, 2007). The concentration of mycotoxins in drinking water is likely to be very diluted and, for the time being, has not been identified as the source of symptoms attributable to mycotoxins.

For an in-depth understanding of the occurrence, ecology and physiology of fungal contaminants in drinking water, Babič et al. (2017; 2018) recently compiled this information in reviews. It focuses on reports from European water sources in the last 30 years, including surface-, ground- and tap-water intended for human consumption.

1.6 Interkingdom relationships between fungi and bacteria: emphasis to DWDS biofilms

Although intraspecific and intrageneric interactions may play an important role in the coexistence of some microbiomes, biotic interactions between distantly related organisms across the kingdoms of life can also regulate the composition of these communities (Kastman et al., 2016). In many microbiomes bacteria can coexist with different eukaryotic microorganisms, including fungi (Frey-Klett et al., 2011). Previously, there has been a clear separation of microbiological research between fungi and bacteria, which has led to the study of each individual group in exclusively axenic settings. However, this division is not representative of reality since fungi and bacteria interact with each other in real environments (Frey-Klett et al., 2011).

The interactions between fungi and bacteria are of interest for several different fields of study such as agriculture, food-processing, forestry, environmental protection, biotechnology and medicine (Kobayashi and Crouch, 2009; Frey-Klett et al., 2011). The most common reports about fungal-bacterial relationships show that usually the bacterial partner exploits resources from the associated fungi through a parasitic or commensalism type of interaction. Nonetheless, there are examples where the fungal partner is able to take advantage of bacterial resources in mutualistic interactions (Kobayashi and Crouch, 2009).

The physical interactions between fungi and bacteria can reach highly specific symbiotic associations of fungal hyphae and bacterial cells. These associations encompass, of course, biofilms. As the microorganisms are closely embedded in an extracellular matrix, inter-kingdom biofilms containing fungi (filamentous or non-filamentous) can be considered a closer level of fungal bacterial interaction, but this aspect is still poorly understood (Frey-Klett et al., 2011). There are several outcomes for the fungal-

bacterial interactions (Figure 1.6). This is due to the combination of different physical and molecular interactions between the two microorganisms that can influence each one in a variety of ways.

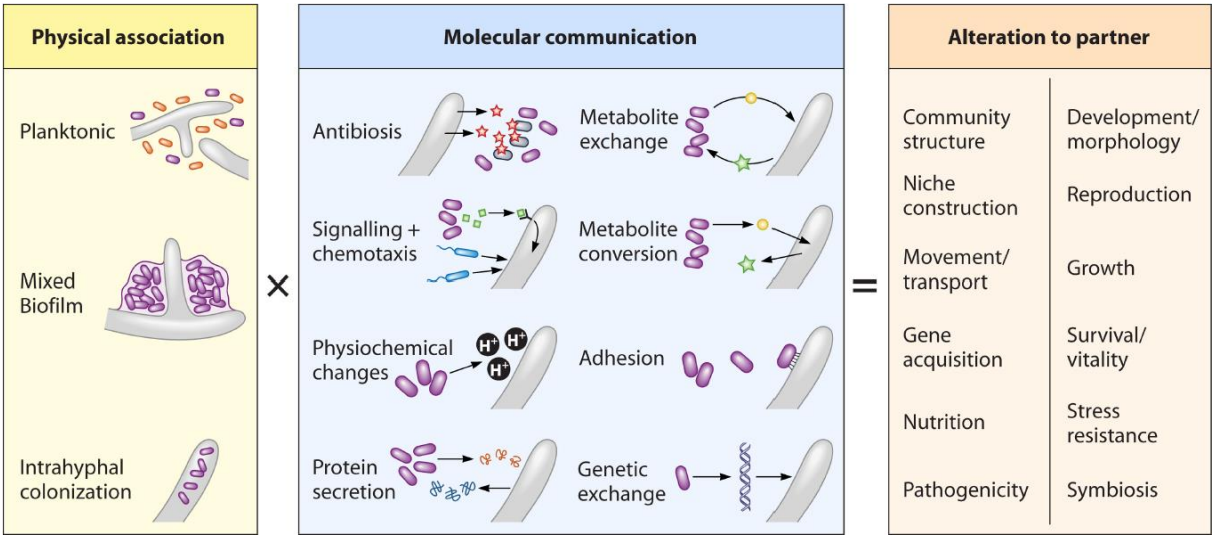


Figure 1.6 Variety of fungal-bacterial interactions and the possible outcomes for each partner. These changes may affect the influence of the fungal-bacterial complex on their biotic and abiotic environment (Frey-Klett et al., 2001).

In DWDS is important to fully understand the different interactions between fungi and bacteria, as different studies have found different relationships between them. In mixed biofilms, both organisms can exist as dual complexes or fungi may offer biotic support for the establishment of a bacterial biofilm (Seneviratne et al., 2007; Lahaye et al., 2016). However, it has also been shown that fungi often colonize pre-established bacterial biofilms, and due to their different ecological requirements, it has been suggested that it can lead to a positive relationship between these microorganisms (Doggett, 2000). Reports of negative relationships may be observed due to culturing processes, where both fungi and bacteria are in direct competition for resources (Gonçalves et al., 2006). This variety in findings could, in sum, be a consequence from several factors, such as the difference in the composition of isolated species from the water systems, differences in methodologies or different biological mechanisms at play (Sonigo et al., 2011). For this reason, a need arises for further research to explore the different correlations between fungi and bacteria in DWDS and what are the factors influencing these interactions.

To demonstrate the heterogeneity in findings, in a single study, performed on fungal-bacterial biofilms in flowing water photo-processing tanks using a model community, it was difficult to determine which interactions were present (Elvers et al., 1998). Some species showed increased growth rate in mixed cultures while others showed a reduction, however, all species were present in a lower number than in single cultures, which was considered to be a result of limitation and competition for the nutrients

available (Elvers et al., 1998). Douterelo et al. (2016) reported a positive coexistence between the bacteria *Pseudomonas* and the fungi *Basidiobolus* in *in situ* biofilms. This could be due to ability of the fungi to produce extracellular enzymes that allow them to degrade high molecular weight compounds, releasing secondary metabolites that can potentially be used, in this case, by *Pseudomonas* (Douterelo et al., 2016). In another study, a correlation was observed between the relative abundance of certain bacterial taxa such as Proteobacteria and *Basidiomycota* (Douterelo et al., 2018b). The same study also confirmed the presence of *Acremonium* and *Neocomospora* from early stages of biofilm formation to a more developed biofilm, forming essential communities with bacteria. Fungal contribution, in particular *Ascomycota*, is very important to the microbial ecology of real DWDS (Douterelo et al., 2018a). One of the roles of filamentous fungi in drinking water biofilms has consequently been associated with providing building blocks and/or biotic support through their hyphae for the establishment and colonization of surfaces by bacteria (Douterelo et al., 2018b). A recent study performed by Del Olmo et al. (2021) detected a core microbial community throughout the network of a DWDS with microorganisms like *Pseudomonas*, *Aspergillus* or *Alternaria* being abundant in underlying and more consolidated material layers. This study revealed a diverse community of fungi which demonstrated a strong contribution to biofilm formation in DWDS, supporting concepts of mutual beneficial fungal-bacterial interactions. In addition, fungal-bacterial communities were found to be highly correlated, with bacteria being more diverse, whilst fungi showing more dominance and stability (Del Olmo et al., 2021).

Flemming et al. (2002) suggested that it is possible that drinking water biofilms can also help to inhibit the propagation of invading pathogens, thus safeguarding water quality. Understanding community dynamics, including the presence of fungi in DWDS can be the key to sustaining a beneficial and ultimately safe microbiome (Del Olmo et al., 2021). A practical example of this statement was observed in a study performed by Lahaye et al. (2016) where the treatment of a DWDS of a pig farm using essential oils lead to the evolution of a positive bacterial biofilm. While the initial biofilms were essentially composed of fungi with hyphae being prominent, after treatment with essential oils, a decay of fungal population was observed to the benefit of new bacterial populations.

There are a variety of factors affecting these inter-kingdom interactions that need to be better understood, including the presence of QS molecules.

1.7 Quorum sensing

1.7.1. Quorum sensing: general overview

Studies performed over the last 50 years have revealed that bacteria can communicate between themselves to carry out a wide range of complex social behaviours, including cooperation (Abisado et al., 2018). These social behaviours are widespread in bacteria and have important consequences in shaping the behaviours and structure of polymicrobial communities (Abisado et al., 2018). The driving force in bacterial community development is the self-organization and cooperation among cells (Parsek and Greenberg, 2005; Li and Tian, 2012). This concept becomes even more emphasized when studying bacterial biofilm communities (Parsek and Greenberg, 2005). Bacteria are far from being considered solitary microorganisms, and in communities, they exploit elaborate systems of intercellular interactions and communications to facilitate their adaptation to environmental stresses (Li and Tian, 2012).

QS is a mechanism employed by microbial species to coordinate community behaviour and is based on the process of autoinduction. It allows the perception of population density by the production, release and detection of small signalling molecules (autoinducers), which in turn modify gene expression (Thomas Bovbjerg Rasmussen et al., 2005). In low population densities, base-level expression of the autoinducer synthase gene leads to the production of low amounts of signalling molecules being released out of the cell resulting in their immediate dilution in the neighbouring environment (González and Keshavan, 2006). As population density increases, an accumulation of these molecules occurs in and around the cells. They will then interact with a receptor protein, causing a coordinated change in gene expression and/or physiological changes in the population (Parsek and Greenberg, 2005; Abisado et al. 2018). QS controls and regulates a variety of population density processes. These include biofilm formation and development, stress resistance, production of EPS, toxins and secondary metabolites and pathogenicity (Quiñones et al., 2005; Antunes et al., 2010; Shrout et al., 2011).

QS systems in bacteria have been generally divided into at least three classes: (1) Gram-negative bacteria rely on the LuxI/LuxR-type QS which utilize *N*-acyl-homoserine lactones (AHLs) as signalling molecules; (2) Gram-positive species use short oligopeptide signals in an oligopeptide-two-component-type QS system; and (3) in both Gram-negative and Gram-positive bacteria, the *luxS*-encoded autoinducer 2 (AI-2). These QS systems are already well understood at the molecular level and deeply described in reviews (Waters and Bassler, 2005; Schuster et al., 2013; Cook and Federle, 2014; Papenfort and Bassler, 2016).

1.7.1. Quorum sensing in Gram-negative bacteria

QS was first described in the 1960s and 1970s in the marine bioluminescent bacterium *Vibrio fischeri* with the AHLs being identified as the autoinducer signals (Fuqua et al., 2001). This bacterium lives in the light organs of fishes and squids and produces luminescence, which helps the animals escape from predators. This is a symbiotic association, as in return, the bacteria gain nutrients and shelter from their host (Dunlap, 1999). Bioluminescence is only exhibited when these bacteria are in this symbiotic mode of life and does not occur when they are in their free-living state. At high cell densities, *V. fischeri* activates bioluminescence through the QS proteins LuxR and LuxI (González and Keshavan, 2006).

As previously mentioned, in Gram-negative bacteria, the primary signalling molecules are AHLs whose synthesis is dependent on the LuxI homologs (Li and Tian, 2012). These molecules differ on the length of their side chains, which can range from C4 to C19 (Churchill and Chen, 2011; Saurav et al., 2020). With the increase in cell density, the extracellular concentration of these molecules increases in parallel as they freely diffuse across the cell membrane. A cognate LuxR-like protein is then responsible for recognising the AHL and when bound to it, the LuxR-like protein binds to a specific promoter resulting in the activation of target gene transcription (Figure 1.7) (Li and Tian, 2012). An important characteristic of this type of QS system is that the autoinducer synthase gene is a target for LuxR. Consequently, activation of the QS process results in increased expression of the autoinducer synthase, leading to the production of more AHLs. This acts a positive feedback loop, significantly amplifying the QS effect (González and Keshavan, 2006).

Similar QS networks homologous to the LuxI/LuxR- system have been identified in several Gram-negative bacteria, such as *Agrobacterium* spp., *Burkholderia* spp. or *Rhizobium* spp., but also *Pseudomonas* spp., the most abundant Gram-negative in DWDS (Table 1.3) (Lewenza et al., 1999; Farrand et al., 2002; Wisniewski-Dyé & Downie, 2002; Juhas et al., 2005).

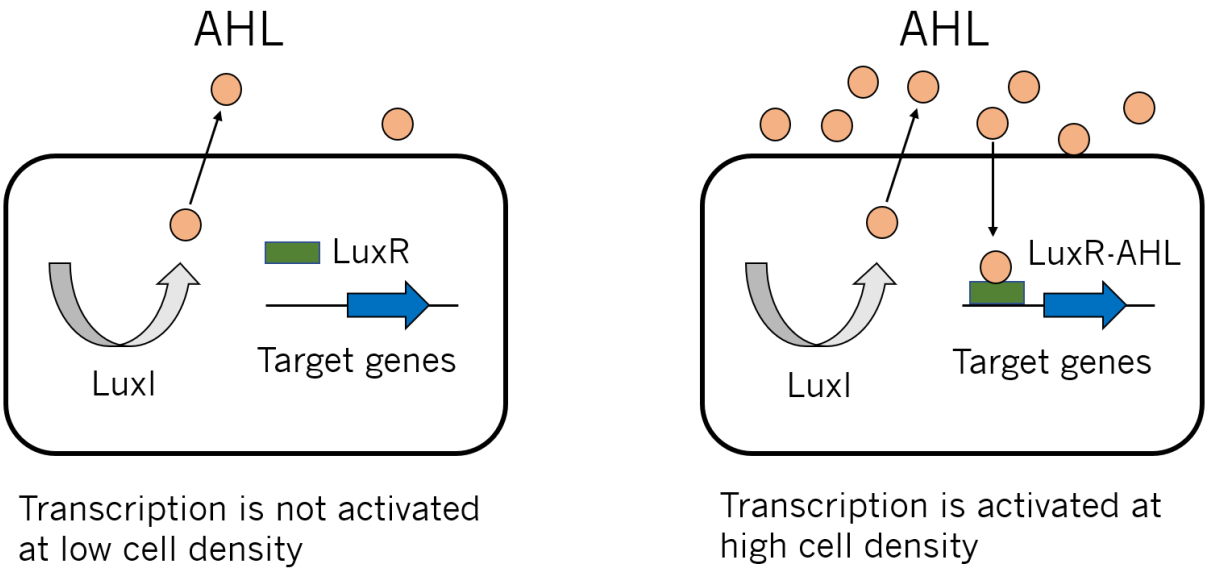


Figure 1.7 Schematic representation of the LuxI/LuxR- type QS in Gram-negative bacteria. At low population densities, the bacterial AHL synthase (LuxI) produces basal amounts of AHLs, that rapidly dilute into the surrounding environment. Higher population densities lead to an accumulation of AHLs beyond a threshold concentration resulting in the activation of the LuxR regulator protein which in turn initiates the QS cascade. Based on Li and Tian (2012).

In 1998, Davies and colleagues, first described the role of the LasI-R type QS in the biofilm formation of *P. aeruginosa* (Davies et al., 1998). The *lasI* gene product directs the synthesis of the diffusible extracellular signal *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL). The structure of this autoinducer is presented in Figure 1.8. These researchers found that a mutant defective in the production of the autoinducer 3-oxo-C12-HSL, formed biofilm cell clusters with 80% less thickness than that of wild-types, while also being more sensitive to detergent removal. When this AHL was added to the system, the mutant was able to, once again, form structured biofilms (Davies et al., 1998). This study demonstrated that QS plays a fundamental role in biofilm development, and more significantly, it makes an inseparable connection between cell-cell signalling and biofilm formation (Li and Tian, 2012).

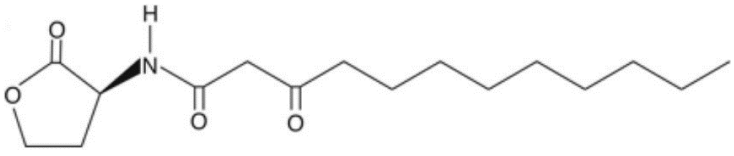


Figure 1.8 Structure of N-(3-oxododecanoyl)-L-homoserine lactone.

Table 1.3 Examples of Gram-negative QS systems and their controlled phenotypic traits.

Bacteria	Regulators	Chain length	Group-derived benefits
Vibrio fischeri	LuxI-LuxR	C6	Bioluminescence
Pseudomonas aeruginosa	LasI-LasR	C12	Biofilm formation
Agrobacterium tumefaciens	TraI-TraR	C8	Conjugal transfer
Burkholderia cepacia	CepI-CepR	C8	Virulence factors

Nowadays, it is well known that this kind of microbial communication is not restricted to members of the same kingdom. In addition to bacterial QS systems, viruses, eukaryotic microorganisms (including filamentous fungi) and even higher order species such as ants, have been reported to also have QS-like systems (Hornby et al., 2001; Pratt et al., 2002; Erez et al., 2017; Mehmood et al., 2019). These examples provide proof that population density responses play important roles across extremely different domains of life (Abisado et al., 2018).

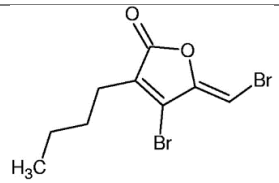
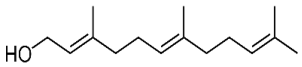
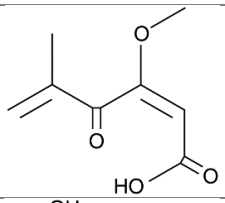
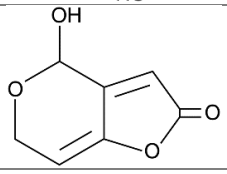
1.8 Quorum sensing inhibition/quorum quenching

QS controls and regulates different bacterial population density processes, including biofilm formation. Biofilms cause detrimental effects in a variety of sectors, and thus, the need arose to search for ways of combating undesirable microorganisms. A promising strategy is based on the interference with the QS systems, also known as quorum quenching (QQ) (Paluch et al., 2020). Eukaryotes, and filamentous fungi included, have the ability to interfere with this bacterial communication by producing molecular signals that interact with bacterial QS. These compounds are called quorum sensing inhibitors (QSI) (González and Keshavan, 2006). They can mimic the structure or function of autoinducers, act as antagonists to the QS molecules as well as interfere with the stability and function of the regulator protein or the autoinducer synthase and hydrolysate signalling molecules (González and Keshavan, 2006; Tang and Zhang, 2014; Delago et al., 2016).

The best studied example of these type of QQ molecules in eukaryotes are the halogenated furanones, naturally produced by the marine macroalgae *Delisea pulchra*. These molecules protect the algae against the overgrowth of bacteria and were the first anti-QS compound identified which activity was based on competitive binding to LuxR-type proteins (González and Keshavan, 2006; Asfour, 2018). Different compounds of fungal origin have also been reported as QSIs (Table 1.4). For example, farnesol, which is produced by *Candida albicans* and can act as a strong QS molecule for this specie, also confers antimicrobial activity against other yeast and bacteria (Weber et al., 2010). Filamentous fungi have also

been studied regarding their potential to produce QSIs, in particular the genus *Penicillium*. Patulin and penicillic acid were identified as being the biologically active QSI compounds (Rasmussen et al., 2005). It was found that these compounds down regulate the expression of numerous QS-regulated genes in *P. aeruginosa* indicating their specificity towards QS-regulated gene expression (Rasmussen et al., 2005). Nevertheless, several other organisms have benefited from the development of QQ mechanisms for protection against pathogenic bacteria. Even bacteria themselves have developed QQ strategies (Weiland-Bräuer et al., 2015). Different studies have identified numerous other compounds and enzymes that function as QSI's (Rasmussen and Givskov, 2006; Jaramillo-Colorado et al., 2012; Chu et al., 2013; Fetzner, 2015).

Table 1.4 Different QSI compounds of fungal origin and their respective role.

QSI compound	Structure	Source	Role	Reference
Halogenated furanone		*Delisea pulchra*	Antifouling and antimicrobial properties. Modulates the cellular concentration of the LuxR protein of *V. fischeri*	Manefield et al., 2002
Farnesol		*Candida albicans*	Antimicrobial activity against non-albican *Candida* species and bacteria. Inhibits biofilm formation	Cerca et al., 2012
Penicillic acid		*Penicillium* spp.	Down-regulates the expression of several QS-regulated genes in *P. aeruginosa*	Rasmussen et al., 2005
Patulin		*Penicillium* spp.	Down-regulates the expression of several QS-regulated genes in *P. aeruginosa*	Rasmussen et al., 2005

The production of multiple QSI compounds by a single organism that can in fact affect the pathogenicity of a variety of bacterial species, reflects the complexity of co-evolution and advocates the existence of elaborate molecular signalling systems in eukaryote-bacterial relationships that need to be further studied (González and Keshavan, 2006).

1.9 Methodology to study mixed biofilms

Biofilm research is a multidisciplinary work, where researchers from different fields of interest, such as molecular biology, microscopy, microbiology, biochemistry and/or physics are involved (Denkhaus et al., 2006). Currently, there are a number of different reviews detailing the methodology to study biofilms as well as methodology for studying the microbial ecology of DWDS (Denkhaus et al., 2007; Douterelo et al., 2014; Franklin et al., 2015; Azeredo et al., 2017). Figure 1.9 summarizes methods for biofilm analysis and respective fields of interest. The following text will only briefly describe the techniques used throughout this work.

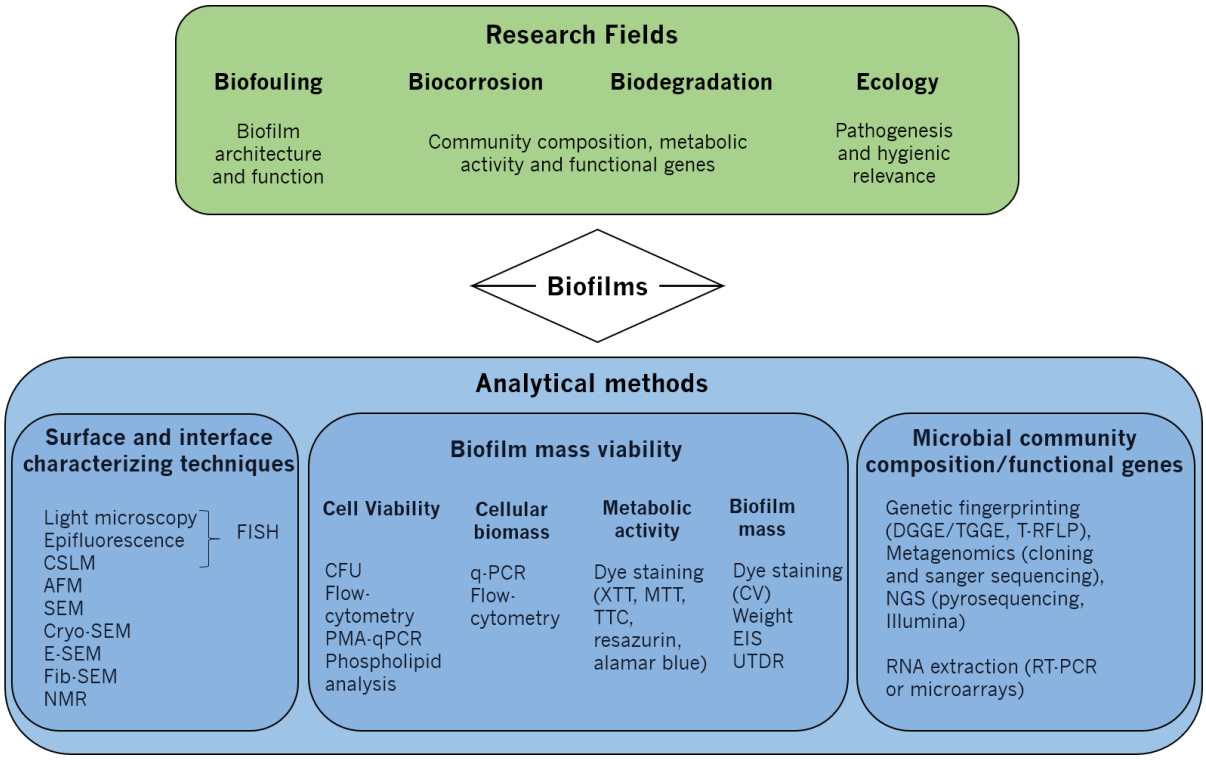


Figure 1.9 Overview of research fields and different methods to characterize biofilms (based on Denkhaus et al., 2006; Douterelo et al., 2014 and Azeredo et al., 2017). AFM – atomic force microscopy, CFU – colony forming units, CSLM – confocal laser scanning microscopy, CV – crystal violet, DGGE - denaturing gradient gel electrophoresis, FISH – fluorescent in situ hybridization, MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, NGS- next generation sequencing, NMR – nuclear magnetic resonance, PMA – propidium monoazide, qPCR – quantitative polymerase chain reaction, RT-PCR – real time PCR, SEM – scanning electron microscopy, Cryo-SEM – cryo scanning electron microscopy, E-SEM – environmental SEM, Fib-SEM – focused ion beam SEM, TGGE – temperature gradient gel electrophoresis, TTC – 2,3,5-triphenyl-2H-tetrazolium chloride, T-RFLP terminal restriction fragment length polymorphism, XTT – 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2Htetrazolium-5-carboxanilide inner salt.

Biofilm characterization can then be performed in a variety of characteristics such as cell numbers, cell viability, production of EPS, spatial organization of particular microorganisms and biofilm architecture. Fluorescent microscopy and fluorescent dyes are of extreme importance for the accomplishment of some of these tasks.

1.9.1 Fluorescence microscopy and fluorescent dyes

Fluorescent microscopy has become an indispensable tool in the field of biology as it has unique features that are not present in other optical microscopy techniques. This type of microscopy requires that the objects of interest fluoresce. Fluorescence is the emission of light that occurs after the absorption of light that is typically of shorter wavelength (Lichtman and Conchello, 2005). By completely filtering out the exciting light without blocking the emitted fluorescence, it is possible see the objects that are fluorescent (Lichtman and Conchello, 2005).

Fluorescent dyes (or fluorochromes) are molecules that are used by virtue of their fluorescent properties. The use of different dyes allows, with a high degree of specificity, the identification of cells and even cellular components. When they absorb light energy, alterations in the molecule vibration, electronic and rotational states can occur, allowing for an "excited state" to be set and fluorescence emitted (Lichtman and Conchello, 2005). The biofilm field, in particular, has benefited greatly from the use of this type of microscopy.

The use of fluorescent dyes provides vast benefits for biofilm research as it allows for non-destructive analysis while providing crucial information about the architecture, microbial diversity and metabolic activity of a given biofilm. These dyes can have specificity for different types or microorganisms, including bacteria and fungi, making this type of microscopy an optimal approach for the analysis of mixed biofilms. CSLM has the advantage of allowing 3-D characterization of the biofilm structure, however it is much more expensive than fluorescence microscopy (Simões et al., 2015; Schlafer and Meyer, 2017).

1.9.1.2 Calcofluor White M2R

Calcofluor White M2R (4,4'-bis[4-anilino-6-bis(2-ethyl)amino-s-triazin-2-ylamino]-2,2'-disulfonic acid) (CW) is a fluorescent fabric-brightener used since the early 1940s in the paper and textile related industries (Fischer et al., 1985). As a selective staining, CW has been particularly useful in the detection and morphological studies of fungi. It allows the visualization of fungal cell walls due of its affinity for β(1-3) and β(1-4) polysaccharides, such as those found in cellulose, carboxylated polysaccharides and chitin

(Harrington and Hageage, 2003). This specificity to binding to chitin, makes it an optimal stain to detect and quantify fungi and has been used for medicine in the rapid detection of pathogenic fungi and for environmental mycology studies (Harrington and Hageage, 2003; Baschien et al., 2008; Dass et al., 2015; Prakash et al., 2016). The absorption spectra for aqueous solutions of CW show absorption over the range 300 to 412 nm, with an absorbance peak at 347 nm, meaning that maximum excitation occurs with ultraviolet (UV) light. Thus, when excited with UV light, CW fluoresces with an intense bluish/white colour (Figure 1.10) (Harrington and Hageage, 2003).

CW has also been widely used to characterize drinking water fungal biofilms (Gonçalves et al., 2006; Siqueira et al., 2011, 2013; Simões et al., 2015).

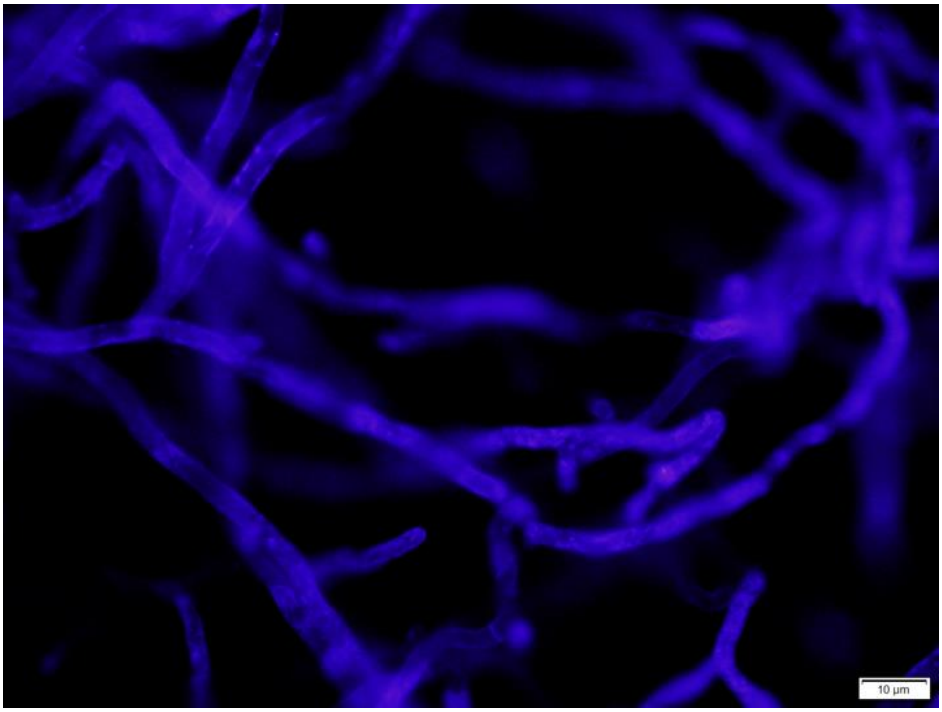


Figure 1.10 CW staining of a *Penicillium expansum* biofilm.

1.9.1.3 DAPI (4,6-diamidino-2-phenylindole)

DAPI is a blue-fluorescent nucleic acid counterstain. This dye is highly stable at UV light and has an absorbance maximum at 340 nm and a fluorescence maximum at 488 nm resulting in a fluorescence of blue colour (Figure 1.11). DAPI has been widely applied in the research of water biofilms to detect bacteria (Deines et al., 2010; Kormas et al., 2010; Siqueira et al., 2013; Gomes et al., 2016).

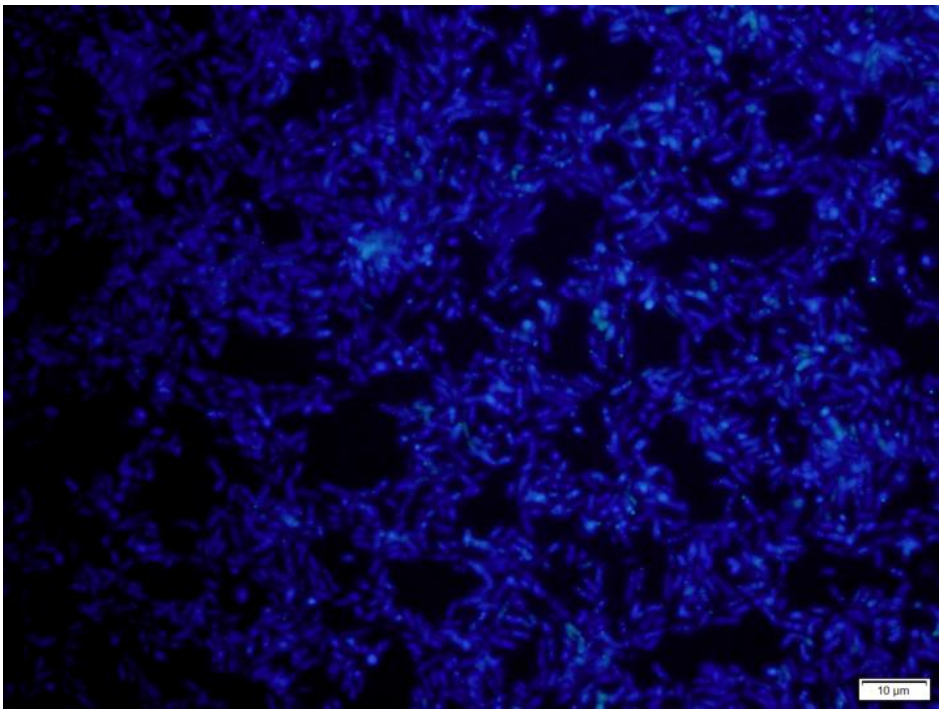


Figure 1.11 DAPI staining of a *Methylobacterium oryzae* biofilm.

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Chapter 2

Inter-kingdom biofilm formation by bacteria and filamentous fungi isolated from a drinking water distribution system

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2.1 Introduction

Biofilms can be considered the main source of microorganisms in drinking water distribution systems (DWDS), which may contribute to the deterioration of drinking water quality (Momba et al., 2000; Berry et al., 2006). The presence of biofilms in DWDS can influence crucial processes including changes in turbidity, taste, odour and colour of the water, increase in cell numbers in the bulk phase, biocorrosion, decay of disinfectant residual, harbouring of pathogens and production of toxins (Paterson and Lima, 2005; Feazel et al., 2009; Li et al., 2015; Wang et al., 2017; Zhou et al., 2017). The study of biofilms in DWDS has been mainly focused on bacteria as they are generally the dominant microorganisms, due to their high growth rates, small size, ability to produce extracellular polymeric substances (EPS) and high adaptation capacities (Characklis and Marshall, 1990). The ecology of a biofilm is a complex function of prevailing growth conditions, hydrodynamic forces, presence of microbial metabolites and molecules excreted by dominant microbial habitants of the biofilm (Bryers and Ratner, 2004). Despite DWDS being considered 'unnatural' habitats to filamentous fungi, they can often be introduced into these environments from different locations (e.g. physical openings in storage facilities, leaking joints and adapters, cracks in pipelines, etc.) and cause additional problems such as unpleasant appearance, pigments, blocked pipes, a source of potentially pathogenic and allergy-causing fungi and the production of mycotoxins (Metzger et al., 1976; Åslund, 1984; Paterson and Lima, 2005, 2010; Gonçalves et al., 2006; Hageskal et al., 2009). Studies considering fungi as pathogenic microorganisms in DWDS are sparse when compared to bacteria, mainly because water contaminated by fungi does not lead to acute disease (Hageskal et al., 2009). While the term "biofilm" has been rarely applied to filamentous fungi, there has been indication that they grow as biofilms in diverse environmental, medical and industrial settings (Anaissie et al., 2003; Gutierrez-Correa and Villena, 2003; Mowat et al., 2007; Siqueira et al., 2013; Simões et al., 2015). This is due to their high ability for growing on surfaces, as shown by their absorptive nutrition mode, secretion of extracellular enzymes to digest complex molecules, and apical hyphal growth (Jones, 1994). Few reports on filamentous fungal biofilms in DWDS can be found in the literature, however, there are already in-depth studies of filamentous fungal biofilms in other environments, especially regarding the fungal pathogen *Aspergillus fumigatus* (Müller et al., 2001; Ramage et al., 2011; Siqueira et al., 2011; 2013; Simões et al., 2015; González-Ramírez et al., 2016; Sav et al., 2018). Harding et al. (2009) proposed a six-step preliminary model for filamentous fungi biofilm formation based on previously published descriptions for filamentous fungi biofilms and drawing from bacterial and yeast models: propagule adsorption, active attachment to a surface, microcolony formation I, microcolony formation II or initial maturation, maturation or reproductive development, and a dispersal or planktonic phase. Simões et al. (2015),

recently demonstrated that similar to bacterial biofilms, it was possible to identify these same phases on filamentous fungi biofilm formation. Filamentous fungi are, therefore, excellent candidates for biofilm formation.

Under natural conditions, true monospecies biofilms are rare, and they are usually considered as complex communities (Douterelo et al., 2018). This diversity leads to a variety of complex relationships involving interspecies and intraspecies interactions (Douterelo et al., 2016). Although intraspecies interactions may play an important role in the coexistence of some microbiomes, biotic interactions between distantly related organisms across the kingdoms of life can also regulate the composition of these communities (Kastman et al., 2016). In many microbiomes, bacteria can coexist with different eukaryotic microorganisms, including fungi (Frey-Klett et al., 2011). One type of these interactions can occur in biofilms. As the microorganisms are closely embedded in an extracellular matrix, inter-kingdom biofilms containing bacteria and fungi (both nonfilamentous and filamentous) can in fact be considered a more intimate level of bacterial-fungal interaction but this aspect is still poorly understood (Frey-Klett et al., 2011; Afonso et al., 2021). In these mixed biofilms both organisms can exist as dual complexes or fungi may offer biotic support for the establishment of a bacterial biofilm (Seneviratne et al., 2008; Lahaye et al., 2016). Understanding the mechanisms of interaction between these organisms is fundamental to unravel the kinetics of inter-kingdom biofilm formation.

As there is a gap in the study of inter-kingdom biofilms between bacteria and filamentous fungi found in DWDS, the purpose of this work was to assess the ability of these microorganisms to interact with each other in biofilm formation.

2.2 Materials and Methods

2.2.1 Microorganisms and culture conditions

Penicillium expansum MUM 00.02 and *Penicillium brevicompactum* MUM 05.17 supplied by Micoteca da Universidade do Minho fungal culture collection (MUM, Braga, Portugal), were used in this work and chosen based on their high occurrence in the tap water of the north of Portugal (Gonçalves et al., 2006). *Penicillium expansum* and *Penicillium brevicompactum* were maintained on malt extract agar (MEA: malt extract 20 g, peptone 5 g, agar 20 g, distilled water 1 l).

Acinetobacter calcoaceticus and *Methylobacterium oryzae* were previously isolated from a model laboratory DWDS by Simões et al. (2007b). They were chosen for being representative of drinking water

bacteria and due to their ability to form complex biofilms (Simões et al., 2010). *A. calcoaceticus* was grown overnight while *M. oryzae* was grown for 72 h before the start of the assay. Bacteria were grown in batch cultures using 100 ml of R2A broth (yeast extract 0.5 g, proteose peptone 0.5 g, casein hydrolysate 0.5 g, glucose 0.5 g, soluble starch 0.5 g, dipotassium phosphate 0.3 g, magnesium sulphate 0.024 g, sodium pyruvate 0.3 g, distilled water 1 l) at room temperature (25 ± 2°C) and under agitation (150 rpm). Afterwards, the bacteria were harvested by centrifugation (10 min at 13,000 ×*g,* room temperature), washed three times in 0.1 M saline phosphate buffer, and resuspended in a volume of R2A to obtain a cellular density of 1×10^8 cells ml^{-1}. This was the bacterial concentration used for biofilm formation assays.

2.2.2 Stock solution of fungal spores

Stock solution of fungal spores were prepared according to Simões et al. (2015). Briefly, spores of *P. expansum* and *P. brevicompactum* were harvested from seven-day old pure cultures in MEA at 25 °C by flooding the surface of the agar plates with 2 ml of TWS solution (0.85% NaCl plus 0.05% Tween 80) and rocking gently. The suspension was then homogenized by vortexing and used for large scale production of spores. The final spore suspension was homogenized by vortexing before quantification using a Neubauer count chamber. Several aliquots of spore suspension with 10% of glycerol were cryopreserved at -80 °C in order to allow the use of the same spore suspension in all the biofilm assays. Stock spore suspensions were resuspended in the volume of R2A broth necessary to achieve a density of 10^5 spores ml^{-1}. This was the spore concentration used for biofilm formation assays. Assays were performed using fresh fungal spores or germinated fungal spores. Spores were pre-germinated in order to stimulate their metabolic state. Germinated fungal spores were prepared by inoculating 10^5 fresh spores ml^{-1} in R2A broth for approximately 14 hours (sufficient time to have > 95% of the spores germinated).

2.2.3 Biofilm formation

Biofilms were developed according to the modified microtiter plate test used by Stepanović et al. (2000) for bacteria and Simões et al. (2015) for fungi. Briefly, 16 wells of sterile polystyrene 96-well flat bottom culture plates (Greiner Bio-One Cellstar®, Kremsmünter, Austria) were filled under aseptic conditions with 200 µl of spore suspension (10^5 spores ml^{-1} in R2A broth) or 200 µl of a cell suspension (10^8 cell ml^{-1} in R2A broth) for the single-species biofilms, which were also used as positive controls. For inter-kingdom biofilm formation, 100 µl of fresh fungal spore suspension or germinated fungal spore

suspension and 100 μl of the bacterial cell suspension was added to each well. The reduction to half-cell density in the inter-kingdom assays was performed to avoid limitation in nutritional factors. Each fungal species grew alongside each bacterium. To promote biofilm formation, all plates were incubated aerobically at room temperature (25 ± 2 °C) and under agitation (150 rpm) for 24, 48 and 72 hours. The medium was renewed each 24 h. At each sampling time, the content of each well was removed and washed two times with 200 μl of sterile distilled water to remove non-adherent and weakly adherent cells. The plates were air dried for 30 min to remove excess water by evaporation. The remaining attached cells were analysed in terms of biomass adhered on the inner walls of the wells, in terms of metabolic activity and colony forming units (CFU) for bacteria. The rinsing and drying procedures were as previously used with bacterial and fungal biofilms (Simões et al., 2007a; 2010; 2015). The morphology of inter-kingdom biofilms was also qualitatively characterized by epifluorescence microscopy. Negative controls were obtained by incubating the wells with only R2A broth without adding any fungal spores or bacterial cells. Experiments were performed in triplicate with at least 3 repeats.

2.2.4 Biofilm monitoring by spectrophotometric methods

2.2.4.1 Biofilm mass quantification by crystal violet

The biomass adhered on the inner walls of the wells was quantified by the crystal violet (CV) method according to the procedure described by Stepanović et al. (2000). The fungal biofilms in the 96-well plates were fixed with 200 μl $well^{-1}$ of 98% methanol (VWR, Carnaxide, Portugal), for 15 min. Afterwards, the methanol was discarded, the plates left to dry and then the fixed biofilm was stained with 200 μL $well^{-1}$ of CV (Merck, Algés, Portugal) for 5 min. Excess stain was rinsed out by placing the plate under slow running tap water. After this, the plates were air dried and the dye bound to the adherent cells was resolubilized by adding 200 μl $well^{-1}$ of 33% (v v^{-1}) glacial acetic acid (Panreac, Cascais, Portugal). The optical density of the obtained solution was measured at 570 nm using a microtiter plate reader (BIO-TEK, Winooski, VT, USA) and the fungal biofilm mass was presented as $OD_{570\ nm}$ values.

2.2.4.2 Biofilm metabolic activity assessment by resazurin

Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) (Sigma-Aldrich, Sintra, Portugal) is a viability dye used to assess the metabolic activity of biofilms (Borges et al., 2012). For each biofilm growth period, fresh R2A broth (190 μl) was added to the plates. A volume of 10 μl of resazurin (400 μM) indicator solution was added to each well, in order to obtain a final resazurin concentration of 20 μM. Plates were

incubated in the dark for 3 hours at 25 °C. The staining protocol was optimized for inter-kingdom biofilms containing fungi and bacteria, in terms of time and temperature of incubation (data not shown). Fluorescence was measured after excitation at 530 nm and emission at 590 nm using a microtiter plate reader (BIO-TEK). The biofilm specific metabolic activity was expressed as Fluorescence/$OD_{570\ nm}$ (biofilm metabolic activity/biofilm mass).

2.2.5 Number of bacteria in single and inter-kingdom biofilms

The number of bacterial cells present in either *A. calcoaceticus* or *M. oryzae* single species biofilms and in inter-kingdom biofilms was determined in terms of CFU using a plate count assay upon biofilm release. Briefly, bacterial single-species biofilms and inter-kingdom biofilms were grown in a 96-well plate as previously described. After each incubation period, the supernatant was removed, and the plate was washed three times with sterile water. A volume of 200 µl of sterile phosphate buffer saline (pH 7.4) was added into each well and the 96-well plate was covered with the lid and placed into an ultrasonic bath (Bandelin electronic GmbH & Co. KG). To release bacterial cells from biofilm, the 96-well plate was sonicated for 1 min (5 sec sonicate, 10 sec interval) at 35 kHz. Three replicates were used for each sample and bacterial cells were plated onto R2A agar plates for CFU determination.

2.2.6 Biofilm monitoring by epifluorescence microscopy

At each sampling time, a coupon of polystyrene (1 cm^2) that had been inserted in a 24-well microtiter plate was inspected by an Olympus BX51 epifluorescence microscope (Olympus, Tokyo, Japan) with different stains: calcofluor white M2R (CW) (Invitrogen/Molecular Probes, Leiden, the Netherlands) to stain the fungi and 4,6-diamino-2-phenylindole (DAPI) (Sigma, USA) to stain the bacteria. Washed fungal biofilms were stained with 10 µl of 25 µM CW at room temperature for 15 min, in the dark. Washed bacterial biofilms were stained with 10 µl of 100 µg ml^{-1} DAPI at room temperature for 15 min, in the dark. Washed inter-kingdom biofilms were stained with 5 µl of 25 µM CW and 5 µl of 100 µg ml^{-1} DAPI at room temperature for 15 min, in the dark. After staining, biofilm samples were observed under epifluorescence microscopy. The optical filter combination used for CW and DAPI were a 365-370 nm excitation filter, a LP421 nm emission filter and a 400 nm barrier filter. Biofilms images were acquired with a microscope camera (Olympus DP71) using the cellSens software (Olympus Corporation).

2.2.7 Statistical analysis

The data were analysed using the software GraphPad Prism version 8.0.2 (GraphPad Software, La Jolla, California, USA). The mean and standard deviation (SD) within samples were calculated for all cases. Statistical significance of results was determined by unpaired *t* test. $P < 0.05$ was considered to be statistically significant.

2.3 Results

2.3.1 Biofilm mass quantification and specific metabolic activity of biofilms

In order to assess biofilm formation and specific metabolic activities of the selected single-species (used as controls) and inter-kingdom biofilms, the standard 96-well microtiter plates with CV and resazurin were used. Results for biofilm mass quantification are presented in Figure 2.1, while results for specific metabolic activity are presented in Figure 2.2. Results are divided for *P. expansum* (Figure 2.1a, 2.2a) and *P. brevicompactum* (Figure 2.1b, 2.2b). Biofilm mass results revealed an increase over time for all single-species (bacteria and fungi) and inter-kingdom biofilms combinations ($P < 0.05$), except for *P. brevicompactum* spores + *M. oryzae* ($P > 0.05$). Fungal species using germinated spores resulted in a higher biofilm mass yield when compared to fresh spores for the first 48 h ($P < 0.05$). At 72 h this difference was no longer observed. Inter-kingdom biofilm formation between *P. expansum* spores + *A. calcoaceticus* revealed significant differences compared to *P. expansum* spores single-species biofilms for all time points ($P < 0.05$) while there was only a difference at 72 h when compared with *A. calcoaceticus* single-species biofilms (Figure 2.1a). This displays an inhibitory effect of the bacteria towards *P. expansum* spores. When *P. expansum* germinated spores were present, this inhibitory effect was still significant at 24 h, less prominent at 48 h ($P < 0.05$) and null at 72 h ($P > 0.05$). Biofilm formation between *P. expansum* spores + *M. oryzae* revealed significant differences when compared with *P. expansum* spores single-species biofilms for all time points ($P < 0.05$) while there was a difference at 48 and 72 h when compared with *M. oryzae* single-species biofilms (Figure 2.1a). This also demonstrates an inhibitory effect of this bacterium towards *P. expansum* spores germination. When germinated spores were present, this effect was less pronounced ($P < 0.05$), similarly to inter-kingdom biofilms with *A. calcoaceticus*. Inter-kingdom biofilm formation between *P. brevicompactum* spores and germinated spores with both bacteria followed the same pattern of inhibition as *P. expansum* spores and germinated spores ($P < 0.05$) (Figure 2.1b).

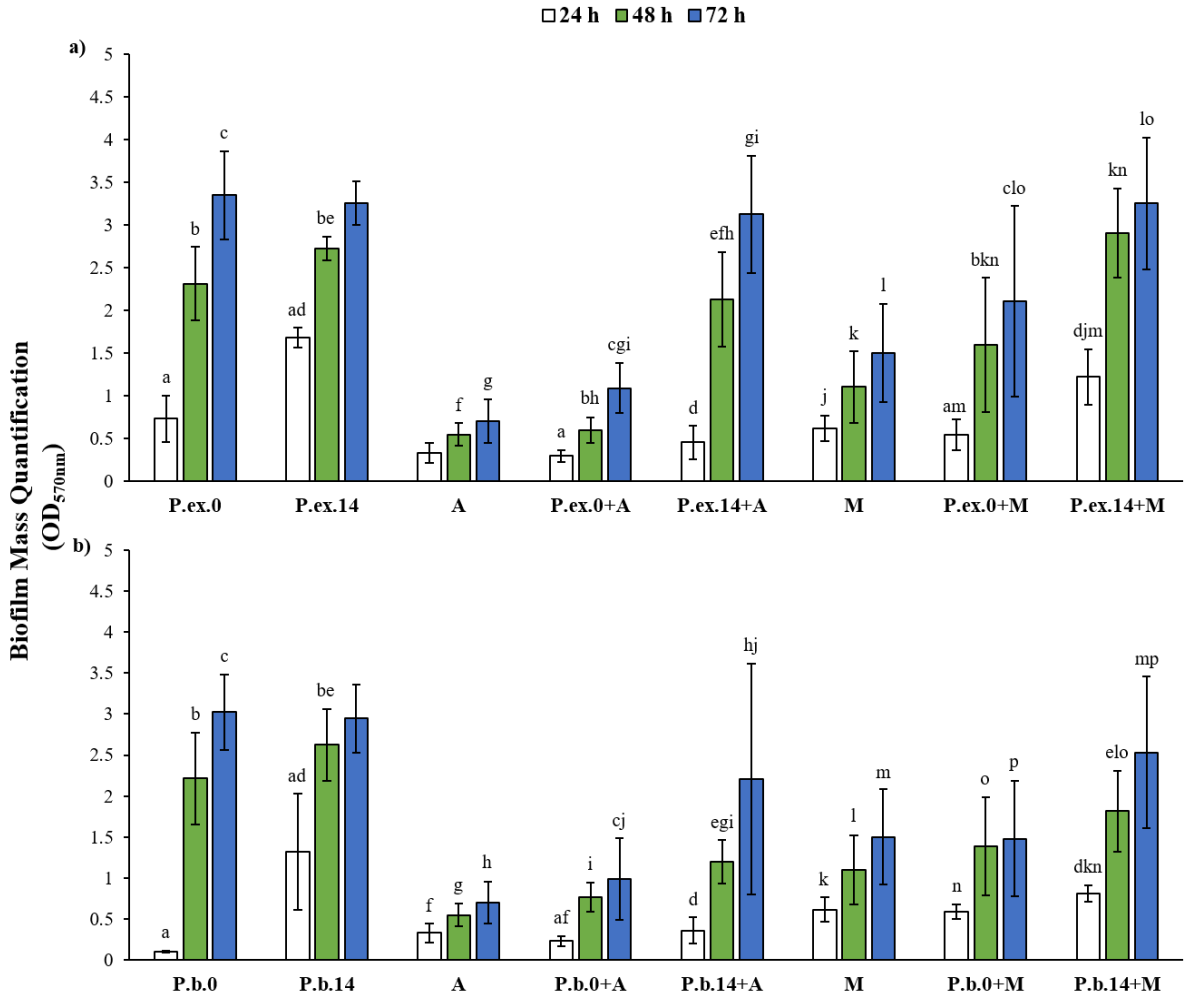

Figure 2.1 Biofilm mass quantification for single-species and inter-kingdom biofilm formation over time with *P. expansum* (a) or *P. brevicompactum* (b). Legend is as follows: 'P.ex.0' - *P. expansum* spores; 'P.ex.14' - *P. expansum* germinated spores at 14 hours; A - *A. calcoaceticus*; M - *M. oryzae*; 'P.b.0' - *P. brevicompactum* spores; 'P.b.14' - *P. brevicompactum* germinated spores at 14 hours. The means ± SDs for three independent experiments are illustrated. Data labelled with the same letters represent significant differences at $P < 0.05$.

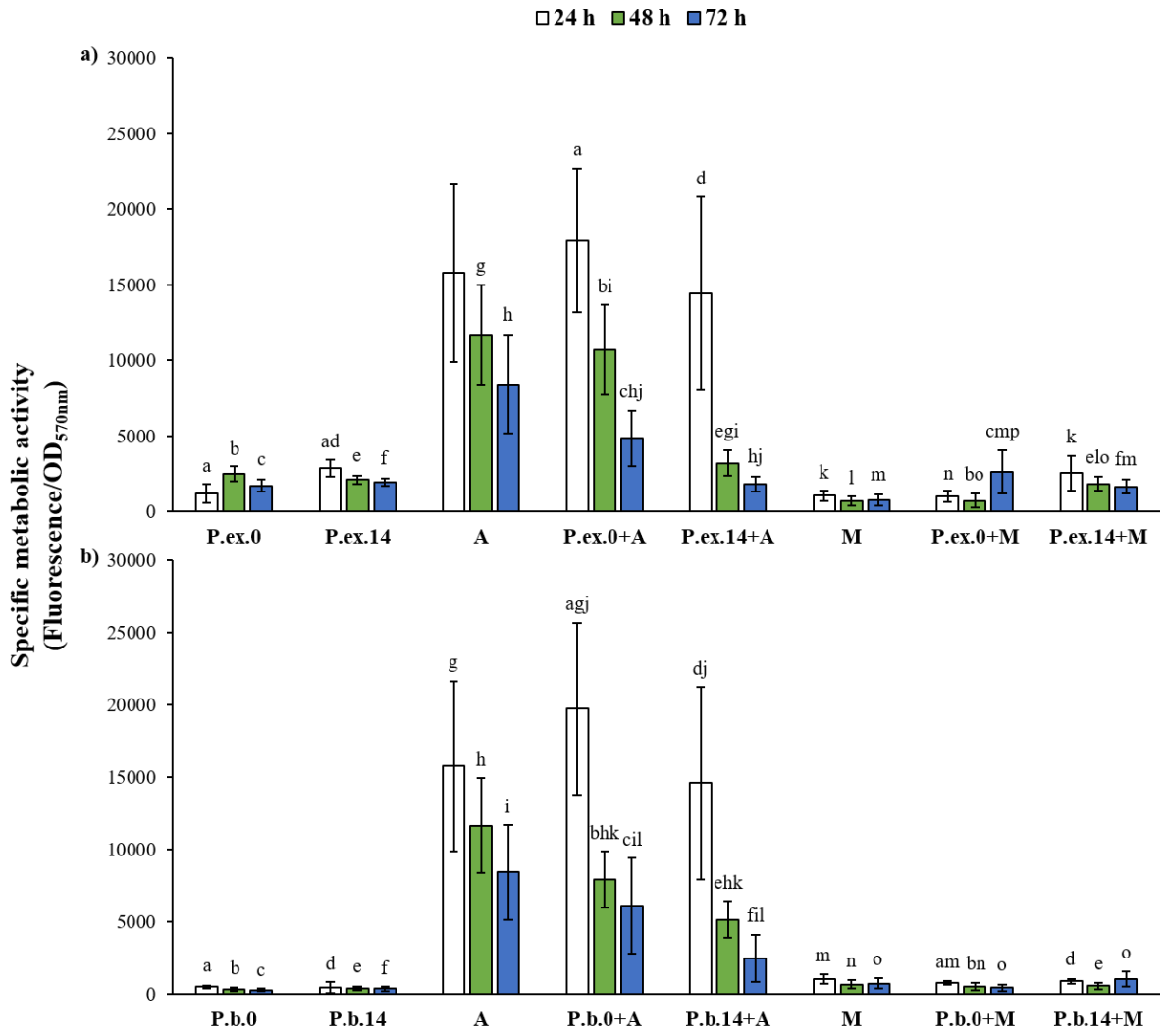

Figure 2.2 Specific metabolic activity for single-species and inter-kingdom biofilm formation over time with *P. expansum* (a) or *P. brevicompactum* (b). Legend is as follows: 'P.ex.0' - *P. expansum* spores; 'P.ex.14' - *P. expansum* germinated spores at 14 hours; A - *A. calcoaceticus*; M - *M. oryzae*; 'P.b.0' - *P. brevicompactum* spores; 'P.b.14' - *P. brevicompactum* germinated spores at 14 hours. The means ± SDs for three independent experiments are illustrated. Data labelled with the same letters represent significant differences at $P < 0.05$.

Figure 2.2 shows the results obtained for specific metabolic activity of *P. expansum* (Figure 2.2a) and *P. brevicompactum* (Figure 2.2b) biofilms. Specific metabolic activity (per biomass) increased in *P. expansum* spores single-species biofilms from 24 to 48 h and then decreased at 72 h ($P < 0.05$). Using germinated spores, the specific metabolic activity of *P. expansum* increased at 24 h relatively to the use of fresh spores. Metabolic activity of *P. expansum* germinated spores biofilms was at its highest at 24 h and then decreased over time ($P < 0.05$). This loss in specific metabolic activity per biomass was also emphasized in *A. calcoaceticus* single-species biofilms. This bacterium had the highest values for metabolic activity registered in this study. For this reason, in inter-kingdom biofilms containing of *A. calcoaceticus*, data for metabolic activity was compared with *A. calcoaceticus* single-species biofilms as

it outshined the metabolic activity of the fungi. In *P. expansum* spores + *A. calcoaceticus* biofilms, there was a maintenance in metabolic activity for the first 48 h ($P > 0.05$) while there was a decrease at 72 h ($P < 0.05$). When *P. expansum* germinated spores were present with *A. calcoaceticus*, there was no change in the metabolic activity of the biofilm at 24 h, but a decrease was observed at 48 and 72 h ($P < 0.05$).

Specific metabolic activity related to *M. oryzae* single-species biofilms showed a decrease from 24 to 48 h ($P < 0.05$) and then a maintenance between 48 and 72 h ($P > 0.05$). In inter-kingdom biofilm formation of *P. expansum* spores + *M. oryzae* there was a maintenance in metabolic activity at 24 h when compared with the single-species biofilms of both microorganisms, a decrease at 48 h when compared with *P. expansum* spores single-species biofilm and an increase at 72 h when compared with both microorganisms single-species biofilms ($P < 0.05$). In the presence of *P. expansum* germinated spores there was an activity maintenance at 24 h compared to *P. expansum* germinated spores single-species biofilm and then a slight decrease at 48 and 72 h ($P < 0.05$).

Analysing the results with *P. brevicompactum* revealed a maintenance in specific metabolic activity for the duration of the experiments when using fungal spores or fungal germinated spores ($P > 0.05$) (Figure 2.2b). In the presence of *A. calcoaceticus*, the same outshine effect was observed due to the difference in the metabolic activities of the microorganisms. In *P. brevicompactum* spores + *A. calcoaceticus* biofilms, metabolic activity was higher at 24 h compared with *A. calcoaceticus* single-species biofilms and then a decrease was observed at 48 and 72 h ($P < 0.05$). With *P. brevicompactum* germinated spores the increase in metabolic activity at 24 h was not observed ($P > 0.05$), but the decrease at 48 and 72 h was more pronounced ($P < 0.05$). In inter-kingdom biofilms containing *P. brevicompactum* spores + *M. oryzae* there was a decrease in metabolic activity for all time points when compared with *M. oryzae* single-species biofilms ($P < 0.05$). For *P. brevicompactum* germinated spores + *M. oryzae* biofilms, there was a maintenance in metabolic activity for the first 48 h compared with *M. oryzae* single specie biofilms, but an increase was observed at 72 h ($P < 0.05$).

2.3.2 Cell density in single-species and inter-kingdom biofilms

The numbers of bacterial cells present in either single-species biofilms and in inter-kingdom biofilms was determined as CFU and the results obtained are presented in Figure 2.3. Bacterial single-species biofilms were used as controls. Analysing *A. calcoaceticus* results (Figure 2.3a), an increase in CFU at 24 h was observed for *A. calcoaceticus* + *P. expansum* germinated spores and *A. calcoaceticus* + *P. brevicompactum* spores ($P < 0.05$). At 48 h, compared with *A. calcoaceticus* single-species biofilms, an

increase in CFU for this bacterium was observed ($P < 0.05$) for all inter-kingdom biofilms, except for *A. calcoaceticus* + *P. brevicompactum* germinated spores ($P > 0.05$). At 72 h, there was an increase in CFU when this bacterium grew alongside *P. expansum* spores or germinated spores ($P < 0.05$). No significant changes were observed for inter-kingdom biofilm formation with *P. brevicompactum* spores, but with *P. brevicompactum* germinated spores, an increase in CFU was observed. With *P. expansum* germinated spores, the number of CFU was higher when compared with fresh spores for all sampling times ($P < 0.05$).

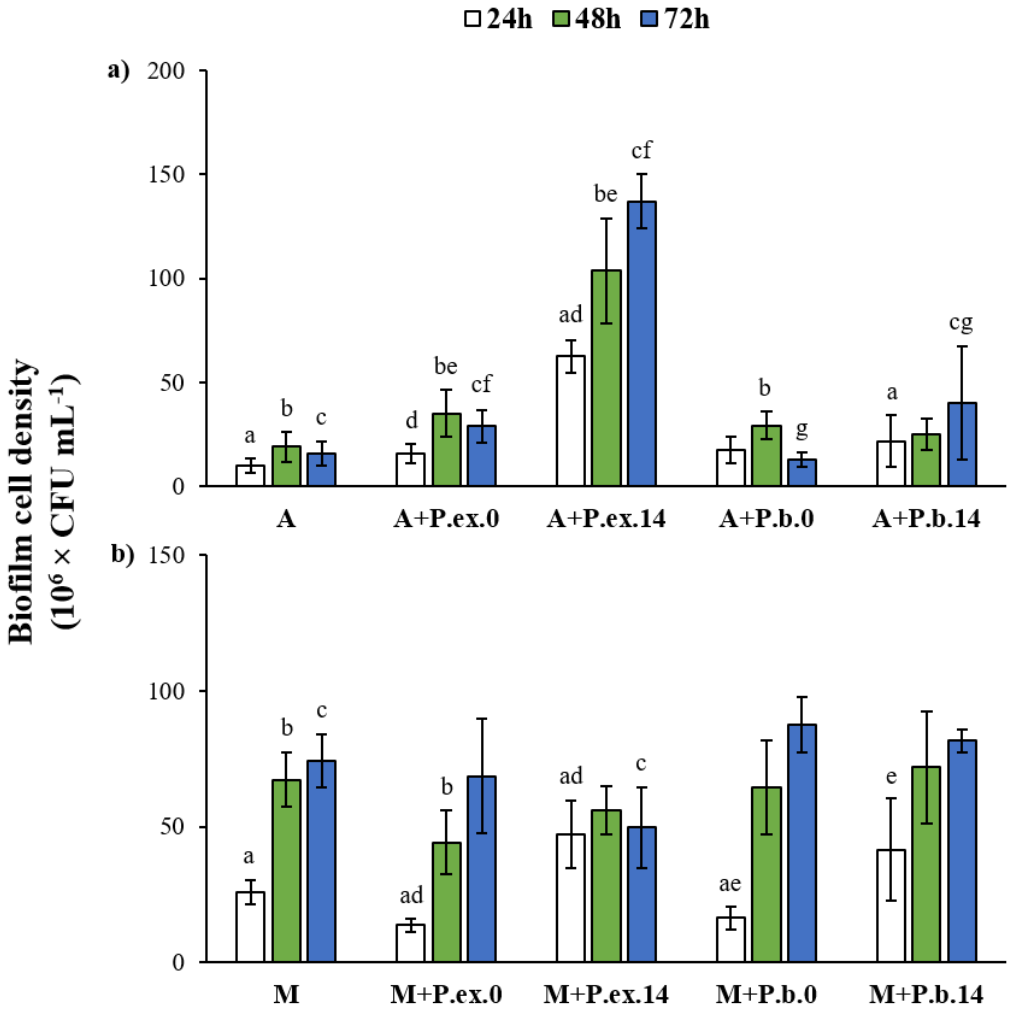

Figure 2.3 CFU counts for *A. calcoaceticus* (a) or *M. oryzae* (b) while forming single-species or inter-kingdom biofilm formation over time. Legend is as follows: A - *A. calcoaceticus*; M - *M. oryzae*; 'P.ex.0' - *P. expansum* spores; 'P.ex.14' - *P. expansum* germinated spores at 14 hours; 'P.b.0' - *P. brevicompactum* spores; 'P.b.14' - *P. brevicompactum* germinated spores at 14 hours. The means ± SDs for two independent experiments are illustrated. Data labelled with the same letters represent significant differences at $P < 0.05$.

Regarding *M. oryzae* results (Figure 2.3b) a decrease in bacterial CFU was seen at 24 h when forming inter-kingdom biofilms with both fungi fresh spores while there was an increase with *P. expansum* germinated spores and no significant changes with *P. brevicompactum* germinated spores. At 48 h, there was only a decrease with *P. expansum* spores while for the other conditions a maintenance in CFU was observed ($P > 0.05$). At 72 h a decrease in *M. oryzae* CFU was detected in inter-kingdom biofilm formation with *P. expansum* germinated spores while no significant changes were observed for the remaining conditions.

2.3.3 Microscopic visualization of biofilms

Biofilms were also qualitatively characterized by epifluorescence microscopy. The results obtained are presented in Figures 2.4 and 2.5 for the presence of *A. calcoaceticus* and in Figures 2.6 and 2.7 for the presence of *M. oryzae*. Microscopic data visually confirmed the biofilm mass increase over time. The differences in inoculating *P. expansum* spores or germinated spores with *A. calcoaceticus* (Figure 2.4a-2.4c) or *M. oryzae* (Figure 2.6a-2.6c) was noticeable at 24 h with the visual increase in fungal density. At 24 h it was not possible to detect fungal mass for *P. brevicompactum* spores + *A. calcoaceticus* nor *P. brevicompactum* germinated spores + *A. calcoaceticus*. With higher magnification it was possible to detect bacterial cells growing around or on top the fungal hyphae (Figure 2.5 and 2.7). It was also possible to see that bacteria are the primary colonizers, as they form the first biofilm layer - they are usually on a lower focal point. However, due to the increase in fungal mass over time, bacterial cells became harder to detect, and in some cases, their detection was not achieved. For this reason, images of 72 h biofilms were not added.

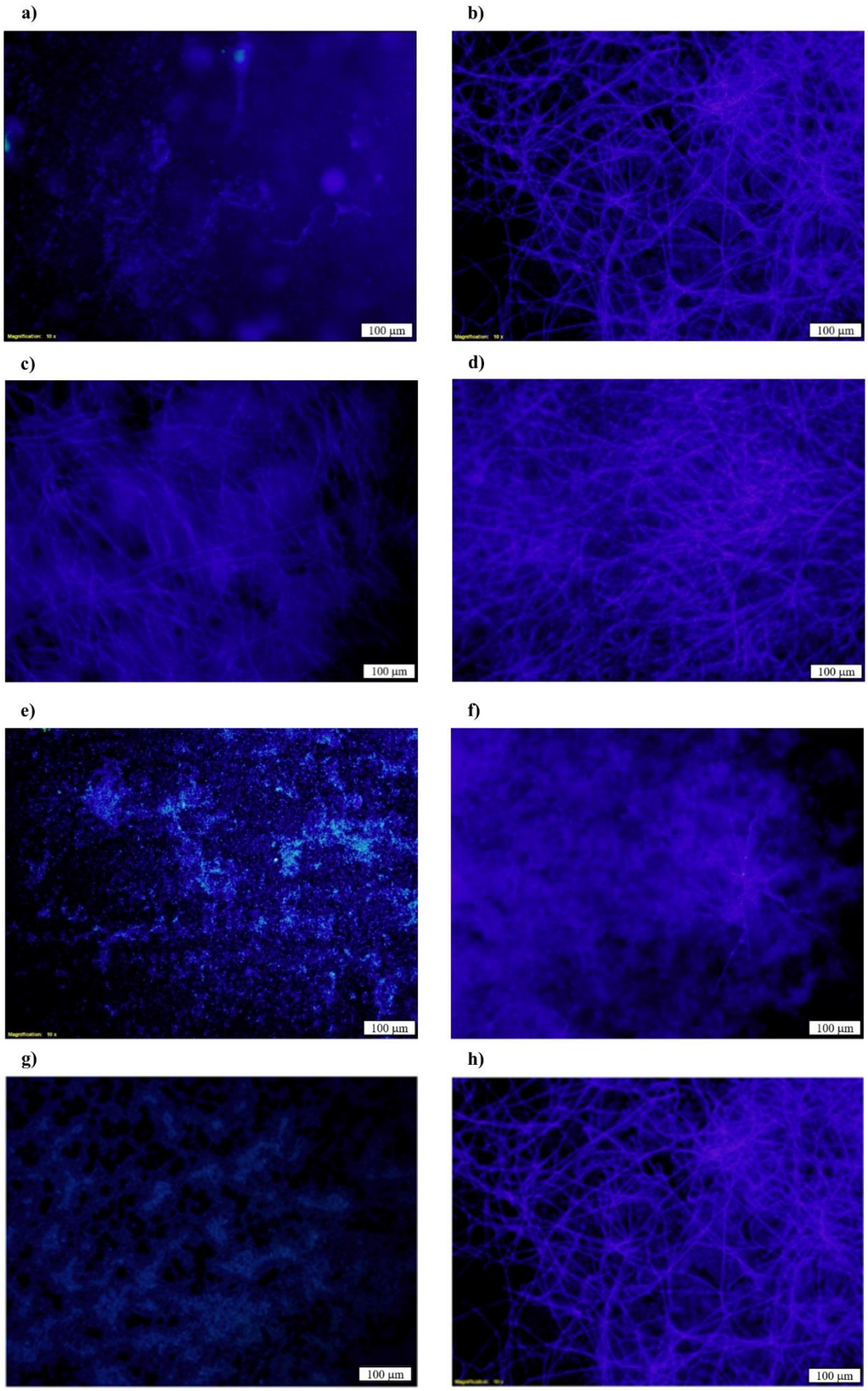

Figure 2.4 Epifluorescence photomicrographs of inter-kingdom biofilm formation with *A. calcoaceticus* on polystyrene over time with CW plus DAPI: (a) *A. calcoaceticus* + *P. expansum* spores at 24 hours; (b) *A.*

calcoaceticus + *P. expansum* spores at 48 hours; (c) *A. calcoaceticus* + *P. expansum* germinated spores at 24 h; (d) *A. calcoaceticus* + *P. expansum* germinated spores at 24 h; (e) *A. calcoaceticus* + *P. brevicompactum* spores at 24 h; (f) *A. calcoaceticus* + *P. brevicompactum* spores at 48 h; (g) *A. calcoaceticus* + *P. brevicompactum* germinated spores at 24 h; (h) *A. calcoaceticus* + *P. brevicompactum* germinated spores at 48 h. × 200 magnification/bar = 100 μm.

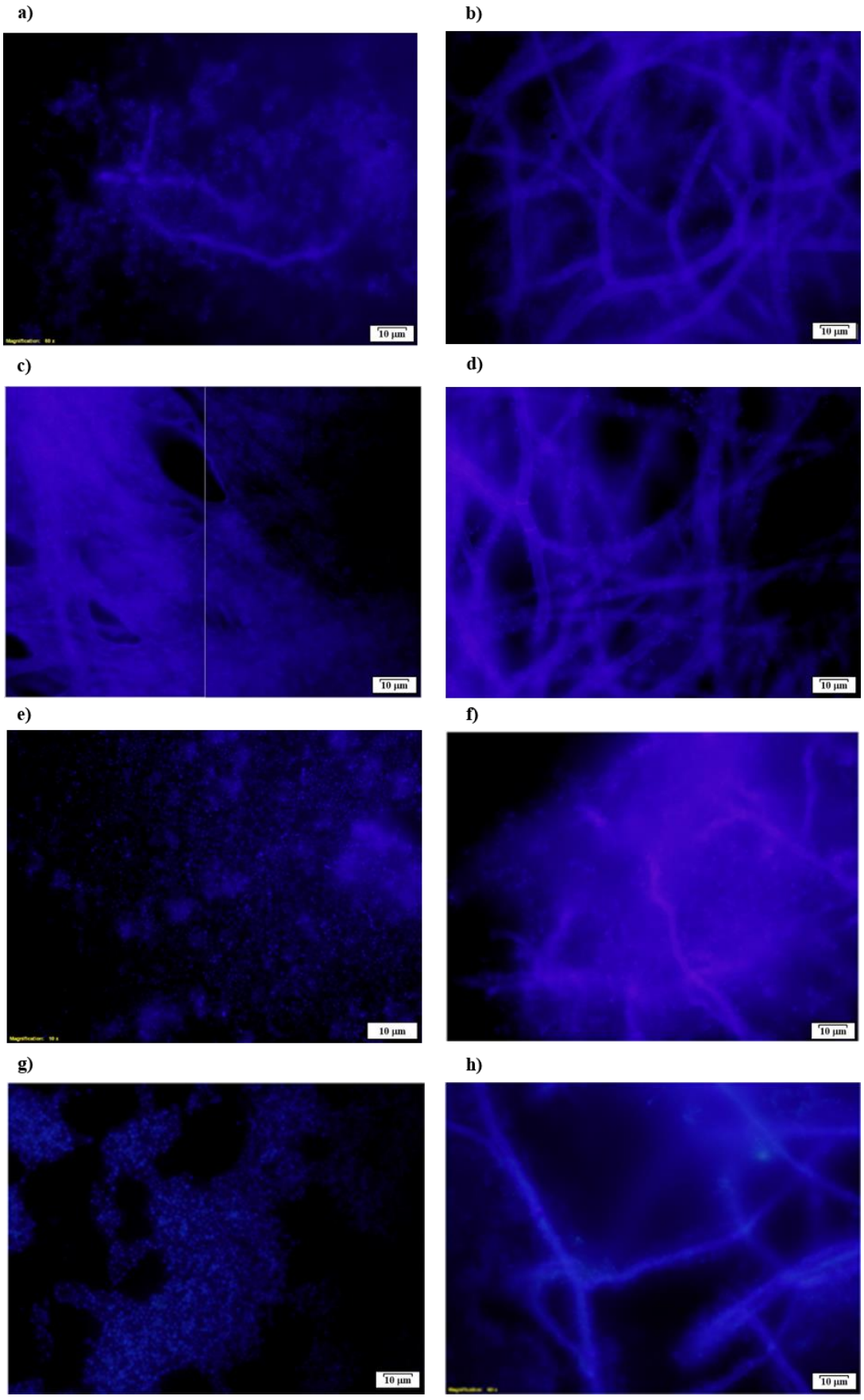

Figure 2.5 Epifluorescence photomicrographs of inter-kingdom biofilm formation with *A. calcoaceticus* on polystyrene over time with CW plus DAPI: (a) *A. calcoaceticus* + *P. expansum* spores at 24 hours; (b) *A.*

calcoaceticus + *P. expansum* spores at 48 hours; (c) *A. calcoaceticus* + *P. expansum* germinated spores at 24 h; (d) *A. calcoaceticus* + *P. expansum* germinated spores at 48 h; (e) *A. calcoaceticus* + *P. brevicompactum* spores at 24 h; (f) *A. calcoaceticus* + *P. brevicompactum* spores at 48 h; (g) *A. calcoaceticus* + *P. brevicompactum* germinated spores at 24 h; (h) *A. calcoaceticus* + *P. brevicompactum* germinated spores at 48 h. × 600 magnification/bar = 10 μm.

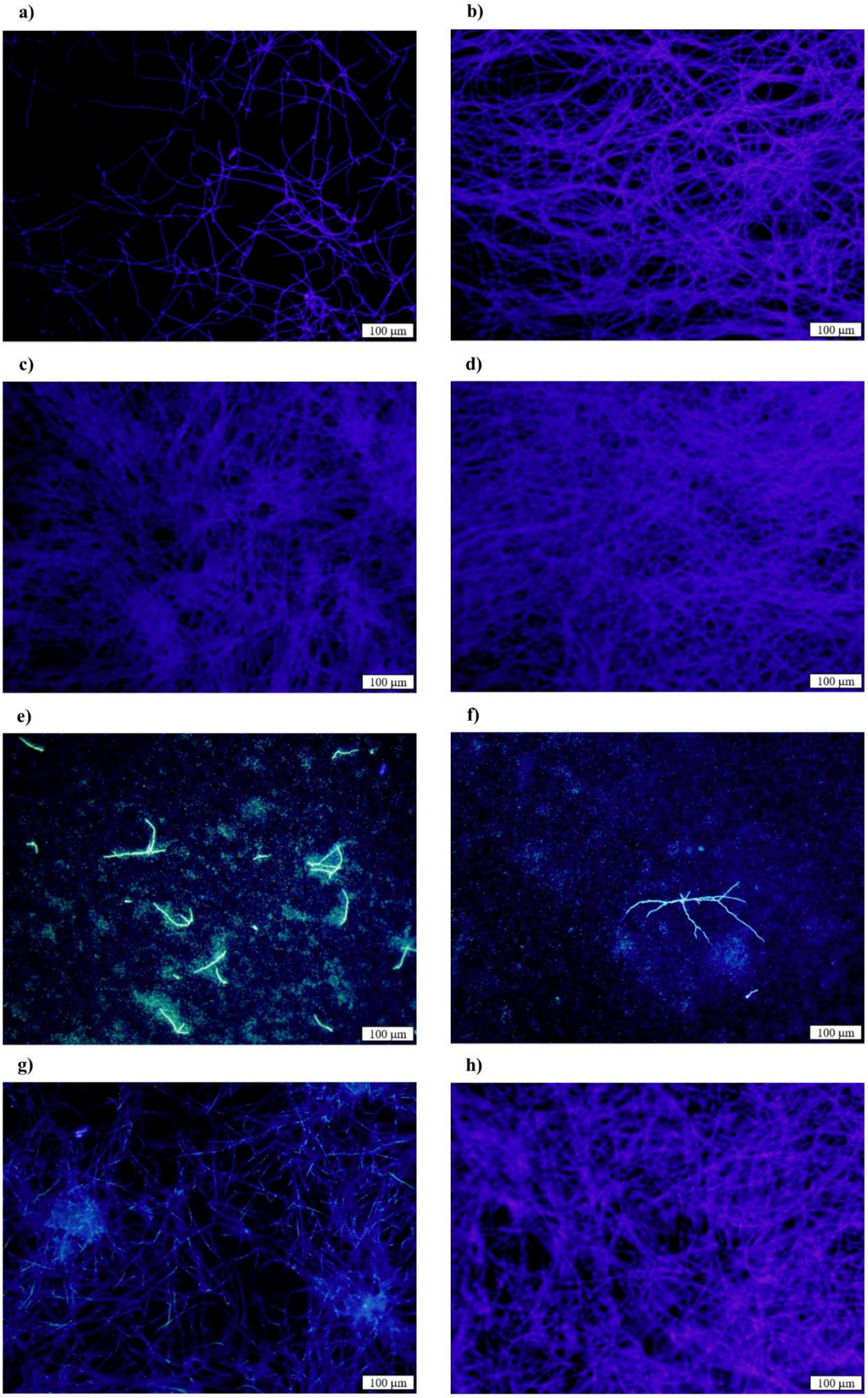

Figure 2.6 Epifluorescence photomicrographs of inter-kingdom biofilm formation with *M. oryzae* on polystyrene over time with CW plus DAPI: (a) *M. oryzae* + *P. expansum* spores at 24 hours; (b) *M. oryzae*

+ *P. expansum* spores at 48 hours; (c) *M. oryzae* + *P. expansum* germinated spores at 24 h; (d) *M. oryzae* + *P. expansum* germinated spores at 48 h; (e) *M. oryzae* + *P. brevicompactum* spores at 24 h; (f) *M. oryzae* + *P. brevicompactum* spores at 48 h; (g) *M. oryzae* + *P. brevicompactum* germinated spores at 24 h; (h) *M. oryzae* + *P. brevicompactum* germinated spores at 48 h. × 200 magnification/bar = 100 μm.

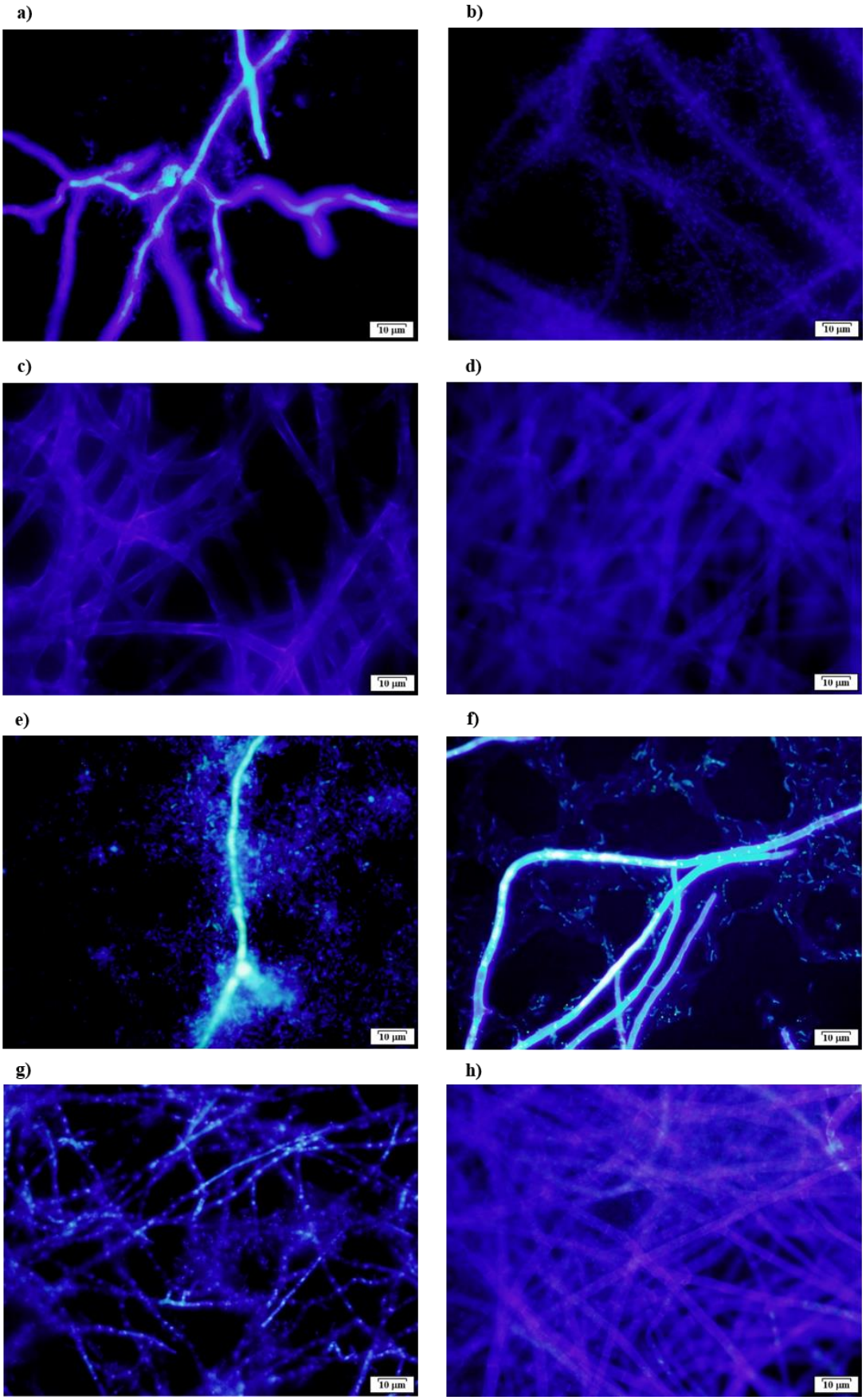

Figure 2.7 Epifluorescence photomicrographs of inter-kingdom biofilm formation with *M. oryzae* on polystyrene over time with CW plus DAPI: (a) *M. oryzae* + *P. expansum* spores at 24 hours; (b) *M. oryzae*

+ *P. expansum* spores at 48 hours; (c) *M. oryzae* + *P. expansum* germinated spores at 24 h; (d) *M. oryzae* + *P. expansum* germinated spores at 48 h; (e) *M. oryzae* + *P. brevicompactum* spores at 24 h; (f) *M. oryzae* + *P. brevicompactum* spores at 48 h; (g) *M. oryzae* + *P. brevicompactum* germinated spores at 24 h; (h) *M. oryzae* + *P. brevicompactum* germinated spores at 48 h. × 600 magnification/bar = 10 μm.

2.4 Discussion

Microbial growth and biofilm formation are dependent on a variety of factors, both biological and physico-chemical. Drinking water biofilms are composed of a complex microbial community comprised by very different organisms (bacteria, fungi, protozoa, viruses, and algae) but all adapted to grow under oligotrophic conditions and in the presence of a disinfectant (Gonçalves et al., 2006; Simões et al., 2008, 2010; Siqueira et al., 2011). Studies on the microbial ecology of DWDS have shown that the resistance of microorganisms to disinfectants, particularly chlorine, is affected by this microbial diversity and interspecies interactions (Berry et al., 2006; Simões et al., 2010). This information was, however, largely obtained from studies with bacteria. Very few reports have been published on filamentous fungi biofilms (Siqueira et al., 2011; Simões et al., 2015; Fernandes et al., 2019). The influence of these organisms in drinking water biofilms, particularly in their interaction with bacteria, is still poorly understood.

Since DWDS are very complex systems, of difficult accessibility, biofilm studies *in situ* are not very common (Douterelo et al., 2014, 2018). In order to study drinking water biofilms apart from DWDS several devices and methodologies have been developed (Gomes et al., 2014). Microtiter plates are the most frequently used *in vitro* model for studying biofilm formation under strictly laboratorial conditions, including with drinking water microorganisms (Gomes et al., 2014). Despite the disadvantages they have on reproducing conditions found in DWDS (i.e. hydrodynamic forces), polystyrene microtiter plates are commonly used as the standard bioreactor system to study adhesion and biofilm formation by bacteria and fungi isolated from these systems (Simões et al., 2010, 2015; Gomes, et al., 2014). They can be used as a rapid and simple method for analysing the effects of a variety of parameters on biofilm formation as equivalent biofilms can be produced simultaneously (Simões et al., 2007a, 2010, 2015). Polystyrene has also physicochemical surface properties similar to those of other materials used in DWDS, such as polyvinylchloride and stainless steel (Simões et al., 2007b). Regions of low shear stress easily reproduced by microtiter plates are common in DWDS particularly in reservoirs, households pipes, corners and valves. These areas are associated with zones of abundant biofilm formation due to the high accumulation of organic material (Simões and Simões, 2013). For the reasons depicted, microtiter plates were selected

to study biofilm formation and behaviour of inter-kingdom biofilms containing fungi and bacteria isolated from drinking water.

Spores were used as the starting inoculum for biofilm formation as they comprise the primary source of viable propagules (Dogget, 2000). However, hyphal fragments or other fungal propagules could also be used as they can be functionally equivalent to planktonic bacterial cells (Harding et al., 2009). The seeding spore density has also been a reportedly important factor in filamentous fungi biofilm development with an optimal concentration of 10^5 spores ml^{-1}, which was why this concentration was used (Mowat et al., 2007; Simões et al., 2015). Higher spore concentration results in poor germination of adhered spores while lower concentrations have a detrimental effect on hyphal density (Mowat et al., 2007).

Both spectrophotometric methods used in this study to quantify single-species and inter-kingdom biofilms have been used to study bacterial, yeast and filamentous fungal biofilms in microtiter plates (Goughenour et al., 2015; Simões et al., 2015; Dalecki et al., 2016; Xu et al., 2016). For the study of inter-kingdom biofilm formation their application is very scarce. Methods aimed at analysing inter-kingdom biofilm formation between fungi and bacteria are yet to be standardized. In the present study, the dynamics of inter-kingdom biofilms on polystyrene surfaces over 72 h were assessed by biomass (CV) and metabolic activity (resazurin) measurements as well as epifluorescence microscopy. CV staining is one of the main methods used for biofilm mass quantification. CV binds to negatively charged microorganisms as well as extracellular polymeric material. The absorbance values are directly proportional to the amount of biofilm biomass (Pantanella et al., 2013). However, this staining method has some limitations, including the low reproducibility and not providing information on the number and viability of cells as it stains both living and dead, along with other components of the biofilm matrix (Simões et al., 2015). For this reason, resazurin was used as a complementary method to assess the viability of biofilms. Resazurin was chosen over other methods, because according to Simões et al. (2015), it was the most sensitive method tested, able to detect lower numbers of active cells. Resazurin is a non-fluorescent dye of blue colour (water-soluble, stable, non-toxic, and permeable through cell membranes) that is reduced to the highly fluorescent pink coloured resorufin. This acts as an intermediate electron acceptor in the electron transport chain without interference by the normal transfer of electrons (Rampersad, 2012). The resazurin assay is, however, very dependent on the growth phase and the age and thickness of the biofilm as they influence cell respiratory efficiency (Pantanella et al., 2013). In addition to the time of resazurin reduction being species- and strain-related (Pantanella et al., 2013), method optimization (particularly incubation time) was required to measure the metabolic activity of inter-

kingdom biofilms. Due to the spectrophotometric limitations and adding to the inter- and intra-variability of the experiments, a high standard deviation was observed in some cases.

Epifluorescence microscopy is commonly used to obtain rapid, inexpensive qualitative information on fungal cells (Ahmad and Khan, 2012). Despite the fact that confocal laser scanning microscopy (CSLM) provides a clear advantage by allowing a 3-D characterization of biofilm structure, it is still much more expensive compared to fluorescence microscopy (Simões et al., 2015; Schlafer and Meyer, 2017). Consequently, epifluorescence microscopy was used as a first approach to qualitatively analyse inter-kingdom biofilm development over time. CW stain allows the visualization of fungal cell walls because of its affinity for β(1-3) and β(1-4) polysaccharides in cellulose, carboxylated polysaccharides, and chitin (Siqueira et al., 2011, 2013; Simões et al., 2015). DAPI was selected due to its frequent use for microscopy as a nuclear counterstain to assist in morphological observations of the bacteria (Kormas et al., 2010; Siqueira et al., 2013). The excitation wavelength for CW and DAPI was the same and both signals were blue, however, they were distinctive in colour intensity and brightness under microscopic observations. In inter-kingdom biofilms, the stronger fluorescent signal coming from the fungal staining by CW made the acquisition of clear images showing both organisms more difficult. This happened especially when germinated spores were used as the starting inoculum due to the higher number of fungal hyphae. On top of the difference in fluorescence intensity between microorganisms, bacteria usually had to be focused on a different focal plane, usually lower, which difficulted in obtaining inter-kingdom biofilm images. Despite these drawbacks, the use of these stains allowed the discrimination of the microorganisms in inter-kingdom biofilms.

In the present study, fresh spores and germinated spores were used assuming that spores in different developmental stages might be present in DWDS and provide an advantage in the metabolic state of the fungi. Through the results, it was observed that fungal stage development is important in the early stages of biofilm formation as demonstrated by the increase in mass and specific metabolic activity at 24 h when using germinated spores versus using fresh spores. Germinated spores, due to the presence of appendages, conferred an initial advantage to biofilm formation corresponding to phase II (adhesion) of the model for filamentous fungal biofilm formation proposed by Harding et al. (2009) and updated by Fernandes et al. (2019). In inter-kingdom biofilms, an increase in biomass while using germinated spores instead of fresh spores was also reported, but only for mature biofilms (≥ 48 h). The bacteria used in this study inhibited fungal sporulation and hyphal growth at 24 h independent of the fungal starting inoculum. *A. calcoaceticus* was the bacterium where this effect was most pronounced. Similar results were obtained in studies of co-culture biofilm formation between *A. fumigatus* and *Pseudomonas aeruginosa*, where, for

24 h, direct contact of the fungi with the bacterium as well as its supernatant resulted in an inhibition of fungal biofilm formation (Mowat et al., 2010). Co-culturing *Rhizopus microsporus* and *Pseudomonas aeruginosa* for 24 h also resulted in the inhibition of spore germination (Kousser et al., 2019). Additionally, Nogueira et al. (2019) demonstrated that co-culture experiments of *Klebsiella pneumoniae* with several *Aspergillus* species resulted in the inhibition of fungal spore germination and hyphal development. *Aspergillus* spores were also pre-germinated for 12 h, followed by addition of *K. pneumoniae* strains, and revealed that the inhibitory effect of this bacterium on these fungal species is independent of the fungal growth stage (Nogueira et al., 2019). In the present study, a similar effect at 24 h was observed. However, using germinated spores as the starting material resulted in a less pronounced inhibition effect towards fungal growth in already mature biofilms (48 h), and at 72 h no inhibition was detected, as revealed by the biofilm mass increase. The removal of planktonic cells and the renewal of the medium might be the reason for this increase in fungal viability. Nogueira et al. (2019) also showed that *Aspergillus* species remain viable upon interaction with *K. pneumoniae*. The inhibitory effect at 24 h, can be due to the direct competition for nutrients; the higher growth rate and metabolic activity of the bacteria; the production of metabolites that might inhibit fungal sporulation or a combination of all these factors (Mowat et al., 2010; Kousser et al., 2019; Nogueira et al., 2019). Nutritional competition between fungi and bacteria is well documented in other environments. For example, in the rhizosphere where bacterial competition for nutrients can be an effective biocontrol mechanism against fungal pathogens (Elad and Baker, 1985; Lemanceau et al., 1993). The occurrence of multiple interspecies interactions or even the simple production of a metabolite can also interfere with biofilm formation and development (Kives et al., 2005; Valle et al., 2006; Mowat et al., 2010).

The specific metabolic activity of inter-kingdom biofilms containing *A. calcoaceticus,* when compared with *A. calcoaceticus* single-species biofilms*,* was reduced in the presence of fungal spores and again the stage of spore germination was substantial in these changes. Inoculation with germinated spores was responsible for causing the highest decrease in the metabolic activity of these biofilms. This seems to be connected with the ability of the fungi to grow and form biofilm as higher fungal biofilm mass is correlated with lower biofilm metabolic activity. However, regardless the loss in specific metabolic activity, a high increase in *A. calcoaceticus* CFU was observed when inoculated alongside *P. expansum* germinated spores. This indicates that, despite the effect on specific metabolic activity, a higher quantity of *P. expansum* mass helps this bacterium to increase in numbers. The fact that *A. calcoaceticus* has been reported to perform a bridging function, facilitating biofilm formation in drinking water as it coaggregates with other bacteria, may also have a role in this increase (Simões et al., 2008).

Coaggregation provides advantages to microorganisms such as transfer of chemical signals, metabolic cooperation between different species and protection from adverse environmental conditions (Wimpenny and Colasanti, 2004). This might be helped by the biotic support and protection that intertwined fungal hyphae confer to bacteria (Frey-Klett et al., 2011). The growth around fungal hyphae was visually confirmed by epifluorescence microscopy. In the presence of *M. oryzae* the correlation between biofilm mass and specific metabolic activity was not observed. The basal metabolic activity of each microorganism might also be a variable to take in consideration when performing inter-kingdom biofilm studies. In terms of CFU, differences were also seen when inoculated with spores in different developmental stages, as they decreased with fresh fungal spores but increased with germinated spores in early biofilms. In more mature biofilms, when inoculated with *P. expansum* germinated spores, opposite to *A. calcoaceticus,* a decrease occurred. All these differences observed suggest a variability in interspecific interactions that are microbial dependent. The results also suggest that *M. oryzae* might be more susceptible to *P. expansum* than *A. calcoaceticus.*

In conclusion this study shows that fungal spore development stage is important in the first 24 h of water fungal biofilm formation. Using germinated spores, an advantage is provided in the first 24 h towards the establishment of a denser biofilm. It was observed that inter-kingdom biofilm formation between these specific filamentous fungi and bacteria isolated from DWDS is a possibility, as they can interact with each other and form mature biofilms. Nevertheless, inter-kingdom biofilm formation and development are microbial dependent as shown by the variability in interspecific interactions. It was also shown that if both inter-kingdom microorganisms are inoculated at the same time, in the first 24 h of interaction, bacteria can, in fact, inhibit filamentous fungal spore germination and its development into a biofilm. However, with time and with the renewal of the medium, these fungi can begin to germinate and develop into mature biofilms. This is particularly interesting because if the possibility of biofilm formation is provided to the fungi, or there is a chance of existing a pre-established fungal biofilm, consequently, an advantage may also be given to opportunistic bacteria to replicate and proliferate in inter-kingdom biofilms inside DWDS.

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Chapter 3

Effect of quorum sensing and quenching molecules on inter-kingdom biofilm formation by *Penicillium expansum* and bacteria

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3.1 Introduction

Drinking water distribution systems (DWDS) represent a system of reservoirs, pipes, and treatment facilities employed to transport water to the public and are known to harbour biofilms which in turn represent more than 95% of the biomass in these systems (Flemming et al., 2002; Siqueira et al., 2013). Biofilms are responsible for influencing different factors affecting the quality of drinking water. They can be a reservoir of pathogens and lead to an increase in cell numbers in the bulk phase; cause changes in the organoleptic properties of the water; increase the corrosion rate of pipes and be responsible for the production of toxins (Paterson and Lima, 2005; Feazel et al., 2009; Li et al., 2015; Wang et al., 2017; Zhou et al., 2017). Biofilm studies in DWDS have had bacteria as their main focus. However, an increasing number of studies have also been performed regarding total microbial ecology and, in particular, the presence and biofilm formation ability of filamentous fungi (Simões et al., 2015; Douterelo et al., 2016; Fernandes et al., 2019; Afonso et al., 2019). Due to their absorptive nutrition mode, secretion of extracellular enzymes to digest complex molecules and their apical hyphal growth, these fungi have a high aptitude to grow on surfaces (Jones, 1994). They can be introduced into DWDS from the water reservoirs as natural contaminants or other different locations, such as leaking joints and adapters, cracks in pipelines, physical openings in storage facilities and/or during maintenance. Fungi have also been associated with the production of mycotoxins in water (Paterson et al., 1997, 2007; Siqueira et al., 2013).

Under natural conditions, biofilms are considered complex communities with a high diversity of complex relationships involving inter- and intraspecies interactions (Douterelo et al., 20016, 2018). In many microbiomes bacteria can coexist with different eukaryotes, including fungi (Frey-Klett et al., 2011). These interactions can occur in biofilms. The ecology of a biofilm is dependent on a complex mix of prevailing growth conditions, hydrodynamic forces and presence of microbial metabolites and molecules, including cell-cell signalling communication molecules, excreted by the microbial inhabitants of the biofilm (Bryers and Ratner, 2004). QS is a mechanism employed by microbial species to coordinate community behaviour. It allows perception of population density by the production, release and detection of small signalling communication molecules which in turn modify bacterial gene expression (Rasmussen et al., 2005a). In Gram-negative bacteria, the primary signalling molecules involved in QS mechanism are *N*-acyl homoserine lactones (AHLs). These molecules differ on the length of their side chains (C4 to C19) (Churchill and Chen, 2011; Saurav et al., 2020). The *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL) is considered a representative autoinducer-1, involved in the LasI/LasR system – the most common QS system in Gram-negative bacteria (Papenfort and Bassler, 2016). QS controls and regulates

different bacterial population density-dependent processes, including, biofilm formation, stress resistance, production of toxins and secondary metabolites and pathogenicity (Antunes et al., 2010; Shrout et al., 2011). In contrast, eukaryotes have the ability to interfere with bacterial communication by producing molecular signals that interact with bacterial QS. These compounds are called quorum sensing inhibitors (QSI) and they can mimic autoinducer structure or function, act as antagonists to the QS molecules, interfere with the stability and function of the regulator protein or the autoinducer synthase and hydrolysate signalling molecules (González and Keshavan, 2006). Filamentous fungi, in particular the genus *Penicillium*, have been studied regarding their QSI potential (Rasmussen et al., 2005b). As they coexist with bacteria and do not have active immune systems, they must rely instead on chemical defence mechanisms. Patulin, a well-known polyketide fungal secondary metabolite (mycotoxin) with low molecular weight (154.12 g mol^{-1}) and high polarity, is one of these biologically active QSI compounds (Rasmussen et al., 2005b).

As there is a gap in the study of the effects of the fungal secondary metabolite patulin and AHLs on inter-kingdom biofilm formation between filamentous fungi and bacteria, the purpose of this work was to assess, under laboratory conditions, the effect of patulin and 3-oxo-C_{12}-HSL on single and inter-kingdom biofilm formation by filamentous fungi and bacteria isolated from a DWDS.

3.2 Materials and Methods

3.2.1 Microorganisms and culture conditions

Penicillium expansum MUM 00.02 and *Penicillium brevicompactum* MUM 05.17 supplied by Micoteca da Universidade do Minho fungal culture collection (MUM, Braga, Portugal), were used in this work and chosen based on their high occurrence in the tap water of the north of Portugal (Gonçalves et al. 2006). Both fungi were maintained on malt extract agar (MEA: malt extract 20 g, peptone 5 g, agar 20 g, distilled water 1 l).

Acinetobacter calcoaceticus and *Methylobacterium oryzae* were previously isolated from a model laboratory DWDS by Simões et al. (2007b). These bacteria were chosen for being representative of drinking water bacteria and due to their ability to form complex single species biofilms as well as inter-kingdom biofilms with this fungus (Simões et al., 2010, Afonso et al., 2019). *Acinetobacter calcoaceticus* was *g*rown overnight while *M. oryzae* was grown for 72 h before the start of the assay. Bacteria were grown in batch cultures using 100 ml of R2A broth (yeast extract 0.5 g, proteose peptone 0.5 g, casein

hydrolysate 0.5 g, glucose 0.5 g, soluble starch 0.5 g, dipotassium phosphate 0.3 g, magnesium sulphate 0.024 g, sodium pyruvate 0.3 g, distilled water 1 l) at room temperature (25 ± 2 °C) and under agitation (150 rpm). Afterwards, the bacteria were harvested by centrifugation (10 min at 13,000 *g*, room temperature), washed twice in 0.1 *M* saline phosphate buffer, and resuspended in a volume of R2A broth to obtain a cellular density of 10^8 cells ml^{-1}. This was the bacterial concentration used for biofilm formation assays.

3.2.2 Fungal screening for QSI production (patulin production).

The reporter strain *Chromobacterium violaceum* (ATCC 12472) was used to determine the QSI activity of *P. expansum and P. brevicompactum*, following the protocol developed by Mclean et al. (2004) with slight modifications. Briefly, *P. expansum* and *P. brevicompactum* were middle point inoculated onto Yeast Extract Sucrose (YES) agar medium (yeast extract 10.0 g, sucrose 75.0 g, agar 10.0 g, 500 mL distilled water) or R2A agar and grown at 25 °C for 7 days. The reporter strain *C. violaceum* (ATCC 12472) was inoculated in 50 mL of Luria-Bertani (LB) broth (Difco) at an $OD_{600\ nm}$ of 0.1 and grown overnight, at 37 °C, in order to reach the exponential phase. The overnight culture was added to molten LB agar (10 mL; 0.5% w/v), cooled to 45 °C and poured over the surface of YES and R2A agar plates containing *P. expansum* or *P. brevicompactum* 7-day colonies. The plates were then incubated overnight at 37 °C. QS inhibition was evaluated by the inhibition of violacein pigment production, represented by a colourless/opaque halo with viable bacterial growth. Growth inhibition representing antibacterial activity is indicated by a transparent halo around the colonies with no bacterial growth. As YES medium is a medium used to induce patulin production, it was used as positive control while R2A agar with no fungal growth was used as the negative control.

3.2.3 Stock solution of fungal spores

Stock solution of fungal spores was prepared according to Simões et al. (2015). Briefly, spores of *P. expansum* were harvested from seven-day old pure cultures in MEA at 25 °C by flooding the surface of the agar plates with 2 ml of TWS solution (0.85% NaCl plus 0.05% Tween 80) and rocking gently. The suspension was then homogenized by vortexing and used for large scale production of spores. The final spore suspension was homogenized by vortexing before quantification using a Neubauer count chamber. Aliquots of spore suspension with 10% of glycerol were cryopreserved at -80 °C in order to allow the use of the same spore suspension in all the biofilm assays. Stock spore suspensions were resuspended in the volume of R2A broth necessary to achieve a density of 10^5 spores ml^{-1}. This was the spore

concentration used for biofilm formation assays. Assays were performed with germinated fungal spores. Spores were pre-germinated in order to stimulate their metabolic state. Germinated fungal spores were prepared by inoculating 10^5 fresh spores ml^{-1} in R2A broth for approximately 14 h (sufficient time to have > 95% of the spores germinated).

3.2.4 Preparation of quorum sensing and quenching molecules

Patulin and 3-oxo-C_{12}-HSL were purchased from Cayman Chemical (Ann Arbor, MI, USA) and Sigma-Aldrich (St. Louis, MO, USA) respectively. Stock solutions of 32.5 m*M* were prepared for each individual molecule in dimethyl sulfoxide (DMSO) (Merck KGaA, Darmstadt, Germany) and stored at -20 °C until use. From the original stock solutions, dose working solutions of 50 and 500 µ*M* were prepared using R2A broth.

3.2.5 Determining the effect of quorum sensing and quenching molecules on biofilm formation

Biofilms were developed according to the modified microtiter plate test used by Stepanović et al. (2000) for bacteria and Simões et al. (2015) for fungi. Briefly, for the single-species biofilms, wells of sterile polystyrene 96-well flat bottom culture plates (SARSTEDT AG & Co. KG, Nümbrecht, Germany) were filled under aseptic conditions with 190 µl of spore suspension (10^5 spores ml^{-1} in R2A broth) or 190 µL of a cell suspension (10^8 cells ml^{-1} in R2A broth) supplemented with 10 µl of each molecule dose working solution of 50 and 500 µ*M* to yield a final concentration of 2.5 and 25 µ*M* respectively. For inter-kingdom biofilm formation, 95 µl of germinated fungal spore suspension and 95 µl of the bacterial cell suspension was added to each well supplemented with 10 µl of each molecule dose working solution. Positive controls were done by adding 10 µl of the same amount of plain DMSO in R2A broth to each well. The reduction to half-cell density in the inter-kingdom assays was performed to avoid limitation in nutritional factors. *P. expansum* grew alongside each bacterium. To promote biofilm formation, all plates were incubated aerobically at room temperature (25 ± 2 °C) and under agitation (150 rpm) for 24, 48 and 72 h. Biofilms at 48 and 72 h were considered as matured, well developed biofilms. Every 24 h, depleted medium was removed and renewed with fresh, clean medium, supplemented with the respective concentration of each molecule. At each sampling time, the content of each well was removed and washed two times with 200 µl of sterile distilled water to remove non-adherent and weakly adherent cells. The plates were air dried for 30 min to remove excess water by evaporation. The remaining attached cells were analysed in terms of biomass adhered on the inner walls of the wells, in terms of metabolic activity and colony forming units (CFU) for bacteria. The rinsing and drying procedures were as previously used

with bacterial and fungal biofilms (Simões et al., 2007a; 2010; 2015; Afonso et al., 2019). Negative controls were obtained by incubating the wells with only R2A broth without adding any fungal spores or bacterial cells. Biofilm assays were performed thrice with five replicates per experiment.

3.2.6 Biofilm monitoring by spectrophotometric methods

3.2.6.1 Biofilm mass quantification by crystal violet

The biomass adhered on the inner walls of the wells was quantified by the crystal violet (CV) method according to the procedure described by Stepanović et al. (2000). The biofilms in the 96-well plates were fixed with 200 µl well^{-1} of 98% methanol (VWR, Carnaxide, Portugal), for 15 min. Afterwards, the methanol was discarded, the plates left to dry and then the fixed biofilm was stained with 200 µl well^{-1} of CV (Merck KGaA) for 5 min. Excess stain was rinsed out by placing the plate under slow running tap water. After this, the plates were air dried and the dye bound to the adherent cells was resolubilized by adding 200 µl well^{-1} of 33% (v v^{-1}) glacial acetic acid (Panreac, Cascais, Portugal). The optical density of the obtained solution was measured at 570 nm using a microtiter plate reader (BioTek, Winooski, VT, USA) and the biofilm mass was presented as $OD_{570\ nm}$ values.

3.2.6.2 Biofilm metabolic activity assessment by resazurin

Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) (Sigma-Aldrich) is a viability dye used to assess the metabolic activity of biofilms (Borges et al., 2012). For each biofilm growth period, fresh R2A broth (190 µl) was added to the plates. A volume of 10 µl of resazurin (400 µ*M*) indicator solution was added to each well, in order to obtain a final resazurin concentration of 20 µ*M*. Plates were incubated in the dark for 3 hours at 25 °C. Fluorescence was measured after excitation at 530 nm and emission at 590 nm using a microtiter plate reader (BioTek). The biofilm specific metabolic activity was determined as metabolic activity per biofilm mass and expressed as Fluorescence/$OD_{570\ nm}$.

3.2.6.3 Monitoring of washed bacterial cells.

For each biofilm growth period, after removing the content of each well from the microtiter plates, 200 µl of sterile distilled water were added to remove non-adherent and weakly adherent bacterial cells. Of these 200 µl of distilled water, half was then placed in a new microtiter plate. The optical density of the obtained solution was measured at 600 nm using a microtiter plate reader (BioTek) and the results presented as CFU. Standard curves correlating the $OD_{600\ nm}$ with CFU were done and the equations $y =$

$1\times10^{9}x - 5\times10^{6}$ (R^2 = 0.9949) and $y = 6\times10^{8}x - 3\times10^{6}$ (R^2 = 0.9931) were obtained for *A. calcoaceticus* and *M. oryzae*, respectively.

3.2.7 Number of bacteria in single and inter-kingdom biofilms

The number of bacterial cells present in either *A. calcoaceticus* or *M. oryzae* single species biofilms and in inter-kingdom biofilms was determined in terms of CFU using a plate count assay upon biofilm release. Briefly, bacterial single-species biofilms and inter-kingdom biofilms were grown in a 96-well plate as described above. After each incubation period, the supernatant was removed, and the plate was washed three times with sterile water. A volume of 200 µl of sterile phosphate buffer saline (pH 7.4) was added into each well. Afterwards, biofilms were scrapped with a pipette tip and, in addition, the 96-well plate was covered with the lid and placed into an ultrasonic bath (Bandelin electronic GmbH & Co. KG, Berlin, Germany). To release bacterial cells from biofilm, the plate was sonicated for 1 min (5 s sonicate, 10 s interval) at 35 kHz. Three replicates were used for each condition and bacterial cells were plated onto R2A agar plates for CFU determination.

3.2.8 Patulin extraction from biofilms

Patulin extraction method for biofilms was based and adapted from Sargenti and Almeida (2010). The contents of 8 wells of each condition (1.6 mL), including medium and biomass, were scrapped and transferred to 15 mL centrifuge tubes (Orange Scientific, Braine-l'Alleud, Belgium). Double volume of ethyl acetate was then added to the Falcon tube. The extraction procedure as follows: 5 min vortexing, followed by 10 min in an ultrasonic bath (Bandelin electronic GmbH & Co. KG, Berlin, Germany) at 35 kHz, followed by vortexing for another 5 min. The ethyl acetate phase was separated and transferred to a 4 mL vial and placed under at 40 °C under a flow of gentle nitrogen stream to evaporate the solvent. When the residue was completely dry, it was kept at -20 °C until all samples were extracted. The residues were then resuspended in 1 mL of HPLC-grade water adjusted to pH 4.0 with acetic acid and vortexed for 1 min. Finally, the extracts were filtered into microvials using a 2 mL Omnifix syringe (B. Braun, Melsungen, Germany) coupled with a 0.22-µm nylon syringe filter (Nantong FilterBio Membrane, Nantong City, China) and analysed by liquid chromatography. Quantification was performed by means of internal calibration curves of increasing concentrations: one in a range from 0.1 to 2.5 µg ml^{-1} and another one from 10 to 100 µg ml^{-1} of pure patulin standards. Patulin recovery percentage using the present method was calculated by spiking the medium with two different concentrations of patulin, in this case, 1 and 10 µg

ml^{-1}, in triplicates and the chromatographic analysis was performed in duplicates. Recovery percentage varied from 68.74% to 84.23% with a mean of 79.78 ± 5.65%.

3.2.8.1 UHPLC apparatus and conditions

Liquid chromatography was performed using a Nexera X2 UHPLC System equipped with a DGU-20A5R degassing unit, LC-30AD pump, SIL-30AC autosampler, CTO-20AC column oven and an SPD-M20A photodiode array detector set at 276 nm (Shimadzu Corp., Kyoto, Japan). Chromatographic separation was carried out with a Chromolith® Performance RP-18 endcapped 100-4.6 HPLC column (Merck KGaA). Oven temperature was maintained at 25 °C. The injection volume was set at 5 μL and the sample flow rate at 0.75 mL min^{-1}. Run time was 10 min with a mobile phase of H_2O:ACN (95:5, *v/v*). Patulin average retention time was obtained at around 4.4 min.

3.2.8 Statistical analysis

Data were analysed applying the two-way analysis of variance (2way ANOVA) and the comparisons between and within experimental groups were carried out using Tukey's multiple comparisons test. The software used for statistical analysis was GraphPad Prism (GraphPad Software, La Jolla, California, USA) version 8.3.0. A $P < 0.05$ was regarded as statistically significant.

3.3 Results

3.3.1 Anti-quorum sensing activity of the fungi and patulin production by *P. expansum*

The results from the anti-QS activity of *P. expansum* and *P. brevicompactum* are shown in Figure 3.1. From the results obtained, a high QSI activity was detected for *P. expansum* (Figure 3.1b and 3.1c) in both agar mediums, as seen by the halo formed around the colonies. On the contrary, *P. brevicompactum* showed no QSI activity (Figure 3.1d and 3.1e). For this reason, only *P. expansum* was selected for further experiments. However, in *P. expansum* biofilms developed until 72 h in R2A, there was no detectable amounts of patulin being produced.

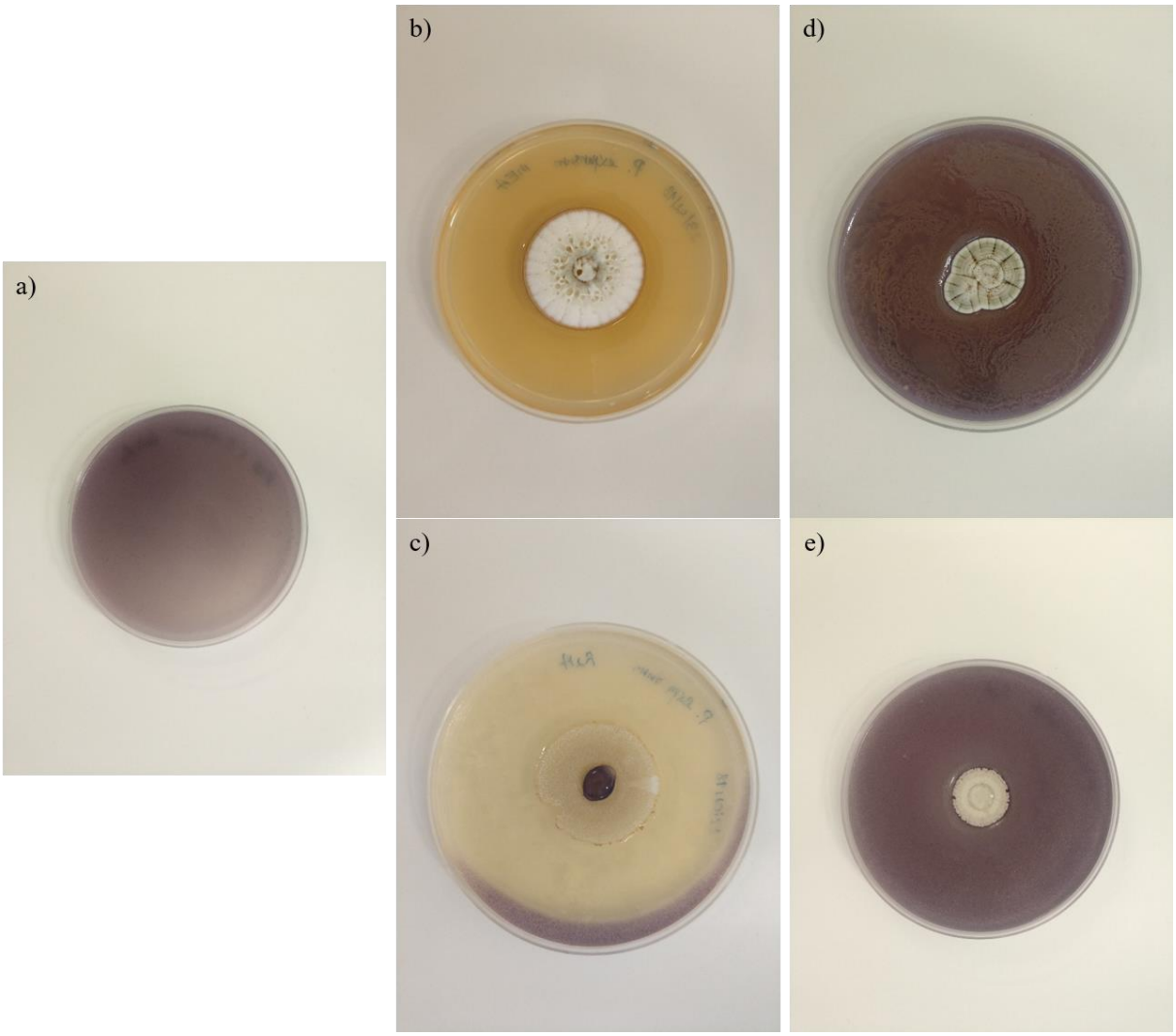

Figure 3.1 Anti-quorum sensing activity test. a) Negative control with R2A agar and no fungal growth; b) *P. expansum* grown on YES agar for 7 days (Positive control); c) *P. expansum* grown on R2A agar for 7 days; d) *P. brevicompactum* grown on YES agar for 7 days; e) *P. brevicompactum* grown on R2A agar for 7 days.

3.3.2 Biofilm mass quantification and specific metabolic activity of biofilms

The results for *P. expansum* and *M. oryzae* biofilm mass quantification and specific metabolic activity are presented in Figure 3.2a and 3.2b respectively, while results for *P. expansum* and *A. calcoaceticus* are presented in Figure 3.3a and 3.3b. In inter-kingdom biofilms, data features the global biomass and metabolic activities of both fungus and bacteria, not only one specific microorganism. Statistical analysis of the data is shown in Tables 3.1 and 3.2 for *P. expansum* and *M. oryzae* or *A. calcoaceticus*, respectively.

Regarding *P. expansum* single-species biofilms, neither patulin nor 3-oxo-C_{12}-HSL influenced its biofilm mass or specific metabolic activity. *M. oryzae* single-species biofilm mass was reduced at all times in the presence of 25 μM of patulin ($P < 0.05$). In contrast, both concentrations of 3-oxo-C_{12}-HSL had a

beneficial effect, increasing this bacterium biofilm mass (Figure 3.2a). Despite both molecules effects on biofilm mass, the specific metabolic activity of single-species biofilms was not affected (Figure 3.2b). In inter-kingdom biofilms an increase in biofilm mass was also observed in the presence of 3-oxo-C_{12}-HSL, probably due to the increase of *M. oryzae* mass. This increase correlated with a decrease in specific metabolic activity. At 72 h, both concentrations of patulin showed an increased metabolic activity of inter-kingdom biofilms in relation to *P. expansum* biofilms. The results show a slight inhibition of *M. oryzae* towards the fungus for the first 24 h.

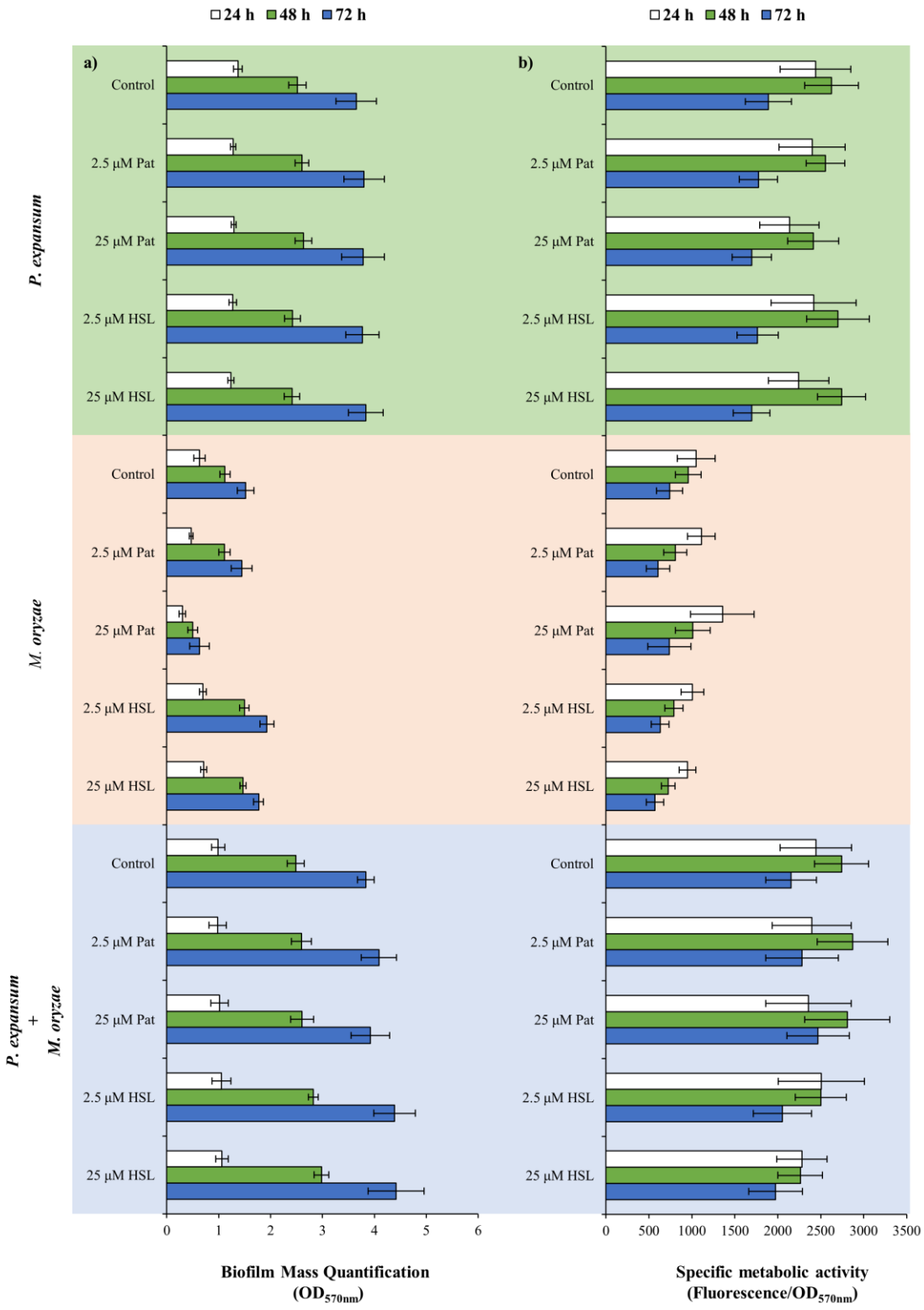

Figure 3.2 a) Biofilm mass quantification and b) Specific metabolic activity for single-species and inter-kingdom biofilm formation over time for *P. expansum* and *M. oryzae*. Legend is as follows: 'Pat' - Patulin; 'HSL' - 3-oxo-C_{12}-HSL. The means ± SDs for at least three independent experiments are illustrated. Each single-species biofilms of *P. expansum* and *M. oryzae* are used as controls against inter-kingdom biofilms.

Table 3.1 Tukey's multiple comparison test for *P. expansum* and *M. oryzae* regarding biofilm mass and specific metabolic activity. Significant differences are depicted with: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Tukey's multiple comparisons test	Adjusted P value					
	Biofilm mass			Specific metabolic activity		
	24 H	48 H	72 H	24 H	48 H	72 H
P Control vs. P 2.5 μ*M* Pat	ns	ns	ns	ns	ns	ns
P Control vs. P 25 μ*M* Pat	ns	ns	ns	ns	ns	ns
P Control vs. P 2.5 μ*M* HSL	ns	ns	ns	ns	ns	ns
P Control vs. P 25 μ*M* HSL	ns	ns	ns	ns	ns	ns
P 2.5 μ*M* Pat vs. P 25 μ*M* Pat	ns	ns	ns	ns	ns	ns
P 2.5 μ*M* HSL vs. P 25 μ*M* HSL	ns	ns	ns	ns	ns	ns
M Control vs. M 2.5 μ*M* Pat	ns	ns	ns	ns	ns	ns
M Control vs. M 25 μ*M* Pat	**	****	****	ns	ns	ns
M Control vs. M 2.5 μ*M* HSL	ns	****	****	ns	ns	ns
M Control vs. M 25 μ*M* HSL	ns	***	ns	ns	ns	ns
M 2.5 μ*M* Pat vs. M 25 μ*M* Pat	ns	****	****	ns	ns	ns
M 2.5 μ*M* HSL vs. M 25 μ*M* HSL	ns	ns	ns	ns	ns	ns
P+M Control vs. P+M 2.5 μ*M* Pat	ns	ns	ns	ns	ns	ns
P+M Control vs. P+M 25 μ*M* Pat	ns	ns	ns	ns	ns	ns
P+M Control vs. P+M 2.5 μ*M* HSL	ns	*	****	ns	ns	ns
P+M Control vs. P+M 25 μ*M* HSL	ns	****	****	ns	*	ns
P+M 2.5 μ*M* Pat vs. P+M 25 μ*M* Pat	ns	ns	ns	ns	ns	ns
P+M 2.5 μ*M* HSL vs. P+M 25 μ*M* HSL	ns	ns	ns	ns	ns	ns
P Control vs. P+M Control	****	ns	ns	ns	ns	ns
P 2.5 μ*M* Pat vs. P+M 2.5 μ*M* Pat	**	ns	*	ns	ns	**
P 25 μ*M* Pat vs. P+M 25 μ*M* Pat	*	ns	ns	ns	ns	****
P 2.5 μ*M* HSL vs. P+M 2.5 μ*M* HSL	ns	****	****	ns	ns	ns
P 25 μ*M* HSL vs. P+M 25 μ*M* HSL	ns	****	****	ns	**	ns
M Control vs. P+M Control	***	****	****	****	****	****
M 2.5 μ*M* Pat vs. P+M 2.5 μ*M* Pat	****	****	****	****	****	****
M 25 μ*M* Pat vs. P+M 25 μ*M* Pat	****	****	****	****	****	****
M 2.5 μ*M* HSL vs. P+M 2.5 μ*M* HSL	*	****	****	****	****	****
M 25 μ*M* HSL vs. P+M 25 μ*M* HSL	***	****	****	****	****	****

Results for *A. calcoaceticus* single-species biofilms show that neither molecule caused a significant effect on biofilm mass (Figure 3.3a). However, both concentrations of patulin significantly decreased its specific metabolic activity at 24 h, while 25 μ*M* of 3-oxo-C_{12}-HSL caused an increase at 48 and 72 h (Figure 3.3b). In general, specific metabolic activity of *A. calcoaceticus* biofilms substantially decreased along time for all conditions. This general decrease is to be expected. Bacteria are more active in initial growth steps (24 h) to either adhere to the substratum or to multiply, resembling the log phase of a growth curve. This will eventually culminate in a stationary or death phase, in which metabolic activity

is reduced. The presence of *A. calcoaceticus* resulted in a higher inhibition of fungal biofilm formation and development than *M. oryzae*, in particular at 24 and 48 h. However, in the presence of 25 μ*M* of patulin, this effect was less pronounced, as *P. expansum* was able to form a denser biofilm. In inter-kingdom biofilms, the presence of patulin decreased the specific metabolic activity, while 3-oxo-C_{12}-HSL had no effect at any time point.

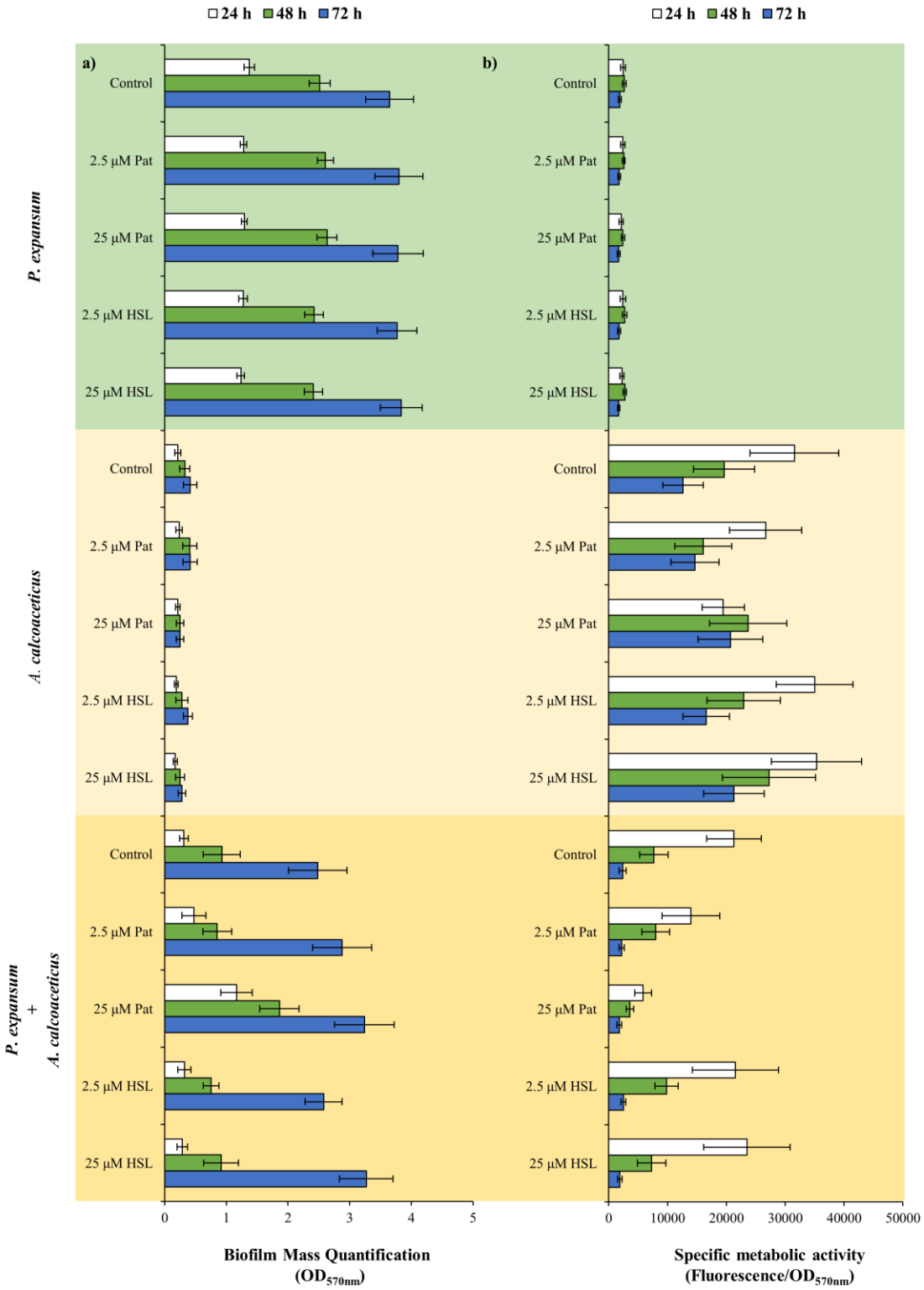

Figure 3.3 a) Biofilm mass quantification and b) Specific metabolic activity for single-species and inter-kingdom biofilm formation over time for *P. expansum* and *A. calcoaceticus*. Legend is as follows: 'Pat' - Patulin; 'HSL' - 3-oxo-C_{12}-HSL. The means ± SDs for at least three independent experiments are illustrated. Each single-species biofilms of *P. expansum* and *A. calcoaceticus* are used as controls against inter-kingdom biofilms.

Table 3.2. Tukey's multiple comparison test for *P. expansum* and *A. calcoaceticus* regarding biofilm mass and specific metabolic activity. Significant differences are depicted with: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Tukey's multiple comparisons test	Adjusted P value					
	Biofilm mass			Specific metabolic activity		
	24 H	48 H	72 H	24 H	48 H	72 H
P Control vs. P 2.5 μ*M* Pat	ns	ns	ns	ns	ns	ns
P Control vs. P 25 μ*M* Pat	ns	ns	ns	ns	ns	ns
P Control vs. P 2.5 μ*M* HSL	ns	ns	ns	ns	ns	ns
P Control vs. P 25 μ*M* HSL	ns	ns	ns	ns	ns	ns
P 2.5 μ*M* Pat vs. P 25 μ*M* Pat	ns	ns	ns	ns	ns	ns
P 2.5 μ*M* HSL vs. P 25 μ*M* HSL	ns	ns	ns	ns	ns	ns
A Control vs. A 2.5 μ*M* Pat	ns	ns	ns	**	ns	ns
A Control vs. A 25 μ*M* Pat	ns	ns	ns	****	ns	***
A Control vs. A 2.5 μ*M* HSL	ns	ns	ns	ns	ns	ns
A Control vs. A 25 μ*M* HSL	ns	ns	ns	ns	****	****
A 2.5 μ*M* Pat vs. A 25 μ*M* Pat	ns	ns	ns	****	****	*
A 2.5 μ*M* HSL vs. A 25 μ*M* HSL	ns	ns	ns	ns	ns	ns
P+A Control vs. P+A 2.5 μ*M* Pat	ns	ns	**	****	ns	ns
P+A Control vs. P+A 25 μ*M* Pat	****	****	****	****	ns	ns
P+A Control vs. P+A 2.5 μ*M* HSL	ns	ns	ns	ns	ns	ns
P+A Control vs. P+A 25 μ*M* HSL	ns	ns	****	ns	ns	ns
P+A 2.5 μ*M* Pat vs. P+A 25 μ*M* Pat	****	****	**	****	ns	ns
P+A 2.5 μ*M* HSL vs. P+A 25 μ*M* HSL	ns	ns	****	ns	ns	ns
P Control vs. P+A Control	****	****	****	****	*	ns
P 2.5 μ*M* Pat vs. P+A 2.5 μ*M* Pat	****	****	****	****	*	ns
P 25 μ*M* Pat vs. P+A 25 μ*M* Pat	ns	****	****	ns	ns	ns
P 2.5 μ*M* HSL vs. P+A 2.5 μ*M* HSL	****	****	****	****	**	ns
P 25 μ*M* HSL vs. P+A 25 μ*M* HSL	****	****	****	****	ns	ns
A Control vs. P+A Control	ns	****	****	****	****	****
A 2.5 μ*M* Pat vs. P+A 2.5 μ*M* Pat	*	****	****	****	****	****
A 25 μ*M* Pat vs. P+A 25 μ*M* Pat	****	****	****	****	****	****
A 2.5 μ*M* HSL vs. P+A 2.5 μ*M* HSL	ns	****	****	****	****	****
A 25 μ*M* HSL vs. P+A 25 μ*M* HSL	ns	****	****	****	****	****

3.3.3 Effect of quorum sensing and quenching molecules on bacterial cell density in single species and inter-kingdom biofilms

The results on the number of bacterial cells, in either single-species biofilms or inter-kingdom biofilms, are presented in Figure 3.4a and 3.4b, for *M. oryzae* and *A. calcoaceticus* respectively. Statistical analysis of the data for *M. oryzae* and *A. calcoaceticus* is shown in Tables 3.3 and 3.4, respectively.

Regarding *M. oryzae* cell density in single-species biofilms, the results correlated with its biofilm mass. Patulin caused a decrease in cell density, specially at 25 μ*M*, while the presence of both

concentrations of 3-oxo-C_{12}-HSL resulted in an increase (Figure 3.4a). The highest concentration was responsible for a higher increase in cell density. In inter-kingdom biofilms, an overall reduction was observed in *M. oryzae* cell density at 48 and 72 h when compared to each condition in single-species biofilms. Despite this reduction, the effect of both molecules was similar to that observed in *M. oryzae* single-species biofilms.

Results for *A. calcoaceticus* cell density in single-species biofilms reveal significant effects that were not noticeable when analysing biofilm mass results. A reduction in cell density was observed in the presence of 25 μM of patulin at all time points (Figure 3.4b). The presence of 3-oxo-C_{12}-HSL also caused a reduction in this bacterium cell density, specially at the concentration of 25 μM and at 24 and 72 h. In inter-kingdom biofilms, *A. calcoaceticus* cell density was always higher than in single-species biofilms. In relation to *P. expansum* + *A. calcoaceticus* control, there was a general decrease in this bacterium cell density in the presence of both molecules. The highest decrease was observed in the presence of 25 μM of patulin.

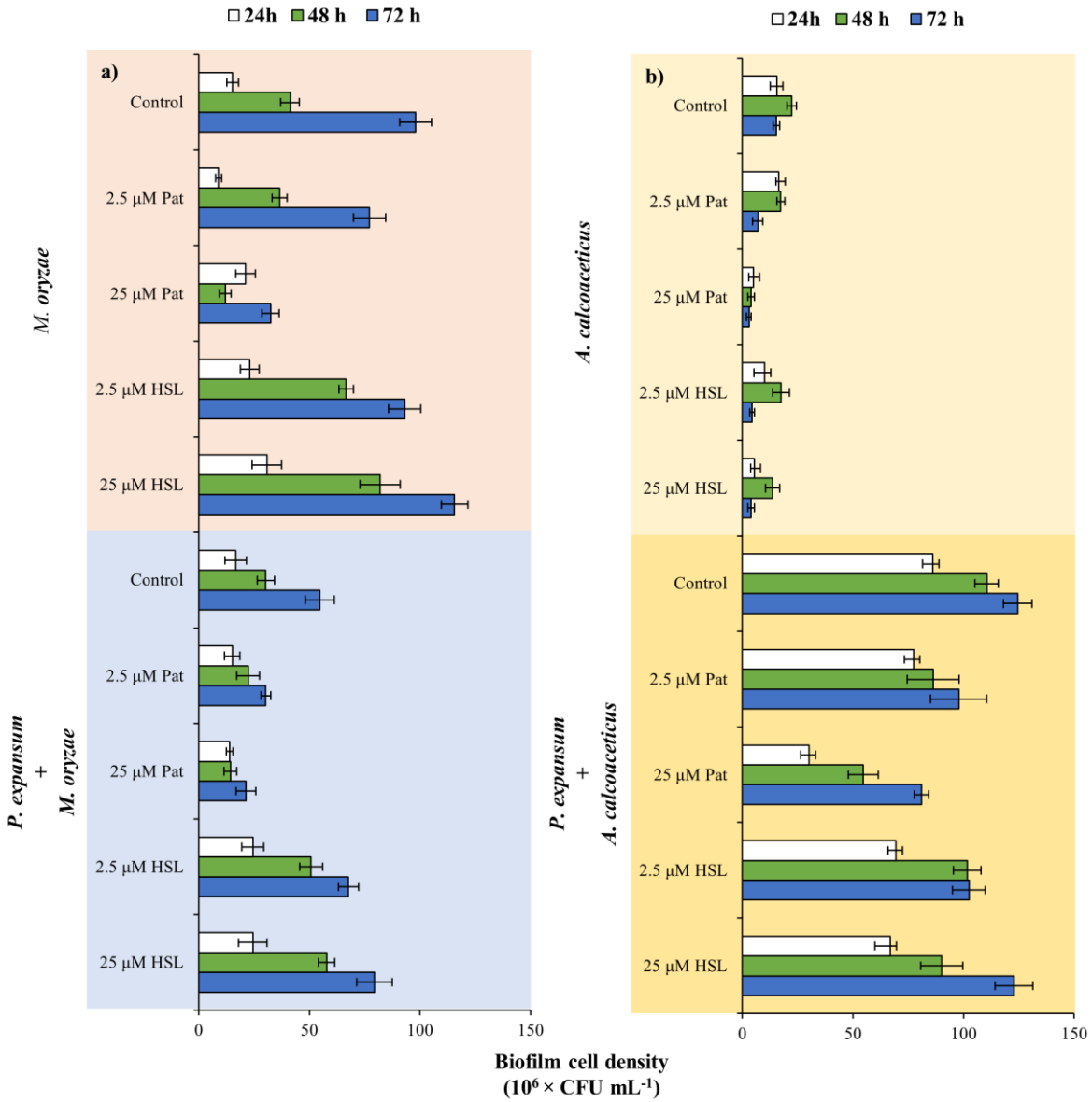

Figure 3.4 Biofilm cell density for (a) *M. oryzae* or (b) *A. calcoaceticus* while forming single-species or inter-kingdom biofilms over time. Legend is as follows: Legend is as follows: 'Pat' - Patulin; 'HSL' - 3-oxo-C_{12}-HSL. The means ± SDs for two independent experiments are illustrated.

Table 3.3 Tukey's multiple comparison test regarding *M. oryzae* biofilm cell density. Significant differences are depicted with: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Tukey's multiple comparisons test	Adjusted P value		
	CFU		
	24 H	48 H	72 H
M Control vs. M 2.5 µ*M* Pat	ns	ns	****
M Control vs. M 25 µ*M* Pat	ns	****	****
M Control vs. M 2.5 µ*M* HSL	ns	****	ns
M Control vs. M 25 µ*M* HSL	****	****	****
M 2.5 µ*M* Pat vs. M 25 µ*M* Pat	**	****	****
M 2.5 µ*M* HSL vs. M 25 µ*M* HSL	ns	****	****
P+M Control vs. P+M 2.5 µ*M* Pat	ns	ns	****
P+M Control vs. P+M 25 µ*M* Pat	ns	****	****
P+M Control vs. P+M 2.5 µ*M* HSL	ns	****	***
P+M Control vs. P+M 25 µ*M* HSL	ns	****	****
P+M 2.5 µ*M* Pat vs. P+M 25 µ*M* Pat	ns	ns	ns
P+M 2.5 µ*M* HSL vs. P+M 25 µ*M* HSL	ns	ns	**
M Control vs. P+M Control	ns	**	****
M 2.5 µ*M* Pat vs. P+M 2.5 µ*M* Pat	ns	***	****
M 25 µ*M* Pat vs. P+M 25 µ*M* Pat	ns	ns	**
M 2.5 µ*M* HSL vs. P+M 2.5 µ*M* HSL	ns	****	****
M 25 µ*M* HSL vs. P+M 25 µ*M* HSL	ns	****	****

Table 3.4 Tukey's multiple comparison test regarding A. calcoaceticus biofilm cell density. Significant differences are depicted with: * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.

Tukey's multiple comparisons test	Adjusted P value		
	Biofilm mass		
	24 H	48 H	72 H
A Control vs. A 2.5 µ*M* Pat	ns	ns	ns
A Control vs. A 25 µ*M* Pat	*	****	**
A Control vs. A 2.5 µ*M* HSL	ns	ns	*
A Control vs. A 25 µ*M* HSL	*	ns	*
A 2.5 µ*M* Pat vs. A 25 µ*M* Pat	*	**	ns
A 2.5 µ*M* HSL vs. A 25 µ*M* HSL	ns	ns	ns
P+A Control vs. P+A 2.5 µ*M* Pat	ns	****	****
P+A Control vs. P+A 25 µ*M* Pat	****	****	****
P+A Control vs. P+A 2.5 µ*M* HSL	****	ns	****
P+A Control vs. P+A 25 µ*M* HSL	****	****	ns
P+A 2.5 µ*M* Pat vs. P+A 25 µ*M* Pat	****	****	****
P+A 2.5 µ*M* HSL vs. P+A 25 µ*M* HSL	ns	*	****
A Control vs. P+A Control	****	****	****
A 2.5 µ*M* Pat vs. P+A 2.5 µ*M* Pat	****	****	****
A 25 µ*M* Pat vs. P+A 25 µ*M* Pat	****	****	****
A 2.5 µ*M* HSL vs. P+A 2.5 µ*M* HSL	****	****	****
A 25 µ*M* HSL vs. P+A 25 µ*M* HSL	****	****	****

3.3.4 Washed bacterial cells in single-species and inter-kingdom biofilms

The results on the number of washed bacterial cells, in either single-species or inter-kingdom biofilms, are presented in Figure 3.5a and 3.5b, for *M. oryzae* and *A. calcoaceticus* respectively. Statistical analysis of the data for *M. oryzae* and *A. calcoaceticus* is shown in Tables 3.5 and 3.6, respectively.

Washed bacterial cells were monitored by CFU. This was done to understand if the effect of the molecules on the number of cells that were loosely attached to the substratum after removing the planktonic cells was significant. They would not be quantified by CV as part of the biofilm after the washing steps. With the removal of depleted medium and its renewal, the equilibrium that had been previously maintained is altered with the removal of free-living cells. The results obtained for *M. oryzae* revealed that the presence of the molecules caused an increase in the amount of bacterial cells that were washed out along time. This was more significant with the highest concentration of each molecule. In general, a significant decrease in bacterial CFU was observed in inter-kingdom biofilms compared to single-species biofilms.

Regarding *A. calcoaceticus*, the results showed that only 25 μM of patulin at 24 h reduced the number of washed cells in single species biofilms. In inter-kingdom biofilms, the number of *A. calcoaceticus* washed cells was inferior to single-species biofilms.

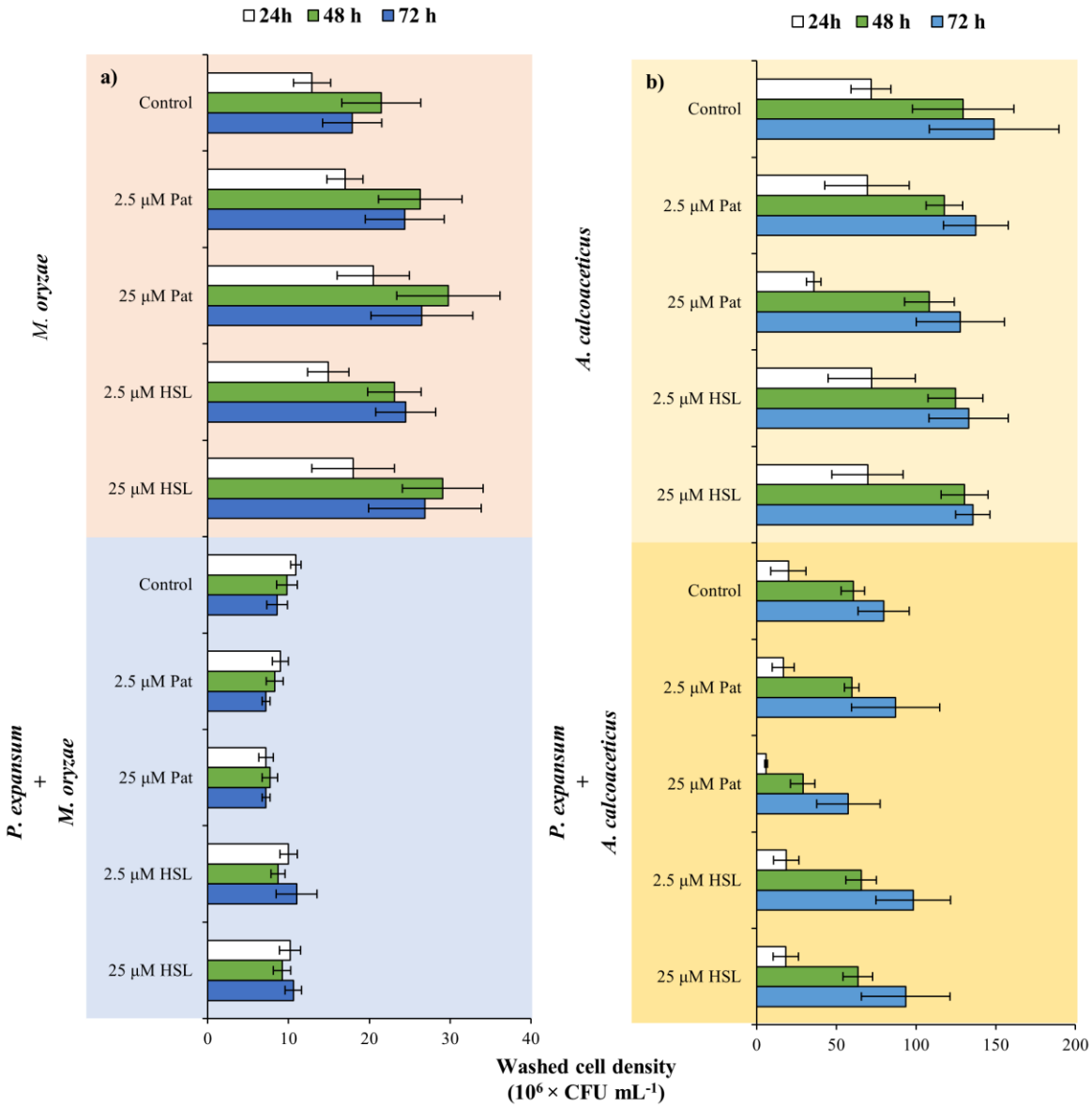

Figure 3.5 Washed cell density for (a) *M. oryzae* or (b) *A. calcoaceticus* while forming single-species or inter-kingdom biofilms over time. Legend is as follows: Legend is as follows: 'Pat' - Patulin; 'HSL' - 3-oxo-C_{12}-HSL. The means ± SDs for two independent experiments are illustrated.

Table 3.5 Tukey's multiple comparison test regarding M. oryzae washed cell density. Significant differences are depicted with: * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.

Tukey's multiple comparisons test	Adjusted P value		
	CFU		
	24 H	48 H	72 H
M Control vs. M 2.5 μ*M* Pat	ns	ns	*
M Control vs. M 25 μ*M* Pat	**	**	***
M Control vs. M 2.5 μ*M* HSL	ns	ns	*
M Control vs. M 25 μ*M* HSL	ns	**	***
M 2.5 μ*M* Pat vs. M 25 μ*M* Pat	ns	ns	ns
M 2.5 μ*M* HSL vs. M 25 μ*M* HSL	ns	ns	ns
P+M Control vs. P+M 2.5 μ*M* Pat	ns	ns	ns
P+M Control vs. P+M 25 μ*M* Pat	ns	ns	ns
P+M Control vs. P+M 2.5 μ*M* HSL	ns	ns	ns
P+M Control vs. P+M 25 μ*M* HSL	ns	ns	ns
P+M 2.5 μ*M* Pat vs. P+M 25 μ*M* Pat	ns	ns	ns
P+M 2.5 μ*M* HSL vs. P+M 25 μ*M* HSL	ns	ns	ns
M Control vs. P+M Control	ns	****	***
M 2.5 μ*M* Pat vs. P+M 2.5 μ*M* Pat	**	****	****
M 25 μ*M* Pat vs. P+M 25 μ*M* Pat	****	****	****
M 2.5 μ*M* HSL vs. P+M 2.5 μ*M* HSL	ns	****	****
M 25 μ*M* HSL vs. P+M 25 μ*M* HSL	**	****	****

Table 3.6 Tukey's multiple comparison test regarding A. calcoaceticus washed cell density. Significant differences are depicted with: * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.

Tukey's multiple comparisons test	Adjusted P value		
	Biofilm mass		
	24 H	48 H	72 H
A Control vs. A 2.5 μ*M* Pat	ns	ns	ns
A Control vs. A 25 μ*M* Pat	*	ns	ns
A Control vs. A 2.5 μ*M* HSL	ns	ns	ns
A Control vs. A 25 μ*M* HSL	ns	ns	ns
A 2.5 μ*M* Pat vs. A 25 μ*M* Pat	ns	ns	ns
A 2.5 μ*M* HSL vs. A 25 μ*M* HSL	ns	ns	ns
P+A Control vs. P+A 2.5 μ*M* Pat	ns	ns	ns
P+A Control vs. P+A 25 μ*M* Pat	ns	ns	ns
P+A Control vs. P+A 2.5 μ*M* HSL	ns	ns	ns
P+A Control vs. P+A 25 μ*M* HSL	ns	ns	ns
P+A 2.5 μ*M* Pat vs. P+A 25 μ*M* Pat	ns	ns	ns
P+A 2.5 μ*M* HSL vs. P+A 25 μ*M* HSL	ns	ns	ns
A Control vs. P+A Control	***	****	****
A 2.5 μ*M* Pat vs. P+A 2.5 μ*M* Pat	***	****	***
A 25 μ*M* Pat vs. P+A 25 μ*M* Pat	ns	****	*
A 2.5 μ*M* HSL vs. P+A 2.5 μ*M* HSL	***	****	ns
A 25 μ*M* HSL vs. P+A 25 μ*M* HSL	***	****	**

3.4 Discussion

The present study reports the effect of patulin and 3-oxo-C_{12}-HSL on single and inter-kingdom biofilm formation and development by *P. expansum* and two bacteria, *A. calcoaceticus* and *M. oryzae*. These microorganisms were chosen based on different characteristics: their ability to form mature single-species biofilms between 48 and 72 h growth, and because they have been assessed while forming inter-kingdom biofilms (Simões et al., 2010; 2015; Afonso et al., 2020). *P. expansum*, in particular, was also selected due to its well-known ability for producing patulin as a secondary metabolite (Santos et al., 2002; Reddy et al., 2010; De Clercq et al., 2016). However, it produces this mycotoxin in certain favourable conditions (i.e. appropriate medium and longer periods of incubation time) (Figure 3.1). In R2A broth, and for the duration of the experiment, there was no detectable amounts of this toxin being produced. For this reason, there was a need to add the mycotoxin in the medium throughout the duration of the experiment. Biofilm formation and development of this filamentous fungus was not affected by the exogenous presence of patulin. At the concentrations used in this work, 3-oxo-C_{12}-HSL did not influence *P. expansum* biofilm formation. To date, the effect of this compound has not been studied on filamentous fungi.

The bacteria used in this study are Gram-negative and representative of drinking water bacteria. In addition, 3-oxo-C_{12}-HSL has been found in *Acinetobacter* (Bhargava et al., 2010) and *Methylobacterium* species (Pomini et al., 2009). In a previous study, both species used presently were screened for the presence of AHLs. Small and long side chained AHLs were detected in *M. oryzae* while no AHLs were detected in *A. calcoaceticus* (Simões et al., 2007a). The absence of the production of AHLs in *A. calcoaceticus* in this study could be related to the media composition, suggesting that the QS molecules produced could be modulated by culture medium conditions (González et al., 2001). Besides their biofilm formation ability, these were the reasons to include both bacteria in this study. *M. oryzae* single-species biofilm mass showed a correlation with this bacterium CFU. Patulin at the highest concentration tested in this work caused a decrease in both biofilm mass and CFU, while 3-oxo-C_{12}-HSL caused an increase. The highest concentration of 3-oxo-C_{12}-HSL was, in general, responsible for a highest increase in CFU. In inter-kingdom biofilms, the effect of the molecules on *M. oryzae* followed the same pattern as for single-species biofilms. The decrease in cell density of planktonic *M. oryzae* cells as well as in biofilms in the presence of *P. expansum,* reveals that the presence of this fungi causes a slight inhibition towards this bacterium. Despite no amounts of patulin being detected, there might be a small quantity being produced, or other molecules and/or other quorum quenching mechanisms that could hinder *M. oryzae* QS mechanism.

In *A. calcoaceticus* single-species biofilms, it was shown that neither patulin nor the QS molecule had a significant effect on biofilm mass despite a slight decrease with 25 μ*M* of patulin. However, looking at this bacterium CFU, a significant reduction was observed in the presence of the same concentration of this molecule. Specific metabolic activity was also reduced in the first 24 h of biofilm formation in the presence of 25 μ*M* of patulin, indicating that this compound may act on the metabolic activity of this bacterium. Opposite to the effect on *M. oryzae,* the QS molecule did not cause an increase in either *A. calcoaceticus* biofilm mass or CFU. As mentioned above, the absence of AHLs in *A. calcoaceticus* and the presence in *M. oryzae* may explain this difference (Simões et al., 2007a). In inter-kingdom biofilms with *P. expansum*, CFU for *A. calcoaceticus* increased significantly for all conditions when compared to single-species biofilms. In contrast, the reduction in CFU when analysing the washed cells in the presence of the fungi reveals a protection effect of the fungi towards this bacterium. This biotic support and protection offered by intertwined fungal hyphae, in addition to the ability of *A. calcoaceticus* to coaggregate, confers an advantage to this bacterium to proliferate (Simões et al., 2008; Frey-Klett et al., 2011). Patulin, in comparison to the effect on this bacterium CFU in single-species biofilms, also caused a reduction in inter-kingdom biofilms, especially at 25 μ*M*. However, the number of *A. calcoaceticus* CFU in inter-kingdom biofilms, was still higher than in any condition for single-species biofilms. These results emphasize the protection and biotic support that the fungal hyphae confer towards this bacterium. Analysing the effect of *A. calcoaceticus* on *P. expansum* biofilm formation, an inhibition was detected towards the fungi, especially at 24 and 48 h. Similar results were observed in other studies where bacteria inhibited fungal spore germination and hyphal growth (Mowat et al., 2010; Kousser et al., 2019; Nogueira et al., 2019; Afonso et al., 2019). With the removal of planktonic cells and the renewal of the medium, viability and fungal growth increased over time as seen by the increase in biofilm mass. Nogueira et al. (2019) showed that *Aspergillus* species remain viable upon interaction with *Klebsiella pneumoniae.* The initial inhibitory effect may be due to direct competition for nutrients; the production of metabolites that might inhibit fungal development; the higher growth rate and metabolic activity of the bacteria or a combination of these factors (Mowat et al., 2010; Nogueira et al., 2019). As the presence of 25 μ*M* of patulin causes a reduction in the *A. calcoaceticus* metabolic activity and CFU, it might be the reason for the increase in fungal biofilm mass in inter-kingdom biofilms.

The effect of both molecules had, as expected, opposite effects. Patulin has been a known QSI. Rasmussen et al. (2005b), has shown, using DNA micro-array-based transcriptomics, that patulin down-regulates QS-regulated genes in *Pseudomonas aeruginosa* by 45%, indicating that it shows specificity for QS-regulated gene expression. More recently, it has also been shown that patulin interferes with ATP

binding cassette transporter of another QS signalling molecule, the auntoinducer-2, in *Salmonella enterica* serovar Typhi (Vijayababu et al., 2018). A high reduction on the biofilm formation ability and QS signalling system of *Halomonas pacifica* was also reported in the presence of 25 μ*M* of patulin (Liaqat et al., 2014). However, studies conducted on biofilm formation of different isolates from dental unit water lines have shown an opposite effect. Up to 25 μ*M* of this compound promoted biofilm formation by *Bacillus cereus*, *P. aeruginosa* and *Achromobacter* sp. (Liaqat et al., 2008, 2010). These reports, in combination with the present results, indicate that patulin effect on biofilm formation is species dependent.

The increase in biofilm formation in the presence of 3-oxo-C_{12}-HSL is in accordance to other studies. Bhargava et al. (2012) showed that an exogenous concentration of 20 μ*M* of this molecule was responsible for a significant increase in the biofilm formation of *Acinetobacter baumannii* and *P. aeruginosa*. In a study on the changes in *Salmonella enterica* phenotypes in the presence of AHLs, it was observed that 3-oxo-C_{12}-HSL, although at lower concentrations (50 n*M*), caused an increase in biofilm formation and promoted expression of biofilm formation genes (Campos-Galvão et al., 2016). However, 3-Oxo-C_{12}-HSL at 50 μ*M* significantly supressed *Legionella pneumoniae* biofilm formation (Kimura et al., 2009). These results indicate that 3-oxo-C_{12}-HSL plays a role in intraspecies communication but also in inter-kingdom interactions.

In conclusion, our results show that, in general and under laboratory conditions, *P. expansum* biofilm formation and development were not affected by the exogenous presence of either patulin or 3-oxo-C_{12}-HSL at the tested concentrations. *M. oryzae* biofilm formation and development were more affected by the presence of the molecules than *A. calcoaceticus.* In inter-kingdom growth, *M. oryzae* cell density was generally lower than in single-species growth which indicates a detrimental effect of the fungi towards this bacterium. However, the increase of *A. calcoaceticus* cell density in inter-kingdom is an indication of the physical protection that the fungi provide to this opportunistic bacterium. Our study finds that inter-kingdom biofilm formation and development is highly species-specific. Opportunistic bacteria such as *A. calcoaceticus* may benefit from the fungal presence whereas *M. oryzae* does not. QS and QQ molecules have a significant effect in inter-kingdom biofilm formation and especially on bacterial cell numbers. Moreover, we report a possible protection role that filamentous fungi may provide to opportunistic bacteria inside complex biofilms.

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Chapter 4

Methylobacterium oryzae influences isoepoxydon dehydrogenase (*idh*) gene expression and patulin production by *Penicillium expansum* mature biofilms

The work presented here is being prepared for submission

4.1 Introduction

Water biofilms can be considered the main source of microorganisms in drinking water distribution systems (DWDS) representing over 95% of biomass in these systems (Flemming et al., 2002; Siqueira et al., 2013). They can cause the deterioration of drinking water quality through a variety of factors: changes in the organoleptic properties of the water, act as a reservoir for pathogenic microorganisms, increase corrosion and blockage of pipes and be responsible for the production of toxins (Paterson and Lima, 2005; Feazel et al., 2009; Li et al., 2015; Wang et al., 2017; Zhou et al., 2017). In DWDS, under natural conditions, biofilms are considered complex communities ruled by complex relationships involving inter- and intraspecies interactions (Douterelo et al., 2016, 2018a). Fungi and bacteria are known to coexist inside DWDS by forming and developing biofilms (Douterelo et al., 2016, 2018b). The inter-kingdom interaction is dependent on a complex mix of prevailing growth conditions, hydrodynamic forces and the presence of microbial metabolites and molecules (Bryers and Ratner, 2004; Afonso et al., 2019, 2020).

QS is a mechanism employed by microbial species that allows perception of population density by the production, release, and detection of small molecules (autoinducers), which in turn modify bacterial gene expression (Rasmussen and Givskov, 2006). QS is used to control and regulate different bacterial population-density processes, including pathogenicity, stress resistance, biofilm formation and production of secondary metabolites and toxins (Antunes et al., 2010; Shrout et al., 2011). In contrast, fungi, have the ability to produce metabolites that interfere with bacterial QS (Venkatesh and Keller, 2019). Some of these are mycotoxins and they can act as QS inhibitors (QSI) by mimicking autoinducer structure or function, acting as antagonists to, or hydrolysing QS molecules, and by interfering with the stability and function of the regulator protein or autoinducer synthase (González and Keshavan, 2006). Filamentous fungi have also been associated with the production of mycotoxins in water (Paterson et al., 1997, 2007; Siqueira et al., 2013). The term mycotoxin refers to harmful secondary metabolites produced by fungi. Initially thought to be waste products, fungal secondary metabolites are now being considered as important players in ecological settings allowing the fungus to secure its environmental niche by providing protection against other microbes (Venkatesh and Keller, 2019). Fungal secondary metabolites are known to be involved in the disruption of QS in bacteria (Martín-Rodríguez et al., 2014). For instance, fusaric acid acts as a quorum quencher against *Pseudomonas chlororaphis* (van Rij et al., 2005). Furthermore, two other mycotoxins produced by the genus *Fusarium,* zearalenone and fumonisin, have been shown to inhibit QS in the bacterium *Chromobacterium violaceum* (Bacon et al., 2017). Filmanentous fungi from the genus *Penicillium*, have also been investigated for their ability to produce QSIs (Rasmussen et al.,

2005). Patulin, a well-known mycotoxin with a low molecular weight (154.12 g mol^{-1}) and high polarity was shown to down-regulate genes related with QS in *Pseudomonas aeruginosa* by 45% indicating specificity for QS-regulated gene expression (Rasmussen et al., 2005). In addition, this mycotoxin has been reported to reduce biofilm formation and QS signalling system by *Halomonas pacifica* as well as modulating biofilm formation by *P. aeruginosa* and *Achromobacter* sp. (Liaqat et al., 2010, 2014). Furthermore, patulin has been shown to reduce biofilm formation by *Methylobacterium oryzae* (Afonso et al., 2020).

Despite some known effects of patulin on biofilm formation and/or bacterial QS-regulated gene expression, little to no information is available on the effect and possible modulation that bacteria themselves cause on the expression of genes involved in the patulin biosynthesis and/or patulin production. The patulin biosynthetic pathway consists of approximately ten steps (Puel et al., 2010; Li et al., 2019). The enzyme isoepoxydon dehydrogenase has been identified as a precursor in the late steps of the patulin biosynthetic pathway and the *idh* gene encoding this enzyme has been cloned and sequenced from *P. expansum* (White et al., 2006). Real-time reverse transcription polymerase chain reaction (RT-qPCR) is the method of choice for sensitive, specific, and reproducible quantification of mRNA levels of a transcribed gene (Bustin, 2000).

The goal of this study was to investigate the effect of the Gram-negative bacterium *M. oryzae* on the *idh* gene expression levels and patulin production of *P. expansum* mature biofilms. For this purpose, an RT-qPCR method to quantify *idh* mRNA levels was adapted from De Clercq et al. (2016). In addition, the *idh* expression levels were compared with the patulin production.

4.2 Materials and Methods

4.2.1 Microorganisms and culture conditions

Penicillium expansum MUM 00.02, supplied by Micoteca da Universidade do Minho fungal culture collection (MUM, Braga, Portugal), was used in this work and chosen based on its occurrence in the tap water of the north of Portugal and because it is able to form single and inter-kingdom biofilms (Gonçalves et al., 2006; Simões et al., 2015; Afonso et al., 2019, 2020). It is also a well-known producer of patulin. *P. expansum* was maintained on malt extract agar (MEA: malt extract 20 g, peptone 5 g, agar 20 g, distilled water 1 l).

Methylobacterium oryzae was previously isolated from a model laboratory DWDS by Simões et al. (2007). This bacterium was chosen for being representative of drinking water bacteria and due to its ability to form complex single species biofilms as well as inter-kingdom biofilms with this fungus (Simões et al., 2010; Afonso et al., 2019, 2020). *M. oryzae* was grown for 72 h before the start of the assay. It was grown in batch cultures using 100 mL of R2A broth (yeast extract 0.5 g, proteose peptone 0.5 g, casein hydrolysate 0.5 g, glucose 0.5 g, soluble starch 0.5 g, dipotassium phosphate 0.3 g, magnesium sulphate 0.024 g, sodium pyruvate 0.3 g, distilled water 1 l) at room temperature (25 ± 2 °C) and under agitation (150 rpm). Afterwards, the bacteria were harvested by centrifugation (10 min at 13,000 *g*, room temperature), washed twice in 0.1 *M* saline phosphate buffer, and resuspended in a volume of 50% R2A broth and 50% apple juice (APJ) to obtain a cellular density of 10^8 cells mL^{-1}. This was the bacterial concentration used for biofilm formation assays.

4.2.2 Stock solution of fungal spores

Stock solution of fungal spores was prepared according to Simões et al. (2015). Briefly, spores of *P. expansum* were harvested from seven-day old pure cultures in MEA at 25 °C by flooding the surface of the agar plates with 2 mL of TWS solution (0.85% NaCl plus 0.05% Tween 80) and rocking gently. The suspension was then homogenized by vortexing and used for large scale production of spores. The final spore suspension was homogenized by vortexing before quantification using a Neubauer count chamber. Aliquots of spore suspension with 10% of glycerol were cryopreserved at -80 °C in order to allow the use of the same spore suspension in all the biofilm assays. Stock spore suspensions were resuspended in the volume of R2A broth necessary to achieve a density of 10^5 spores ml^{-1}. This was the spore concentration used for biofilm formation assays.

4.2.3 Apple Puree Agar Medium (APAM) and Apple Juice (APJ) preparation

APAM was prepared as described by Baert et al. (2007). Briefly, apples (cultivar Golden Delicious) were cut into small pieces (with the peel, but without the core) and mixed until a fine puree was obtained. This puree was stored was stored at -20 °C. To prepare APAM, 100 g of apple puree was weighed in a sterile Schott bottle and heated during 45 minutes in a hot water bath at 99 °C. This was done to kill vegetative cells. 50 ml sterile agar solution was then added to the puree to make the APAM. Under aseptic conditions, Petri dishes were filled with 20g APAM.

To prepare APJ, 50 g of apple puree was weighed in a sterile Schott bottle and 300 ml of sterile H_2O was added to the puree. The Schott was heated at 99 °C for 10 min to help mix the puree with the

water. After this step, the solution was filtered using a first layer of gauze followed by a second filtration using a 12-15 μm qualitative filter paper (VWR, Radnor, PA, USA) into a new sterile Schott bottle. After the filtering process, the Schott bottle containing the final APJ, similarly to APAM, was heated during 45 minutes in a hot water bath at 99 °C.

4.2.4 Biofilm formation

Biofilms were developed according to the modified microtiter plate test used by Stepanović et al. (2000) for bacteria and Simões et al. (2015) for fungi. Briefly, wells of sterile polystyrene 96-well flat bottom culture plates (Greiner Bio-One Cellstar®, Kremsmünter, Austria) were filled under aseptic conditions with 200 μL of spore suspension (10^5 spores ml^{-1} in 50% R2A broth and 50% APJ) or 200 μl of a cell suspension (10^8 cell ml^{-1} in 50% R2A broth and 50% APJ) for the single-species biofilms, which were also used as positive controls. To promote biofilm formation, all plates were incubated aerobically at room temperature (25 ± 2 °C) and under agitation (150 rpm) for 120 h. *P. expansum* biofilms were developed for 96 h. Each 24 h, the depleted medium was removed, and fresh, clean medium was added to each well. In parallel, at 48 and 72 h in different batches of *P. expansum* biofilms, the depleted medium was also removed, but fresh medium inoculated with 10^8 cells ml^{-1} of *M. oryzae* was added. This co-growth was analysed for 24 h, meaning that the interaction between these two microorganisms was evaluated from 48 to 72 h and from 72 to 96 h. M. oryzae single species biofilms at 24 h were also used as positive controls. At each sampling time (72 and 96 h) the content of each well was removed and washed two times with 200 μl of sterile distilled water to remove non-adherent and weakly adherent cells. The plates were air dried for 30 min to remove excess water by evaporation. The remaining attached cells were analysed in terms of biomass adhered on the inner walls of the wells, in terms of colony forming units (CFU) for bacteria. The rinsing and drying procedures were as previously used with bacterial and fungal biofilms (Simões et al., 2007, 2010, 2015). Negative controls were obtained by incubating the wells with only 50% R2A broth and 50% APJ without adding any fungal spores or bacterial cells. Experiments were performed in triplicate with at least 3 repeats.

4.2.5 Biofilm mass quantification by crystal violet

The biomass adhered on the inner walls of the wells was quantified by the crystal violet (CV) method according to the procedure described by Stepanović et al. (2000). The biofilms in the 96-well plates were fixed with 200 μL $well^{-1}$ of 98% methanol (VWR, Carnaxide, Portugal), for 15 min. Afterwards, the methanol was discarded, the plates left to dry and then the fixed biofilm was stained with 200 μl well

$^{-1}$ of CV (Merck KGaA, Darmstadt, Germany) for 5 min. Excess stain was rinsed out by placing the plate under slow running tap water. After this, the plates were air dried and the dye bound to the adherent cells was resolubilized by adding 200 µl well^{-1} of 33% (v v^{-1}) glacial acetic acid (Panreac, Cascais, Portugal). The optical density of the obtained solution was measured at 570 nm using a microtiter plate reader (BioTek, Winooski, VT, USA) and the biofilm mass was presented as $OD_{570\,nm}$ values.

4.2.6 Number of bacteria in single and inter-kingdom biofilms

The number of bacterial cells present in *M. oryzae* single-species biofilms and in inter-kingdom biofilm after 24 h of co-growth with *P. expansum* was determined in terms of CFU using a plate count assay upon biofilm release. Briefly, *M. oryzae* single-species biofilms and inter-kingdom biofilms were grown in a 96-well plate as described above. After 24 h of incubation period, the supernatant was removed, and the plate was washed three times with sterile water. A volume of 200 µl of sterile phosphate buffer saline (pH 7.4) was added into each well. Afterwards, biofilms were scrapped with a pipette tip and, in addition, the 96-well plate was covered with the lid and placed into an ultrasonic bath (Bandelin electronic GmbH & Co. KG, Berlin, Germany). To release bacterial cells from biofilm, the plate was sonicated for 1 min (5 s sonicate, 10 s interval) at 35 kHz. Three replicates were used for each condition and bacterial cells were plated onto R2A agar plates for CFU determination.

4.2.7 RT-qPCR method to measure *idh* gene expression of *P. expansum*

4.2.7.1 RNA extraction and DNase treatment

Biofilms were prepared as described in section 4.2.4. At each sampling time (72 and 96 h) the content of each well was removed and washed two times with 200 µl of sterile distilled water to remove non-adherent and weakly adherent cells. After this washing step, the contents of at least 3 wells were scrapped off the microtiter plate and transferred to a 2 ml microcentrifuge tube (Frilabo, Maia, Portugal) with 0.6 g of 710-1180 µm glass beads, acid-washed (Sigma-Aldrich, St. Louis, MO, USA). The microcentrifuge tube was immediately placed on ice until all samples to be tested were collected. When all samples were collected (time taken < 5 min), RNA was extracted by means of the PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, CA, USA). Briefly, 600 µl of Lysis Buffer was added to each microtube containing fungal biomass and glass beads, which were then homogenized in a FastPrep-24™5G Instrument (MP Biomedicals, Irvin, CA, USA). Homogenization procedure was as follows: one homogenization cycle for 45 s at 6 m s^{-1}, followed by 30 s on ice, followed by a second homogenization cycle for 45 at 6 m s^{-1}. The

microtubes were again placed on ice and the rest of the RNA extraction procedure followed the manufacturer's instructions. Total RNA was eluted in a final volume of 50 µl nuclease-free H_2O (Invitrogen, Carlsbad, CA, USA).

The DNase I, RNase-free (Thermo Scientific, Waltham, MA, USA) was used to remove any residual DNA. Two microliters of DNase I buffer and 1 U DNase I were added to a 200 µl centrifuge tube containing 20 µl total RNA. After gentle inversion, the mixture was incubated for 30 min at 37 °C. After the incubation period, 2 µl EDTA was added to the RNA mixture and thoroughly resuspended by smooth pipetting, and incubated for 5 min at 65 °C to inactivate DNase I. The tube was then stored at -20 °C until the next day when further analysis was conducted.

RNA samples were visually checked after staining a 1.5% (w/v) agarose gel (NZYTech, Lisbon, Portugal) with GreenSafe Premium (NZYTech). Non-denaturing electrophoresis was carried out at 80 V for 45 min. The Nanodrop® ND-1000 spectrophotometer (Thermo Scientific) was used to measure the RNA concentration and nucleic acid purity based on the A_{260}/A_{280} and A_{260}/A_{230} absorbance ratios for protein contamination and polysaccharide and/or chaotropic salt contamination, respectively.

4.2.7.2 cDNA synthesis

cDNA synthesis was done with the Xpert cDNA synthesis kit (GRiSP, Porto, Portugal) according to the manufacturer's instructions. All DNase treated TNA extracts were $1/10^{th}$ diluted before converting to cDNA to prevent the possible presence of sample inhibition (De Clercq et al., 2016). Prior to cDNA synthesis by means of reverse transcription, a heat shock treatment was applied to the mixture of template RNA, dNTPs, and primers for sequence denaturation. In this way, the absence of secondary structures of RNA and/or primers is assured, guaranteeing a more efficient cDNA synthesis. Hence, a mixture of 50 ng of total RNA, 1 µl of random hexamer primers (10 µM) , 1 µl of dNTP's (10 mM each) and RNase-free water up to 14.5 µl (GRiSP) were subjected to heat shock for 5 min at 65 °C followed by 2 min cooling on ice. After the heat shock, 4 µl of 5x reaction buffer, 0.5 µl of RNase inhibitor (40 U μl^{-1}) and 1 µl of Xpert reverse transcriptase (200 U μl^{-1}) were added to the reaction mixture obtaining a final volume of 20 µl. Reverse transcription was carried out with a C1000™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) with the following conditions: 10 min at 25 °C, 15 min at 50 °C and 5 min at 85 °C. To check for residual genomic DNA (gDNA), each RNA sample was also subjected to a cDNA synthesis reaction without the addition of reverse transcriptase enzyme (noRT) (Werbrouck et al., 2007). cDNA's were then stored at -20° C until qualitative real-time PCR assay was performed.

4.2.7.3 Quantitative real-time PCR (qPCR) assay

Optimization of the qPCR is essential for an accurate quantification (Werbrouck et al. 2007). *Idh* gene expression levels in each sample were quantified relative to the expression of the reference gene *β-tubulin*. The set of primers used in this study were selected from De Clerc et al. (2016) (Table 4.1).

Table 4.1 Primers and primer efficiency (De Clercq et al., 2016).

Gene	Primer set	F primer sequence (5' to 3')	R primer sequence (5' to 3')	Product length (bp)	qPCR efficiency (%)
idh	EK-RT-2F/EK-RT-2R	GCAGTTTCGCGATCGATGT	GTAGGGAGTAGCCGCCTTGA	59	92.1
β-tubulin	BTub-2F/BTub-2R	GGTCCCTTCGGCAAGCTT	TGTTACCAGCACCGGACTGA	64	93.0

For the qPCR itself, a reaction mixture containing 5 μl Xpert Fast SYBR Mastermix (GRiSP), 300 nM (10 mM μl^{-1}) of each of the appropriate gene-specific primers, 2 μl of template cDNA ($1/10^{th}$ diluted to prevent the presence of inhibitors) and ultra-pure water up to 10 μl was prepared for each sample. Reaction mixtures were added to a clear, low profile 96-well PCR plate (Thermo Scientific) and sealed using adhesive sealing foil (Frilabo). For each sample (duplicates), a relative quantification of the mRNA levels of the gene of interest against the reference gene was done using a CFX96™ real-time system (Bio-Rad) coupled to a C1000™ Thermal Cycler (Bio-Rad). Thermal cycling conditions were as follows: 2 min at 95 °C to activate the polymerase enzyme, followed by 40 cycles of denaturation at 95 °C for 5 s, and a one-step annealing and elongation during 20 s at 60 °C. The data were collected during each elongation step. A melting-curve analysis between 65 °C and 95 °C was performed after each PCR. To check the specificity of the amplification product. Samples were tested for residual gDNA by comparing the Cq values of RT cDNA and noRT cDNA reactions. When $\Delta Cq > 7$ was obtained, gDNA contamination was considered negligible (De Clercq et al., 2016). In each run, a negative control (without cDNA) was included. Both genes were quantified with biological duplicates for each independent assay. The expression of *idh* was normalized to the expression of the reference housekeeping gene *β-tubulin* using the Pfaffl method and considering *P. expansum* single-species biofilms at 72 and 96h as controls (Pfaffl, 2001).

Preliminary results of qPCR amplification in the conditions selected for this work, revealed high Ct values (> 28 cycles) for the *idh* gene, which would not allow for enough 10× serial dilutions of cDNA to recalculate primer efficiency. For this reason, efficiency was recalculated in house for the *P. expansum* strain used in this study in APAM. Briefly, *P. expansum* MUM 00.02 was middle-point inoculated onto

APAM Petri dishes and incubated at 25 °C for seven days. Sporulating mycelium was then scrapped into a 2 mL microcentrifuge tube and RNA was extracted by the method described in section 4.2.6.1. After cDNA synthesis and qPCR analysis, efficiency was determined with the CFX Manager™ Software version 3.1 (Bio-Rad) using the dilution curve method. cDNA was 10× serial diluted, and the amplification curves were used for efficiency calculation (Table 4.1).

4.2.8 Patulin extraction and quantification by UHPLC

4.2.8.1 Reagents and Chemicals

Pure patulin standard (≥98 %) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Stock solution of 5 mg mL^{-1} was prepared in dimethyl sulfoxide (DMSO) (Merck KGaA) and stored at -20 °C until use. Ethyl acetate (AcOEt) and acetonitrile (ACN) (HPLC gradient grades) were supplied by Fischer Chemical (Hampton, NH, USA). HPLC-grade water was generated by a Milli-Q-grade purification system (Millipore, Darmstadt, Germany).

4.2.8.2 Patulin extraction

Patulin extraction method was based and adapted from Sargenti and Almeida (2010). The contents of 8 wells of each condition (1.6 ml), including medium and biomass, were scrapped and transferred to 15 mL centrifuge tubes (Orange Scientific, Braine-l'Alleud, Belgium). Double volume of ethyl acetate was then added to the Falcon tube. The extraction procedure as follows: 5 min vortexing, followed by 10 min in an ultrasonic bath (Bandelin electronic GmbH & Co. KG, Berlin, Germany) at 35 kHz, followed by vortexing for another 5 min. The ethyl acetate phase was separated and transferred to a 4 mL vial and placed under at 40 °C under a flow of gentle nitrogen stream to evaporate the solvent. When the residue was completely dry, it was kept at -20 °C until all samples were extracted. The residues were then resuspended in 1 ml of HPLC-grade water adjusted to pH 4.0 with acetic acid and vortexed for 1 min. Finally, the extracts were filtered into microvials using a 2 mL Omnifix syringe (B. Braun, Melsungen, Germany) coupled with a 0.22-μm nylon syringe filter (Nantong FilterBio Membrane, Nantong City, China) and analysed by liquid chromatography. Quantification was performed by means of internal calibration curves of increasing concentrations: one in a range from 0.1 to 2.5 μg ml^{-1} and another one from 10 to 100 μg ml^{-1} of pure patulin standards. Patulin recovery percentage using the present method was calculated by spiking the medium with two different concentrations of patulin, in this case, 1 and 10 μg

ml^{-1}, in triplicates and the chromatographic analysis was performed in duplicates. Recovery percentage varied from 68.74% to 84.23% with a mean of 79.78 ± 5.65%.

4.2.8.3 UHPLC apparatus and conditions

Liquid chromatography was performed using a Nexera X2 UHPLC System equipped with a DGU-20A5R degassing unit, LC-30AD pump, SIL-30AC autosampler, CTO-20AC column oven and an SPD-M20A photodiode array detector set at 276 nm (Shimadzu Corp., Kyoto, Japan). Chromatographic separation was carried out with a Chromolith® Performance RP-18 endcapped 100-4.6 HPLC column (Merck KGaA). Oven temperature was maintained at 25 °C. The injection volume was set at 5 µl and the sample flow rate at 0.75 ml min^{-1}. Run time was 10 min with a mobile phase of H_2O:ACN (95:5, *v/v*). Patulin average retention time was obtained at around 4.4 min.

4.2.9 Statistical analysis

The data were analysed using the software GraphPad Prism version 8.0.2 (GraphPad Software, La Jolla, California, USA). The mean and standard deviation (SD) within samples were calculated for all cases. Statistical significance of results was determined by unpaired *t* test. $P < 0.05$ was considered to be statistically significant.

4.3 Results

4.3.1 Biofilm mass quantification

In order to assess the effect of *M. oryzae* on pre-established biofilms of *P. expansum*, the standard 96-well microtiter plates with CV was used. Results for biofilm mass quantification are presented in Figure 4.1. *P. expansum* single-species biofilms were used as controls. The results revealed that *P. expansum* single-species biofilms increased in mass until 96 h. The introduction of *M. oryzae* into pre-established *P. expansum* biofilms at 48 and 72 h, and consequent 24 h interaction until 72 and 96 h, respectively, caused a significant decrease of *P. expansum* biofilm mass at both times ($P < 0.05$).

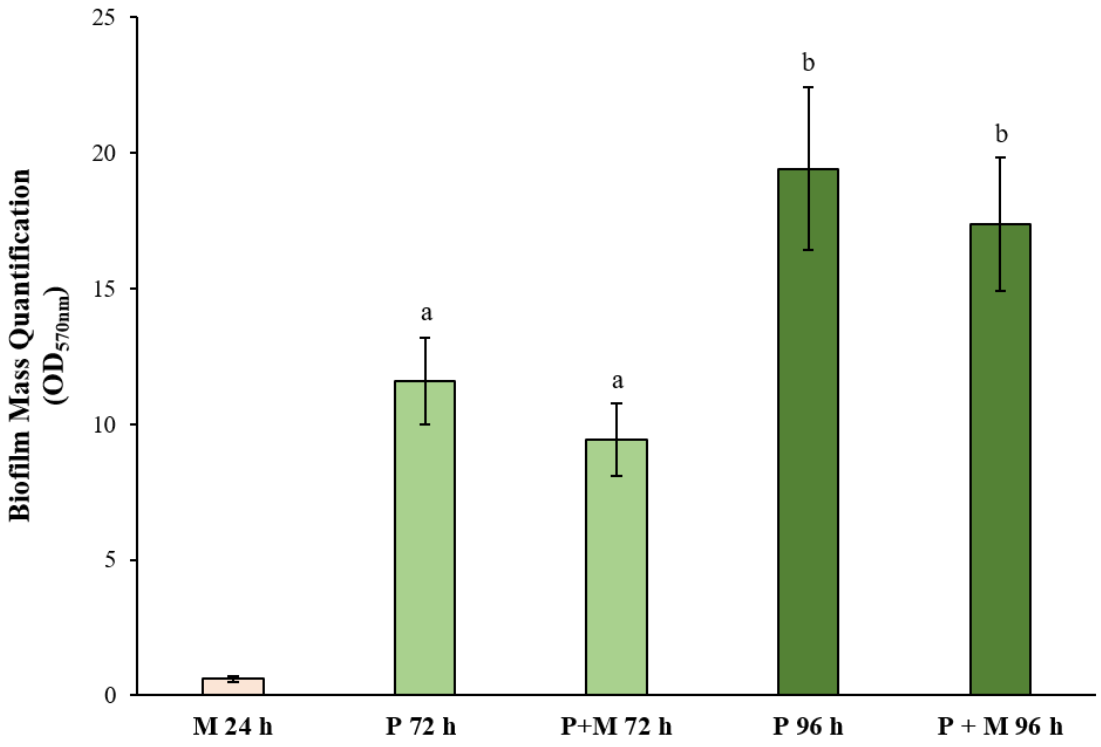

Figure 4.1 Biofilm mass quantification for single-species and inter-kingdom biofilm formation over. Legend is as follows: 'M' - *M. oryzae*; 'P' - *P. expansum*. The means ± SDs for three independent experiments are illustrated. Means with the same letter are significantly different.

4.3.2 Bacterial cell density in single and inter-kingdom biofilms

The number of *M. oryzae* cells present in either single-species biofilms and in inter-kingdom biofilms after 24 h interaction with 48 or 72 h pre-established *P. expansum* biofilms was determined as CFU and the results obtained are presented in Figure 4.2. CFU from *M. oryzae* single-species biofilms at 24 h was used as control. Analysing the results, the interaction of *M. oryzae* with pre-established *P. expansum* biofilms from 48 to 72 h did not cause a statistically significant change ($P > 0.05$) in this bacterium CFU. However, a significant decrease ($P < 0.05$) in CFU was observed from the interaction with pre-established *P. expansum* biofilms from 72 to 96 h indicating a higher inhibition of *P. expansum* against *M. oryzae* at this time. This inhibition was also observed when comparing the interaction from 72 to 96 h with 48 to 72 h ($P < 0.05$).

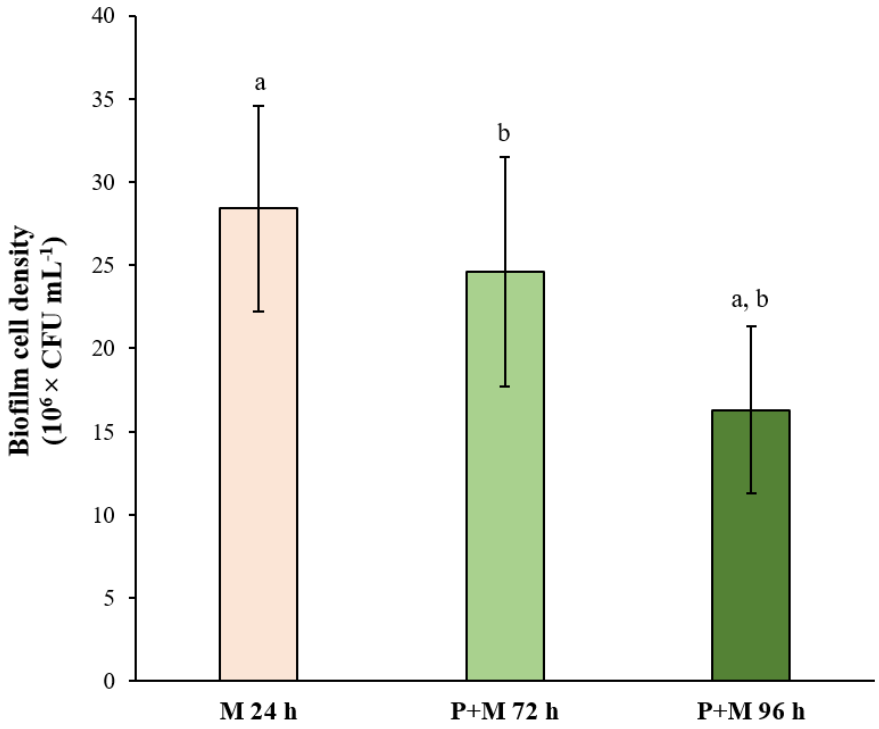

Figure 4.2 Biofilm cell density for *M. oryzae* while forming single-species or inter-kingdom biofilms with pre-formed *P. expansum* biofilms. Legend is as follows: 'M' - *M. oryzae*; 'P' - *P. expansum*. The means ± SDs for three independent experiments are illustrated. Means with the same letter are significantly different.

4.3.3 *Idh* gene expression in single and inter-kingdom biofilms

The effect of the introduction of *M. oryzae* on the *idh* gene expression of pre-established *P. expansum* biofilms was investigated *in vitro* on 50% R2A + 50% APJ. Figure 4.4 shows the results of the normalized expression levels (fold change) of the *idh* gene in relation to the reference gene *β-tubulin*. *P. expansum* single-species biofilms at 72 and 96 h were set as controls, while the treatment was the presence of *M. oryzae* at each respective time (Figure 4.4a and 4.4b). Through the results it is possible to observe a decrease in the expression of the *idh* gene when *M. oryzae* cells were introduced to *P. expansum* biofilms from 48 to 72 h ($P < 0.05$). The normalized *idh* expression levels at this time, in the different independent assays, varied from a minimum of 0.12 to a maximum of 0.45 which is equivalent to a fold decrease of 8.3 and 2.2, respectively. At 96 h, there was a slight increase in *idh* gene expression in the presence of *M. oryzae*, however, it was not considered statistically significant ($P = 0.099$). *Idh* normalized expression levels at 96 h varied from a minimum of 1.3 to a maximum of 3.1-fold increase.

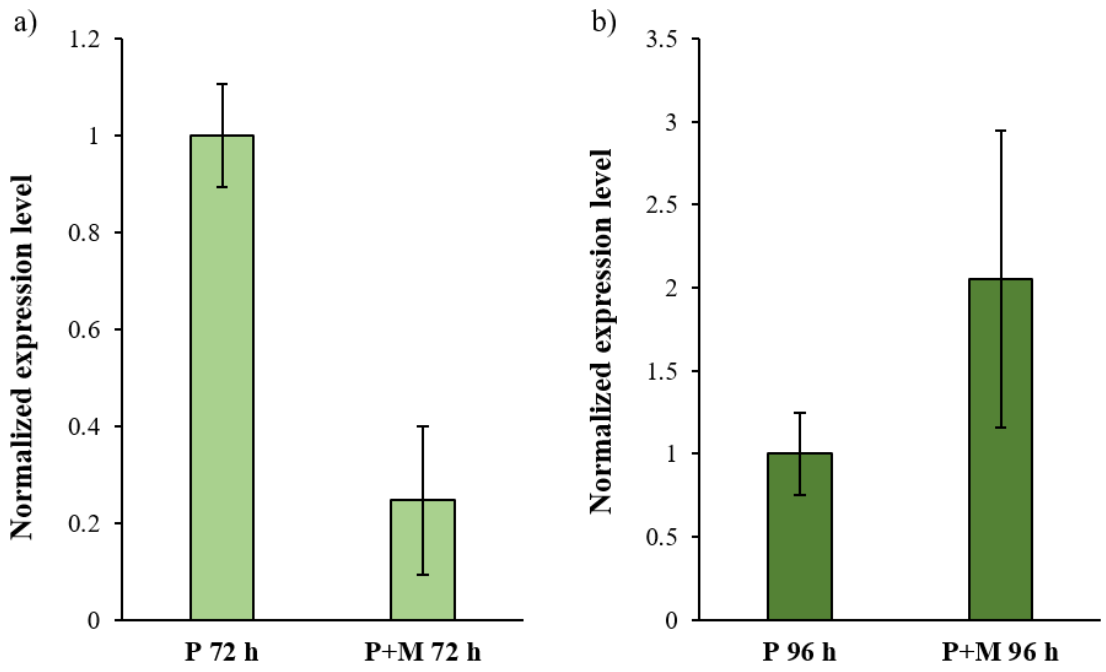

Figure 4.3 *Idh* gene normalized expression levels relative to the reference gene *β-tubulin* in *P. expansum* biofilms at 72 h (a) and 96 h (b) in the presence of *M. oryzae*. Legend is as follows: 'M' - *M. oryzae*; 'P' - *P. expansum*. The means ± SDs for four independent experiments are illustrated.

4.3.4 Patulin production in single and inter-kingdom biofilms

The effect of the introduction of *M. oryzae* on patulin production of 48 and 72 h pre-established *P. expansum* biofilms was investigated *in vitro* on 50% R2A + 50% APJ. Figure 4.3 shows the results of the relative fold change of patulin in the presence of *M. oryzae* in relation to *P. expansum* single-species biofilm controls at 72 and 96 h, Figure 4.3a and 4.3b, respectively. The individual results of patulin production from the three independent assays revealed a high variation of patulin concentration between them. For instance, among three independent assays, concentrations ranged from 1.14 -7.09 μg mL^{-1} in *P. expansum* 72 h biofilms, 0.19-2.61 μg mL^{-1} in *P. expansum* + *M. oryzae* 72 h biofilms, 0.59-1.05 μg mL^{-1} in *P. expansum* 96 h biofilms and 1.43-5.14 μg mL^{-1} in *P. expansum* + *M. oryzae* 96 h biofilms. For this reason, relative fold change difference in relation to each individual control was calculated in order to normalize the results.

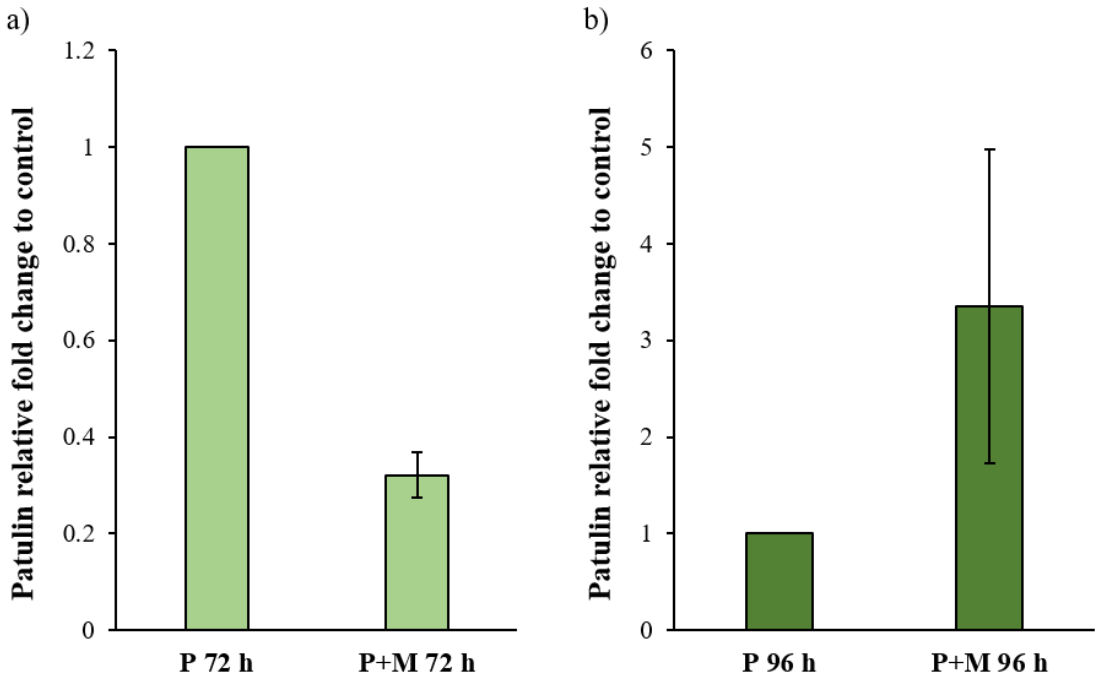

Figure 4.4 Patulin relative fold change production by *P. expansum* biofilms at 72 h (a) and 96 h (b) in the presence of *M. oryzae*. Legend is as follows: 'M' - *M. oryzae*; 'P' - *P. expansum*. The means ± SDs for three independent experiments are illustrated.

Through the normalization we can observe that *P. expansum* biofilms growing in the presence of *M. oryzae* from 48 to 72 h results in a decrease of patulin production. However, the inverse is observed when *M. oryzae* is introduced to 72 h *P. expansum* biofilms, where an increase in patulin is observed at 96 h.

A trend between *idh* gene expression and patulin production values is observed. The results obtained from both patulin production and *idh* gene expression, reveal a tendency of decrease in both of these parameters when *M. oryzae* is introduced to *P. expansum* from 48 to 72 h biofilms, while the opposite is detected when this bacterium is introduced to *P. expansum* biofilms at 72 h.

4.4 Discussion

The present study aimed at analysing the changes that occur when *M. oryzae,* a Gram-negative bacterium found in DWDS is introduced to mature *P. expansum* biofilms. For this purpose, *M. oryzae* was introduced to pre-established fungal biofilms at 48 and 72 h, the interaction was allowed to occur for 24 h, and different parameters were evaluated at 72 and 96 h, respectively. The parameters analysed to understand this interaction were biofilm mass, bacterial CFU, *idh* gene expression and patulin production

from the developed biofilms. These microorganisms were chosen based on different characteristics: their ability to form mature single-species biofilms between 48 and 72 h growth and because they have been assessed while forming inter-kingdom biofilms (Simões et al., 2010, 2015; Afonso et al., 2019, 2020). *P. expansum* was also selected due to its well-known ability to produce patulin as a secondary metabolite (Santos et al., 2002; Reddy et al., 2010; De Clercq et al., 2016). Preliminary assays leading to this work revealed that *P. expansum* biofilms developed in R2A did not produce detectable amounts of patulin for as long as 120 h of biofilm formation. For this reason, apple juice made from apple extract was added to induce patulin production. In the conditions tested in the present work (50% R2A-50% APJ), patulin was detected from 72 h onwards, thus, the time-points selected to evaluate the effect of *M. oryzae* on *P. expansum* biofilms patulin production and *idh* gene expression were from 48 to 72 h and 72 to 96 h.

Regarding biofilm mass quantification, we can observe that at 72 and 96 h, after 24 h of co-growth with *M. oryzae*, a decrease in *P. expansum* biomass was detected at both times, indication an inhibitory effect of *M. oryzae* towards *P. expansum*. This inhibition has been observed in previous studies where *M. oryzae* was inoculated at the same time as *P. expansum* (Afonso et al., 2019; 2020). This could mean that, despite the beginning of interaction with *M. oryzae*, albeit inoculated at the same time or added to pre-established *P. expansum* biofilms, this bacterium has the ability to interfere with fungal growth. This could be due to direct competition for available nutrients, the production of metabolites that might inhibit fungal growth or a combination of both factors (Mowat et al., 2010; Kousser et al., 2019).

To analyze the effect that pre-established *P. expansum* biofilms had on *M. oryzae* itself, bacterial CFU were determined with *M. oryzae* single-species biofilms at 24 h being used as control. A reduction in this bacterium CFU was observed when it was added to 48 and 72 h *P. expansum* biofilms, although it was only statistically significant when it was added to the latter. A significant reduction was also observed when comparing bacterial CFU at 96 with 72 h, meaning a stronger inhibitory effect of the fungus towards *M. oryzae* in more mature biofilms. This inhibitory effect had also been observed in previous studies where these two microorganisms were inoculated at the same time (Afonso et al., 2019; 2020). The presence of exogenous concentrations of patulin highlighted this inhibitory effect (Afonso et al., 2020). In the present study, the inhibitory effect accentuated at 96 h might be related to the increased concentrations of patulin detected at this time (0.19-2.61 $\mu g\ ml^{-1}$ compared to 1.43-5.14 $\mu g\ ml^{-1}$).

RT-qPCR is a molecular technique that allows the quantification of fungi in environmental samples and the study of host-pathogen interactions and changes in gene expression in response to certain conditions (Schena et al., 2004). Its application has become the method of choice for quantitatively assessing steady state mRNA levels (De Clercq et al., 2016). In the present study, the primers selected

for qPCR were selected from a study by De Clercq et al. (2016). An optimization process for the conditions required for this study was performed, as well as the recalculation of primer efficiency for both sets of primers used presently. The presence of PCR inhibitors is a major drawback of the PCR, as it decreases sensitivity and can give false-negative results. For this reason, a $1/10^{th}$ dilution of both RNA prior to cDNA synthesis as well as cDNA prior to the qPCR assay were done to remove possible inhibitors (Schrader et al., 2012; De Clercq et al., 2016).

Several studies regarding the expression of fungal genes involved in mycotoxin biosynthesis have been quantified relative to one reference gene (Sweeney et al., 2000; Mayer et al., 2003; Jiao et al., 2008; Sanzani et al., 2009). In our study, we used *β-tubulin* as the reference housekeeping gene as it maintained very stable Ct values throughout the different conditions tested.

Overall, the results of our *in vitro* study show that the presence of *M. oryzae* affects the patulin production of *P. expansum* mature biofilms on 50% R2A-50% APJ (Figure 4.4), by influencing the transcriptional level of the *idh* gene (Figure 4.3). This influence is observed by a decrease of the *idh* expression level and consequent patulin production from a 24 h interaction with 48 h *P. expansum* biofilms while the opposite is observed from the 24 h interaction with 72 h *P. expansum* biofilms until 96 h. To the best of our knowledge, this is the first study evaluating the effect of a bacterium on the *idh* gene expression and patulin production of *P. expansum* biofilms. Nevertheless, comparing with studies in different matrixes/conditions, a good trend was also found between *idh* expression and patulin production in a study performed by De Clercq et al. (2016). The use of RT-qPCR to monitor gene expression related to the biosynthesis of mycotoxins has also been described for other fungal species. For instance, the mRNA levels of two genes involved in the biosynthetic pathway of aflatoxin in *Aspergillus parasiticus* revealed an absence in the transcription of both genes when this fungus was grown in a medium that did not support aflatoxin production (Sweeney et al., 2000). Jiao et al. (2008) investigated the effect of different carbon sources on the trichothecene production and induction mechanisms of *Fusarium graminearum* and suggested that this fungus recognizes the sucrose molecules, activates the expression of a specific gene, and induces trichothecene production.

As previously stated, in the present study, *M. oryzae* caused a decrease in *idh* gene expression and patulin production after 24 h interaction with a 48 h pre-established *P. expansum* biofilm. Different studies have reported the use of bacteria to inhibit the growth and mycotoxin production of different fungal species. Taheur et al. (2019) demonstrated that using different lactic acid bacteria (LAB) in agar medium lead to a growth inhibition of *Aspergillus flavus* and *Aspergillus carbonarius* as well as the reduction of different mycotoxins in almonds. In this study, aflatoxin B1, B2 and ochratoxin A were reduced by 97.22%,

95.27% and 75.26%, respectively. Another study using LAB, revealed a growth inhibition of *F. graminearum* and *A. parasiticus* by *Lactobacillus rhamnosus* and *L. plantarum* (Dogi et al., 2013). In addition, a decrease in zearalenone production was observed as a result of the interaction of both *Lactobacillus* strains with *F. graminearum,* while the interaction between *A. parasiticus* and *L. plantarum* resulted in an increased aflatoxin B1 production (Dogi et al., 2013). The use of viable and nonviable bacteria has also been studied regarding the reduction of patulin from different matrixes, including apple juice. For instance, Hatab et al. (2012a), demonstrated that LAB have the ability to remove patulin from aqueous solution. In this study, the maximum patulin uptake was achieved by *Bifidobacterium bifidum* and *L. rhamnosus* (52.9% and 51.1% for viable and 54.1% and 52.0% for nonviable cells) after 24 h incubation. The same authors reported in another study that incubation for 24 h with *L. rhamnosus* and *Enterococcus faecium* caused a decrease of patulin in apple juice by 80.4% and 64.5%, respectively (Hatab et al., 2012b). Yuan et al. (2014), also reported the use of inactivated *Alicyclobacillus* spp. cells to reduce patulin concentration in apple juice. These authors reported patulin reduction rates of 88.8% and 81.6% by two strains of *A. acidoterrestris* achieved after a 24 h incubation period (Yuan et al., 2014).

The increase observed in the expression of the *idh* gene and consequent increase in patulin production from the 24 h interaction of *M. oryzae* with the 72 h pre-established *P. expansum* biofilm, might be related to the overall metabolic state of the fungus being more active, allowing it to develop better strategies, namely by increasing the production of secondary metabolites (i.e. patulin), to contest the presence of *M. oryzae*.

A drawback observed from the results of patulin production was the high variablility in the concentration of patulin obtained among the independent experiments. Different concentration values were obtained between each independent experiment, despite the good recovery percentage of the extraction method (Sargenti et al., 2010). This variability in the patulin concentration values, despite all efforts to control the conditions, could be due to differences in the composition of APJ medium, differences in the starting metabolic state of *P. expansum* or the low sample volume used in each extraction. However, regardless of this drawback, when the results were normalized to each independent experiment control, a clear tendency in the results was observed.

In conclusion, our results show that the effect of *M. oryzae* on pre-established *P. expansum* biofilms is dependent on the time of interaction as demonstrated by the different resulting tendencies when the bacterium is introduced to 48 or 72 h *P. expansum* pre-established biofilms. More mature *P. expansum* biofilms appear to be more resistant to the inhibitory effect that *M. oryzae* causes towards *idh* gene expression and patulin production. This is a result of the increased concentration of patulin at 96 h

and reciprocates an inhibitory effect of *P. expansum* towards *M. oryzae*. A trend was observed between the *idh* expression and patulin production values. The results indicate that *M. oryzae* affects patulin production by acting at the transcriptional level of the *idh* gene. These findings indicate that patulin plays a vital role in inter-kingdom communication and/or microbial assembly processes that influence biofilm formation between fungi and bacteria.

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Chapter 5

Concluding remarks and perspectives for future research

5.1 General conclusions

The aim of this thesis was to gain deeper insights into the role of filamentous fungi in drinking water biofilms, emphasising inter-kingdom interactions with bacteria as well as the specific role of signalling molecules and secondary metabolites on biofilm formation and microbial interactions. In order to achieve these goals, several aspects were studied throughout this thesis, namely: the ability of these distinct microorganisms to interact with each other in inter-kingdom biofilms, including the influence of fungal stage development; the ability of the fungi to produce QSI; the effect of a QS molecule and a fungal secondary metabolite on inter-kingdom biofilm formation; and the effect of bacteria on gene expression of mycotoxin-related genes and mycotoxin production by filamentous fungi biofilms.

The main conclusions that can be highlighted from the work presented in this thesis are the following:

Fungal spore development stage is important in the first 24 h of water fungal biofilm formation. Using germinated spores, an advantage is provided in the first 24 h towards the establishment of a denser biofilm. Inter-kingdom biofilm formation between these specific filamentous fungi and bacteria isolated from DWDS is a possibility, as they can interact with each other and form mature biofilms. Nevertheless, inter-kingdom biofilm formation and development are microbial dependent as shown by the variability in interspecific interactions. It was also shown that if both inter-kingdom microorganisms are inoculated at the same time, bacteria can inhibit filamentous fungal spore germination and its development into a biofilm. However, with time and with the renewal of the medium, these fungi can begin to germinate and develop into mature biofilms. This is particularly interesting because if the possibility of biofilm formation is provided to the fungi, or there is a chance of existing a pre-established fungal biofilm, consequently, an advantage may also be given to opportunistic bacteria to replicate and proliferate inside inter-kingdom biofilms.

From the results of the production of QSIs, *P. expansum* was the selected filamentous fungi for further studies. Analysing the effect of the exogenous amounts of patulin or the 3-oxo-C_{12}-HSL, *P. expansum* biofilm formation and development was not affected by their presence. *M. oryzae* biofilm formation and development were more affected by the presence of the molecules than *A. calcoaceticus*, indicating a specificity for the AHL mediated QS system. In inter-kingdom growth, *M. oryzae* cell density was generally lower than in single-species growth which indicates a detrimental effect of the fungi towards this bacterium. However, the increase of *A. calcoaceticus* cell density in inter-kingdom is an indication of the physical protection that the fungi provide to this opportunistic bacterium. QS and QQ molecules have a significant effect in inter-kingdom biofilm formation and especially on bacterial cell numbers. Moreover,

we report a possible protection role that filamentous fungi provide to opportunistic bacteria inside complex biofilms.

The studies on the effect of *M. oryzae* on pre-established *P. expansum* biofilms revealed that this interaction is time dependent as demonstrated by the different resulting tendencies when the bacterium is introduced to 48 or 72 h *P. expansum* pre-established biofilms. More mature *P. expansum* biofilms appear to be more resistant to the inhibitory effect that *M. oryzae* causes towards *idh* gene expression and patulin production. This is a consequence of the increased concentration of patulin and reciprocates an inhibitory effect of *P. expansum* towards *M. oryzae*. A trend was observed between the *idh* expression and patulin production values. The results indicate that *M. oryzae* affects patulin production by acting at the transcriptional level of the *idh* gene.

These conclusions indicate that patulin plays a vital role in inter-kingdom communication and/or microbial assembly processes that influence biofilm formation between filamentous fungi and bacteria.

5.2 Perspectives for future research

The results from this work have begun to highlight the role of filamentous fungi in water biofilms and the importance of QS molecules and secondary metabolites on inter-kingdom biofilm formation. However, the information here obtained is only the beginning and much more needs to be studied to fully understand the impacts of these microorganisms on drinking water biofilm formation and on the microbial ecology of these systems.

The study of bacterial intrageneric diversity in co-growth with filamentous fungi should be performed to elucidate the impacts of filamentous fungi on bacterial communities. As observed in this thesis, depending on the bacteria being studied the results can be quite different, so a mixture of inoculated bacteria could provide better insights into this aspect.

It would be interesting to evaluate biofilm formation between these two distantly related microorganisms in a flow cell system to understand how stronger hydrodynamic forces influence inter-kingdom biofilm formation and its development.

The study of other filamentous fungi secondary metabolites (i.e. penicillic acid) and the genes involved in their regulation could be provide newer insights into this fungal-bacterial interaction. The evaluation of the expression of QS-regulated genes in these bacteria (especially on *M. oryzae*) while forming inter-kingdom biofilms should also be done. This could be achieved through RT-qPCR or the use

of next generation sequencing through RNA-seq. The latter would provide wider insights into the changes occurring in both microorganisms while interacting in biofilms.

In biofilms, and in the system used throughout this thesis (microtiter plates), the detection of specific metabolites may be strongly influenced by the techniques being used, as they may not be sensible enough. For this reason, the use of more sensible techniques such as MALDI-TOF mass spectrometry should be implemented to accurately study the microbial metabolites being produced.

The study on the efficacy of disinfectants (i.e. sodium hypochlorite) on the biofilm formation and biofilm removal of inter-kingdoms biofilms would provide a better understanding of the protection role that filamentous fungi may confer to opportunistic bacteria.

All of these insights could then be used to modulate beneficial drinking water biofilms as they can help to inhibit the propagation of invading pathogens, thus safeguarding water quality. Understanding community dynamics, including the presence of filamentous fungi, can be the key to sustaining a beneficial and ultimately safe drinking water microbiome.