Universidade do Minho
Escola de Engenharia

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The role of fungi in drinking water biofilm formation and behavior

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Dissertação de Mestrado
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Trabalho efetuado sob a orientação da
Doutora Lúcia da Conceição Diogo Chaves Simões
Professor Nelson Manuel Viana da Silva Lima

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

ABSTRACT

Drinking Water Distribution Systems (DWDS) is essential for the delivery of high-quality and safe drinking water (DW). However, DWDS allows the establishment of a dynamic microbiological ecosystem, where microorganisms are present in both planktonic and biofilm states. Microorganisms adhered to the surfaces of pipes are dominant. A biofilm can be defined as a sessile community of microorganisms irreversibly attached to a surface or substratum and each other, which are embedded in an extracellular polymeric substances (EPS) matrix that they produce and excrete. The biofilms formed in a DWDS are an interkingdom complex community since under natural conditions is rare the formation of monospecies biofilms. This diversity leads to a multiplicity of complex relationships involving intraspecies and interspecies interactions. In addition, biofilm formation in DWDS can be affected by a variety of biotic and abiotic factors, namely: environmental factors (temperature, pH), residual concentration of disinfectant, type and availability of nutrients, hydrodynamic conditions, design of the network, pipe material and sediment accumulation. The control of biofilm formation in DWDS is essential to make sure that the water delivered to the consumer is microbiologically safe.

In this work, six different fungi were first evaluated in terms of growth kinetics and biofilm formation. The six fungi were: *P. expansum, P. brevicompactum, F. oxysporum, A. versicolor, Alternaria* sp, and *Mucor* sp. A stock of spore suspension of each fungus was made, and the biofilm assay was executed. For biofilm formation, 200 µL of spore suspension R2B was added into each well and spectrophotometric-based methods (crystal violet method for biomass quantification and resazurin method for metabolic activity quantification) were used to monitor the biofilm formation over time. Macro and Microscopic characterization were also performed for each fungus. In the end, the fungus which presented higher biomass formation and metabolic activity was *Alternaria* sp., and the values were 20.070 ± 3.825 and 3695.625 ± 802.910, respectively. This fungus was chosen for further studies to understand its behavior under different process conditions.

The conditions chosen were the hydrodynamics, the nutrient concentration, the presence of chlorine, and an interkingdom association. Relative to the hydrodynamics, four conditions were evaluated (static, 30, 150, and 200 rpm). After three days of incubation, some significant differences between conditions were observed. The use of 200 rpm caused a significant difference ($\rho<0,05$) compared to the static and 30 rpm conditions, meaning that high rotations might influence the fungus growth and metabolic activity. In this condition, there was an increase in biomass and metabolic activity, suggesting that the high rotations had a positive influence. Comparative to the nutrient's concentration, four different

conditions were evaluated (synthetic tap water, ¼ R2B, ½ R2B, and R2B medium). After three days of incubation, that was observed that STW caused significant differences ($\rho<0,05$) compared to the others, meaning that the oligotrophic environment influences the fungus growth and metabolic activity negatively. The exposure to chlorine was studied under 5 conditions (without chlorine, 2,4 ppm, 6,03 ppm, 12,06 ppm, 24,12 ppm) and revealed no significant impact of the chlorine levels in biofilm formation and activity, suggesting the fungi resistance to chlorine. For the interkingdom factor, a strain of *Stenotrophomonas maltophilia* was used. Three assays were performed (Fungus and bacterium alone and associated). The results revealed that microbial association affected biofilm formation and activity, showing a decrease in biomass and metabolic activity compared to the fungi assay alone.

Lastly, the species identification of two fungi used in this work was executed as well. The *Alternaria* sp. and *Mucor* sp. were the fungi that were not previously identified until the species group. For this identification, the fungi were incubated in R2B medium, and the DNA was extracted. After running some tests, such as the NanoDrop, the electrophoresis gel of the sample, and PCR cycle, the Fungi DNA was sequenced. Then, a phylogenetic tree was created based on the genetic sequences, with the same genetic marker (ITS), of different fungi species related to the samples. Thus, the phylogeny tree of the *Mucor* seems to direct the *Mucor* sp. toward the *Mucor plumbeus* and the phylogeny tree of the *Alternaria* put the sample in the *Alternata* section.

RESUMO

Os Sistemas de Distribuição de Água Potável (DWDS) são essenciais para o fornecimento de água potável de alta qualidade e segura (DW). Contudo, os DWDS permitem o estabelecimento de um ecossistema microbiológico dinâmico, onde os microrganismos estão presentes tanto em estados planctónicos como de biofilme. Os microrganismos aderidos nas superfícies das tubagens são dominantes. Um biofilme pode ser definido como uma comunidade séssil de microrganismos irreversivelmente ligados a uma superfície ou substrato e uns aos outros, que estão embutidos numa matriz extracelular de substâncias poliméricas (EPS) que produzem e excretam. Os biofilmes formados numa DWDS são uma comunidade complexa do interreino, uma vez que em condições naturais é rara a formação de biofilmes monoespécie. Esta diversidade conduz a uma multiplicidade de relações complexas envolvendo interações intraespécies e interespécies. Além disso, a formação de biofilmes no DWDS pode ser afetada por uma variedade de fatores bióticos e abióticos, nomeadamente: fatores ambientais (temperatura, pH), concentração residual de desinfetante, tipo e disponibilidade de nutrientes, condições hidrodinâmicas, conceção de rede, material de tubagem e acumulação de sedimentos. O controlo da formação do biofilme no DWDS é essencial para garantir que a água entregue ao consumidor é microbiologicamente segura.

Neste trabalho, seis fungos diferentes foram primeiramente avaliados em termos de cinética de crescimento e formação de biofilme. Os seis fungos foram: P*. expansum, P. brevicompactum, F. oxysporum, A. versicolor, Alternaria* sp, e *Mucor* sp. Foi feito um stock de suspensão de esporos de cada fungo, e o ensaio do biofilme foi executado. Para a formação do biofilme, foram adicionados 200 µL de suspensão de esporos e caldo R2A em cada poço e foram utilizados métodos espectrofotométricos (método violeta cristal para quantificação da biomassa e método de resazurina para quantificação da atividade metabólica) para monitorizar a formação do biofilme ao longo do tempo. Foi também realizada a caracterização macroscópica e microscópica de cada fungo. No final, o fungo que apresentou maior formação de biomassa e atividade metabólica foi a *Alternaria* sp., e os valores foram de 20,070 ± 3,825 e 3695,625 ± 802,910, respetivamente. Este fungo foi escolhido para mais estudos a fim de compreender o seu comportamento sob diferentes condições de processo.

As condições escolhidas foram a hidrodinâmica, a concentração de nutrientes, a presença de cloro, e uma associação interrelacionada. Em relação à hidrodinâmica, foram avaliadas quatro condições (estática, 30, 150, e 200 rpm). Após três dias de incubação, foram observadas algumas diferenças significativas entre as condições. A utilização de 200 rpm causou uma diferença significativa ($\rho<0,05$)

em comparação com as condições estáticas e 30 rpm, o que significa que altas rotações podem influenciar o crescimento de fungos e a atividade metabólica. Nesta condição, houve um aumento da biomassa e da atividade metabólica, sugerindo que as altas rotações tiveram uma influência positiva. Em comparação com a concentração de nutrientes, foram avaliadas quatro condições diferentes (água da torneira sintética, ¼ R2B, ½ R2B, e meio R2B). Após três dias de incubação, observou-se que o STW causou diferenças significativas ($\rho<0,05$) em comparação com os outros, o que significa que o ambiente oligotrófico influencia negativamente o crescimento do fungo e a atividade metabólica. A exposição ao cloro foi estudada em 5 condições (sem cloro, 2,4 ppm, 6,03 ppm, 12,06 ppm, 24,12 ppm) e não revelou qualquer impacto significativo dos níveis de cloro na formação e atividade do biofilme, sugerindo a resistência dos fungos ao cloro. Para o fator interespécies foi utilizada uma estirpe de *Stenotrophomonas maltophilia*. Foram realizados três ensaios (Fungos e bactérias isolados e associados). Os resultados revelaram que a associação microbiana afetou a formação e atividade do biofilme, mostrando uma diminuição da biomassa e da atividade metabólica em comparação com o ensaio de fungos isoladamente.

Finalmente, foi também executada a identificação das espécies de dois fungos utilizados neste trabalho. As *Alternaria* sp. e *Mucor* sp. foram os fungos que não foram previamente identificados até ao grupo de espécies. Para esta identificação, os fungos foram incubados em meio R2B, e o ADN foi extraído. Após a realização de alguns testes, tais como o NanoDrop, o gel de eletroforese da amostra e o ciclo de PCR, o ADN dos fungos foi sequenciado. Depois, foi criada uma árvore filogenética baseada nas sequências genéticas, com o mesmo marcador genético (ITS), de diferentes espécies de fungos relacionados com as amostras. Assim, a árvore filogenética do Mucor parece dirigir o *Mucor* sp. para o *Mucor plumbeus* e a árvore filogenética da *Alternaria* colocou a amostra na *secção Alternata*.

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1. STATE OF THE ART

1.1. Introduction

Drinking Water Distribution Systems (DWDS) are crucial for the delivery of high-quality and safe drinking water (DW). This is a complex network with a dynamic ecosystem, where some microorganisms dominate by attaching to the inner surface of pipes, forming biofilms (Douterelo et al., 2018). These structures consist in microorganisms, which can be formed by different species of different kingdoms, and a matrix that protects them from environmental stress and gives them antimicrobial resistance (Tian et al., 2021). Thus, biofilms can have some pathogens that if they reach the consumers' tap can cause a waterborne disease (Simões et al., 2015). For this reason, the study of biofilms is important to understand how they can be controlled or inhibited for the delivery of high-quality, accessible, and safe DW.

1.2. Biofilms

A biofilm can be defined as a sessile community of microorganisms irreversibly attached to a surface or substratum and to each other, where the cells are embedded in an extracellular polymeric substances (EPS) matrix that they produce (Blankenship & Mitchell, 2006; Carr et al., 2021; Harding et al., 2009). The first biofilm was observed in 1933 by Arthur Henrici and since then biofilms have been widely explored (Li et al., 2019). These complex structures are home to more than 99% of microorganisms on Earth (Flemming et al., 2002), showing that it is likely to be a positive trait that became a crucial feature for the survival of microbial communities in a diverse and changing environment (Harding et al., 2009).

Biofilms may form on a wide variety of surfaces such as living tissues, indwelling medical devices, industrial or potable water systems, and natural aquatic, sewage, and irrigating systems (Donlan, 2002; Li et al., 2019; Yao & Habimana, 2019). Hence, some of these biofilms are harmless to humans, having a roleplay in some areas, such as bioremediation, wastewater treatment, nontoxic leaching of copper from, ore, and production of biofuels and bioethanol (Krsmanovic et al., 2021; Zabiegaj et al., 2021).

Despite the benefits that some biofilms can present, sometimes the presence and dispersal of it can cause severe damage and have negative effects in some areas, for example in medical devices resulting in chronic diseases (Krsmanovic et al., 2021; Zabiegaj et al., 2021). Moreover, the detachment of biofilms particles to the DW stream can lead to deterioration of water quality, changing

the turbidity, taste, odor, and color of the water, as well as potential accumulation and dispersion of pathogens, such as bacteria and viruses, and production of toxins. Consequently, there is a potential risk of waterborne diseases, which include gastroenteritis, legionellosis, giardiasis, hepatitis, and salmonellosis, among others. (Fernandes, 2018; Simões et al., 2015).

1.3.Biofilms in Drinking Water Distribution Systems

DWDS can be considered an environment for the proliferation of different types of microorganisms, such as bacteria, fungi, protozoa, algae, and viruses that interact and cohabit together, resulting in the formation of extremely complex systems. Moreover, each microorganism has its own key role in the environment and should not be underestimated (Chaves, 2014). This diversity leads to a multiplicity of complex relationships involving intraspecies and interspecies interactions (Douterelo et al., 2018). Furthermore, the biofilm formed by this microbiome can be affected by some abiotic factors for instance: pipe material, pH, nutrient level, temperature, water flow, and concentration of disinfectant. So, these microorganisms living in association and forming biofilms get better conditions to survive and thrive in this environment, since the structure of the biofilm gives resistance and protection to the microorganisms against these factors (Chaves, 2014; Harding et al., 2009).

1.3.1. Biofilm formation in DWDS

Bacterial biofilms compared to fungi biofilms, are well known by the scientific community and the formation of these biofilms typically follows five stages: (1) reversible attachment, (2) irreversible attachment, (3) microcolony formation, (4) mature biofilm, and (5) dispersal (Zabiegaj et al., 2021). Figure 1 shows the scheme of bacterial biofilm formation.

Figure 1: Different phases of a bacterial biofilm formation (1) reversible attachment, (2) irreversible attachment, (3) microcolony formation, (4) mature biofilm, and (5) dispersal phase. Adapted from Krsmanovic et al. (2021)

The first and second stages are essential for bacterial biofilm formation, consisting of leaving the motile state to a sessile one. The adhesion of cells to the surface is complex and dynamic (Yao & Habimana, 2019), depending on the bacterial species, the surface, and the environmental factors that involve those two. These phenomena are initiated by type IV pili, flagella, fimbriae, hydrophobins, and adhesin proteins (Carr et al., 2021). Thus, the irreversible attachment can be described by the secondary minimum theory. That theory says that the cells when approaching the surface become entrapped by electrostatic and Van der Waals forces and if the cells continue to move toward the surface, they become irreversibly attached to it (Krsmanovic et al., 2021).

The third and fourth stages are related to biofilm growth and maturation. Since the cells are irreversibly attached to the surface, they start to proliferate and form the EPS matrix, increasing the thickness of the biofilm, and at the same time, other cells in the environment aggregate to the biofilm. During these stages, particularly in multi-species biofilms, microcolonies and cell differentiation occur, resulting in the formation of micro niches and the development of stress resistance mechanisms (Carr et al., 2021).

The final stage may occur because of the applied mechanical forces or changes in the surroundings and the detachment of cells arises due to deliberate signaling, quorum sensing, and physiological changes (Krsmanovic et al., 2021).

As said earlier, the bacterial biofilm compared to the fungal biofilms are well known in the scientific community, and that is why the fungi capable of biofilm formation have started to gain special attention. These microorganisms are adapted for growth on surfaces, evidenced by their absorptive nutrition mode and secretion of extracellular enzymes (Afonso et al., 2020; Harding et al., 2009). One example of apathogenic fungi capable of biofilm formation is *Candida albicans*. In the present day, these microorganisms became a problem for colonizing clinical and implanted devices and sometimes have fatal consequences (Blankenship & Mitchell, 2006).

Paralleled to the model of bacterial biofilm formation, fungal biofilm formation is more complex and Harding et.al. (2009) proposed the next model: 1) propagule adsorption, 2) active attachment to a surface, 3) microcolony formation I, 4) microcolony formation II (or initial maturation), 5) maturation and 6) dispersal (planktonic). Figure 2 shows the main stages of fungal biofilm formation.

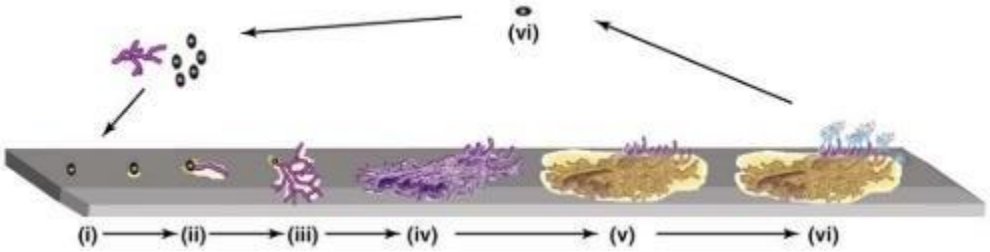


Figure 2: Harding *et al.* model for filamentous fungi biofilm formation: (i) adsorption, (ii) active attachment, (iii) microcolony I (germling and/or monolayer), (iv) microcolony II (initial maturation), (v) development of the mature biofilm, and (vi) dispersal or planktonic phase. Adapted from Harding et al. (2009)

These stages are all comparable to the bacterial biofilm stages but have some details that make them different. The first stage consists of the deposition of spores or other propagules such as hyphal fragments or sporangia. This stage involves the physical contact between the filamentous fungi (ff) and the surface. When comparing it with the bacterial models it represents the reversible attachment stage (Harding et al., 2009).

The second stage is the active attachment to the surface. In this phase usually the ff secrete adhesive substances by germinating spores and active germlings. Also, it is comparable to the fixed attachment phase in bacteria (Harding et al., 2009).

In the microcolony formation starts the initial stages of growth and surface colonization. In this phase, the cells produce an extracellular polymeric matrix that allows the growing colony to adhere tenaciously to the substrate (Harding et al., 2009). After the microcolony formation, the initial maturation happens.

The initial maturation encompasses the formation of compacted hyphal networks or mycelia and hypha–hypha adhesion. Additionally, includes the layering, the formation of hyphal bundles bonded together by an exopolymeric matrix, and the formation of water channels via hydrophobic repulsion between hyphae or hyphal bundles (Harding et al., 2009). After the initial maturation, a stage of maturation occurs, which consists of the formation of fruiting bodies, sporogenous cells, sclerotia, and other survival structures. Aerial growth is often a crucial feature of fungal fruiting and dispersal (Harding et al., 2009).

The last stage of this preliminary model is the dispersal or planktonic phase. This phase is characterized by the dispersal or release of spores or biofilm fragments. The detached cells can act as new propagules to re-initiate the cycle (Harding et al., 2009)

It is important to note that although most ff does not normally exist as single cells, spores, hyphal fragments, and other fungal propagules can be considered functional equivalents to planktonic bacterial cells (Harding et al., 2009).

1.3.2. Composition of Biofilms in DWDS

The biofilm composition consists of a microbial (e.g. bacteria, fungi, viruses, protozoa, and/or algae) community embedded by an EPS matrix that they excrete to the environment and can present some inorganic particles (e.g. corrosion products, clays, sand, etc.) and water (Fernandes, 2018; Simões & Simões, 2013). In these communities, bacteria are generally the dominant group due to their high growth rates, small size, adaptation capacities, and ability to produce the EPS, but viruses, protozoa, fungi, and algae may also be present in these biofilms (Simões & Simões, 2013). Because of the capacity of biofilm formation of some microorganisms, for instance, *Acinetobacter, Aeromonas, Alcaligenes, Arthrobacter, Corynebacterium, Bacillus, Burkholderia, Citrobacter, Enterobacter, Flavobacterium, Klebsiella, Methylobacterium, Moraxella*, *Pseudomonas* sp., *Acremonium, Alternaria, Aspergillus, Cladosporium, Fusarium, Penicillium and Trichoderma* (Fernandes, 2018), some pathogenic species that takes advantage of these species for protection and the interaction between the pathogens and the biofilm microorganisms has been the main concern in DWDS (Simões & Simões, 2013). For instance, some pathogens that can be found in the DWDS *are Legionella pneumophila, Mycobacterium* spp*., Pseudomonas aeruginosa, Klebsiella* spp*., Burkholderia* spp*., Giardia* and *Cryptosporidium*, among others (Simões & Simões, 2013).

Not only bacterial pathogens are found in DWDS, but there are also some viruses within the water systems, so-called enteric viruses. These viruses are known to cause gastrointestinal problems such as calicivirus, rotavirus, astrovirus, Hepatitis A virus, Norwalk virus, and the small round viruses and the symptoms are normally nausea, vomiting, and diarrhea, among others (Flemming et al., 2002). Relative to the viruses. Skraber et al. (2009) studied the occurrence of enteroviruses and noroviruses in natural wastewater biofilms and verified that the viruses were detected in all samples. Besides, Skraber (2009) showed that viruses are able to transfer from the environment to the biofilms and show great stability in these biofilms.

This matrix is essentially composed of proteins and polysaccharides involved in microbial protection against antimicrobial and mechanical stress (Fernandes, 2018). The EPS matrix acts as a

glue that fixes the cells to the surface and allows the development of a stable community of microorganisms, which can stay together for an extended period of time (Flemming et al., 2002). Thus, this matrix will provide chemical and mechanical protection against the environment, prevents antimicrobial penetration (Tian et al., 2021) and at the same time, will trap nutrients and facilitate the microorganisms' growth (Krsmanovic et al., 2021; Yao & Habimana, 2019). This matrix is composed by polysaccharides as a variety of proteins, glycoproteins, glycolipids, and extracellular DNA (Yao & Habimana, 2019).

Each species of microorganisms might produce a different type of EPS, which varies in the type of polysaccharides. Polysaccharides can be divided into homopolysaccharides and heteropolysaccharides according to their monosaccharide composition (Sun & Zhang, 2021). Whereas homopolysaccharides are composed of one kind of monosaccharide, for example, dextran, curdlan, and cellulose. Heteropolysaccharides are composed of two or more different monosaccharides into regular repeating units, such as xanthan, alginate, and hyaluronic acid (Sun & Zhang, 2021). Because of the variety of polysaccharides that bacteria can produce, the EPS matrix has been receiving special attention since is more reliable economically to produce those substances at an industrial level rather than extract from other organisms, for example, higher plants. Furthermore, with gene editing, it is possible to optimize the production of these substances and be applied in some areas, like pharmaceuticals, medical devices, and cosmetics (Sun & Zhang, 2021).

1.3.3. Factors that affect biofilms in DWDS

Biofilm formation in DWDS can be affected by a variety of biotic and abiotic factors, namely: environmental factors (temperature, pH), residual concentration of disinfectant, type and availability of nutrients, hydrodynamic conditions, design of the network, pipe material and sediment accumulation (Fernandes, 2018).

The environmental factors influence the electrostatic interaction between the microorganisms and the surface, as well as the microbial growth, the enzymatic activity, kinetic and equilibrium of reaction, and other properties, such as diffusivity, tortuosity, and solubility (Fernandes, 2018). These environmental factors are crucial to the formation of the biofilm, being observed at water temperatures of 15-25°C the highest rates of biofilm formation (Sonigo et al., 2011).

Relative to the residual disinfectant level factor, chlorine is the most common disinfectant,

and the residual concentration of this chemical kills the microorganisms in the DWDS that survive the earlier treatment processes, preventing microbial growth in the water network (Fernandes, 2018). For human consumption, the council directive (WHO, 2012) advises to use of 0.2 to 0.6 mg/L of free chlorine in the DWDS but is complicated to control this concentration in all DWDS, which may cause episodes of an absence of disinfectant, enabling the biofilm formation (Fernandes, 2018).

Another factor that influences biofilm formation is the presence and concentration of nutrients. There is a positive correlation between nutrient levels and the number of heterotrophic organisms such as fungi and bacteria (Fernandes, 2018; Sonigo et al., 2011). These organisms demand nutrients for survival and growth, including organic carbon, phosphorus, and ammonium, which they entrap and accumulate, and when favorable conditions appear, they start growing (Sonigo et al., 2011). Usually, the DWDS are characterized by low concentrations of these nutrients and a residual disinfectant concentration which prevents biofilm growth (Fernandes, 2018; Sonigo et al., 2011). Usually, the carbon: nitrogen: phosphorus ratio to allow microbial growth is (100C:10N:1P), so if this ratio is changed microbial growth may be limited (X. Luo et al., 2021).

The hydrodynamic conditions can influence the cellular adhesion, growth, structure and detachment of biofilm, nutrient availability, and loss of EPS (Sonigo et al., 2011). Under turbulent conditions, there are more shear forces, the biofilm mass and thickness can decrease, and cellular density can increase. Despite the rate of mass transfer being higher, which enhances biofilm growth, the biofilm becomes more compact, and consequently mass transfer is lower (Fernandes, 2018). Compared to crippled cells, motile bacteria have some advantages relative to the formation of biofilms. The crippled cells are heavily dependent on the fluid flow to attach and form a biofilm, whereas the motile bacteria can spend energy autonomously moving around until finding a location to colonize (Krsmanovic et al., 2021; Scheuerman et al., 1998). So, the general shear stress of gravity pipes should be in the range of 1.0–2.0 N/m^2, equivalent to a velocity of 0.60–0.75 m/s, to guarantee the self-cleaning function happens and the accumulation of sediments can be significantly avoided. Thus, high shear stress reduces biofilm diversity and slows down the procession of biofilm maturation, which leads to relatively young biofilm (Li et al., 2019).

The last factor is the surface material with different characteristics, such as composition, charge, hydrophobicity, and roughness. Some examples of DWDS surface material are [ethylene-propylene rubber (EPDM), natural latex, stainless steel (SS), mild steel (carbon steel), polypropylene (PP), polyethylene (PE), polyvinyl chloride (PVC)] (Fernandes, 2018). These materials can influence

the deposition and presence of fungi and these materials propitiate biofilm growth, although with different structures (Sonigo et al., 2011). At this moment, the metal plumbing was replaced by plastic pipes owing to low-cost maintenance, easy handling, and implementation. However, these pipes might release organic compounds, increasing the nutrient level and consequently enabling biofilm development and growth. Even though, the bacterial growth in plastic pipes is lower than in cast iron, steel, or cement surfaces (Fernandes, 2018). Furthermore, those pipes which present roughness are more likely to be colonized by microorganisms than smooth surface pipes because of the greater surface area and reduction in shear forces (Fernandes, 2018; Sonigo et al., 2011). Another characteristic of the pipes is the hydrophobicity they can present. In theory, biofilm formation would be more likely to happen in hydrophobic pipes than hydrophilic ones, but it seems that this characteristic does not specifically assess biofilm formation in DWDS (Sonigo et al., 2011).

1.4. Filamentous fungi in oligotrophic systems

Fungi belong to the Kingdom *Eumycota*, and then can be divided in seven recognized *phyla*: *Basidiomycota, Ascomycota, Glomeromycota, Microsporidia, Blastocladiomycota, Neo-callimastigomycota* and *Chytridiomycota* (Hibbett et al., 2007). These organisms appeared billion years ago, and they are abundant today and consequently there is some fungi that proliferate in aquatic environments, whereas some fungi are adapted to terrestrial environments (Afonso et al., 2021)

The microorganisms naturally interact with each other and in the DWDS is not different. These microorganisms compete with each other for nutrition and space and normally produce bioactive secondary metabolites, such as mycotoxins (Chaves, 2014). The most important mycotoxins are aflatoxins, deoxynivalenol, ochratoxin, fumonisins, and zearalenone (Rebellato et al., 2021). These metabolites are usually diluted and present a minor concern, but can be harmful to human health, especially when water is stored in cisterns or reservoirs because the concentration of mycotoxins can increase in these places (Chaves, 2014). The most serious and prevalent chronic adverse effect in humans is hepatocellular carcinoma, which is the most common of liver cancers and is caused by aflatoxins (Jin et al., 2021). Moreover, the mycotoxins can cause a wide range of toxicities, and disturb the gut homeostasis by inducing intestinal damage, inflammation and gut microbiota dysbiosis (Jin et al., 2021). A wide range of fungi can produce mycotoxins, for instance *Alternaria, Aspergillus, Claviceps, Fusarium* and *Penicillium* genera (Rebellato et al., 2021). In table 1 is possible to see the different effects that each mycotoxin has in human health.

Table 1: Effects on human health of different mycotoxins			
Mycotoxins	**Species**	**Effects on human health**	**Reference**
Aflatoxins	*Aspergillus flavus* *Aspergillus parasiticus*	Chronic exposure to Aflatoxins may cause hepatocellular carcinomas, and 4.6–28.2% of all new hepatocellular carcinoma cases worldwide might result from AFs exposure. Can cause acute hepatic necrosis, bile duct proliferation, lethargy, and edema, and in some cases death when exposed to high doses.	(Jin et al., 2021)
Deoxynivalenol	*Fusarium graminearum,* *Fusarium moniliforme,* *Fusarium culmorum*	Acute effects include nausea, vomiting, Gastrointestinal tract upset, dizziness, diarrhoea, and headache. Can cause intestinal pathologies, such as lesions and disturbing the barrier function.	(Jin et al., 2021) (Luo et al., 2021)
Ochratoxin	*Aspergillus ochraceus*	Considered nephrotoxic and the possible cause of porcine nephropathy, chronic interstitial nephropathy (CIN) and human Balkan endemic nephropathy (BEN). Has mutagenic, teratogenic, neurotoxic, hepatotoxic and immunotoxic properties.	(Jin et al., 2021)
Fumonisins	*Fusarium moniliforme,* *Fusarium verticillioides* *Fusarium roliferatum*	Can interfere with myelin synthesis and cause leukoaraiosis. liver necrosis or even death. Have carcinogenic, hepatotoxicity, renal toxicity, and embryo toxicity.	(Luo et al., 2021)
zearalenone	*Fusarium graminearum,* *Fusarium culmorum;* *Fusarium cerealis;* *Fusarium equiseti.* *Fusarium semitectum*	Can cause alterations in the reproductive system. Can cause ovarian disease, reduce the number of litters or infertility, and induce sows to have masochism, pseudopregnancy, and endometrial disease.	(Jin et al., 2021) (Luo et al., 2021))

The biofilms are a problem in the DWDS, contributing for the deterioration of water quality, and the bacteria are generally dominant in these systems due to their high growth rates, small size, and ability to produce the EPS matrix (Simões et al., 2015). However, there is a gap in this field of

knowledge when we think about the ff role in the formation of biofilms, since one of major reasons is due to the pipes of DWDS are inaccessible to take a sample of this microbial ecosystem and study (X. Luo et al., 2021). Another reason for the rare debate about this organism is owing to the fact that the diseases and symptoms caused by fungi are less frequent than diseases caused by pathogenic bacteria, viruses and protozoa (Chaves, 2014). The most common ff genera that appear in the DWDS are *Acremonium, Alternaria, Aspergillus, Cladosporium, Fusarium, Penicillium and Trichoderma* sp. (Afonso et al., 2020; Chaves, 2014).

1.5. Interactions between bacteria and filamentous fungi

The biofilms in nature can be composed by different species from different kingdoms. These structures lead to a complex relationship between different species, such as bacteria and fungi (Carr et al., 2021). These interactions are interesting for several fields of study, for example agriculture, forestry, environmental protection, food processing biotechnology and medicine (Chaves, 2014). Furthermore, these interactions between bacteria and ff and adhesion are crucial for the formation of multispecies bacterial-fungi biofilms (Chaves, 2014). Despite the lack of understanding of these closed interactions, some factors seem to affect them, for instance quorum sensing (Afonso et al., 2021). Quorum sensing is a cell to cell signaling process which enables bacteria to respond environmental conditions by producing and detecting extracellular molecules, coordinating the community behavior (Afonso et al., 2020; Tian et al., 2021). Hence, these molecule signals modify the gene expression in microorganisms and controls and regulates different bacterial population density-dependent processes, including biofilm formation, stress resistance, production of toxins and secondary metabolites and pathogenicity (Afonso et al., 2021).

The ff can interfere with the bacterial communication by producing quorum sensing inhibitors (Afonso et al., 2021) inhibiting the microorganisms' growth or reducing the virulence. One example of this is the interaction between *C. albicans* and *P. aeruginosa.* The *C. albicans* produce farnesol which inhibits the production of phenazine pyocyanin by *P. aeruginosa* reducing its virulence. On the other hand, *P. aeruginosa* produces a lactone which inhibits the hyphal growth in *C. albicans* (Carr et al., 2021). The interactions between fungi and bacteria can differ depending on several factors, for example difference in the composition of isolated species from the water systems, differences in methodologies or different biological mechanisms at play (Sonigo et al., 2011). The most common interactions reported are the bacteria exploits recourses from the associated fungi through a parasitic

or commensalism interaction (Chaves, 2014). However, there are some examples of fungi taking advantage of bacteria by mutualistic interaction (Chaves, 2014). Afonso *et al* (2020), reviewed possible interactions, that have been reported in the literature, between fungi and bacteria and these interactions can be symbiotic and beneficial to both, for example protection, degradation of molecules facilitating nutrition. However, some negative interactions may occur, such as competition for nutrients or space and inhibition of proliferation. Although is needed to further explore and research this area of knowledge, to understand better these interactions. Moreover, these interactions can have different outcomes depending on the combination of physical associations and molecular interactions between the microorganisms. These changes have influence on the biofilm structure on their biotic and abiotic environment (Chaves, 2014). In Figure 3 is possible to see the different outcomes of these interactions:

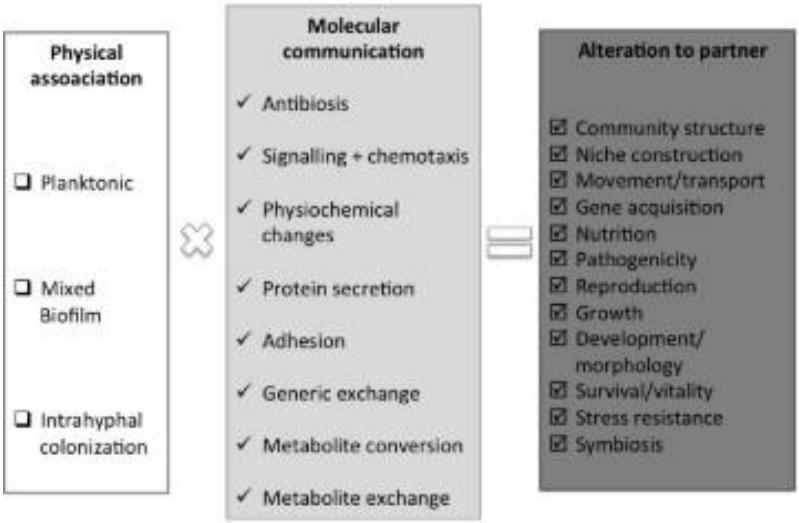


Figure 3: The different and possible outcomes depending on the molecular communication and physical association. Adapted from Frey-Klett et al., (2011)

Bernard et al. (2020) reviewed the current knowledge of interactions between *C. albicans* and the *Staphylococcus* species and the *Streptococcus* species. In this review is said that the interactions between these fungi and bacteria within the biofilm can be antagonistic or synergistic, competitive, or not. It seems that the three main different modes of interaction have been described are physical, chemical, or metabolic interactions and those interactions can influence the growth and viability of certain microbial species and affect the yeast-to-hyphal transition (Bernard et al., 2020). Furthermore, interkingdom interactions may have the capacity to change the activity of the antimicrobial agents that are mostly used to treat patients suffering from infections related to these bacterial or fungal species. One example is the co-presence of *C. albicans* and *Cutibacterium acnes* in a biofilm decreases the efficiency of echinocandins, which is among the most active antifungal agents

against *C. albicans* biofilms (Bernard et al., 2020).

Another example reviewed by Bernard et al., (2020) is the interaction between *C. albicans* and *Staphylococcus aureus*. The interaction between these species enables the biofilm formation in some surfaces that could not form by themselves, for instance *C. albicans* enhances the ability of *S. aureus* to form biofilms on silicon surfaces, although these two species only can form an interkingdom biofilm if the *C. albicans* form hypha (Bernard et al., 2020). On the other hand, the *S. aureus* uses the fungi as a structural scaffold for their growth and it seems that the biomass of the biofilm increases comparing to monospecies biofilms. Thus, the growth of *C. albicans* can be positively influenced by *S. aureus* and these interactions can decreased their susceptibility towards some antibiotics, such as vancomycin, nafcillin, oxacillin, and gentamycin (Bernard et al., 2020).So, it is possible to conclude that the interactions between fungi and bacteria improves the odds to form a biofilm and more resistant against antimicrobial agents.

2.Materials and Methods

2.1. Microorganisms and culture conditions

In the first phase of this work, six different fungal strains were supplied by Micoteca da Universidade do Minho (MUM, Braga, Portugal) and were studied to see their biofilm formation in certain conditions: *Penicillium expansum* (MUM 00.02), *Penicillium brevicompactum* (MUM05.17), *Aspergillus versicolor* (MUM 02.37) *Alternaria* sp. (MUM 02.42) *Mucor* sp. (MUM02.01) and *Fusarium oxysporum* (MUM 17.33). The selection of these fungi was related to their high occurrence in tap water and to health problems that they may cause to humans when digested or inhaled. The first three fungi were grown in malt extract agar [MEA: 14 g of malt; 3,5 g of peptone; 14 g of agar and 700 mL of distilled water] and the other three were incubated in potato dextrose agar [PDA: 39 g of the mixture and 1 L of distilled water] during 7 days in Petri plates at 25°C.

The bacteria used for the interkingdom biofilm formation was *Stenotrophomonas maltophilia*. This bacterium was isolated from tap water in Braga and was selected due to its pathogenicity and opportunistic behavior (Gomes et al., 2019). The bacterium was grown in batch culture overnight before the interkingdom factor assay was executed in 100 mL of R2B [0.5 g of yeast extract; 0.5 g of peptone; 0.5 g of hydrolyzed casein; 0.5 g of glucose; 0.5 g of starch; 0.5 g of sodium pyruvate; 0.3 g of dipotassium phosphate; and 0.024 g of magnesium sulphate, and 1 L of distilled water] at room

temperature at 150 rpm. After, the bacterium was harvested by centrifugation (15 min at 9000 rpm, 4 °C). At the end of the centrifugation, the cells were washed with 0.1M saline phosphate buffer [8.5 g of sodium chloride, 0.272 g of potassium dihydrogen phosphate, and 1.785 g of di-Sodium hydrogen phosphate dihydrate, and 1 L of distilled water] three times, and resuspended in a certain volume of R2B necessary to achieve the bacterial concentration ($1*10^8$ cells/mL) required for each assay (Afonso et al., 2019).

2.2. Stock solution of fungal spores

Stock solutions of fungal spores were performed according to Simões et al. (2015). Spores of each fungal species were harvested from seven days aged pure cultures in specific solid medium grown at 25°C. Using 2 ml of a saline solution [TWS: 0.85 % NaCl; 0.05 % Teen 80 and 500 mL of distilled water] to collect the spores. Then, 1 mL of this solution was used to inoculate the Erlenmeyer flasks, that then were incubated during 7 days at 25°C. After that, the spores were collect using 20 mL of TWS solution and was used agitation to suspend as much as spores as possible in the solution (Afonso et al., 2019). Finally, to ease the conservation of these fungi, spores of each fungus were collected and preserved in Eppendorf tubes with 1.5 mL of semiliquid agar 0.2 % (w/w). The suspension of spores and mycelia obtained were filtered using glass wool to form a solution only with spores. This process was different for *Alternaria* and *F. oxysporum* due to the spore size. Glass wool was replaced by gaze during the filtration of the solution (Afonso et al., 2019). The recovered spore suspension was homogenized and quantified using a Neubauer chamber. After the quantification, the spore solution was divided and preserved in cryovials with 10 % of glycerol at-20°C. For the further assays, the spores were resuspended in a volume of R2B necessary to achieve a final concentration of $1*10^5$ spores/mL (Afonso et al., 2019)

2.3. Fungal Micro and Macroscopic characterization

2.3.1. Macroscopic characterization

For the macroscopic characterization, all fungal strains were inoculated in Czapek's Dox + Yeast Extract Agar [CYA:0.65 g of dipotassium phosphate; 5 mL of Czapek solution; 2.5 g yeast extract; 15 g sucrose; 7.5 g agar and 500 mL of distilled water]. A three-point inoculation using sterile toothpicks immersed in the semisolid agar solutions were used to inoculate the plates. After 7 days of incubation at 25 °C, a macroscopic photograph was taken of each strain.

2.3.2. Microscopic observation

For the microscopic observation, sterile cover slips were inserted directly in the plates inoculated with fungal strains (MEA or PDA according to the better growth medium for each fungus), and left for 7 days at 25 °C. After 7 days, the cover slips were carefully removed from the plate and placed over the glass slide with one drop of cotton blue stain. The preparations were observed at the microscope (Leica DMR, Germany). On the microscope, the goal was to visualize some structures that are representative of the species, such as the reproductive structures.

2.4. Kinetics of fungal biofilm formation in microtiter plates

Fungal biofilms were developed according to the modified microtiter plate test previously used by Simões et al. (2007) to form bacterial biofilms. Briefly, for each condition (different fungal species spores) at least 16 wells of sterile polystyrene 96-well flat tissue culture plates (Greiner Bio-one Cellstar®, Krems-münster, Austria) were filled under aseptic conditions with 200 µL of spore suspension (10^5 spores ml^{-1} in R2B). Additionally, for the *in-situ* biofilm microscope visualizations, 2 ml of spore suspension were added to the wells of a sterile DNAase and RNAase free (Greiner Bio-one Cellstar®) polystyrene 24-well culture. To promote biofilm formation, all the plates were incubated aerobically at 25°C under agitation (150 rpm), for 4, 8, 24, 48, and 72 h. The medium was renewed each 24 h. At each sampling time, to remove non-adherent and weakly adherent cells, the content of each well was removed and washed two times with 200 µL or 2 mL of sterile distilled water for 96 or 24-well plates, respectively. The remaining attached cells were analyzed in terms of biomass adhered on the inner walls of the wells, and in terms of metabolic activity. The morphology of ff biofilms was characterized by microscopy. Negative controls were obtained by incubating the wells only with R2B without adding fungal spores.

2.5. Biofilm monitoring by spectrophotometric methods

2.5.1. Biofilm metabolic activity assessment using resazurin

Resazurin is a blue redox indicator that can be reduced to pink by viable microorganisms in the biofilm (Extremina et al., 2011). To do so, was prepared a stock solution of resazurin (400 µM) and was stored at -20 °C. During the assays, to obtain a final concentration of 20 µM of resazurin, in each well was added 10 µL of resazurin and 190 µL of R2B. Then the plates were incubated for 3 h in the dark at 25 °C. Then the fluorescence was measured (λ_{ex}: 530 nm and λ_{em}: 590 nm) using a microtiter reader

(Cytation3 Imaging Reader, USA) (Afonso et al., 2021). Besides the metabolic activity, the specific metabolic activity was calculated by dividing the metabolic activity by the biomass quantification.

2.5.2. Biofilm mass quantification using crystal violet

Crystal violet is a basic dye capable of binding to negatively charged surface molecules and polysaccharides in the extracellular matrix of both live and dead cells. Therefore, it can be used to quantify the matrix of both live and dead cells. (Extremina et al., 2011). After the fungal biofilm washing step, 200 µL of methanol was added for 15 min to promote the cells/spore's fixation to the wells. Afterwards, the methanol was removed, the plates were left to dry and then 200 µL of crystal violet stain was added for 5 min. Finally, the excess dye is removed by washing carefully using running tap water to keep only the crystal violet attached to the cells/spores (Simões et al., 2015). Finished this process, the microtiter plates were left to dry and then 200 µL 33 % (w/w) of glacial acetic acid in the wells and then, the optical density of obtained solution was measured at 570 nm using a microtiter plate reader (Cytation3 Imaging Reader, USA). Depending on the values obtained, dilutions were made to get reliable results from the measurement. Moreover, since this process tends to create a lot of waste, the final part with the crystal violet and with the acetic acid were executed when the assays were finished.

2.6. Biofilm monitoring by microscopy

2.6.1. Epifluorescence microscopy

The morphology of the biofilm can be characterized by microscopy (Simões et al., 2015). Epifluorescence microscopy was already used to visualize the structure of a ff biofilm with specific fluorochromes, namely calcofluor white M2R (CW) and FUN-1 (Simões et al., 2015). At each sampling time, a small square of the bottom of each well of the microtiter plate was cut off and the biofilms were observed by an epifluorescence microscope. The washed biofilms were stained with 15 µl of 25 µM of FUN-1 at 30°C for 30 min and 10 µl of 25 µM CW at room temperature for 15 min in the dark (Simões et al., 2015). After staining the samples, these were observed under an epifluorescence microscope (Olympus BX51, Germany) using UV light equipped with 10 × / 0.30 and 40 × / 0.75 objective lenses.

The optical filter combinations that are going to be used for FUN-1 are a 470-490 nm excitation filter, a LP516 nm emission filter and a 500 nm barrier filter and for CW are a 365-370 nm excitation filter, a LP421 nm emission filter and a 400 nm barrier filter (Simões et al., 2015). After these steps, the

biofilm images were acquired with an epifluorescence microscope (Olympus BX51, Germany) using the Olympus cellSens software (Simões et al., 2015).

2.6.2. Scanning electron microscopy

Besides epifluorescence microscopy, scanning electron microscopy (SEM) was also used for biofilm characterization. At each sampling time, a small square of the bottom of each well of the microtiter plate with biofilm was cut off and then went through an ethanol treatment to dehydrate the samples. Different increasing ethanol concentrations were used to dehydrate the samples (10 %; 25 %; 40 %; 50 %; 70 %; 80 %, 90 %; and 100 % (v/v) of ethanol). The samples were dipped in each ethanol concentration for 15 min. This process began from the lower concentration to the highest concentration of ethanol. At the end, the samples were preserved in an exicator until it was possible to observe under microscope. Then the samples were characterized using a desktop Scanning Electron Microscope (SEM) (Phenom ProX, Netherlands). All results were acquired using the ProSuite software v.3.0. Non-conductive and uncoated samples were added to aluminium pin stubs with electrically conductive carbon adhesive tape (PELCO Tabs™) on a Phenom Charge Reduction Holder (CRH).

2.7. Identification of two species of fungi used in this work

There two fungi (*Alternaria* sp. and *Mucor* sp.) did not have a complete identification. So, was purposed to do all process for the identification of the *species/ sector/ series* of the fungi that were part of this work.

2.7.1. Biomass collection by filtration

The two strains were incubated in liquid medium at 25 °C and with agitation of 150 rpm. After 1 week, the biomass was collected into a filter paper (5-13 µm, filtraTECH, France) using a vacuum pump to filter the biomass from the medium. The filtration system was disinfected with alcohol between the two filtrations. The biomass was stored at -20°C.

2.7.2. DNA extraction

In this part, 200 mg of biomass of each sample were weighted and put in tubes with sterile acid-washed glass beads (710-1,180 µm, Sigma) to suffer mechanical lysis in the fast-prep (MP Biomedicals, USA). After the cycle, it was added 1 mL of CTAB 2 % [5 mL Tris-HCl pH 8.4; 8.18 g NaCl; 12.48 mL EDTA pH 8.0 and 2 g CTAB] and the samples were centrifuge for 8 min at 14000 ×g. After 8 min, 800

µL of the supernatant of each sample were collected and put it in new tubes with 1mL of cold sodium acetate 3 M pH=5.5 and were mixed carefully then incubated at -20°C for 10 min and centrifuge for 10 min at 14000 ×g. The sodium acetate solution was used for the precipitation of polysaccharides and proteins. After the centrifugation, 1 mL of the supernatant of each sample was transferred to a new tube with 1 mL of isopropanol. The solutions were mixed carefully and were incubated for 1 h at room temperature. When the time was over, the tubes were centrifuged for 10 min at 14000 ×g. At the end, the supernatants were discarded. The remain pellets were washed with 1 mL of cold ethanol 70 %, centrifuged for 7 min at 6000 ×g and the supernatant discarded. This last step was repeated two times for each sample. Finally, the samples were dried in the speed Vac [40°C; 3-5 cycles min] then the samples were resuspended with 100 µL ultrapure water and stored at -20°C. To ease the dissolution of DNA in ultrapure water, the sample were incubated ate 56 °C for 2 h. To remove the RNA present in the samples, 2 µL of RNase (10 mg/mL) solution were added to each sample and incubated for 1 hour at 60 °C.

2.7.3. Gel electrophoresis and PCR

To assess the quality of the samples, these were taken to the NanoDrop to assess the ratio between DNA-RNA/protein and DNA-RNA/residues and conclude if it was needed to do dilutions. Also, an agarose gel (1%) was prepared using 0.3 g of agarose dissolved in 30 mL of TAE 0.5X prepared from a stock solution of TAE 50X (242 g of Tris Free base; 18.61 g of Disodium EDTA; 57.1 mL of Glacial acetic acid; and dissolve in distilled water) and 2 µl of GreenSafe Premium (NZYTech). The samples were loaded in the gel and run was 5 min at 120V plus 30 min at 80V. PCR amplification of the ITS1+5.8S+ITS2 rDNA region was performed with 50 µL of a reaction mixture containing 25 µL of NZYTaq II 2x Green Master Mix (NZTtech, Lisbon, Portugal), 1 µL of primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), 1 µL of DNA, and 22 µL of sterile ultra-pure water. The used PCR (Biorad, Hercules, USA) conditions were as follows: denaturation step at 94 °C for 3 min; 35 cycles of the annealing step: 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C; and a final elongation step of 5 min at 72 °C. Amplification success was verified in 1% agarose gel

2.7.4. Samples purification

To purify the PCR products, was used 3µL of ExoSAP in 7.5µL of each sample to disintegrate the primers in the solution. These new solutions were taken to the thermocycle for 10 min at 37°C plus 10 min at 80°C. When the time was over, 10 µL of each sample was transferred to new tubes with 3 µL of

reverse primers, in these cases was ITS4. Sequencing of the products was conducted in the STAB Vida Lda (Caparica, Portugal) using the Sanger/capillary method.

2.7.5. Sequence analysis and phylogeny of the species

Preliminary BLAST searches in GenBank with ITS sequences of the present isolates indicated that they had a close phylogenetic relation to the strains that were studied. Ten *Alternaria* species and *Stagonosporopsis hortensis* CBS 04.42 (outgroup) were retrieved from National Center of Biotechnology Information (NCBI) mostly published in Dettman & Eggertson. (2021), and Lawrence et al. (2013). Nine *Mucor* species and *Rhizopus arrhizus var.arrhizus* CBS 112.07 (outgroup) were also retrieved from NCBI, mostly published in Walther et al. (2013), and Hurdeal et al. (2021). Table 2 shows the strains used for the phylogenetic analysis of the strains studied.

Table 2: Species used for phylogeny analysis of *Alternaria* sp. (MUM 02.42) and *Mucor* sp.(MUM 02.01)

Species	Strain Number	Accession number (ITS)
Alternaria limoniasperae	-	FJ266476
Alternaria arborescens	CBS 102605	NR135927
Alternaria longipes	-	AY278835
Alternaria tenuissima	EGS 34-015	AF347032
Alternaria alternata	EGS 34-016	AF347031
Ulocladium atrum	ATCC 18040	AF229486
Ulocladium cucurbitae	-	FJ266483
Alternaria consotialis	CBS 104.31	KC584247
Altenaria multiformis	CBS 102060	NR077187
Alternaria terricola	CBS 202.67	NR103600
Stagonosporopsis hortensis	CBS 04.42	GU237730
Mucor plumbeus	CBS 284.78	JN205914
Mucor brunneogriseus	CBS 129.41	JN205910
Mucor circinelloides f. circinelloides	CBS 239.35	JN205942
Mucor bainieri	CBS 293.63	JN205995
Mucor circinelloides f. lusitanicus	CBS 108.17	JN205980
Mucor luteus	CBS 243.35	HM999954
Mucor hiemalis f. hiemalis	CBS 107.19	JN206137
Mucor irregularis	CBS 700.71	JN206154
Mucor grandis	CBS 186.87	JN206252
Rhizopus arrhizus var. arrhizus	CBS 112.07	JN206323

The gene sequences were concatenated and edited manually according to ITS for the two strains using MEGA v.11.0.11. To each fungal strain studied (MUM 02.01 and MUM02.42) they were aligned to the other respective strain species and estimated the best model to each case. The bootstrap values (BS)

with 100 replicates were performed to determine branch support. Parsimony scores of tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency (RC) were calculated for each generated tree.

2.8. Evaluation of biotic and abiotic factors on fungal biofilm formation and behavior

2.8.1. Interkingdom biofilm formation and number of bacteria in single and interkingdom biofilms

To study the effects of a biotic factor on biofilm formation, namely the effects of the presence of bacterium *S. maltophilia* on *Alternaria* sp. biofilm formation, single and interkingdom biofilms were performed. In this part three biofilm experiments were done: an assay where fungi and bacteria grew simultaneously (interkingdom biofilms), and other two assays where fungi (*Alternaria* sp.) and bacterium (*S. maltophilia*) grew separately (single biofilms). Relative to the fungal biofilm formation, the procedure was already described above (2.4.). Regarding the bacterial biofilm formation, 200 µL of cell suspension ($1x10^8$ cell/mL in R2B) were inoculated in a sterile polystyrene 96-well flat tissue culture plates (Greiner Bio-one Cellstar®, Krems- münster, Austria).

For the inter-kingdom biofilm formation, 100 µL of fungal spores' suspension (1×10^5 spores/mL) and 100 µL of bacterial cell's suspension ($1x10^8$ cell/mL) was added to each well of a sterile polystyrene 96-well flat tissue culture plates. The reduction to half-cell density in the inter-kingdom assays was performed to avoid limitations in nutritional factors. To promote single and inter-kingdom biofilm formation, all the plates were incubated aerobically at 25°C under agitation (150 rpm) for 24, 48, and 72 h. The medium was renewed each 24 h. Afterwards, the methods used for fungal biofilms described above (2.4 and 2.5), namely the biofilm mass determination by crystal violet and the metabolic activity by resazurin, were followed.

Additionally, for bacterial and inter-kingdom biofilms the number of bacterial cells in biofilms were determined by counting colony-forming units (CFUs) in R2A agar upon biofilm release. Briefly, after each incubation period, the supernatant was removed, and the biofilms were washed two times with sterilized water as previously described. Then 200 µL of phosphate buffer saline (pH7.4) were added into each well and the microtiter plate were covered with the lid and put into an ultrasonic bath (Bandelin electronic GmbH &Co. KG, Berlin, Germany). To release the bacterial cells from biofilms, the microtiter plates were sonicated for 1 minute (5s sonication, 10s interval) at 35kHz. After this, the bacterial suspension of the

16 wells were collected and saved in a 2 mL Eppendorf to be used for serial dilutions in order to inoculate R2A agar plates for CFU determination (Afonso, *et al.,* 2019).

2.8.2. Evaluation of abiotic factors on the fungal biofilm formation

Three different abiotic factors were studied to evaluate their influence on fungal biofilm formation, namely on *Alternaria* biofilms. The three factors evaluated were: hydrodynamics conditions, the presence of disinfectant, and nutrient concentration. To study the effects of hydrodynamics conditions on biofilm development and behavior, four different hydrodynamic conditions were used to perform the fungal biofilm formation assays (static, 30, 150 and 200 rpm). In terms of presence of disinfectant, five different conditions were evaluated (without free chlorine; 2.4 ppm of free chlorine; 6.03 ppm of free chlorine; 12.06 ppm of free chlorine and 24.12 ppm of free chlorine). To prepare these different chlorine concentrations, a stock solution of chlorine of 603 ppm of active chlorine in 500 mL of sterilized water was prepared. Then, depending on the condition a work solution of chlorine in R2B medium was prepared in a Falcon and then added with the fungi in the microtiter plate. Regarding the nutrients concentration, four different conditions were evaluated which were synthetic tap water [100 mg/L of sodium bicarbonate; 13 mg/L of magnesium sulfate heptahydrate; 0.7 mg/L of dipotassium phosphate; 0.3 mg/L of monopotassium phosphate; 0.01 mg/L of sodium chloride; 0.01 mg/L of ammonium sulfate; 0.001 mg/L of iron sulfate heptahydrate; 1 mg/L of sodium nitrate; 27 mg/L of calcium sulfate ; and 1 mg/L of humic acids] as medium, R2B medium diluted by factor of two and four, and R2B medium without being diluted.

The fungal biofilms were performed according to procedure described above. The medium was renewed each 24 h. But depending on the conditions the medium was renewed supplemented with chlorine or diluted as an appropriated concentration of nutrients. For each condition tested and for different biofilm sampling times (24h, 48h, and 72h), the medium was removed of wells of the microtiter plate, and the wells were washed with 200 µL of sterilized distilled water to remove non-adherent and weakly adherent cells. The remaining attached cells were analyzed in terms of biomass adhered on the inner walls of the wells, and in terms of metabolic activity by crystal violet and resazurin methods, respectively.

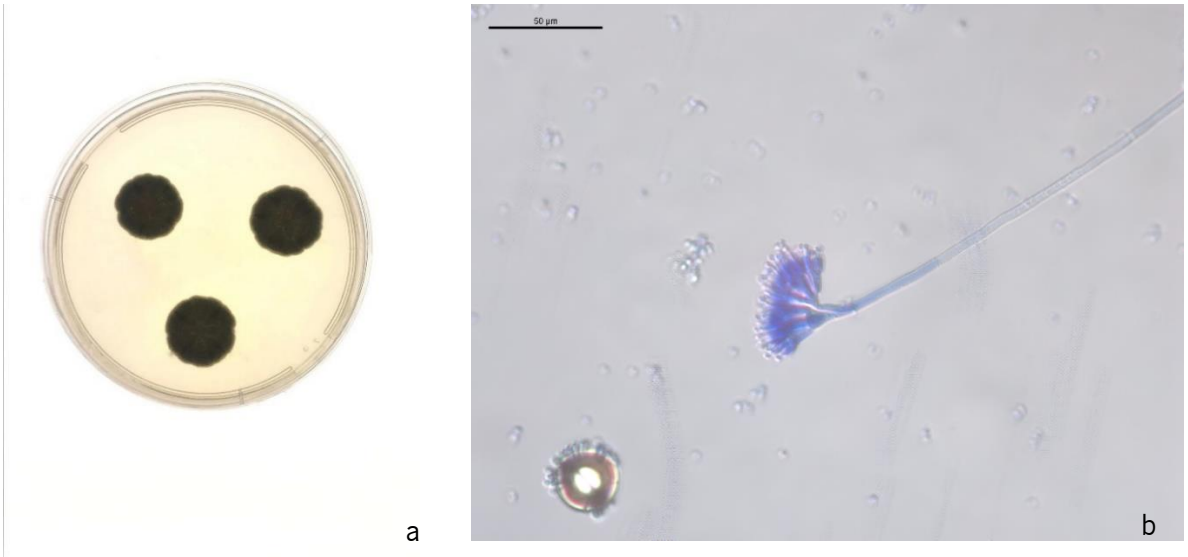
2.9. Statistical analysis

The data were analyzed using the software Excel and GraphPad Prism version 9.4.1. The mean and standard deviation were calculated for all cases and the statistical significance of results was determined by non-parametrical ANOVA test $p<0,05$ was considered to be statistically significant.

3.Results and Discussion

3.1. Fungal Micro and Macroscopic characterization

These six fungi were chosen for this study due to their prevalence in the DWDS of Braga, Portugal and due to the capacity of some fungi to produce mycotoxin and to form interkingdom biofilms which can lead to host viruses and other pathogens that can cause harmfulness to human health. Additionally, these fungi can form biofilm in the DWDS put in cause the quality of water, changing the taste, odor and color (Del Olmo et al., 2021; Simões & Simões, 2013). Some of these fungi have been appearing in hospitals, such as *A. versicolor* and *F. oxysporum* (Litvinov et al., 2015; Navale et al., 2021) and the latter cause an outbreak of fusariosis in the hospital. The Figure 4 are the result of macroscopic and microscopic analysis of the *P. brevicompactum* and *P. expansum*.



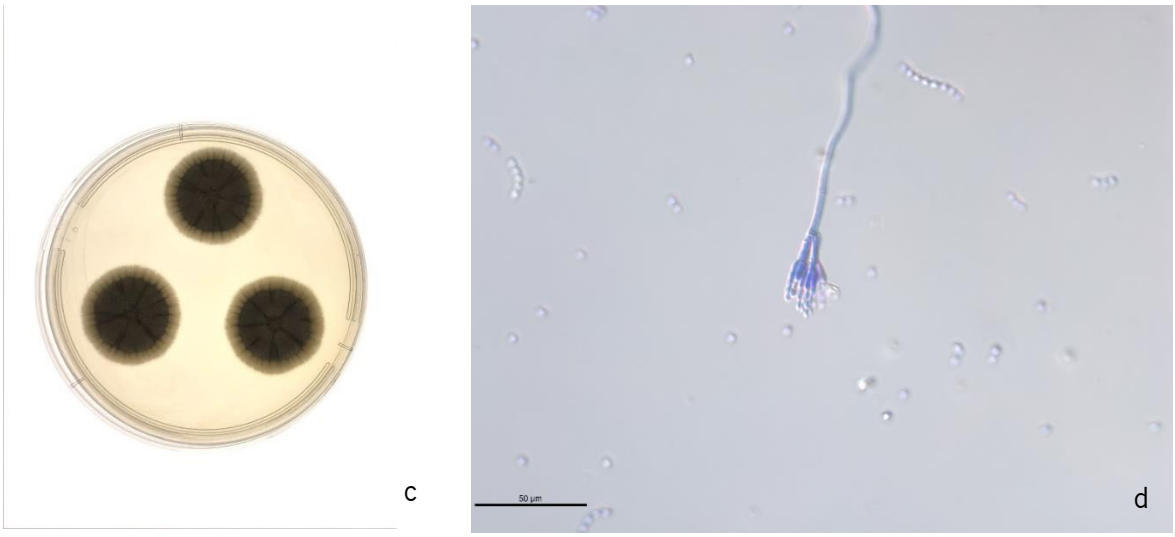
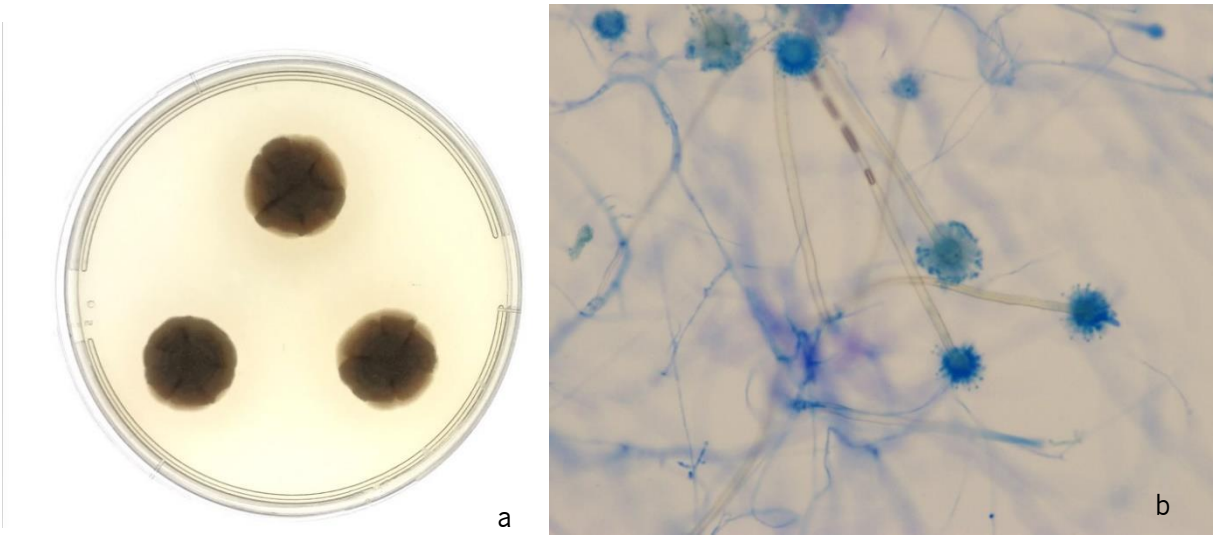


Figure 4: *Penicillium brevicompactum* (a) colonies on CYA medium; (b) phialides with conidia. *Penicillium expansum* (c) on CYA medium; (d) phialides with conidia. x 50 magnification; bars = 50 µm.

P. brevicompactum (Figure 4.a) produce compact penicilli, short and broad metulae and often apically inflated (Figure 4.b). This fungus can produce mycophenolic acid which is a weakly compound (Pitt & Hocking, 1997). *P. expansum* (Figure 4.c) is distinguished by the dull green conidia (Figure 4.d) and are capable of producing patulin and citrinin and can be harmful for human due to their cytotoxicity, genotoxicity and immunosuppressive properties (Pang et al., 2022).

Figure 5 is the result of macroscopic and microscopic analysis of the *F. oxysporum* and *A. versicolor*.



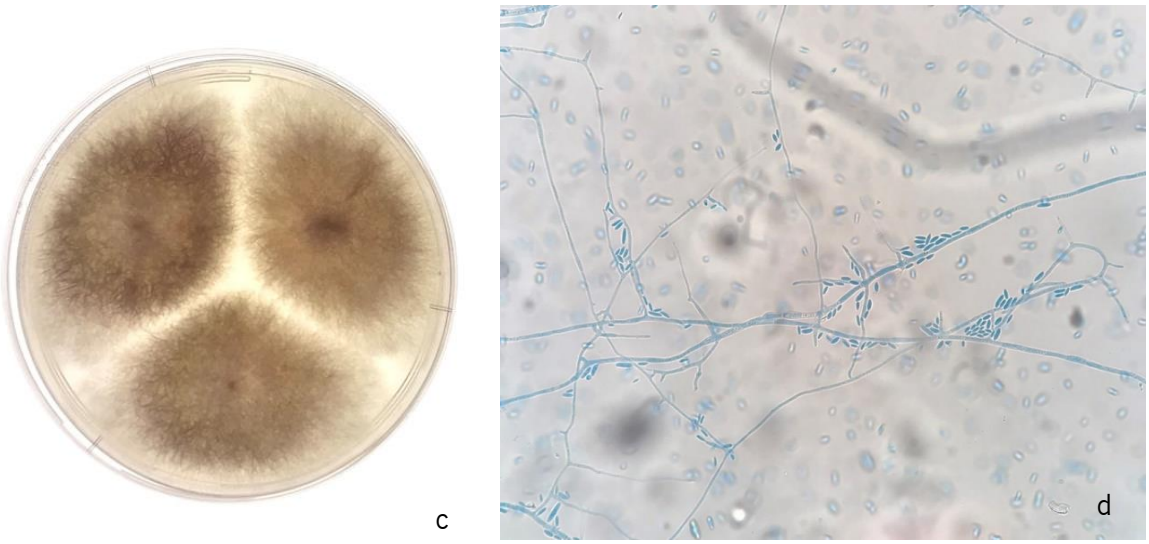


Figure 5: *Aspergillus versicolor* (a) colonies on CYA medium; (b) conidia and phialides. *Fusarium oxysporum* (c) on CYA medium; (d) conidiophore. x 50 magnification.

A. versicolor (Figure 5.a) grows slowly, produces metulae and phialides from vesicles (Figure 5.b) and is distinctive characteristic is the different pigmentations that can present during the incubation. Fungus from this genus can produce various life threatening mycotoxins such as aflatoxins, ochratoxins, patulin, citrinin, aflatrem, selonic acids, cyclopiazonic acid, terrain, sterigmatocystin and gliotoxin (Navale et al., 2021). *F. oxysporum* (Figure 5.c) is distinctive by the production of fusiform to kidney shaped microconidia and flask shaped phialides in the aerial mycelium (Figure 5.d). This fungus is mainly plant pathogens, but they can infect humans and cause diseases depending on the health status of the host. Keratitis, onychomycosis, and fusariosis are examples of diseases caused by this fungus (Litvinov et al., 2015; Moretti et al., 2018).

Figure 6 is the result of macroscopic and microscopic analysis of the *Alternaria* sp. and *Mucor* sp.

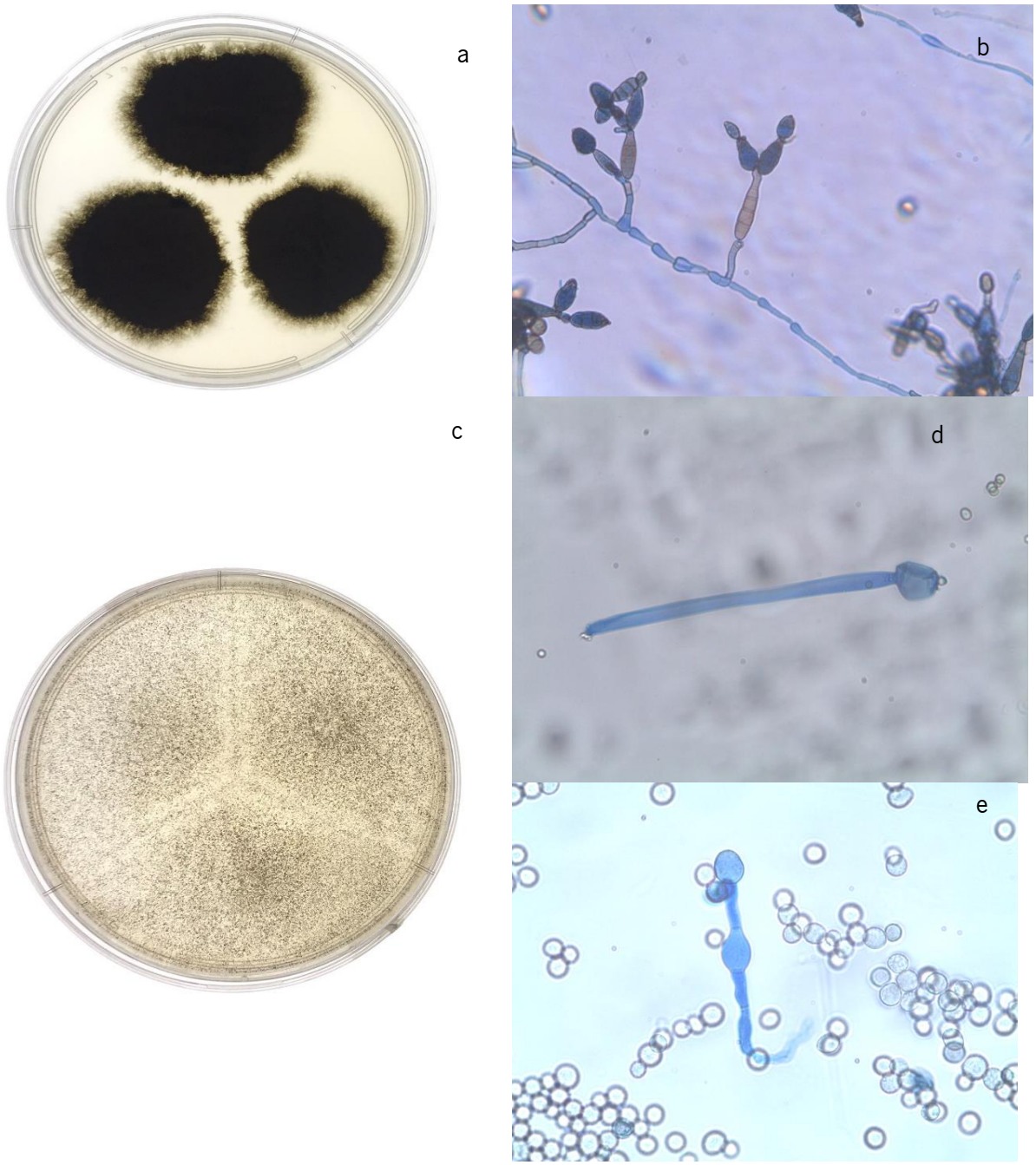


Figure 6: *Alternaria* sp. (a) colonies on CYA medium; (b) conidia and phialides. *Mucor* sp. (c) on CYA medium; (d) columella x 50; (e) chlamydospore. x 50 magnification.

Alternaria sp.(Figure 6.a) is characterized by the large brown club shaped conidia with both longitudinal and transverse septa (Figure 6.b). This fungus can produce several mycotoxins, which the most important is tenuazonic acid (King & Schade, 1984) and the most frequent toxins produced by this fungus are alternariol, alternariol monomethyl ether and alertoxins (Tralamazza et al., 2018). The *Mucor* sp. (Figure 6c) is characterized by primarily grow as hyphae and some may form columella (Figure 6.d), which at the end normally exists the spores. And also, may form chlamydospores (Figure 6.e). Moreover

they present fast growth and spores are abundantly produced (Morin-Sardin et al., 2017). Also, some fungus of this genus can cause human diseases, such as mucormycosis since their capacity to tolerate high temperature (Morin-Sardin et al., 2017).

3.2. Kinetics and biofilm formation by filamentous fungi isolated from drinking water.

In this first part, six fungi that can be found in drinking water environments were chosen to evaluate their biofilm formation ability and behavior in controlled specific conditions. The six fungi chosen were *P. expansum* (MUM 00.02), *P. brevicompactum* (MUM05.17), *A. versicolor* (MUM 02.37) *Alternaria* sp. (MUM 02.42) *Mucor* sp. (MUM02.01) and *F. oxysporum* (MUM 17.33). Furthermore, were created six measure points to evaluate the metabolic activity and the biomass formed over time (t=4h; t=8h; t=11h; t=24h; t=48h; and t=72h). The Figure 7 shows the biomass of the different fungi for single species biofilm formation over time.

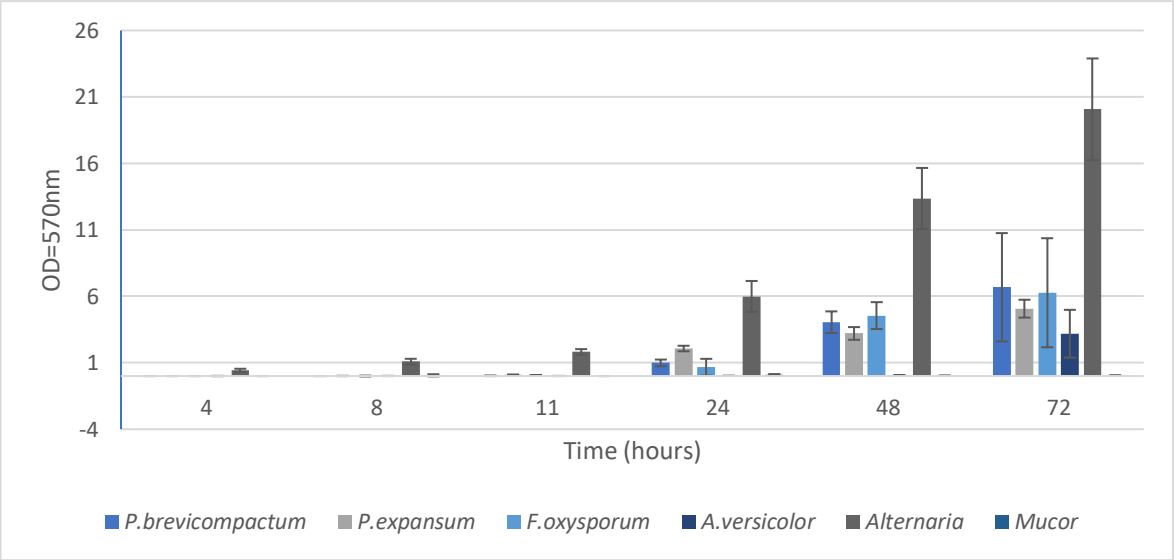


Figure 7: Biomass productivity in terms of OD_{570nm} values for single-species biofilm formation over time (t=4 h, t=8 h, t=11 h, t=24 h, t=48 h, and t=72 h). The mean ± SDs for two independent experiments are illustrated.

In general, the biomass productivity increased over time, but with differences among the several fungi studied. *P. brevicompactum* showed an increase of biomass productivity along all experiment, which the maximum value of biomass productivity is verified at 72 h of 6.680 ± 4.074. At the biofilm sampling times of 4 and 8 h, the values of biomass productivity were equal to zero, meaning that at these times the fungus did not adhere to the surface or even if it adheres, the fungi passed through a process of adaptation to the surface and environment. *P. expansum* showed an increase of biomass productivity until the end of the experiment, which the maximum value of biomass productivity is verified at 72 h of

5.069 ± 0.673. In the measure 4 h, the biomass value is zero, which means the fungus is adapting to the environment and the surface to adhere and start to form biofilm. These two fungi, *P. brevicompactum* and *P. expansum*, in some reports are described as non-adherent and weakly adherent which can explain the delay in biomass productivity in the first measure times (Stepanović et al., 2000).

Relative to *F. oxysporum*, this fungus showed an increase of biomass during all experiment. The maximum value of biomass productivity presented by this fungus is at 72 h of 6.266 ± 4.101. The same way as *P. expansum*, *F. oxysporum* shows a biomass productivity of zero at t=4h and this can indicate that the fungus is adapting to the environment and the surface to adhere and start to form biofilm. Furthermore, this fungus only started to show a mature biofilm after 48 h, and this behavior is reported in some studies as Imamura et al. (2008) and Mukherjee et al. (2012) Another fungus studied is *A. versicolor*, and this fungus showed an increase of biomass productivity during all experiment. At 72 h, the *A. versicolor* biomass productivity was the highest showing a value of 3.183 ± 1.798. Besides, *A. versicolor* presented a decline of biomass productivity at t=11 h that can indicate a longer adaptation of this fungus to the environment, but after 4 h this fungus already presented biomass productivity, in contrast to the prior fungi. This fungus, compared to others, showed lower biomass productivity which can be explained since this fungi may not had the most favorable conditions to its growth. Kaur & Singh. (2014) reported that another species of *Aspergillus* finds static better for biofilm formation than when with rotation.

Alternaria sp. showed a biomass productivity increase from the first biofilm sampling at 4 h until the end of the assay. As the *A. versicolor*, this fungus shows biomass productivity since the beginning and the highest verified was at 72 h, a value of 20.070 ± 3.825. Moreover, *Alternaria* sp. is the fungus that presented the highest biomass production among all fungi studied during this work. Siqueira & Lima (2013) verified that *Alternaria* sp. was the only fungi available of growing in oligotrophic conditions, showing the high adaptation of this fungi to form biofilm. Lastly, the *Mucor* sp. shows a non-growing tendency during all assays. During the three days, the biofilm formation could be noticed in suspension, but during the changes of medium and the washing process the biofilm was almost washed out from the microtiter plate. Furthermore, one more measure point is formed in an attempt to understand if three days of the experiment is sufficient for adhesion and biofilm formation for this fungus.

However, after four days (t=96h) the biofilm is also washed out from the plate. This is explained by the hydrophobicity that the fungal' spores present and this hydrophobicity influences the adhesion of the spores to the surface (Karimi & Zamani, 2013). For this reason, *Mucor* sp. has only an assay related

to biomass quantification because of its inability to adhere to the microtiter plate well's surface, explaining why does not exist results the metabolic activity.

Figure 8 shows the metabolic and specific metabolic activity of the different fungi for single-species biofilms formation over time. Furthermore, were created six measure points to evaluate the metabolic activity and the biomass formed over time(t=4h; t=8h; t=11h; t=24h; t=48h; and t=72h).

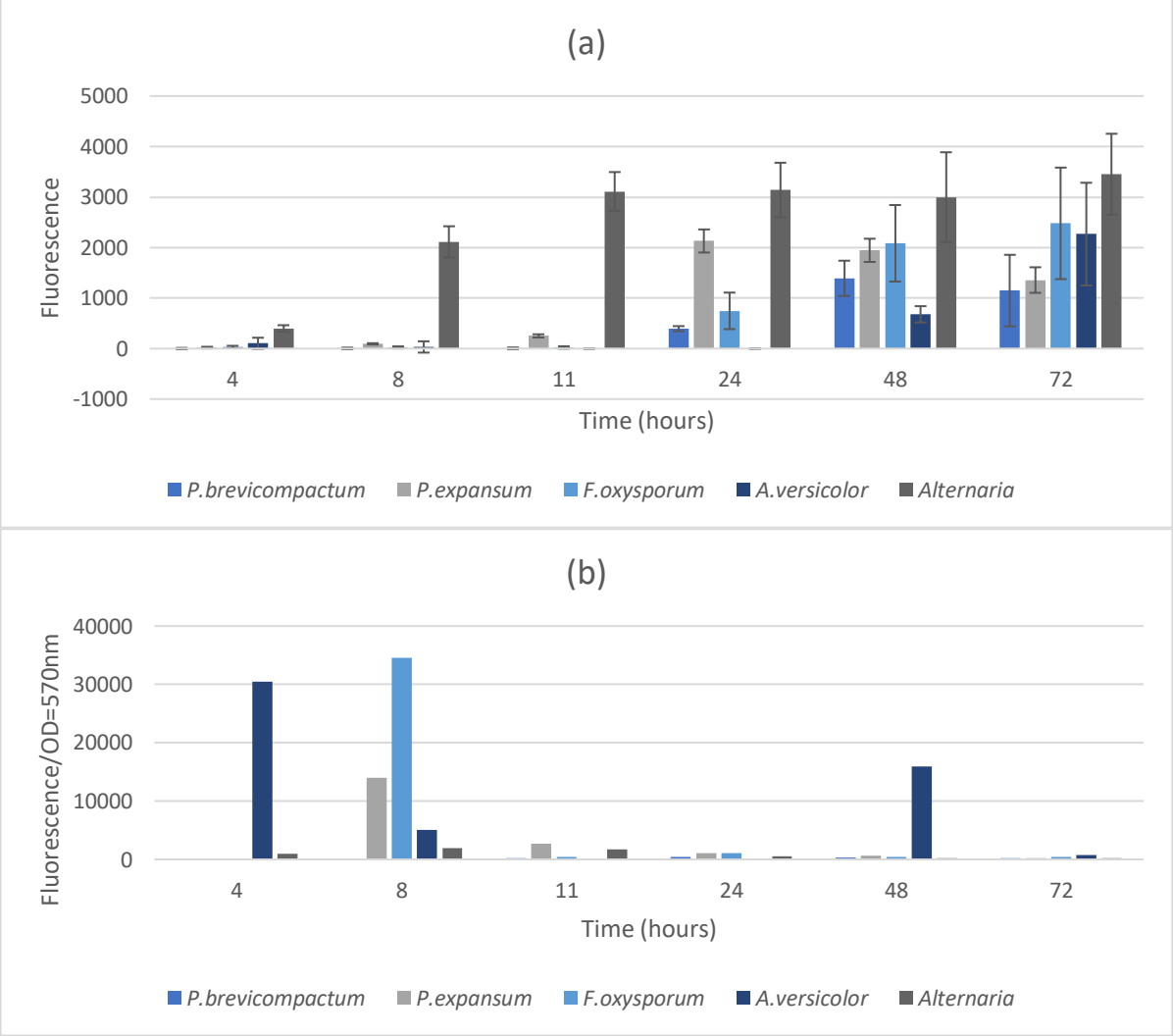


Figure 8: Metabolic activity(a) and specific metabolic activity (b) for single-species biofilm formation over time (t=4 h, t=8 h, t=11 h, t=24 h, t=48 h, and t=72 h). The mean ± SDs for two independent experiments are illustrated.

The metabolic activity varied depending on the fungi that were studied, but in general the behavior of the fungi showed an increase in metabolic activity over time. *P. brevicompactum* showed an increase in metabolic activity in the first 48 h and then showed a decrease at 72 h. This metabolic activity decrease can indicate that *P. brevicompactum* entered a plateau phase and stop developing the biofilm or can

indicate that the biofilm entered a log phase, but to certify this hypothesis would be needed to measure a point a day after. Another reason for this decrease in metabolic activity can be a change in an abiotic factor, such as nutrients or temperature that might influence the development of the biofilm (Fernandes, 2018). At 48 h, the metabolic activity presented by *P. brevicompactum* was 1390.523 ± 349.936 and was the highest value presented by *P. brevicompactum*. Comparative to the specific metabolic activity, *P. brevicompactum* showed an activity increase until 24 h, and then a decrease. In the first two measure points, the specific metabolic activity was zero, which can be explained by the fact that the biomass measured in this fungus was zero as well. Then, *P. brevicompactum* showed an increase in the first 24 h and then a decrease until the end of the assay. Compared with the values of biomass quantification and metabolic activity of this fungus, these values indicate that the biofilm formation and growth of the fungus occurred during all experiments.

P. expansum showed an increase in metabolic activity in the first 24 h and then showed a decrease at 48 and 72 h. As *P. brevicompactum*, the decrease of metabolic activity in *P. expansum* can suggest that the fungus entered a plateau phase and stop developing the biofilm or that the biofilm entered a log phase. A different reason for this decrease can be a change in an abiotic factor, such as nutrients or temperature that might influence the development of the biofilm (Fernandes, 2018). At 24 h, the metabolic activity presented by *P. expansum* was 2130.633 ± 228.637 and was the highest value verified during the *P. expansum* experience. Relative to the specific metabolic activity, *P. expansum* showed a higher level of activity at 8 h and then decreased until the end of the experiment. This can indicate that at the beginning of the experiment the fungus had a higher metabolic activity to grow and form biofilm and at the end, the metabolic activity was less due to lack of nutrients or space to grow, and the metabolism activity is only being used to the maintenance of the microorganism and the proliferation.

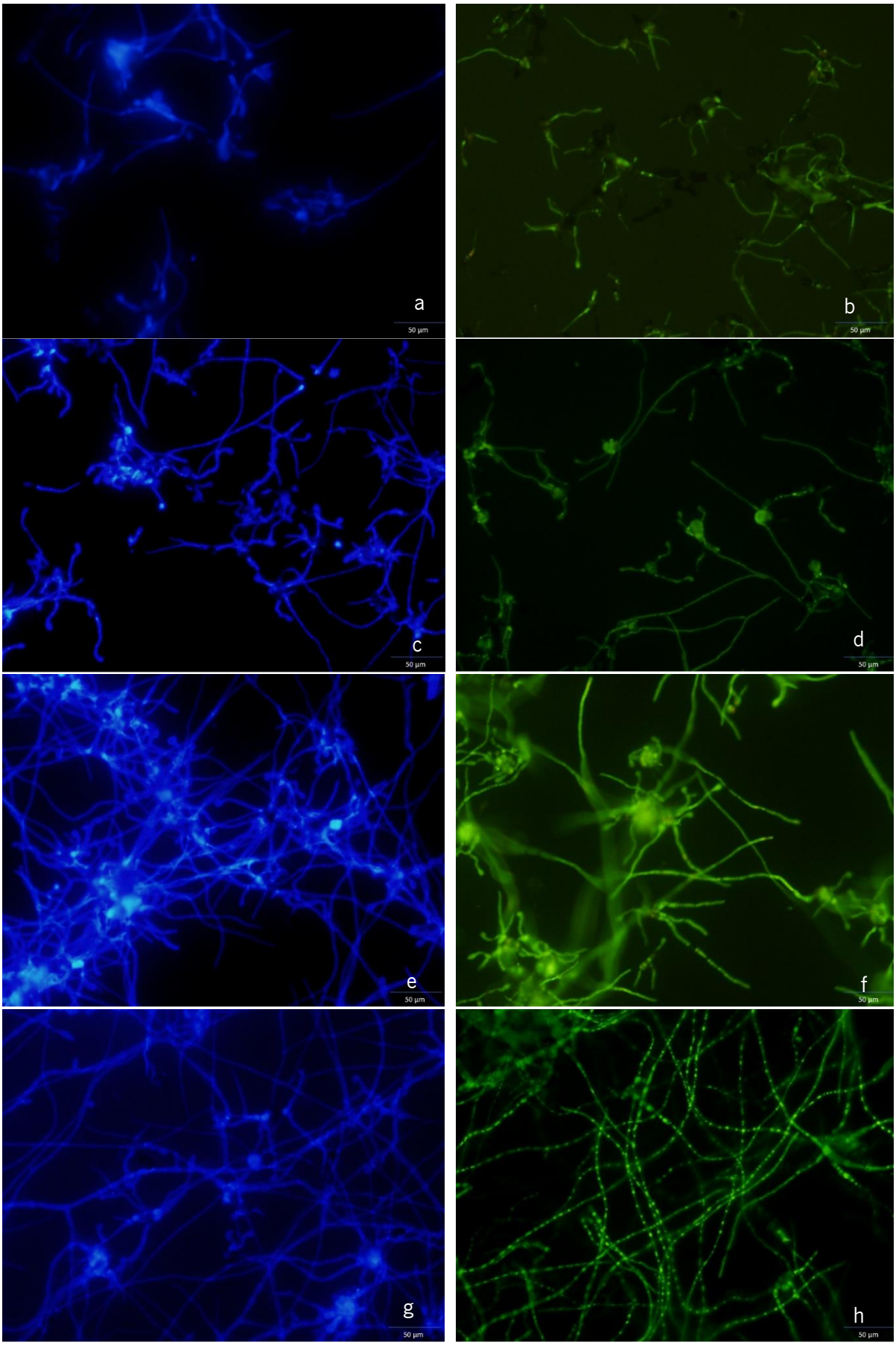
Relative to *F. oxysporum*, this fungus showed an activity decrease in the first 11 h, but then was verified a metabolic activity increase at 24 h and continued until 72 h. This decrease in activity at the beginning of the experience suggests that the *F. oxysporum* had a long phase of adaptation than the other fungi which did not present this decrease in an initial phase. Another explanation for this metabolic activity decrease can be related to some abiotic factors that are influenced an initial stage of biofilm formation (Fernandes, 2018; Imamura et al., 2008). At 72 h, *F. oxysporum* metabolic activity was the highest showing a value of 2479.183 ± 1104.545. About the specific metabolic activity, this fungus followed a same behavior as *P. expansum. F. oxysporum* showed a great specific metabolic activity at 8 h and then these values tend to decline until the end of the experiment. This suggest, that in the beginning the

metabolism was directed to the biofilm proliferation and formation and at the end the energy of the microorganisms was directed to the maintenance of the biofilm and not grow.

Relative to *A. versicolor*, this fungus showed a decrease in metabolic activity in the first 24 h, but then was verified an increase at 48 h and continued until the 72 h. At 72 h, *A. versicolor* metabolic activity was the highest showing a value of 2266,467 ± 1016,384. Besides, *A. versicolor* presented negative values of metabolic activity at t=11h and t=24h. In terms of specific metabolic activity, this fungus showed a high activity at t=4h and decline until zero at t=24h. In the second day showed a high activity and started to decline until the end the of the experiment. This suggest that in the beginning the biofilm formation was greater, but for some reason, such as temperature, lack of nutrients or any kind of stress made the fungus stop the biofilm formation. Then, the fungus started again the biofilm formation and proliferation in the second day (Fernandes, 2018; Kaur & Singh, 2014).

Lastly, *Alternaria* sp. has shown a metabolic activity increase since the first checkpoint at 4 h until the 24 h checkpoint. Then, at 48 h the metabolic activity of *Alternaria* decreased and at 72 h increased again. The highest metabolic activity value measured was 3451.425 ± 802.910 at 72 h. In terms of specific metabolic activity, the *Alternaria* sp. showed an increase of activity in the first 8 h and then started to decline until the end of the experiment. This suggest that the metabolism of this fungus was directed to the biofilm formation in the beginning and at the end the specific metabolism was directed to the maintenance of the microorganism. Comparing the fungi to each other, *Alternaria* sp. presented the highest values of metabolic activity in all checkpoints, whereas *P. brevicompactum* present lowest values among all fungi. Moreover, this method is highly sensitive and highly dependent on cell respiratory efficiency, which in turn is related to the growth phase, and to the age and thickness of the biofilm (Simões et al., 2015).

After completing these assays, the fungus chosen for the next assays was the *Alternaria* sp. since presented a high metabolic activity and biomass during the three days of the experiment. Since this fungus presented the better biomass productivity and metabolic activity values, different microscopy technics were applied to study the biofilm morphology and to try to identify the different stages of the biofilm formation process. So, epifluorescence and SEM microscopy methods were used to analyze the biofilm formed by *Alternaria* and try to identify the different stages of biofilm formation of this fungus. The Figure 9 shows several of epifluorescence microscopy of *Alternaria* biofilm formation over time () stained with CW and FUN 1 (t=4 h; t=6 h; t=8 h; t=13 h; t=30 h; t=48h; and t=61 h).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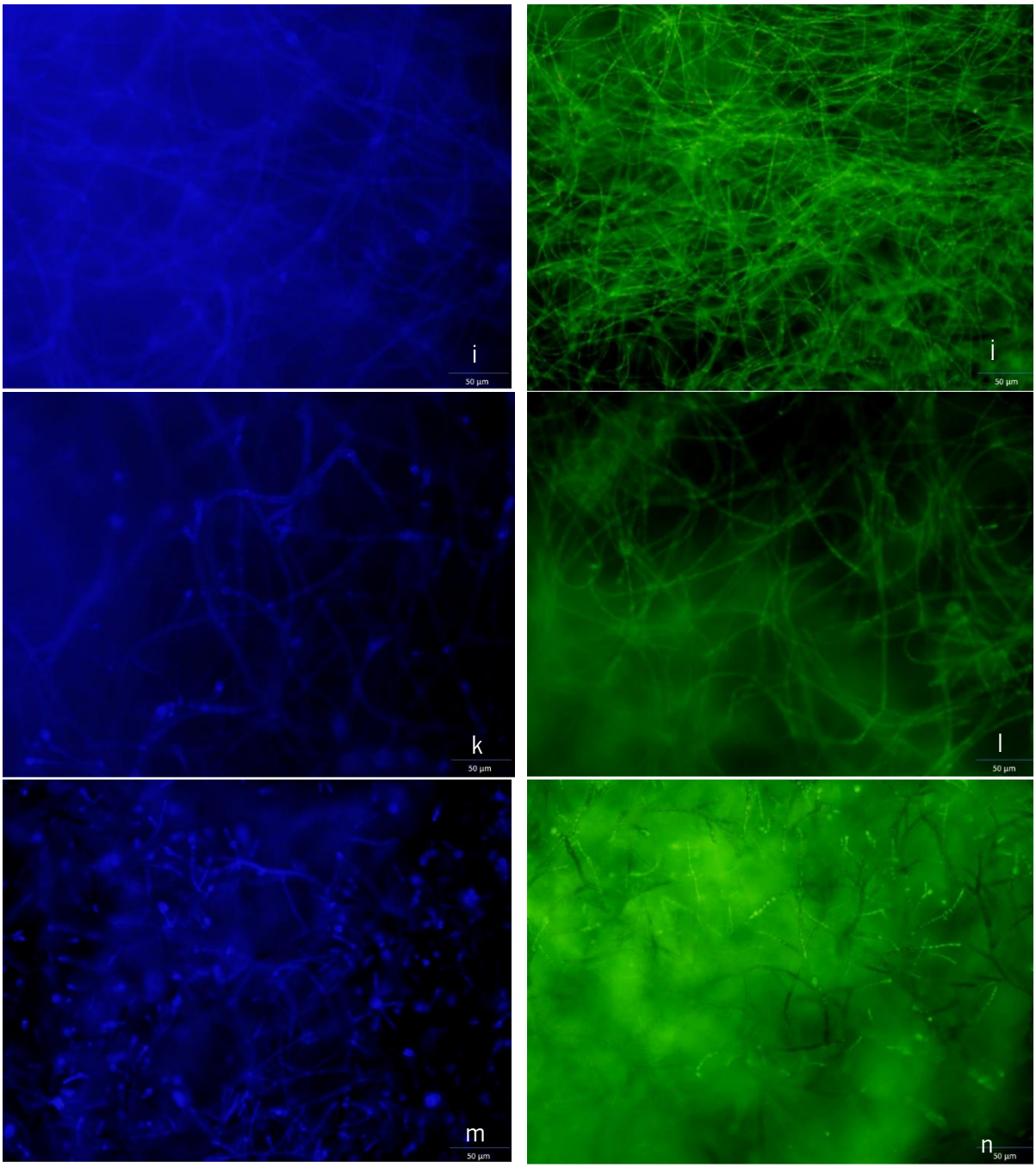
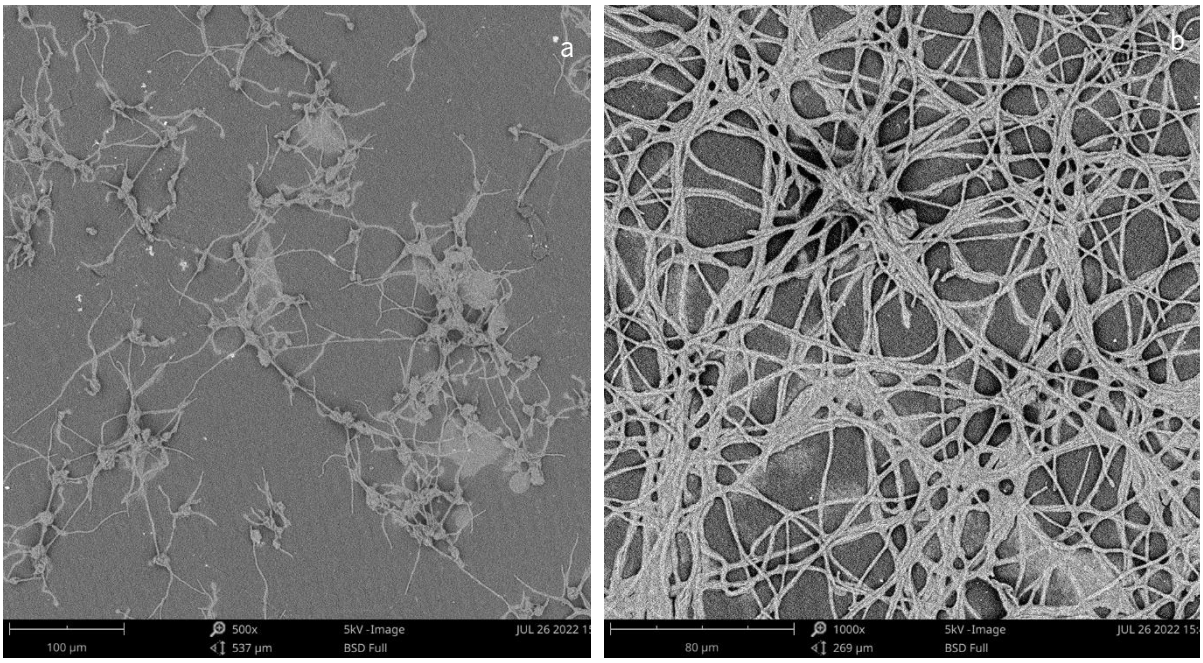


Figure 9: Epifluorescence photomicrographs of *Alternaria* sp. (MUM02.42) biofilm formation on polystyrene using specific fluorochromes, namely CW (pictures on the left side) and FUN1 (pictures on the right side) over time: (a) and (b) 4 h, (c) and (d) 6 h, (e) and (f) 8 h, (g) and (h) 13 h, (i) and (j) 30 h, (k) (l) 48 h, (m) and (n) 61 h. Magnification ×20. Bars= 50 µm.

Relative to the epifluorescence microscopy, the use of the CW stain allows the morphological characterization of the cell walls of fungi due to its affinity for the β(1–3) and β(1–4) polysaccharides in cellulose, carboxylated polysaccharides and chitin. Thus, the FUN-1 stain acts as metabolic indicator stains provide information on the viability of fungal biofilm (Simões et al., 2015). Moreover, using this technic, was possible to verify different stages of biofilm formation. Harding et al. (2009) purposed a

model for filamentous fungi biofilm formation, consisting in six stages: adsorption, active attachment, microcolony I (germling and/or monolayer), microcolony II (initial maturation), development of the mature biofilm, and dispersal or planktonic phase. In this case, was possible to see some of the stages of biofilm formation. In the Figure 9.a and 9.b may correspond to an initial stage of attachment. At 4 h, was seen the different spores that started to attach to the surface and other spores were germinating. In the Figure 9.c and 9.d, corresponding to 6 h of biofilm formation may correspond to an active attachment phase of the spores, since the spores were germinating and spreading their hyphae through the surface. Relative to the Figure 9.e, 9.f, and the Figure 9.g, and 9.h the fungal biofilm has 8 and 13h of biofilm formation, may correspond to an initial microcolony phase, which can be explained by the formation of a monolayer of mycelia and the germination of different spores (Harding et al., 2009). Until this point, was possible to see several stages of the biofilm formation within 13 h of incubation, showing how fast this fungus can proliferate. Regarding the Figure 9.i, 9.j 9.k, 9.l, which correspond to 30 and 48 h of biofilm formation, the biofilm was so thick that is difficult to get good images. These images may correspond to a mature biofilm, and it was possible to find some brighter and thicker dots compared to the mycelia and may correspond to sexual structures such as chlamydospores (Harding et al., 2009; He et al., 2021). Lastly, the Figure 9.m and 9.n may also correspond to a mature biofilm, with 61 h of incubation, and it showed some sexual structures that might correspond to the formation of the conidia that will transform into spores.



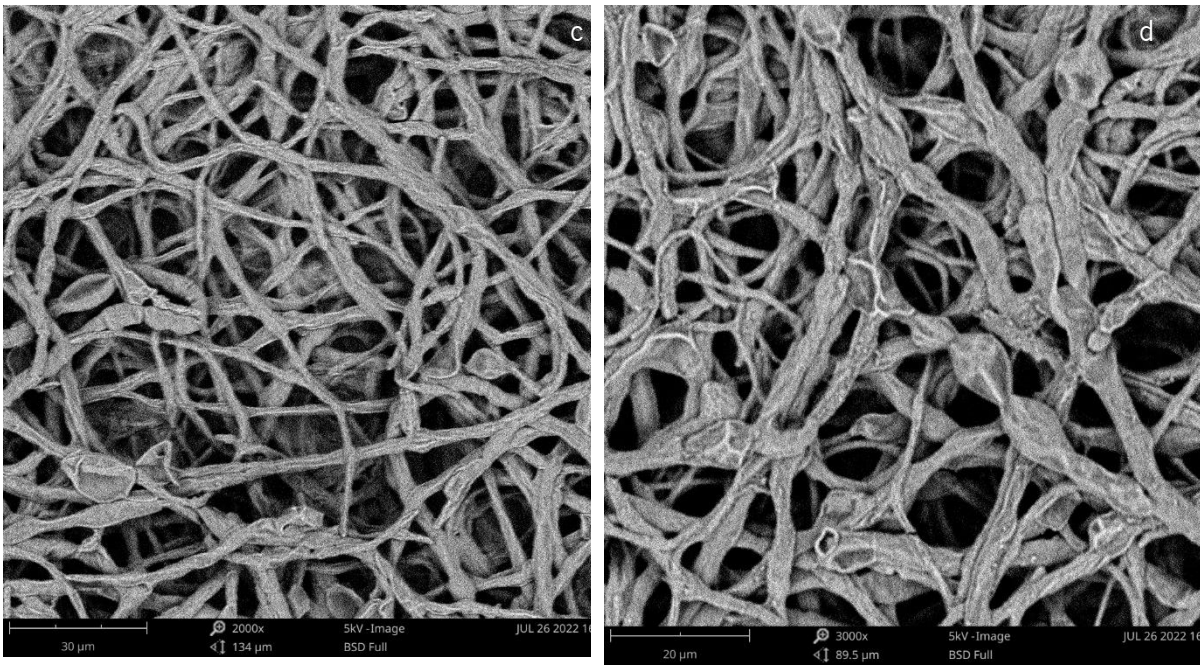


Figure 10: SEM photomicrographs of *Alternaria* sp. (MUM02.42) biofilm formation on polystyrene over time:(a) 4 h. x500 magnification; bars= 100 µm; (b) 8 h. x1000 magnification; bars= 80 µm; (c) 14 h. x2000 magnification; bars =30 µm and (d) 48 h. x3000 magnification; bars =20 µm

Due to the rapid growth and proliferation of the fungus, and the thick biofilm that he forms, SEM microscopy technics were used to get higher resolution of different structures that the biofilm can present. In the figure 10.a was possible to see different spores at different stages, where some of them were in an attachment phase and other spores started to germinate and form hyphae (Harding et al., 2009). Since the *Alternaria* sp. spores were septate, was possible to see one spore form several hyphae (Pitt & Hocking, 1997). In the figure 10.b, the fungus was forming a monolayer of the biofilm and presented some uncommon structures, specially at the end to the hyphae. This might correspond, as in plants, to a zone of high proliferation and growth of cells, making the hyphae and, consequently the mycelia grow. Relative to the figure 10.c, this stage of the biofilm might correspond to the mature biofilm and already showed some sexual structures such as chlamydospores (Pitt & Hocking, 1997). Lastly, the figure 10.d showed a biofilm with 48 h of incubation and must correspond to a mature biofilm. In this image, was possible to see some sexual structures, such as chlamydospores and some conidia at the end of the hyphae.

3.2. Influence of biotic and abiotic factors on the biofilm formation.

Regarding the different biotic and abiotic factors were evaluated to try to understand the behavior of the biofilm. The fungus chosen was the *Alternaria* sp. (MUM 02.42) and the abiotic factors were the

hydrodynamics, the nutrients concentration, and the presence/absence of disinfectant. Relative to the biotic factor, the bacteria chosen for this part was the *Stenotrophomonas maltophilia*.

3.2.1. Hydrodynamics

In terms of hydrodynamics factor, four conditions were evaluated (Static; 30 rpm; 150 rpm; and 200 rpm). These conditions were chosen in an attempt to try to simulate the different hydrodynamics forces, stresses that biofilm can suffer in some parts of DWDS (Afonso et al., 2020; Krsmanovic et al., 2021). The assays were executed at room temperature, for three days and were created three measure points to evaluate the metabolic activity and the biomass formed (t=24h; t=48h; and t=72h) in R2B medium. Figure 11 shows the biomass of the *Alternaria* sp. biofilms formed for at different hydrodynamic conditions over time.

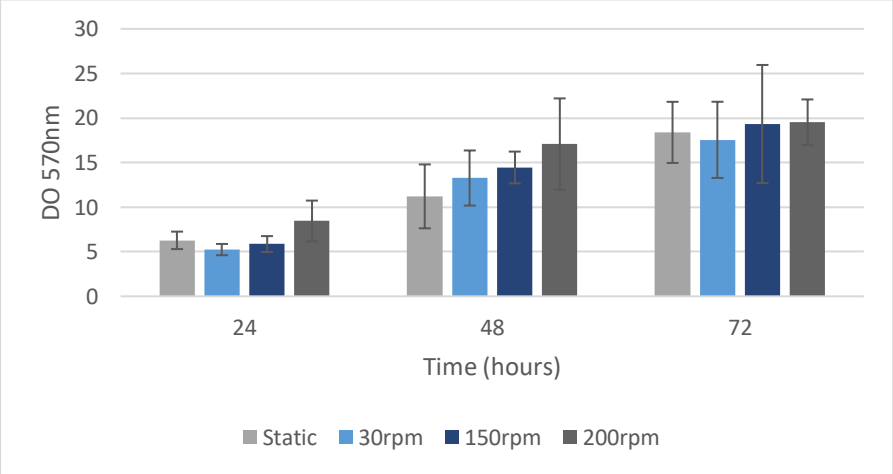
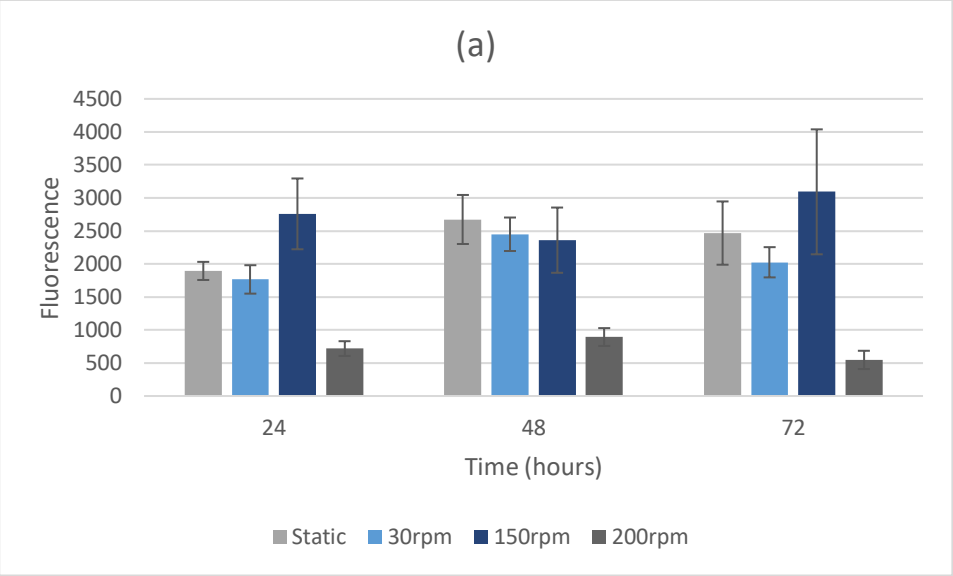


Figure 11: Biomass productivity in terms of OD_{570nm} values for *Alternaria* sp. biofilm formation over time (t=24 h, t=48 h, and t=72 h) for the different conditions considered for the hydrodynamics factor. The mean ± SDs for two independent experiments are illustrated.

In general, the biomass productivity increased at each measure point for each condition. The condition that showed the highest of biomass productivity is 200 rpm and the value is 19.517 ± 2.565. Relative to the other conditions, at 72 h the static experiment got a value of biomass quantification of 18.387 ± 3.430; the 30 rpm got a value of 17.549 ± 4.272 and the 150 rpm got a value of 19.326 ± 6.623. This biomass increase can indicate that the fungus was in a lag phase, which means that the fungus was using is energy to biofilm formation. To understand if there was a significance different between the conditions examined, an ANOVA test was made and the 200 rpm condition shows a significance difference ($\rho<0,05$) compared to the static and 30 rpm conditions. This can indicate that *Alternaria* sp. formed more biomass at higher rotations rather than lower rotations or static conditions

since the biomass values increased with the increase of the rotation. This can be explained due to higher aeration rate that the 200 rpm condition may have in comparison to the other conditions. Besides, the comparison among the other three conditions (static; 30 rpm; and 150 rpm) showed that they do not have a significance difference (ρ>0,05) and the comparison between 150 rpm condition and 200 rpm condition had no significant difference (ρ>0,05). This suggest that the growth of the fungus and, consequently the biofilm formation did not suffer influence of hydrodynamics forces and the *Alternaria* sp. can grow normally in these different conditions. In the DWDS, low hydrodynamic stress and availability of nutrients could support high filamentous fungi adhesion such as, reservoirs, corners, valves, dead ends, and zones with low DW consumption. These zones are potential places for adhesion of ff spores due to low hydrodynamic conditions and high residence times (Fernandes et al., 2019).

Figure 12 shows the metabolic and specific metabolic activity of *Alternaria* sp. biofilms formed at different hydrodynamic conditions over time.



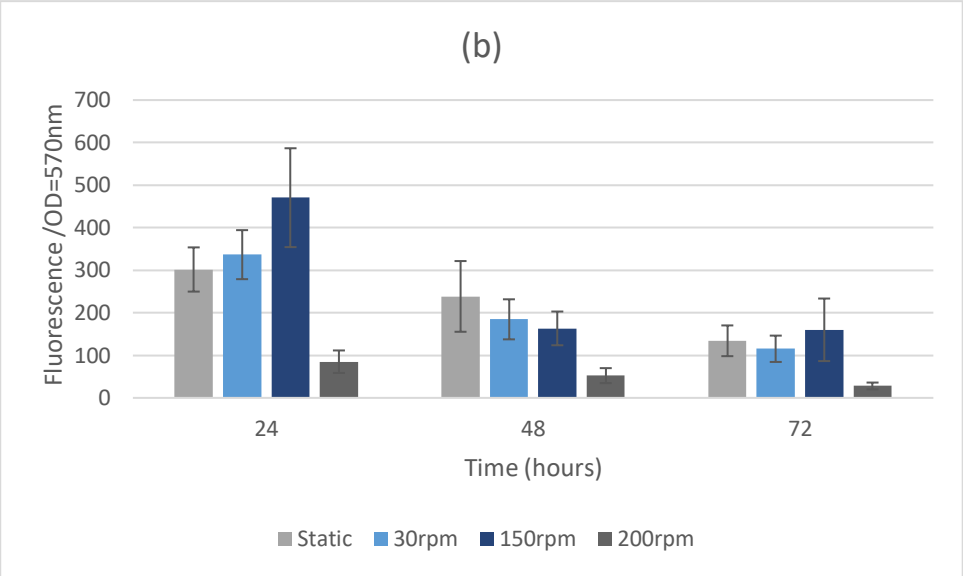


Figure 12: Metabolic activity(a) and specific metabolic activity (b) for *Alternaria* sp. biofilm formation over time (t=24 h, t=48 h, and t=72 h) for the different conditions considered for the hydrodynamics factor. The mean ± SDs for two independent experiments are illustrated.

In relation to the metabolic and specific metabolic activity, the behavior differed between conditions. Relative to the metabolic activity, the static, the 30 rpm, and the 200 rpm condition showed an increase until the second day and then a decrease of metabolic activity. The higher values presented in these three conditions were at 48 h, which were 2673.785 ± 371.343; 2449.642 ± 253.679; and 893.149 ± 135.063 respectively. About the 150 rpm condition, this showed a different behavior since the metabolic activity decreased in the second day and increased again in the third day. At 72 h this condition presented the highest values of metabolic activity of 3092.382 ± 946.210. Regarding this parameter, the 200 rpm showed the lower values of metabolic activity, whereas the 150 rpm showed the highest values of metabolic activity.

Relative to the specific metabolic activity, is noticed a decrease in all conditions through time. Comparing the different figures, it is noticed that the conditions that resulted with higher biomass had a lesser specific metabolic activity. This can indicate that in the beginning the microorganism metabolism was directed to proliferation and growing of the microorganism. This type of behavior is related in some study of fungi biofilm such as Simões et al. (2007) where the specific metabolic activity of the single and interkingdom biofilms decreased when the biomass raised.

The same way in the biomass quantification, an ANOVA test was run to understand if it has significant changes among the different conditions. In this case, the results showed that are significant differences between the 200 rpm and the other conditions ($\rho<0,05$). This means that the hydrodynamics

forces influenced the metabolism of this fungus. Comparing the figures, this factor may influence in a negative way, since the values of metabolic activity decreased in each condition at the end. Lastly, comparing with the ANOVA results of the biomass, is possible to defer that the hydrodynamics forces tend to influence more the metabolic activity rather than the biomass productivity.

3.2.2. Nutrients

Relative to the concentration of nutrients, four conditions were evaluated (STW, 1/4 R2B, 1/2 R2B, and R2B without dilution). These conditions were chosen in an attempt to try to simulate the different nutrient's concentrations that the fungus has to adapt in the DWDS, for example some places with little concentrations or none of nutrients or a normal concentration of nutrients. (Luo et al., 2021). The assays were executed at room temperature, for three days and were created three measure points to evaluate the metabolic activity and the biomass formed (t=24h; t=48h; and t=72h) to all tested conditions Figure 13 shows the biomass of the *Alternaria* sp. biofilms formed for the different conditions over time.

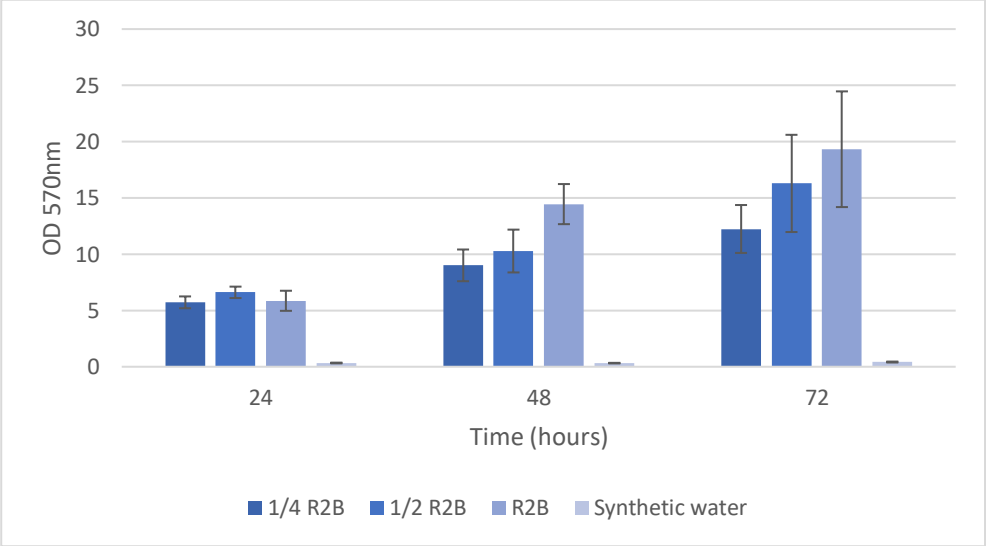


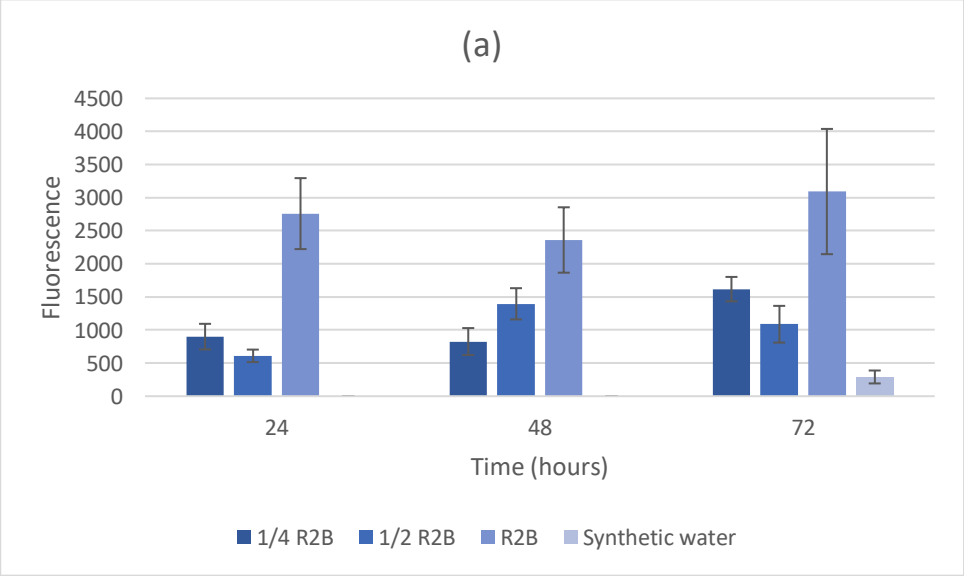
Figure 13: Biomass productivity in terms of OD_{570nm} values for *Alternaria* sp. biofilm formation over time (t=24 h, t=48 h and t=72 h) for the different conditions considered for the nutrients factor. The mean ± SDs for two independent experiments are illustrated.

In general, the biomass productivity increased at each measure point for each condition (Figure 13). The condition that showed the highest of biomass productivity was R2B medium without dilution and the value is 19.326 ± 5.140. Comparative to the other conditions, at 72 h the ¼ R2B experiment got a value of biomass quantification of 12.241 ± 2.129; the ½ R2B got a value of 16.288 ± 4.318 and the STW got a value of 0.419 ± 0.042. This increase of biomass can indicate that the fungus was in a lag phase, which means that the fungus was using is energy to biofilm formation. Moreover, it can be implied

that the experiment where the concentration of nutrients was higher, greater the biomass productivity was. Luo et al. (2021) studied three different fungi that were present in groundwater and studied their behavior when exposed different influencing factors and one of them was different concentrations of nutrients. He concluded that higher the concentration, more biomass was produced, which occurs during our experiments.

To understand if there was a significance different between the conditions examined, an ANOVA test was made and the STW condition showed a significance difference ($\rho>0,05$) compared to the rest conditions examined. This can indicate that *Alternaria* sp. formed less biomass at lower concentrations of nutrients or even in the absence of them, such the STW medium, they present little biofilm formation and values presented may only correspond to the fungus adhesion to the surface. This is corroborated by Siqueira & Lima. (2013) where in their study the *Alternaria sp.* was the only fungus capable of forming biofilm in STW medium, representing its high adaptation to oligotrophic conditions. Besides, the comparison among the other three conditions (1/4R2B; 1/2R2B; and R2B) showed that they did not have a significance difference ($\rho<0,05$). This suggest that the growth of the fungus and, consequently the biofilm formation did not suffer influence within this range of different concentrations of nutrients and the *Alternaria* sp. can grow normally in these different conditions.

Figure 14 shows the metabolic and specific metabolic activity of *Alternaria* sp. over time.



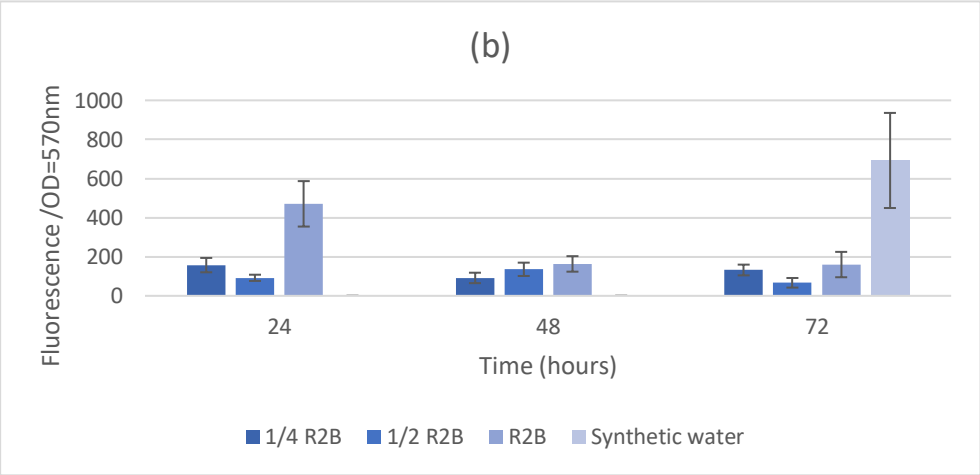


Figure 14: Metabolic activity (a) and specific metabolic activity (b) for *Alternaria* sp. biofilm formation over time (t=24 h, t=48 h, and t=72 h) for the different conditions considered for the nutrients factor. The mean ± SDs for two independent experiments are illustrated.

In relation to the metabolic and specific metabolic activity, the behavior differs between conditions. Relative to the metabolic activity, the ½ R2B condition showed an increase until the 48 h and then a decrease at 72 h in terms of metabolic activity. The higher values presented in this condition was at 48 h, which was 1395.926 ± 236.153. The other two conditions (1/4 R2B; R2B) had a different behavior since the metabolic activity decreased in the second day and increased again in the third day. At 72 h these three conditions presented the highest values of metabolic activity of 1617.736 ± 184.339, and 3092.382 ± 946.210 respectively. Relative to the STW condition, the fungus did not show metabolic activity for two days and in the last day showed a value of metabolic activity of 290.143 ± 102.171.

In terms of the specific metabolic activity, the fungus behavior was different between conditions. The ¼ R2B showed a decrease at 48 h and then an increase at 72 h, whereas the ½ R2B conditions showed an increase of specific metabolic activity in the second day and then a decrease. The R2B condition, showed a decrease of specific metabolic activity during all experiment. However, the STW condition only had specific metabolic activity in the last day. By analyzing these figures is possible to imply that the fungus in these measure points was already in an exponential phase with an increase of biomass productivity and high values of metabolic activity in the conditions with R2B medium. This can indicate that in the beginning the microorganism metabolism was directed to proliferation and growing of the microorganism. This type of behavior is related in some study of fungi biofilm where in the first three days the fungi are in an exponential phase (Luo et al., 2021). Relative to the STW condition, it indicates that there was no biofilm formation, and the metabolic activity was low.

An ANOVA test was run to understand if it had significant changes among the different conditions. In this case, the results showed that were significant differences between the STW condition and the other ones ($\rho<0,05$) and the R2B condition to the other ones. This means that the nutrients' concentration influenced the metabolism of this fungus and comparing to the figures, the lower concentration of nutrients may influence in a negative way, since the values of metabolic activity and biomass decreased in lower nutrients' concentration medium. It's possible to verify that in the STW water, the metabolic activity and biomass was low which means the fungus was using the few nutrients in the environment to maintain itself and not proliferate. In contrast, the R2B condition showed a higher value of biomass and metabolic activity, meaning the fungus was directing these nutrients to the proliferation and biofilm formation when higher the nutrients' concentration, greater the metabolic activity presented by the fungus.

3.2.3. Presence of disinfectant

Another factor evaluated was the influence of chlorine in the growth and metabolic activity of the fungus, where five different conditions were examined (without chlorine, 2.4 mg/L, 6.03 mg/L, 12.06 mg/L, and 24.12 mg/L). These conditions were chosen in an attempt to understand if the chlorine, the most used method to disinfect and inhibit the microorganisms growth in the DWDS (Sonigo et al., 2011), has influence on this particular fungus. The assays were executed at room temperature, for three days and were created three measure points to evaluate the metabolic activity and the biomass formed (t=24 h, t=48 h, and t=72 h) in R2B medium.

Figure 15 shows the biomass of the *Alternaria* sp. biofilms formed for the different conditions over time.

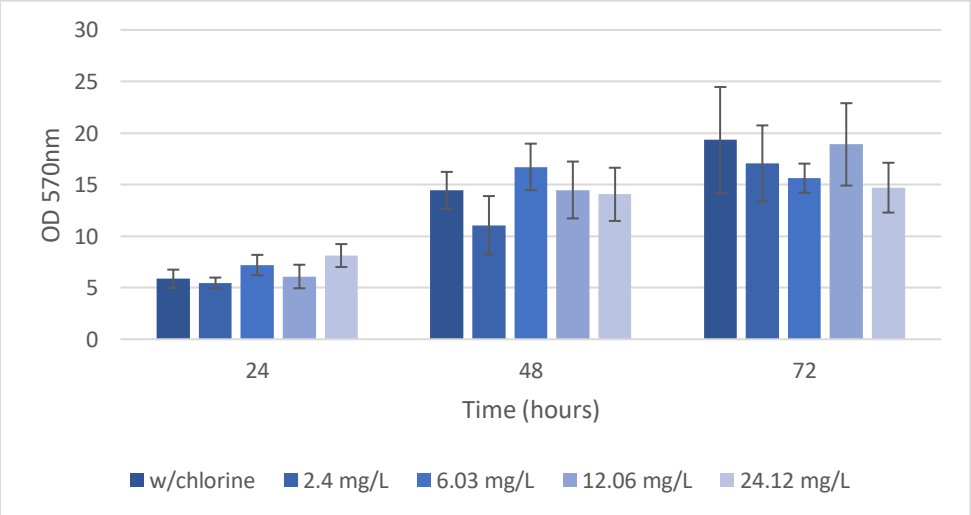


Figure 15: Biomass productivity in terms of OD_{570nm} values for *Alternaria* sp. biofilm formation over time (t=24 h, t=48 h, and t=72 h) for the different conditions considered for the presence of disinfectant factor. The mean ± SDs for two independent experiments are illustrated.

In general, the biomass productivity increased at each measure point for each condition. The condition that showed the highest of biomass productivity was the control group and the value was 19.326 ± 5.140. Comparative to the other conditions, at 72 h the experiment with 12.06 mg/L got a value of biomass quantification of 18.897 ± 3.995; the 6.03 mg/L got a value of 15.620 ± 1.413; the 2.4 mg/L got a value of 17.044 ± 3.693 and the 24.12 mg/L condition got 14.701 ± 2.416. This increase can point out that the fungus was in an exponential phase, which means that the fungus was using energy to biofilm formation. Comparing the values between conditions, was verified that the biomass quantification tend to be lower in the assays with higher concentration of free chlorine, apart from 12.06 mg/L assay where the biomass productivity surpassed the other assays, with exception of the control group. Luo et al. (2022) studied the influence of different concentrations of residual chlorine in different fungi and all have a similar trend like *Alternaria* sp., which is higher concentration of chlorine in the medium less the biomass production and biofilm formation.

To understand if there was a significance different between the conditions examined, an ANOVA test was made and there were no significant differences among the assays ($\rho>0,05$). This can mean that the different concentrations of free chlorine did not affect the biomass productivity and biofilm formation. Since the normal residual concentrations of chlorine in the DWDS have a range value lower than the concentrations used in this assay, this means that the *Alternaria* sp. is not affected by the chlorine in the DWDS and might cause problems to human health if proliferate in the system. In some studies, using different fungi, showed that fungi can be resistant to free chlorine, for example in Ma & Bibby (2017). They conclude that the fungus studied in their report is resistant to the free chlorine concentrations that they use (varying from 1mg/L to 5mg/L) and this behavior can explain the resistance of the *Alternaria* sp. to chlorine. Pereira et al. (2013) had similar results and verified that some fungi are resistant to the free chlorine used in the DWDS and one reason to explain to this resistance can be the chemical change of the fungus cell wall (Ma & Bibby, 2017).

Figure 16 shows the metabolic and specific metabolic activity of *Alternaria* sp. over time.

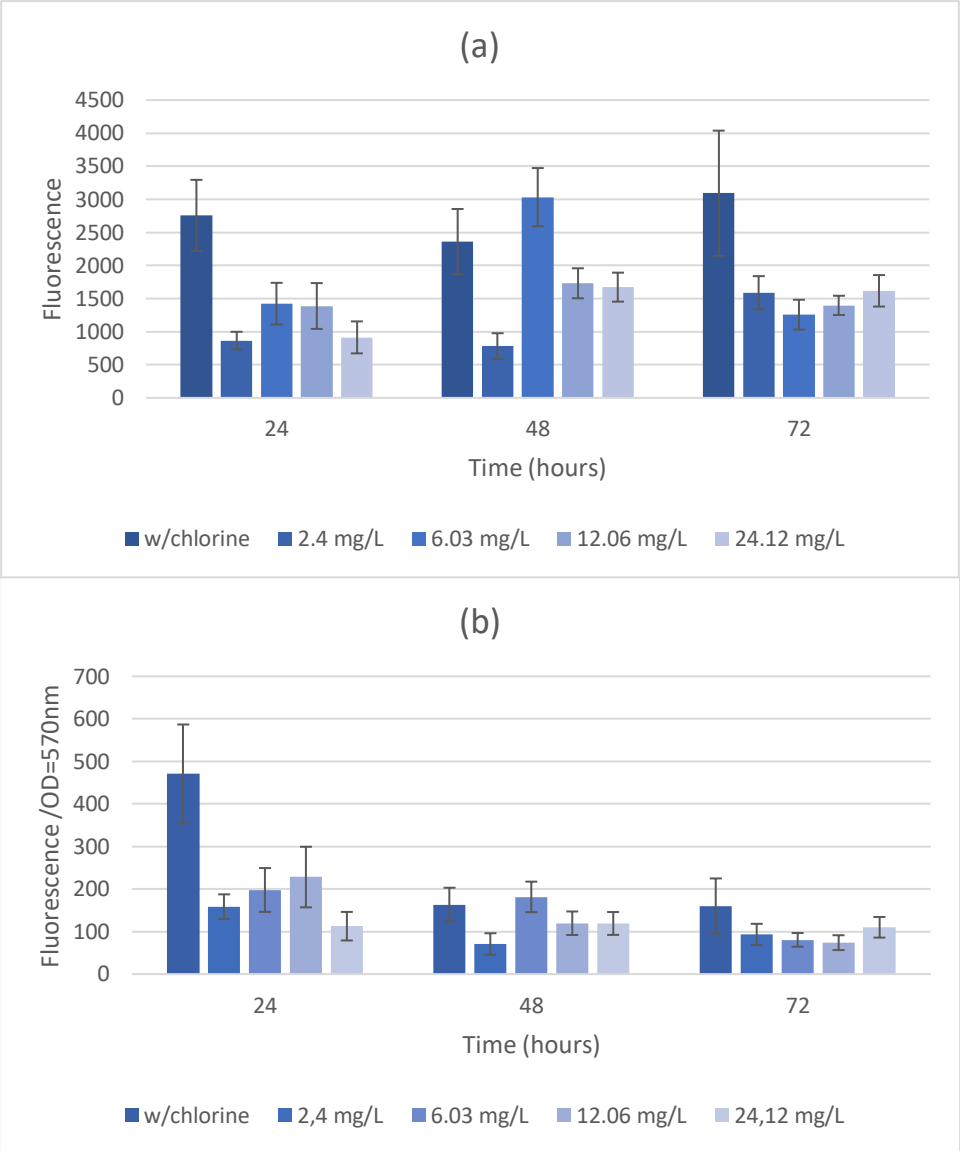


Figure 16: Metabolic activity (a) and specific metabolic activity (b) for *Alternaria* sp. biofilm formation over time (t=24 h, t=48 h, and t=72 h) for the different conditions considered for the presence of disinfectant factor. The mean ± SDs for two independent experiments re illustrated.

In relation to the metabolic and specific metabolic activity, the behavior differed between conditions. Relative to the metabolic activity, the w/chlorine and the 2.4 mg/L conditions showed a decrease until the second day and then an increase of metabolic activity. The higher values presented in these two conditions were at 72 h, which were 3092.382 ± 946.210 and 1588.386 ± 251.628 respectively. The other three (6.03 mg/L, 12.06 mg/L, 24.12 mg/L) conditions showed a different behavior since the metabolic activity increased in the second day and decreased again in the third day. At 48 h this condition presented the highest values of metabolic activity of 3032.744 ± 439.593, 1731.693 ± 226.710 and 1673.438 ± 219.329 respectively.

Relative to the specific metabolic activity, was noticed a decrease in all conditions through time. Comparing the different figures, it was noticed that the conditions that resulted with higher biomass had a lesser specific metabolic activity. This can indicate that in the beginning the microorganism metabolism was directed to proliferation and growing of the microorganism. This type of behavior is seen through the factors already analyzed and can be related in some studies of fungi biofilm such as Simões et al. (2007) and Luo et al. (2022) where the specific metabolic activity of the single and interkingdom biofilms decreased when the biomass raised.

The same way in the biomass quantification, an ANOVA test was run to understand if it had significant changes among the different conditions. In this experiment, the results showed that were significant differences between the w/chlorine assay and the others ($\rho<0,05$) and significant differences between 2.4 mg/L assay and the others ($\rho<0,05$). This means that the residual concentration of chlorine influenced the metabolism of this fungus. Moreover, comparing the ANOVA's results of the biomass and the metabolic activity its verified that the chlorine concentration only influenced the metabolic activity, meaning that the chlorine might influence certain metabolic pathways that do not affect the proliferation of the fungus.

Additionally, the effectiveness of the chlorine was calculated to evaluate the biofilm removal and biofilm inactivation. The following table shows the values in percentage of the biomass removal and the biofilm inactivation.

Table 3- Results of the percentage biomass removal and biofilm inactivation for *Alternaria* sp. biofilms developed in presence of several chlorine concentrations. The values with * means that the biomass and metabolic activity values were higher or equal to the control (w/chlorine).

[Cl] (mg/L)	Biofilm mass removal (%)			Biofilm inactivation (%)		
Time (h)	24	48	72	24	48	72
2.4	6.94	23.51	11.81	68.62	66.84	48.64
6.03	0.00*	0.00*	19.17	48.41	0.00*	59.34
12.06	0.00*	0.00*	2.22	49.67	26.63	54.80
24.12	0.00*	2.75	23.93	66.86	29.10	47.69

Analyzing the results obtained in the biomass removal part, it is possible to ascertain that the *Alternaria* sp. showed different behaviors depending on the free chlorine concentration. Most assays only started to show effectiveness at 72 h, where the higher percentage of biomass removal corresponds to the higher concentration of chlorine. However, the 2.4 mg/L experiment showed a biomass removal at 24 h. These results suggest that, in general, *Alternaria* sp. may not suffer the influence of chlorine in

terms of biofilm formation, and this can be corroborated by the ANOVA test (ρ>0,05). Even though there are no studies about the influence of chlorine in *Alternaria* sp.' biofilm formation, compared to others studies relative to other fungi, the percentages of biomass removal are higher in other fungi. Luo et al. (2022) studied the influence of disinfectants in three fungi species (*Aspergillus niger; Penicillium polonicum;* and *Trichoderma harziadum*) and the percentages of biomass removal in 1 mg/L of chlorine were between 23.6%–27.9%, 31.3%–42.1% and 27.1%–37.3%, respectively. Comparing these values to the *Alternaria* sp.' values is possible to conclude that this fungus might be resistant to the disinfectant due to the low values that present after 72 hours. These differences might reinforce the lower influence of the chlorine on the *Alternaria* sp. biofilm formation.

Concerning the biofilm inactivation values, it is possible to infer that the different concentrations of chlorine started to affect the biofilm since the beginning until the end of the experiment. Moreover, the 2.4 mg/L condition shows higher effectiveness than the other conditions and this is corroborated by the ANOVA test that showed significant differences between this condition and the others (ρ<0,05). These higher values of biofilm inactivation, suggest that *Alternaria* sp. was influenced by the chlorine in some way that is not related to the microorganism proliferation and growth pathways. As can be verified, in the 12.06 mg/L assay there was no biomass removal since the beginning until the end of the experiment, but the biofilm inactivation values are around the 49% and 26%. Therefore, the chlorine may affect some biochemical pathways in the fungus, but not the ones related to the growth and proliferation of the fungus.

3.2.4. Interkingdom biofilm

Regarding the interkingdom factor, where the fungus was inoculated with a bacterium, namely *S. maltophilia*. Three conditions were evaluated (only the fungus (F); only the bacterium (B); and the two species simultaneously (B+F)) and there are two assays of the fungus because one corresponds to the first part values and the second assay corresponds to the second part values. These conditions were chosen in an attempt to try to simulate the inter-kingdoms biofilms , which exist in the DWDS and the interaction between bacteria and fungi can occur (Afonso et al., 2021). The assays were executed at room temperature, for three days and were created three measure points to evaluate the metabolic activity and the biomass formed (t=24h; t=48h; and t=72h) in R2B medium.

Figure 17 shows the biomass of the *Alternaria* sp., *S. maltophilia,* and the multispecies biofilm formed for the different check points at different conditions. The initial spore and bacteria's concentration for the biofilm formation was 10^5 cell/mL and 10^8 cell/mL, respectively.

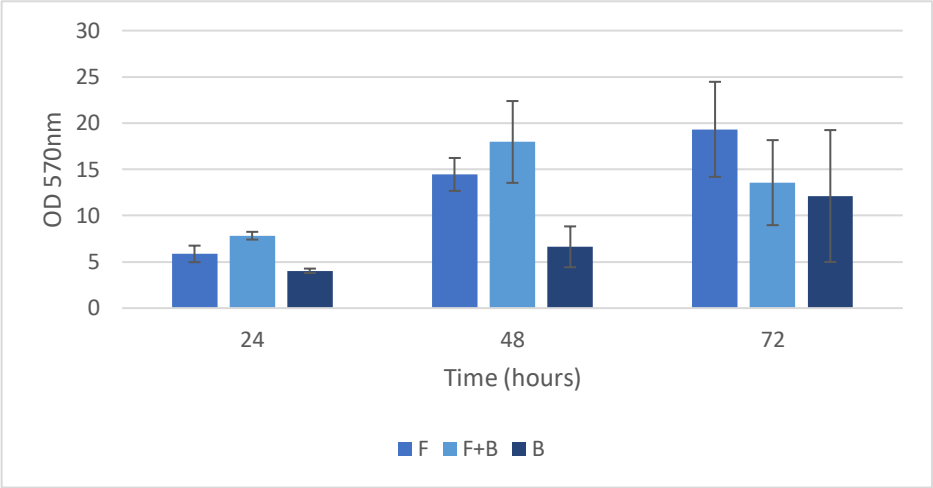


Figure 17: Biomass productivity in terms of OD_{570nm} values for *Alternaria* sp., *S. maltophilia,* and the multispecies assay biofilm formation over time (t=24 h, t=48 h, and t=72 h) for the different conditions considered for the interkingdom factor. The mean ± SDs for two independent experiments are illustrated.

Regarding the biomass quantification, the values increased at each measure point for the single species assays, whereas in the interkingdom assay there was a decrease of biofilm productivity at 72 h. The condition that showed the highest of biomass productivity was the F and the value was 19.326 ± 5.140 at 72 h. Relative to the B condition, at 72 h got a value of biomass quantification of 12.119 ± 7.126. The B+F condition had a different behavior due to the decrease of the biomass productivity at 72 h. The highest value of this condition was at 48 h and got a value of 17.964 ± 4.423. In the F and B assays the increase of biomass indicated that the microorganism was in an exponential phase, which means that the fungus was using energy to biofilm formation. However, the B+F assay showed a decrease which means that the bacterium competed with the fungus for the nutrients, space and proliferation and they might inhibit each other (Gonçalves et al., 2006).

To understand if there was a significant difference between the conditions examined, an ANOVA test was made and the B condition showed a significance difference ($\rho<0,05$) compared to the rest conditions examined. This can indicate that *S. maltophilia* formed less biomass comparing to the fungus and the dual-species biofilm. Besides, the comparison among the other three conditions (F and B+F) showed that they did not have a significant difference ($\rho>0,05$). This suggest that even though the bacterium and the fungus might inhibit the growth of each other and compete for resources, the fungus may be more prevalent to the fungus conditions rather than the bacterium conditions.

Figure 18 shows the metabolic and specific metabolic activity of *Alternaria* sp. over time.

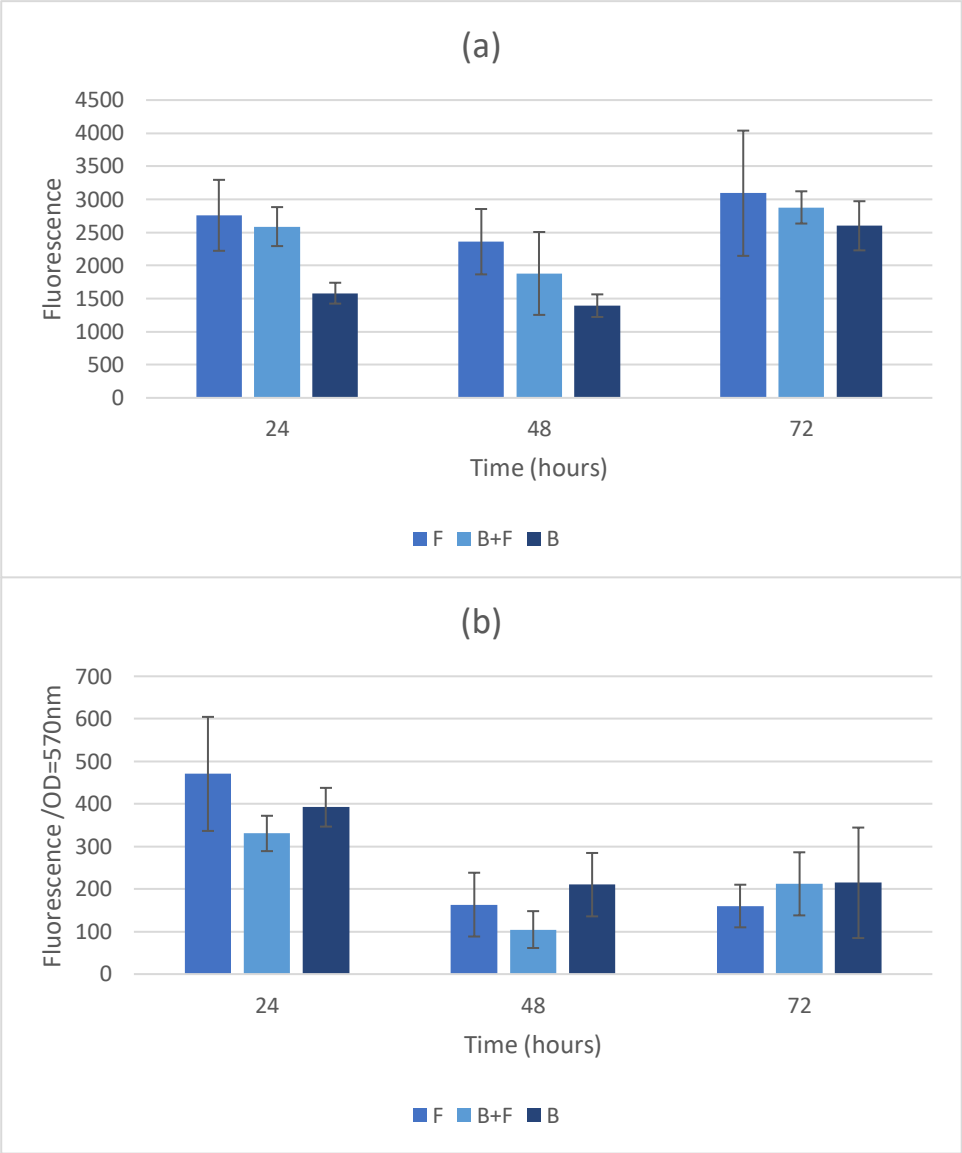


Figure 18: Metabolic activity (a) and specific metabolic activity (b) for *Alternaria* sp., *S. maltophilia,* and the multispecies assay biofilm formation over time (t=24 h, t=48 h, and t=72 h) for the different conditions considered for the interkingdom factor. The mean ± SDs for two independent experiments are illustrated.

In relation to the metabolic and specific metabolic activity, the behavior differed amongst conditions. Relative to the metabolic activity, the F; B+F; and B conditions showed a metabolic activity decrease on the second day and then increased again in the third day. At 72 h this condition presented the highest values of metabolic activity of 3092.382 ± 946.210; 2878.000 ± 243.728; and 2600.556 ± 371.397, respectively. Relative to the specific metabolic activity, is noticed a decrease in the F assays, but in the B and B+F, the specific metabolic activity decreased on the second day and increased on last the day of experiment. Comparing the different figures, it is noticed that the conditions that resulted in higher biomass, such as the F assays, had a lesser specific metabolic activity. This can indicate that in

the beginning the microorganism metabolism was directed to the proliferation and growth of the microorganism. This type of behavior is reported in some studies of fungi biofilms like Simões et al. (2007) where the specific metabolic activity of the single and interkingdom biofilms decreased when the biomass raised. In the case of B+F, is verified a decrease of specific metabolic activity and increase in the last day.

The same way in the biomass quantification, an ANOVA test was run to understand if it had significant changes among the different conditions. In this case, the results showed that are significant differences between the bacterium and the other conditions ($\rho<0,05$). This can result by the fact that the microorganisms were different and presented some differences in the metabolism. Another reason is that the coexistence of these two species influenced each other some way and comparing the results, this coexistence may be unfavorable to the two microorganisms, since the values of metabolic activity and biomass decreased at the end. For example, these two microorganisms may have competed for space and nutrients, and consequently, compromised each other's growth and proliferation. It seems that the fungus had some influence on the bacterium growth and metabolic activity, justifying the statistical differences between the bacterium and the fungus. One reason for that may be a metabolite that the fungus produced to inhibit the bacterium by quorum sensing. Rashmi et al. (2018) studied the anti-quorum sensing effects that *Alternaria alternata* had against *Pseudomonas aeruginosa* and conclude that this fungus can suppress the growth of the bacterium and inhibited the production of several metabolites that can be dangerous if ingested y humans.

Besides the metabolic activity and the biomass productivity, the CFU of the biofilm bacterium, and the CFU of the interkingdom biofilm was calculated and analyzed. The following table shows the CFU/cm^2 of the different biofilms.

Table 4: Results of CFU counts for bacteria biofilm and interkingdom biofilms formation over time.

CFU/cm^2					
B			B+F		
24 h	48 h	72 h	24 h	48 h	72 h
2.45E+07	2.57E+07	4.74E+07	2.63E+07	3.23E+07	5.84E+07

Analyzing the CFU counting results, is possible to ascertain that there was an increase of bacterial CFU over time. The interkingdom had higher values of CFU/cm^2 than the single species biofilm. This can be explained by the fact that in the interkingdom biofilm exists the *Alternaria* sp. which contributes for the CFU counting due to the higher surface area that the fungus provides for the bacterial growth. Moreover,

a Kolmogorov- Smirnov test was executed to find out if there are significant differences between the two conditions and the results indicated there was no differences between them (ρ>0,05).

3.3. Identification of *Mucor* sp. and *Alternaria* sp. species

3.3.1. Sample and PCR electrophoresis gel

After the DNA extraction, the samples were run in the NanoDrop to examine the sample purity and to verify if it was needed to dilute the samples for the PCR. Therefore, the samples were run in the NanoDrop and the ratios of DNA-RNA/protein, corresponding to the 260/280 value and DNA-RNA/residues, corresponding to the 260/230 value. The next table shows the ratio values obtained in the NanoDrop of the samples of *Mucor* sp. (MUM 02.01) *Alternaria* sp. (MUM 02.42).

Table 5: The ratio values obtained of the samples examined to know if it is needed dilutions for the PCR run.

Ratios	MUM 02.01	MUM 02.42
260/280	2.30	2.19
260/230	2.25	1.48
ng/µL	100.1	669.2

Regarding the values, dilutions were made since the standard value to consider a sample pure is 1,8. Considering these values, the dilution made to the MUM 02.01 was 1:10 and to the MUM 02.42 was 1:6. Relative to the MUM 02.01, another dilution was experimented and that was 1:100, but then in the sample gel electrophoresis (Figure 19) the band was thin and almost invisible. Even though, the ratios of these dilution were better, the final selected dilution was 1:10 since presented higher DNA content needed for the PCR cycle. Figure 19 shows the result of the sample electrophoresis gel.

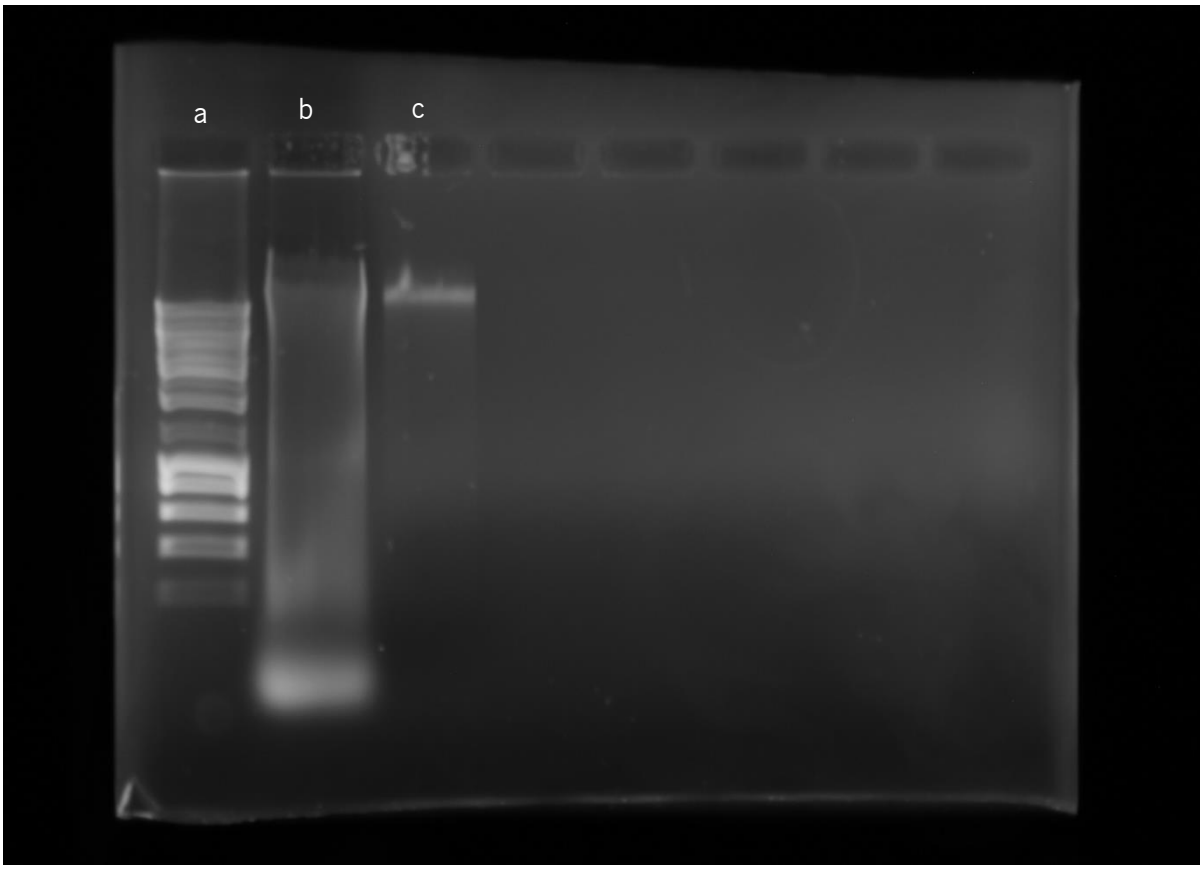


Figure 19: The electrophoresis gel of the sample. In the first well was inoculated (a)the ladder dye, (b) the MUM 02.01+ loading dye sample with a dilution of 1:100, (c) the MUM 02.42 + loading dye sample with a dilution of 1:6.

Is possible to see in the picture the ladder dye with the different bands, corresponding to different molecular weights, and the further the band is from the well less weight the band has. Therefore, is expected that the sample bands stay closer to the well, corresponding to the DNA fragments. This behavior is seen in the MUM 02.42 sample, meaning that the dilution and ratio are good for the PCR cycle, whereas the MUM 02.01 shows a thin band in the same position as the MUM 02.2 sample and a prominent band at the bottom, corresponding to RNA fragments. Due to this bands disposition, the dilution 1:100 was discarded and the 1:10 dilution was chosen.

After this process, the samples were prepared for the PCR cycle, adding the primers (ITS1 and ITS4) to the sample and ultrapure water. At the end, an electrophoresis gel was run to verify if the PCR was successful. Figure 20 shows the electrophoresis gel of the PCR cycle.

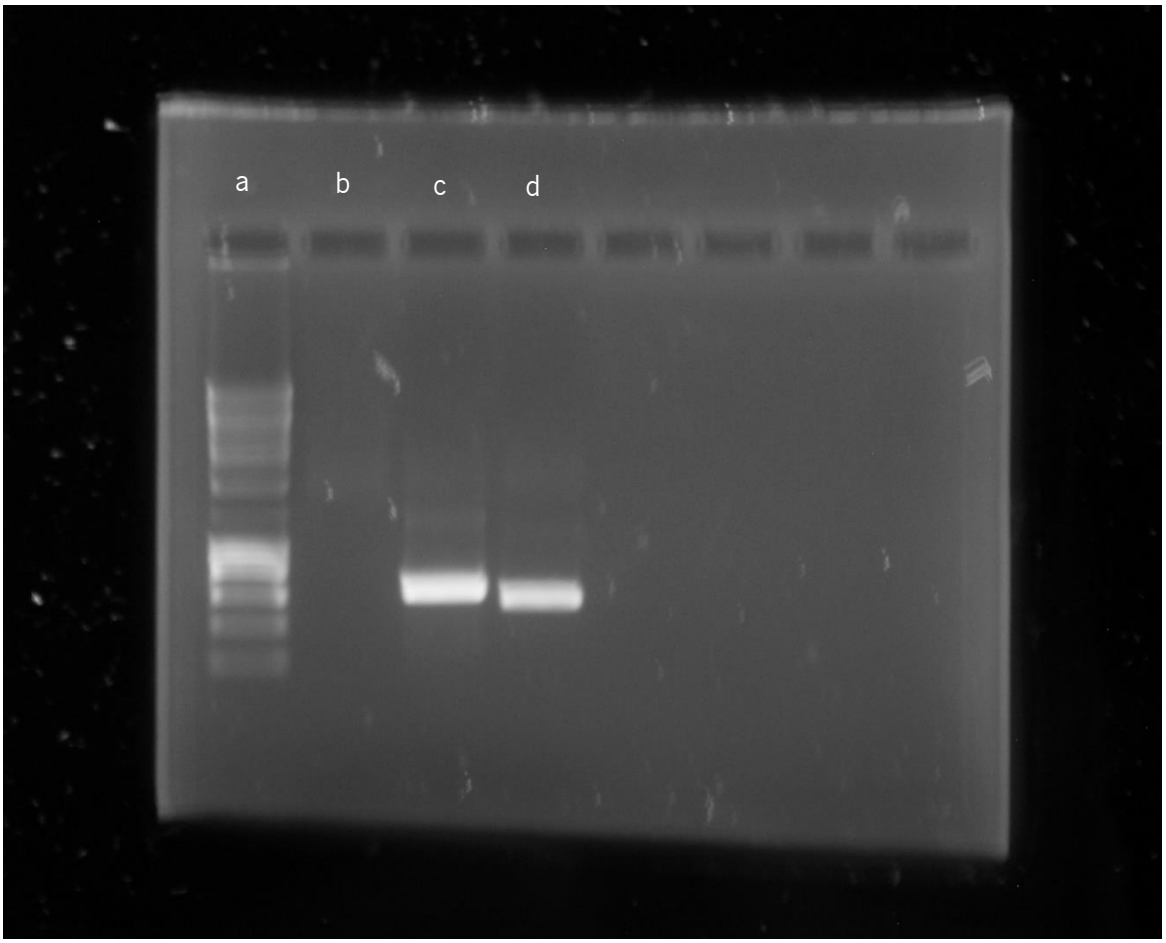


Figure 20: The electrophoresis gel of the PCR cycle. (a) the ladder dye (positive control), (b) the negative control, (c) PCR sample of MUM 02.01, and (d) PCR sample of MUM 02.42.

Regarding the electrophoresis gel, the PCR cycle was successful. In the positive control had amplification of the different bands and in the negative control hadn't any amplification which was expected. Relative to the samples, it is possible to see only one and thick band corresponding to the DNA fragment which is going to be sequenced. The samples were sequenced and treated and using several software and programs, the phylogeny trees were created. Figure 21 shows the phylogeny tree of the MUM 02.01.

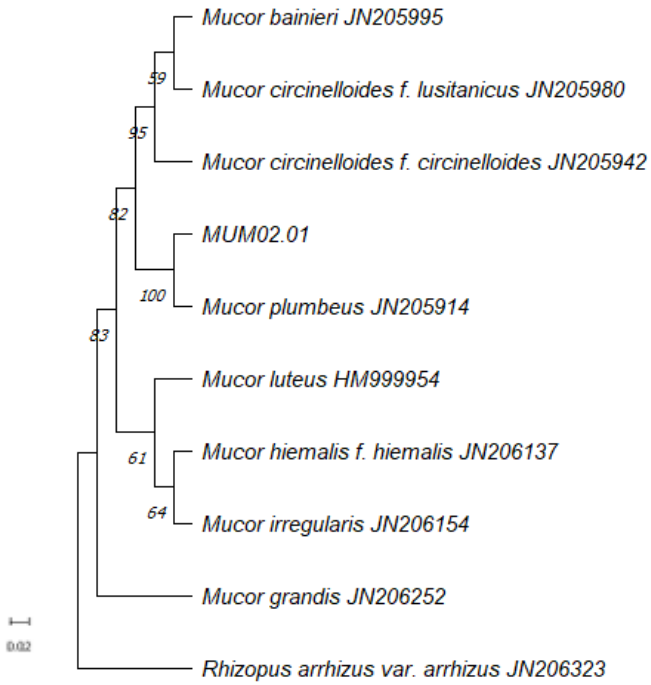


Figure 21: Phylogenetic tree of MUM 02.01 based on the analyzed gene sequence of ITS4 of different species of *Mucor.*

Regarding the fungus MUM 02.01, eight different fungi were used and collected from NCBI database plus de sample to create the phylogeny tree. *Rhizopus arrihizus var. arrhizus* (JN206323) used as the outgroup taxa. The sample had 573 characters and primers used were ITS1 (forward) and ITS4 (reverse). Analyzing the best model that would fit the MUM 02.01 and the gene sequence of the several fungi, the best DNA/Protein model was the Tamura 3-parameter and a gamma distribution. Our sample formed a clade with *Mucor plumbeus* (JN205914) with a bootstrap support of 100 and our sample may be considered a *Mucor plumbeus* as well.

Is possible to infer this statement due to the high reliable the primers ITS-sequences present to identify *Mucor* species (Hurdeal et al., 2021; Walther et al., 2019). This ITS marker usually is a good choice for *Mucor* species identification, although depending on the species used there are better sequences to use in the identification, such as *tsr1* and *rpb1* gene sequences are better for identification when sample is a *Mucor circinelloides* (Walther et al., 2019). Figure 22 shows the phylogeny tree of the MUM 02.42.

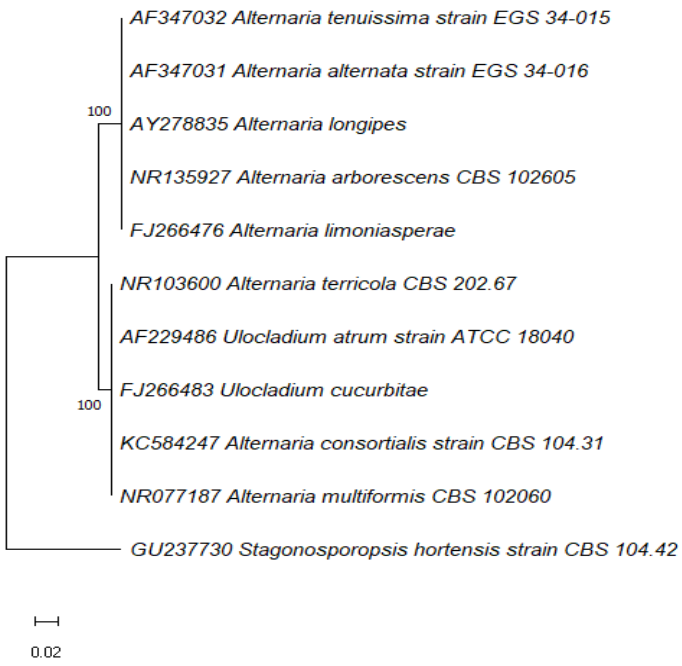


Figure 22: Phylogenetic tree of MUM 02.42 based on the analyzed gene sequence of ITS4 of different species of *Alternaria*

Relative to the fungus MUM 02.42, eleven different fungi were used and collected from NCBI database plus de sample to create the phylogeny tree. *Stagonosporopsis hortensis* strain CBS04.42 (GU237730) used as the outgroup taxa. The sample had 513 characters and primers used were ITS1 (forward) and ITS4 (reverse). Analyzing the best model that would fit the MUM 02.42 and the gene sequence of the several fungi, the best DNA/Protein model was the Kimura 2- parameter and uniform rate. Our sample formed a clade with five more fungi species with a bootstrap support of 100, making it difficult to suggest a definitive species to our sample. Therefore, our sample is considered to belong to the section *Alternata.*

Due to the high homogeneity among *Alternaria* genus, there is some difficulty to identify species of the fungus, being more common to get the section identification. There are some studies which use different genetic marks (such as ITS, TEF 1-α, rDNA, ATPase, and *Tsr1*) in an attempt to identify the Alternaria species provided support to reach the phylogenetic species group, but only reach to the section-species identification (Lawrence et al., 2016). The marker used in this study (ITS), which is used and reliable for most fungi species identification, is no so great for *Alternaria* species identification because some species of *Alternaria,* such as *A. alternata* and *A. tenuissima* are morphologically different, but using

this genetic marker results in 100% identical or nearly so, which is uninformative (Lawrence et al., 2013, 2016)

4.Conclusions

The analysis of biofilm formation by the different ff allowed to conclude that fungus *Alternaria* sp*.* was the most active and prolific biofilm former. *Mucor* sp. could not form biofilms under the process conditions tested, due to their hydrophobicity, and consequently, was discarded in the first phase of the studies. The other fungi, *P. expansum, P. brevicompactum, F. oxysporum* and *A. versicolor* presented a slow growth dynamic with a long adaptation phase, beginning the exponential phase after 11 h of incubation. From these, *A. versicolor* presented the lowest biomass values and *P. brevicompactum* the highest biomass values*. A. versicolor* biofilms had negligible metabolic activity between 11 and 24 h, apparently due to environmental stress.

Regarding the effects of different abiotic and biotic factors on biofilm formation and behavior, *Alternaria* sp*.* biofilm formation shows different behaviors depending on the condition studied. In terms of hydrodynamic forces, this factor significantly influenced the biomass productivity at 200 rpm and the metabolic activity differed for any conditions studied. On the other hand, the nutrients concentration showed that the biomass quantification is significantly lower in STW relative to the other conditions and the metabolic activity as well, meaning that in oligotrophic conditions, the growth and behavior of the fungus is affected. No effects were observed from the presence of chlorine, regardless the concentration used, proposing the remarkable tolerance of the fungus to the disinfectant. Lastly, the biotic condition showed that the interkingdom biofilm was significant different from the bacterium biofilm but was not different from the fungus biofilm. Moreover, there was no differences between the CFU of the bacterium single biofilms and interkingdom biofilms.

The work on the identification of MUM02.01 and MUM02.42 species, revealed that the *Mucor* sp. might be a *Mucor plumbeus* and the *Alternaria* sp. might be *Alternaria alternata* section. The genetic marker used in both fungi identifications, was reliable for MUM 02.01 identification, whereas to the MUM 02.42 was not sufficient to identify the species.

5. Future Perspectives

Regarding the kinetics growth and biofilm formation, there is a lack of knowledge for fungi growth and behavior in DWDS system. Therefore, there is some work there is needed in this area, such as the standardization of the procedures and assays to easily compare results among the scientific community. Moreover, the study of other fungi that are capable of biofilm formation and can be pathogenic to human health and put in cause the water quality is crucial to understand their behavior and then provide a way to avoid its proliferation in DWDS system .

Related to the *Alternaria* sp. used in this work, it might be crucial to study other abiotic factors that can influence the biofilm formation and the fungi growth. Examples of abiotic factors that can be studied are the several surfaces that exists in the DWDS and assess the influence that they may have on biofilm formation. On the other hand, the presence/absence of disinfectant on the environment to evaluate if the fungi can form or not biofilm.

Lastly, relative to the identification one way to improve and narrow the identification of the species fungi may be using different genetic markers to diverge the sample to a simple clade.

6.References

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