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Interactions between *Penicillium brevicompactum/Penicillium expansum* and *Acinetobacter calcoaceticus* isolated from drinking water in biofilm development and control

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ABSTRACT

Bacteria and filamentous fungi (ff) are commonly encountered in biofilms developed in drinking water (DW) distribution systems (DWDS). Despite their intimate ecological relationships, researchers tend to study bacteria and ff separately. This work assesses the impact of bacteria-ff association in biofilm formation and tolerance to chlorination. One strain of *Acinetobacter calcoaceticus* isolated from DW was used as a model bacterium. *Penicillium brevicompactum* and *P. expansum* isolated from DW were the ff selected. Single species and inter-kingdom adhesion and biofilm formation occurred under two shear stress (τ) conditions (0.05 and 1.6 Pa). The sessile structures were further characterized in terms of biomass production, respiratory activity and structure. The results showed that 1.6 Pa of shear stress and *A. calcoaceticus*-ff association favoured biofilm production. Inter-kingdom biofilms produced more biomass than *A. calcoaceticus* single species and reduced *A. calcoaceticus* susceptibility to disinfection, particularly to high sodium hypochlorite (SHC) concentrations. In addition, *P. brevicompactum* formed single species biofilms highly resistant to removal and inactivation by SHC. The presence of *P. brevicompactum* or *P. expansum* in inter-kingdom biofilms significantly decreased SHC removal and inactivation effects in comparison to the bacterial biofilms alone, proposing that using bacteria to form biofilms representative of DWDS can provide inaccurate conclusions, particularly in terms of biofilm production and susceptibility to disinfection.

1. Introduction

Drinking water (DW) is not thought of as a sterile product and can serve as a habitat for diverse microorganisms (Simões et al., 2010a; Simões and Simões, 2013). The water that leaves a treatment plant typically has acceptable quality, but it decreases along with the DW distribution system (DWDS) where biofilms can accumulate on the surface of pipes (Fish and Boxall, 2018; Ling et al., 2018). This happens even in the presence of residual levels of a disinfectant, aiming to guarantee that the DW is delivered without pathogens. Pathogenic microorganisms in biofilms are protected from stress conditions (*i.e.* chlorine, shear stress, temperature, etc), which allows them to remain viable (Huq et al., 2008; Zhang et al., 2021). When the detachment of portions of biofilm occurs microorganisms enter the bulk water, permitting a possible outbreak of disease (Gonçalves et al., 2021). It is well known that biofilms constitute one of the major microbial problems in DWDS that contribute to the deterioration of water quality. However, DW analyses are made from the liquid phase, while about 95 % of the total biomass in water is attached to pipe walls (Flemming et al., 2002). In addition, bacteria are typically assessed for DW microbiological quality monitoring, even if other microorganisms are encountered in DW biofilms, including non-prokaryotic organisms (Buse et al., 2014; Delafont et al., 2016; Ji et al., 2017; Morgan et al., 2016; Simões and Simões, 2013). In particular, filamentous fungi (ff) are among the most disregarded microorganisms in DW biofilm studies (Siqueira et al., 2011; Simões et al., 2015). Ff in DWDS are a source of diverse public health problems, particularly allergies, opportunistic infections and intoxications (Hageskal et al., 2009; Slavin et al., 2015). Their presence in

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DW is increasingly being recognized as an emerging public health issue (Afonso et al., 2021; Babič et al., 2017; Fisher et al., 2018). DW-related and unrelated outbreaks of fungal infections (*i.e.* pneumonia and pulmonary mycoses, sinusitis, endocarditis, onychomycosis, cutaneous and subcutaneous infections, wound infections) are becoming more frequent with mortality rates often higher than 50 % (Afonso et al., 2021; Douglas et al., 2016). Global warming and the decrease in water quality, due to water scarcity, are expected to intensify ff proliferation. These consequences of global warming were already reported by Delgado-Baquerizo et al. (2020) for the proliferation of soil-borne fungal pathogens in large regions of Earth from Asia, Africa, Australia and America.

It is now clear that multispecies/inter-kingdom biofilms are the real scenario in DWDS (Fish and Boxall, 2018; Hull et al., 2019; Ling et al., 2018). However, biofilm studies are typically implemented based on the use of bacterial models. The study of inter-kingdom biofilms will substantially advance the current knowledge on biofilm development and behaviour under realism-based conditions. In this work, single species and inter-kingdom biofilm formation were characterized for two ff (*Penicillium expansum* and *P. brevicompactum*) and one bacterium (*Acinetobacter calcoaceticus*), isolated from the same DWDS (Gonçalves et al., 2006; Simões et al., 2007a), under two shear stress conditions commonly encountered in DWDS (Fernandes et al., 2021; Gomes et al., 2014). The ff were selected based on their prevalence (Gonçalves et al., 2006), while *A. calcoaceticus* was used as a model bacterium due to its increased relevance as an opportunistic microorganism and its association with infections related to hospital water supply (Gales et al., 2001; Gomes et al., 2016). The shear stress is known to affect bacterial biofilm formation and resilience to disinfection (Simões et al., 2022). No data exist on the role of shear stress for ff biofilms. Therefore, this study provides pioneer results on the role of shear stress in ff and inter-kingdom biofilm formation and control. Sodium hypochlorite (SHC) is the most conventional DW disinfectant (Codony et al., 2005; Lee and Nam, 2005; Simões et al., 2010a) and was used to comprehend the effects of inter-kingdom interactions and shear stress on the disinfection effectiveness. Diverse free chlorine concentrations were used (0.1–10 mg/L). These concentrations were below and above the levels recommended by the World Health Organization (between 2 and 4 mg/L) and the recommended minimum concentration-time of 150 mg.min/L (Gallandat et al., 2019; WHO, 2011), to understand the behaviour of the different biofilms to the diverse levels of disinfectant.

2. Materials and methods

2.1. Microorganisms and culture conditions

A. calcoaceticus was isolated from a DWDS and identified by 16S rRNA gene sequencing (Simões et al., 2007a). Bacterial cells were grown overnight in batch cultures using 250 mL shake flasks containing 100 mL of R2A broth (0.5 g/L yeast extract; 0.5 g/L proteose peptone; 0.5 g/L casein hydrolysate; 0.5 g/L glucose; 0.5 g/L starch soluble; 0.3 g/L sodium pyruvate; 0.3 g/L di-potassium hydrogen phosphate; 0.05 g/L magnesium sulfate 7 H_2O) at 25 °C and under agitation (150 rpm) in an orbital incubator (New Brunswick Scientific, I26, Hamburg, Germany). R2A medium is typically used in studies with DW microorganisms (Reasoner and Geldreich, 1985) and favours both bacterial and ff biofilm formation (Fernandes et al., 2019; Simões et al., 2007a). *A. calcoaceticus* was harvested by centrifugation (Eppendorf centrifuge 5810R, Hamburg, Germany) at 3777 × *g* for 12 min, followed by three washes with phosphate-buffered saline (0.1 M PBS, pH 7.2) and resuspended in a certain volume of the same buffer or R2A broth necessary to achieve the bacterial concentration required for each assay.

P. brevicompactum MUM 05.17 and *P. expansum* MUM 00.02 were supplied by Micoteca da Universidade do Minho fungal culture collection (MUM, Braga, Portugal) and were selected based on their high occurrence in the tap water of the north of Portugal (Gonçalves et al., 2006). Both fungi were maintained on Malt Extract Agar (MEA: 20 g/L malt extract; 5 g/L mycological peptone; 20 g/L agar) at 4 °C. Stock solutions of fungal spores were obtained from 7 d-old pure cultures in MEA at 25 °C by flooding the surface of the agar plates with 2 mL TWS solution (0.85 % NaCl plus 0.05 % Tween 80) and rocking gently. The suspension was then homogenized by vortexing (Heidolph Reax 2000, Schwabach, Germany) and used for large scale production of spores (Fernandes et al., 2019). The final suspension of spores was homogenized by vortexing before quantification using a Neubauer count chamber. Several aliquots of spore suspension with 10 % of glycerol were cryopreserved at −80 °C to allow the use of the same suspension of spores in all the assays. Stocks of spore suspensions were resuspended in a certain volume of R2A broth necessary to achieve the required density of spores for each assay.

2.2. Single and inter-kingdom adhesion and biofilm formation

Both adhesion and biofilm formation assays (single and inter-kingdom) were performed using sterile 96-well flat tissue culture plates (polystyrene, Orange Scientific, USA). Microtiter plates are commonly used as the standard platform for studying adhesion and biofilm formation, under strict laboratory conditions providing reliable comparative data, including with DW microorganisms (Gomes et al., 2014; Simões et al., 2010b). For bacterial single species adhesion and biofilm formation, the method described by Gomes et al. (2016) was followed with some modifications. Briefly, *A. calcoaceticus* planktonic cells (1×10^8 cells/mL in R2A broth) were added to at least 16 wells of a sterile 96-well microtiter plate (200 μL per well). To promote bacterial adhesion and biofilm formation, the plates were incubated aerobically in an incubator shaker (New Brunswick Scientific, I26) under 25 or 250 rpm, at 25 °C, for 2 (adhesion), 24 and 48 h (biofilm formation). The fluid flow applied to the sessile cells through the orbital agitation is known to result in uniform application of shear stress across the entire layer, where the majority of the cells are exposed to near maximal shear stress (τ), which can be calculated according to eq. 1:

$$\tau = R\sqrt{\rho\,\mu\,(2\pi N)^3} \quad (1)$$

where R is the radius of orbital rotation (1.25 cm), ρ is the density of the culture medium (997 kg/m^3), μ is the viscosity of the medium (0.89×10^{-3} kg/m.s), and N is the frequency of rotation (rotations/s) (Berson et al., 2008; Dardik et al., 2005). Therefore, 25 and 250 rpm correspond to shear stress values of 0.05 and 1.6 Pa, respectively.

Single ff adhesion and biofilm formation were performed according to Simões et al. (2015). *P. expansum* and *P. brevicompactum* spore suspensions with 1×10^5 spores/mL in R2A broth were used to fill at least 16 wells of a sterile 96-well microtiter plate (200 μL per well). Fungal adhesion was assessed 4 h after incubation (Simões et al., 2015). Biofilm formation was then performed as described for *A. calcoaceticus*. For inter-kingdom adhesion and biofilm formation assays, the 96-well microtiter plate was inoculated with 100 μL of *A. calcoaceticus* (1×10^8 cells/mL) and 100 μL of ff (1×10^5 spores/mL) and the method used for single species adhesion and biofilm formation was followed (Afonso et al., 2020). The initial numbers of bacterial cells and spores were previously validated to study adhesion and biofilm maturation (Simões et al., 2007a, 2015). In this study, bacterial adhesion was assessed after 2 h of incubation while ff spore adhesion was assessed after 4 h of incubation. These incubation times ensured the formation of a monolayer of adhered bacterial cells or spores, avoiding the presence of a three-dimensional structure from the active bacterial growth or spore germination and the production of extracellular polymeric substances encountered in the subsequent biofilm development process, as implemented in previous studies (Afonso et al., 2019; Fernandes et al., 2019; Simões et al., 2007a, 2010a, 2015). Biofilms were allowed to form for 24 and 48 h at 25 °C (Afonso et al., 2020). These incubation times allowed to understand the ability of *A. calcoacticus* and ff to adhere and develop biofilms under the process conditions tested.

For all conditions tested and incubation periods, to remove non-adhered and weakly adhered cells, the content of each well was withdrawn and the wells were washed three times with 250 μL of sterile distilled water. Then, the plates were used for the determination of *A. calcoaceticus* colony forming units (CFU) or air dried for 30 min and analysed in terms of biomass on the surfaces of the microtiter plates and the respiratory activity (Afonso et al., 2019; Gomes et al., 2016; Simões et al., 2015). The morphology of biofilms was characterized by epifluorescence microscopy.

Single and inter-kingdom biofilms (48 h-old) were further tested for disinfection with sodium hypochlorite (SHC).

Negative controls for all assays were obtained by incubating the wells only with R2A broth without adding any bacterial cells/ff spores. All the experiments were independently repeated at least three times.

2.3. Biofilm disinfection

Biofilm disinfection was performed according to Gomes et al. (2016). A stock solution of SHC was prepared by diluting a commercially available solution (Sigma, Sintra, Portugal) in sterile distilled water. The working SHC solutions with the desired free chlorine concentrations (0.1, 0.5, 1 and 10 mg/L) were prepared on the day of use, under aseptic conditions, and stored in the dark at 4 °C. The free chlorine concentration was determined using the HI96701 free chlorine portable photometer (Hanna Instruments) based on the DPD (N,N-diethyl-*p*-phenylenediamine) colourimetric method (Meireles et al., 2017). The free chlorine levels ensured concentration-time factors of 6, 30, 60 and 600 mg.min/L.

Immediately after rinsing, the biofilms (48 h-old) and abiotic control wells (without microorganisms) were exposed to the different free chlorine concentrations. At least 16 wells of the 96-well microtiter plate were filled with 250 μL of each concentration of chlorine. Additionally, untreated biofilm wells (control wells) were characterized for each condition tested. The SHC solutions remained in contact with the biofilms for 1 h, being refreshed every 30 min during the 1 h treatment period. The refreshment of the disinfectant solutions occurred taking into account that the biofilms have typically a high density of cells and low volumes of SHC solutions are applied for treatment (Shakeri et al., 2007; Simões et al., 2010a).

During the disinfection treatment, the microtiter plates were incubated on a shaker under the agitation speed used for biofilm formation. Immediately after treatment, the disinfectant solutions were removed and the wells were rinsed twice with 250 μL of sterile sodium thiosulfate (VWR, Portugal) at 0.5 % (w/v) to quench the SHC activity (Gomes et al., 2016; Simões et al., 2010a). Additionally, the wells were rinsed one time with 250 μL of sterile distilled water. The treated and untreated biofilms were characterized in terms of the biofilm mass and respiratory activity. *A. calcoaceticus* CFU from single and inter-kingdom 48 h-old biofilms were determined after SHC exposure. No CFU counts were performed for ff considering it is microbiologically inaccurate – a mycelial mass may include hundreds of viable ff cells that will appear as a single colony (Kinsey et al., 1998).

2.4. Biomass quantification

The biomass adhered to the inner walls of the 96-well plates was quantified by crystal violet (CV) staining (Fernandes et al., 2019; Gomes et al., 2016; Simões et al., 2007b, 2010b, 2015). For that, the adhered microorganisms and biofilms in the 96-well plates were fixed with 250 μL per well of 98 % methanol (VWR, Carnaxide, Portugal) for 15 min. Afterwards, the 98 % methanol was discarded, the plates were left to dry and then the fixed cells were stained for 5 min with 200 μL/well of 1 % (v/v) CV (Gram colour-staining set for microscopy; Merck, Carnaxide, Portugal). Excess stain was rinsed out by placing the plate under low running tap water. After the plates were air-dried the dye bound to the adhered cells was resolubilized with 200 μL/well of 33 % (v/v) glacial acetic acid (Panreac, Cascais, Portugal). The optical density of the obtained solution was measured at 570 nm using a microtiter plate reader (BIO-TEK, Model Synergy HT, Vermont, USA) and adhesion and biofilm mass were presented as $OD_{570\ nm}$ values. The disinfection results were expressed as biofilm mass removal (BR) percentage calculated using the following equation:

$$BR\ (\%) = \frac{(C - B) - (T - B)}{(C - B)} \times 100 \quad (2)$$

where *B* indicates the average absorbance for the blank wells (negative controls – without microorganisms), *C* indicates the average absorbance for the control wells (untreated biofilms), and *T* indicates the average absorbance for the SHC treated wells.

2.5. Biofilm respiratory activity

Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt) (Sigma-Aldrich, Sintra, Portugal) is a cell-permeable redox indicator dye used to assess the respiratory activity of biofilms and was described as a reliable and reproducible method for evaluating the biofilm susceptibility to antimicrobials (Pettit et al., 2009; Sarker et al., 2007). The staining protocol was optimized for bacteria (Borges et al., 2012) and fungi (Simões et al., 2015) in terms of resazurin concentration and incubation time. For each condition, fresh R2A broth (190 μL) was added to the plates. A volume of 10 μL of resazurin (400 μM) indicator solution was added to each well to obtain a final concentration of 20 μM of resazurin. Plates were incubated at 25 °C, in the dark, for 1 and 18 h, for bacteria and fungi, respectively. For inter-kingdom sessile structures, it was verified that the incubation time required was 1 h. Fluorescence was measured at $\lambda_{excitation} = 530$ nm and $\lambda_{emission} = 590$ nm using a microtiter plate reader (BIO-TEK, Model Synergy HT, Vermont, USA). The specific metabolic activity was presented as the ratio between the biofilm metabolic activities per unit of biofilm mass according to Simões et al. (2007b). The disinfection results obtained were expressed as biofilm inactivation (BI) percentage calculated using the following equation:

$$BI\ (\%) = \frac{(C - B) - (\mathrm{T} - B)}{(C - B)} \times 100 \quad (3)$$

where *B* indicates the average fluorescence for the blank wells (negative controls – without microorganism), *C* indicates the average fluorescence for the control wells (untreated biofilms), and *T* indicates the average fluorescence for the SHC treated wells.

2.6. Epifluorescence microscopy

The bottom of each well of 96-well microtiter plates was inspected by epifluorescence microscopy (Olympus BX51, Tokyo, Japan) using a DNA binding stain, 4,6-diamino-2-phenylindole (DAPI), according to Simões et al. (2015). Washed biofilms were stained with 100 μg/mL of DAPI (Sigma) for 15 min and preparations were stored at 4 °C in the dark until visualization. After staining, biofilm samples were observed under epifluorescence microscopy using UV light equipped with 10×/0.30, 40×/0.75 and 60×/1.25 objective lenses. The optical filter combinations used for DAPI were a 365–370 nm excitation filter, a LP421 nm emission filter and a 400 nm barrier filter. The biofilm images were acquired with a microscope camera using the Olympus CellSens Standard software.

2.7. Culturable bacteria

The number of bacterial cells present in either *A. calcoaceticus* or inter-kingdom biofilms was determined in terms of CFU using a plate count assay upon biofilm release. Briefly, *A. calcoaceticus* single biofilms and inter-kingdom biofilms were grown in a 96- well plate as previously described (Afonso et al., 2019). After each incubation period, the

supernatant was removed, and the plate was washed three times with sterile distilled water. A volume of 200 μL of sterile phosphate buffer saline (pH 7.4) was added to each well and the 96-well plate was covered with the lid and placed into an ultrasonic bath (Bandelin electronic GmbH & Co. KG, Berlin, Germany) for 1 min (5 s sonicate, 10-s interval) at 35 kHz, to release the cells from the surface and to disrupt the biofilm structure (Afonso et al., 2019). Three replicates were used for each sample and bacterial cells were plated onto R2A agar plates for CFU determination. The results are presented as log CFU/cm^2 for biofilm development experiments and log CFU/cm^2 reduction for 48 h-old biofilms exposed to SHC.

2.8. Classification of adhesion/biofilm formation and disinfection efficacy

Bacteria, fungi and their associations were classified for their adhesion and biofilm formation using the scheme of Stepanović et al. (2000): non-adherent/non-biofilm producer (0): OD $\leq$ OD$_c$; weakly adherent/weak biofilm producer (+): OD$_c$ < OD $\leq$ 2 × OD$_c$; moderately adherent/moderate biofilm producer (++): 2 × OD$_c$ < OD $\leq$ 4 × OD$_c$; strongly adherent/strong biofilm producer (+++): 4 × OD$_c$ < OD. This classification was based upon of cut-off of the OD (OD$_c$) value defined as three standard deviation values above the mean of OD of the negative control (Stepanović et al., 2000). The OD values used to obtain this classification were the OD$_{570\ nm}$ values. The mean OD$_c$ value used in the classification was 0.101.

The action of SHC on biofilm mass removal (BR)/inactivation (BI) was classified according to Lemos et al. (2014): BR or BI <25 % - low efficacy; 25 $\leq$ BR or BI <60 % - moderate efficacy; 60 $\leq$ BR or BI <90 % - high efficacy; 90 $\leq$ BR or BI $\leq$100 % - excellent efficacy.

2.9. Statistical analysis

The data was statistically analysed using One-Way Anova followed by Tukey–Kramer multiple comparison method through the statistical program Prism 6. The significance level for the difference between data was set at $P < 0.05$. It should be noticed that for each condition tested at least three independent experiments were performed.

3. Results

3.1. Single and inter-kingdom adhesion and biofilm formation - biomass quantification and productivity classification

The selected microorganisms and consortia adhered and formed biofilms regardless of the shear stress condition used (Fig. 1). Equivalent biomass production ($P > 0.05$) was observed for initial adhesion (incubation time of 2 and 4 h) of single *A. calcoaceticus* and ff (Fig. 1) under both shear stresses. After 24 h of incubation under 0.05 Pa *A. calcoaceticus* and the single ff had similar biofilm productivities ($P > 0.05$). For 1.6 Pa, both single ff had similar biofilm productivities ($P > 0.05$), which were much higher than that of *A. calcoaceticus* ($P < 0.05$). This higher single ff biofilm productivity in comparison to the bacterial biofilms was also observed for the 48 h incubation period under both shear stresses ($P < 0.05$). At 0.05 Pa of shear stress and after 48 h, *P. brevicompactum* produced more biofilm than *P. expansum*, while the opposite was observed under 1.6 Pa ($P < 0.05$).

In terms of bacterium-fungal associations, similar biomass values were found for initial adhesion (2 and 4 h) under both 0.05 and 1.6 Pa ($P > 0.05$). In terms of biofilm development (24 and 48 h), shear stress played a significant role in biofilm productivity. The use of 0.05 Pa reduced *A. calcoaceticus – P. brevicompactum* inter-kingdom biofilm productivity in comparison to the other biofilms formed under that shear stress ($P < 0.05$), except 48 h-old *A. calcoaceticus* single biofilms, which presented equivalent biomass amounts ($P > 0.05$). An increase in inter-kingdom biofilm production was observed for the 48 h incubation period under 0.05 Pa ($P < 0.05$). However, the 48 h-old single ff biofilms produced the highest biomass amounts ($P < 0.05$). The use of 1.6 Pa of shear stress significantly increased 24 h-old *A. calcoaceticus – P. expansum* biofilm productivity and the lowest biofilm amounts were produced by *A. calcoaceticus* ($P < 0.05$). Under that shear stress and for the 48 h incubation period, the inter-kingdom biofilms had higher biofilm amounts than the single species biofilms ($P < 0.05$). The association of *A. calcoaceticus – P. expansum* was the most prolific biofilm producer

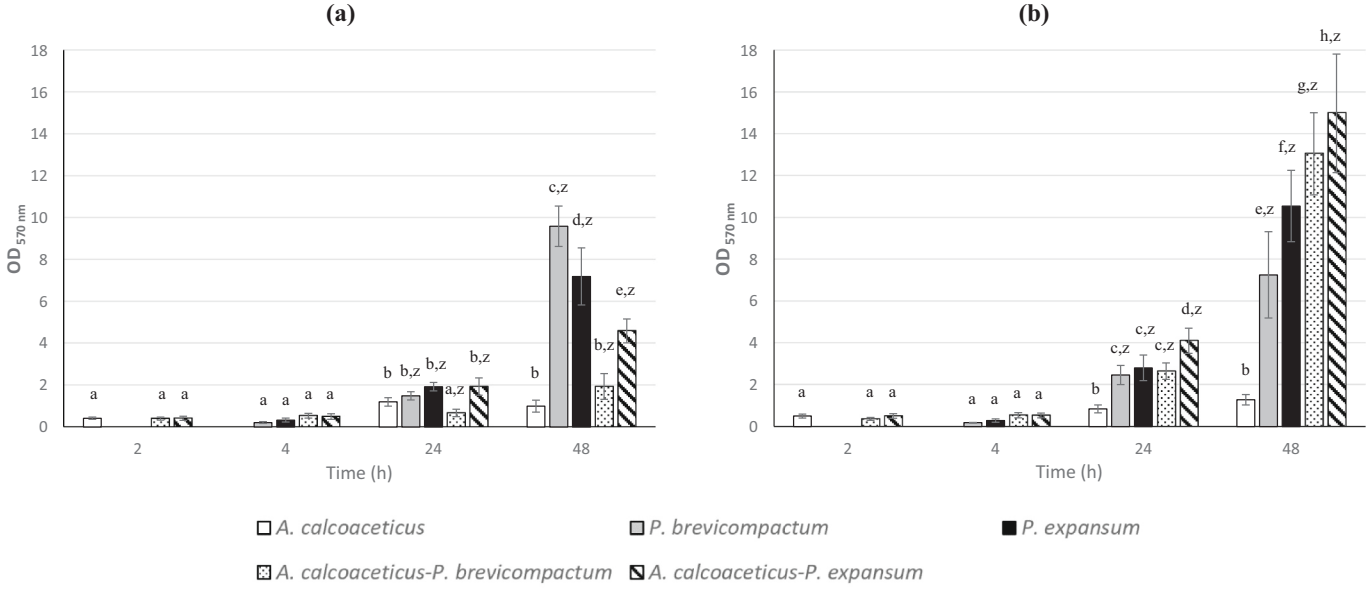

Fig. 1. OD$_{570\ nm}$ as measure of biomass productivity of single and inter-kingdom adhered cells/spores (2 h for *A. calcoaceticus* and 4 h for fungi) and biofilms (24 and 48 h) grown under 0.05 (a) and 1.6 Pa (b). Values are means ± SDs of at least three independent experiments. Non-adherent/non-biofilm producer - OD$_{570\ nm}$ $\leq$ 0.101; Weakly adherent/weak biofilm producer – 0.101 < OD$_{570\ nm}$ $\leq$ 0.202; Moderately adherent/moderate biofilm producer – 0.202 < OD$_{570\ nm}$ $\leq$ 0.404; Strongly adherent/strong biofilm producer (+++): 0.404 < OD$_{570\ nm}$. The mean values with different letters (from a to h) are statistically different for the same shear stress ($P < 0.05$). The letter z shows results statistically different when comparing the same experimental conditions for the two shear stresses. Bacterial single species adhesion was considered up to the initial 2 h while spore single species adhesion was considered up to the initial 4 h.

under 1.6 Pa ($P < 0.05$). The use of 1.6 Pa notably favoured 48 h-old inter-kingdom biofilm formation ($P < 0.05$).

A ranking of adhesion and biofilm formation abilities was produced for all the microorganisms and combinations tested (Table 1), to more easily compare the results obtained. For both shear stresses and considering the adhesion step, *A. calcoaceticus*, *P. expansum* and inter-kingdom biofilms were characterized as weakly adherent and *P. brevicompactum* as non-adherent (4 h incubation). Looking at the biofilm data, both ff and bacterium-fungal associations were classified as strong biofilm producers at 24 and 48 h, under 0.05 and 1.6 Pa, except *A. calcoaceticus – P. brevicompactum* (classified as moderate when formed under 0.05 Pa for 24 h). *A. calcoaceticus* was moderate (48 h at 0.05 Pa and 24 h at 1.6 Pa) or strong (24 h at 0.05 Pa and 48 h at 1.6 Pa) biofilm producer.

Representative epifluorescence microscopy inspections (Fig. 2) show the development of the inter-kingdom sessile structure. The first stage (4 h) highlights the predominance of a layer of *A. calcoaceticus* and the appearance of germinating buds of ff. In the biofilm development stages (24 and 48 h), the active hyphal growth dominated over the bacterial basal layer.

3.2. Single and inter-kingdom adhesion and biofilm formation - respiratory activity

The respiratory activity of single and inter-kingdom adhered cells and biofilms was assessed using the resazurin dye. These values were normalized per biomass amount and are presented in Fig. 3. An increase in biofilm age (24 and 48 h) resulted in decreased biofilm specific respiratory activity ($P < 0.05$), except for *A. calcoaceticus* single biofilms formed under 0.05 Pa of shear stress ($P > 0.05$). The highest specific respiratory activity of 2 h adhered cells was found for *A. calcoaceticus*, *A. calcoaceticus – P. expansum* and *A. calcoaceticus – P. brevicompactum* biofilms under 1.6 Pa and *A. calcoaceticus – P. brevicompactum* under 0.05 Pa ($P < 0.05$). The 4 h adhesion period highlights the increase in the activity of the inter-kingdom under both shear stresses ($P < 0.05$), except *A. calcoaceticus – P. brevicompactum* formed under 0.05 Pa, with respiratory activity values equivalent to that observed for the 2 h incubation period ($P > 0.05$). In terms of 24 h biofilms, the specific respiratory activity of inter-kingdom biofilms was generally lower under 1.6 Pa than under 0.05 Pa, except the *A. calcoaceticus* biofilms ($P < 0.05$). For the 48 h incubation period, the biofilms formed under 0.05 Pa by *A. calcoaceticus* and the inter-kingdom biofilms had higher specific respiratory activity than these formed under 1.6 Pa ($P < 0.05$). *A. calcoaceticus* biofilms (24 and 48 h-old) formed under both 0.05 and 1.6 Pa and *A. calcoaceticus – P. brevicompactum* formed under 0.05 Pa where these with the highest specific respiratory activity values ($P < 0.05$).

Table 1
Adhesion and biofilm formation ability of the microorganisms grown under 0.05 and 1.6 Pa of shear stress. For *A. calcoaceticus* values for the adhesion after 4 h were not collect and the same occurred for *P. brevicompactum* and *P. expansum* for 2 h of adhesion.[a]

Microorganisms	0.05 Pa				1.6 Pa			
	Adhesion at sampling time (h)		Biofilm formation at sampling time (h)		Adhesion at sampling time (h)		Biofilm formation at sampling time (h)	
	2	4	24	48	2	4	24	48
A. calcoaceticus	+	[b]	+++	++	+	[b]	++	+++
P. brevicompactum	[c]	0	+++	+++	[c]	0	+++	+++
P. expansum	[c]	+	+++	+++	[c]	+	+++	+++
A. calcoaceticus - P. brevicompactum	+	+	++	+++	+	+	+++	+++
A. calcoaceticus - P. expansum	+	+	+++	+++	+	+	+++	+++

[a] According to classification proposed by Stepanović et al. (2000): (0) non-adherent/non-biofilm producer; (+) weakly adherent/weak biofilm producer; (++) moderately adherent/moderate biofilm producer; (+++) strongly adherent/strong biofilm producer. The mean OD_c value used in the classification was 0.101.

[b] Bacterial adhesion was only considered up to the initial 2 h.

[c] Spore adhesion was only considered up to the initial 4 h.

3.3. Acinetobacter calcoaceticus culturability

The CFU/cm^2 values of *A. calcoaceticus* in single and inter-kingdom biofilms (Fig. 4) showed an increase in cell density with the increase of the sessile structure age. The values were invariably higher when assessed in both inter-kingdom sessile structures incubated for 24 and 48 h in comparison to the monolayer adhesion incubation stage (2 and 4 h) ($P < 0.05$). The higher shear stress favoured bacterial surface colonization, particularly in both inter-kingdom biofilms incubated for 2 and 4 h, the 24 h-old *A. calcoaceticus – P. expansum* and *A. calcoaceticus – P. expansum* biofilms, and the 48 h-old *A. calcoaceticus – P. expansum* ($P < 0.05$).

3.4. Control of single and inter-kingdom biofilms with SHC

The effects of SHC on 48 h-old biofilms were assessed in terms of reduction in biomass and respiratory activity (Figs. 5 and 6). Total biofilm mass removal was not achieved for any of the concentrations applied against the several biofilms (Fig. 5). The application of SHC caused biofilm mass reductions ranging from low (BR < 25 %) to moderate (25 % ≤ BR < 60 %). Moderate removal was found for *A. calcoaceticus* (0.1, 0.5, 1 and 10 mg/L – 1.6 Pa; 10 mg/L – 0.05 Pa), *P. expansum* (0.1, 0.5, 1 and 10 mg/L – 0.05 Pa; 1 and 10 mg/L – 1.6 Pa) single species biofilms, and *A. calcoaceticus - P. brevicompactum* (10 mg/L – 1.6 Pa) and *A. calcoaceticus - P. expansum* (0.1, 0.5, 1 and 10 mg/L – 1.6 Pa; 0.5 mg/L – 0.05 Pa) biofilms. In general, biofilms formed under 1.6 Pa were more susceptible to removal by SHC. This higher susceptibility was noticeable for *A. calcoaceticus* single biofilms and *A. calcoaceticus – P. expansum* inter-kingdom biofilms ($P < 0.05$). *P. brevicompactum* single biofilms were found to be highly resistant to removal ($P < 0.05$). Moreover, both inter-kingdom biofilms seem to benefit from the microbial association, particularly in comparison to the behaviour of *A. calcoaceticus* single biofilms ($P < 0.05$). The only exceptions were the *A. calcoaceticus - P. expansum* biofilms formed under 1.6 Pa and exposed to free chlorine at 0.5, 1 and 5 mg/L, for which removal was comparable or even higher (5 mg/L) than for the bacterial biofilms ($P < 0.05$).

In terms of biofilm inactivation, SHC caused effects ranging from low (BI <25 %) to excellent (90 % ≤ BI ≤100 %) (Fig. 6). Excellent inactivation was observed for *A. calcoaceticus* (10 mg/L – 0.05 and 1.6 Pa) and *P. expansum* (10 mg/L – 0.05 Pa) single biofilms. High inactivation (60 % ≤ BI <90 %) was observed for *P. expansum* (0.5 and 1 mg/L – 0.05 Pa) single biofilms. SHC caused moderate inactivation (25 % ≤ BI <60 %) of *A. calcoaceticus* (1 mg/L – 0.05 and 1.6 Pa) and *P. expansum* (0.1–0.05 Pa; 0.5, 1 and 10 mg/L – 1.6 Pa) single biofilms and *A. calcoaceticus - P. expansum* (10 mg/L – 0.05 Pa) inter-kingdom biofilms. The inter-kingdom association increased biofilm resistance to inactivation by SHC, particularly when compared to *A. calcoaceticus* single biofilms ($P < 0.05$). *P. brevicompactum* single biofilms demonstrated remarkable resistance to inactivation. Inactivation of *P. expansum* single biofilms and *A. calcoaceticus – P. expansum* inter-kingdom biofilms was consistently and significantly higher under 0.05 Pa ($P < 0.05$). The opposite was observed for *P. brevicompactum* single biofilms and *A. calcoaceticus – P. brevicompactum* inter-kingdom biofilms, where the use of 1.6 Pa favoured inactivation ($P < 0.05$), except when 0.5 mg/L of free chlorine was used (inactivation was found to be negligible in both cases).

The total reduction of *A. calcoaceticus* CFU/cm^2 in the 48 h-old single and inter-kingdom biofilms was not achieved for the diverse free

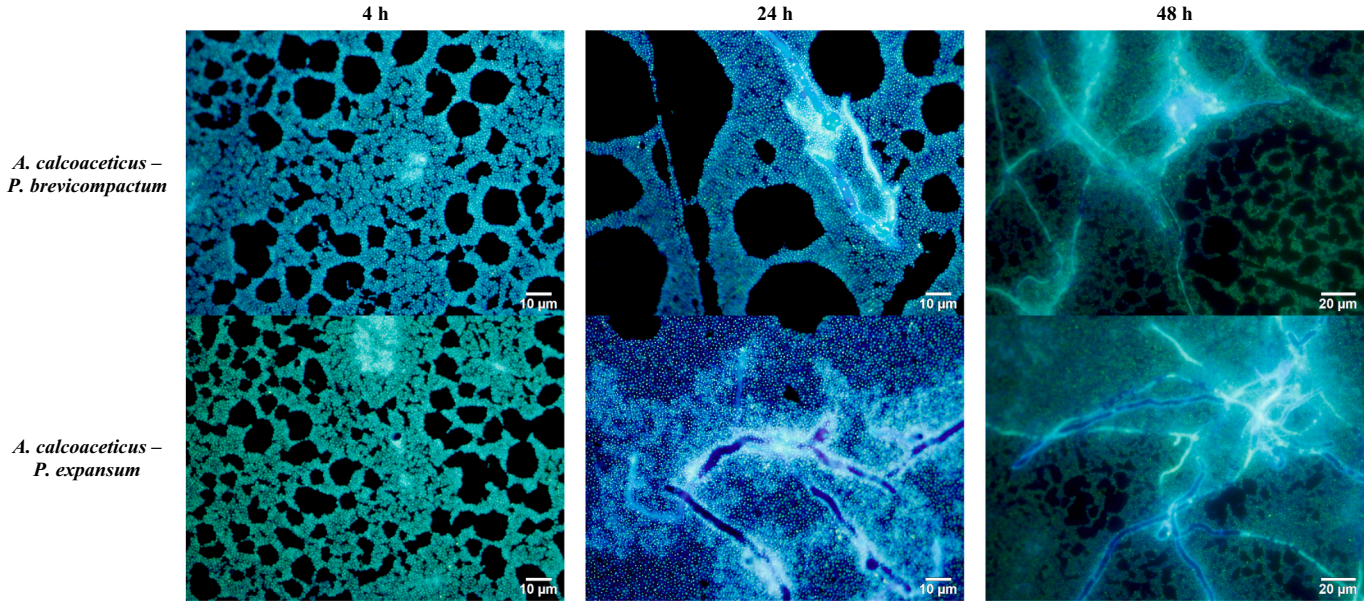

Fig. 2. Epifluorescence photomicrographs with DAPI of inter-kingdom biofilm formation over time (4, 24 and 48 h) under 1.6 Pa. ×400 magnification/bar = 20 μm; 600× magnification/bar = 10 μm.

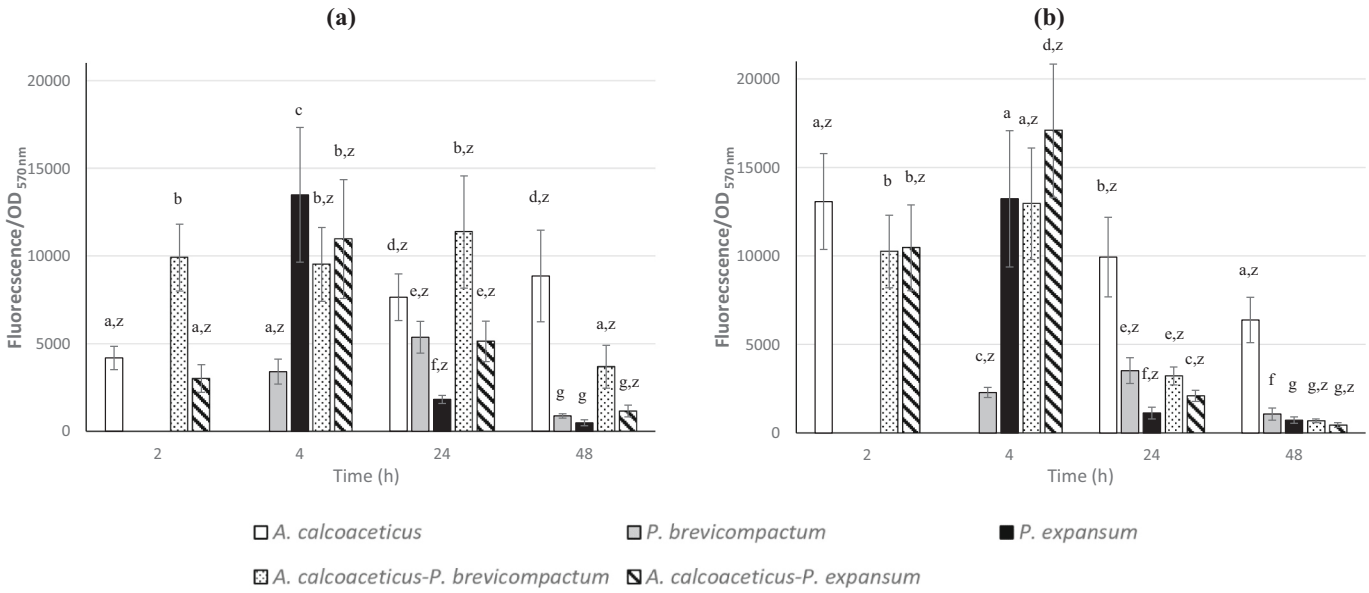

Fig. 3. Fluorescence units per $OD_{570\ nm}$ as a measure of specific respiratory activity of single and inter-kingdom adhered cells/spores (2 h for *A. calcoaceticus* and 4 h for fungi) and biofilms (24 and 48 h) grown under 0.05 (a) and 1.6 Pa (b). Values are means ± SDs of at least three independent experiments. The mean values with different letters (from a to g) are statistically different for the same shear stress ($P < 0.05$). The letter z shows results statistically different when comparing the same experimental conditions for the two shear stresses. Bacterial single species adhesion was considered up to the initial 2 h while spore single species adhesion was considered up to the initial 4 h.

chlorine concentrations tested (Fig. 7). *A. calcoaceticus* single biofilms log reduction was higher when formed under 0.05 Pa, and exposed to 10 mg/L of free chlorine ($P < 0.05$). When in inter-kingdom biofilms, *A. calcoaceticus* log CFU/cm^2 reduction was significantly lower than in the single species biofilms ($P < 0.05$), particularly when the highest free chlorine concentrations were applied (5 and 10 mg/L). In the inter-kingdom biofilms, >1 log reduction was only obtained when 10 mg/L of free chlorine was used.

4. Discussion

Biofilms are commonly studied using cultures of a single species, even if ecosystems, including the DWDS, are typically composed of networks of interacting species. While the single species formalism is attractive to generate easy-to-interpret data, it is far from adequate to understand the role of species interactions in community behaviour. The role of ff in multispecies biofilms remains unclear, even if it is now recognized that these microorganisms can form biofilms under diverse process conditions (Afonso et al., 2021; Fernandes et al., 2019; Simões et al., 2015). In addition, there is a significant lack of scientific data on

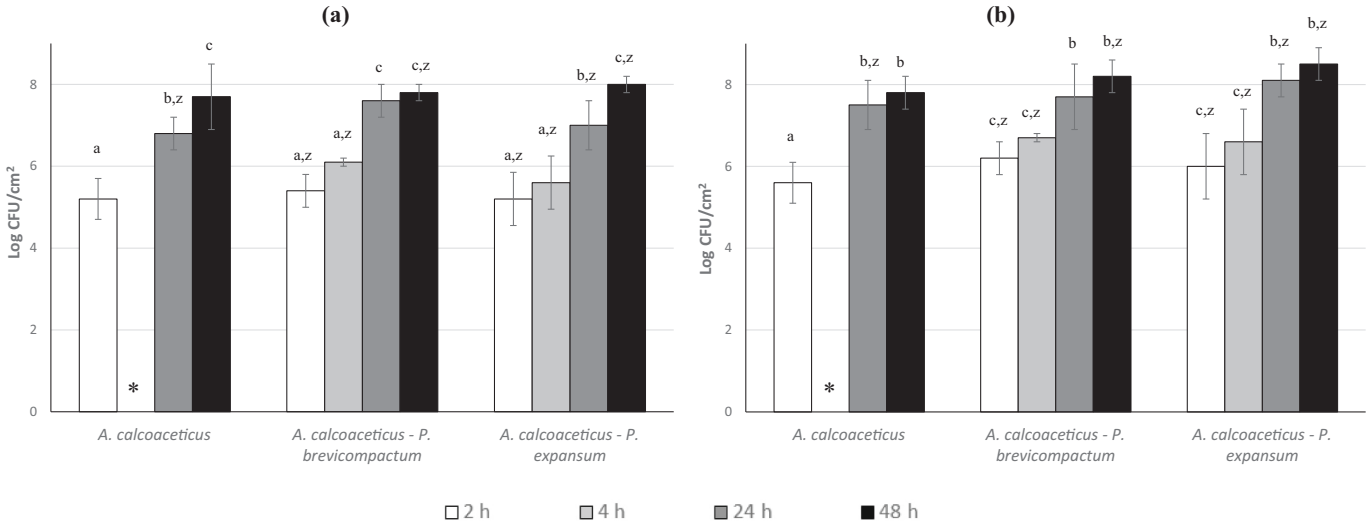

Fig. 4. Log CFU/cm^2 of *A. calcoaceticus* in single and inter-kingdom sessile structures (adhered cells and biofilms) developed under 0.05 (a) and 1.6 Pa (b) for the four sampling times. Values are means ± SDs of at least three independent experiments. The mean values with different letters (from a to d) are statistically different for the same shear stress ($P < 0.05$). The letter z shows results statistically different when comparing the same experimental conditions for the two shear stresses. * Means that bacterial single species adhesion was considered up to the initial 2 h and biofilm were analysed after 24 and 48 h of incubation. For inter-kingdom biofilms, CFU/cm^2 of *A. calcoaceticus* were determined after 2, 4, 24 and 48 h of incubation.

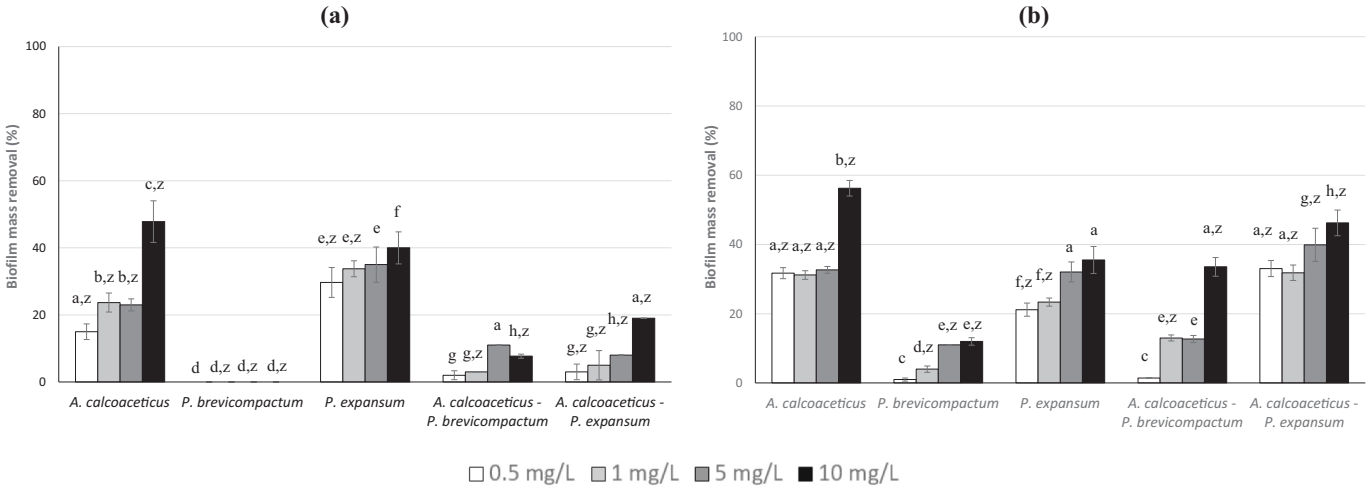

Fig. 5. Percentage of mass removal of 48 h-old single and inter-kingdom biofilms formed under 0.05 (a) and 1.6 Pa (b) and exposed to several SHC concentrations (0.1, 0.5, 1 and 10 mg/L). The results presented were calculated using the biofilm mass values obtained from three independent experiments. Values are means ± SDs. The mean values with different letters (from a to h) are statistically different for the same shear stress ($P < 0.05$). The letter z shows results statistically different when comparing the same experimental conditions for the two shear stresses.

the role of ff in DW microbiological quality and safety (Fish et al., 2016; Hull et al., 2019; Liu et al., 2016; Pinto et al., 2014). This is largely due to underestimated and not fully understood health problems associated with the presence of fungi in DWDS (Hageskal et al., 2009; Paterson and Lima, 2005). This study provides pioneer data demonstrating the association between *A. calcoaceticus* and *P. brevicompactum* or *P. expansum* and how such association affects biofilm establishment and its resilience to SHC exposure.

The single and inter-kingdom adhesion and biofilm formation assays were performed in 96-well PS microtiter plates under 0.05 and 1.6 Pa of shear stress. These are conditions of low shear stress. DWDS have variability of hydrodynamic conditions along with the distribution system. Regions of stagnant or lower shear stress conditions are common (reservoirs, household pipes, dead zones, corners and valves) and these are associated with zones of high organic material sedimentation and consequently, abundant biofilm formation (Gomes et al., 2014; Simões and Simões, 2013). Water stagnation in these regions promotes microbial accumulation in either the sessile or planktonic state, and also a significant vegetative growth and sporulation of ff that compromise the DW microbiological quality (Manuel et al., 2010; Sammon et al., 2011). However, the biofilm development and behaviour of ff in single and inter-kingdom biofilm under distinct hydrodynamic conditions remains to be understood. The present study demonstrates that, even if bacteria are known to be prolific biofilm producers, *P. brevicompactum* and *P. expansum* can form biofilms under 0.05 and 1.6 Pa. Both ff had similar values of biomass adhered ($P > 0.05$), but *P. expansum* was found to be weakly adherent and *P. brevicompactum* non-adherent, regardless of the shear stress used. In a mechanistic study on ff adhesion (Fernandes et al., 2019), it was found that *P. brevicompactum* spores adhered to a higher extent than that of *P. expansum*, regardless of the process conditions used. The higher adhesion ability was found to be related to the spore surface properties, favouring long and short-range interactions between

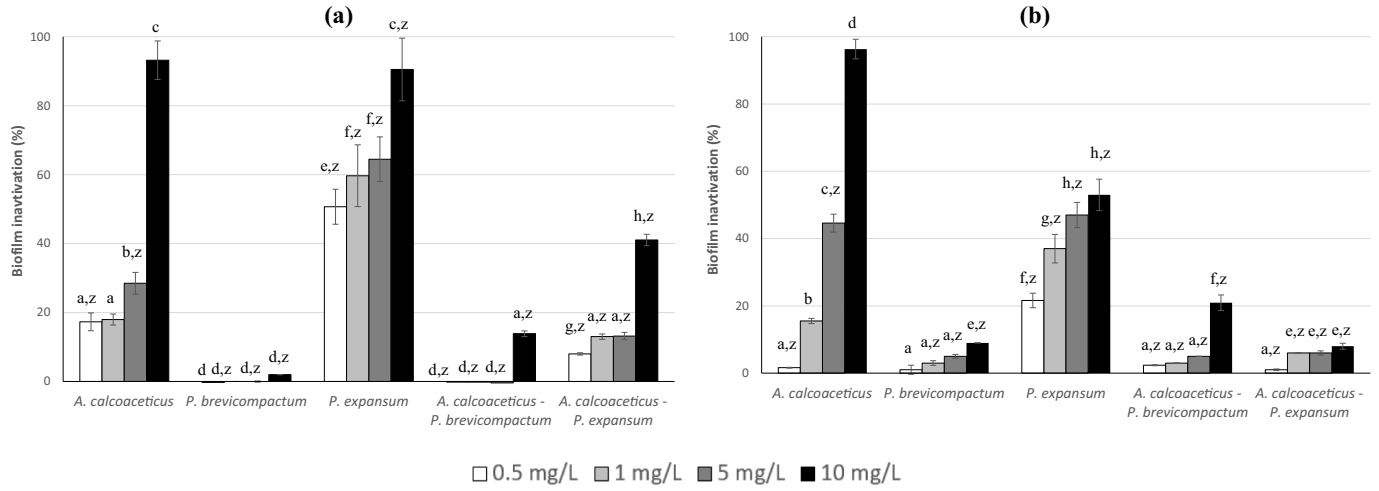

Fig. 6. Percentage of inactivation of 48 h-old single and inter-kingdom biofilms formed at 0.05 (a) and 1.6 Pa (b) and exposed to several SHC concentrations (0.1, 0.5, 1 and 10 mg/L). The results presented were calculated using the respiratory activity values obtained from three independent experiments. Values are means ± SDs. The mean values with different letters (from a to h) are statistically different for the same shear stress ($P < 0.05$). The letter z shows results statistically different when comparing the same experimental conditions for the two shear stresses.

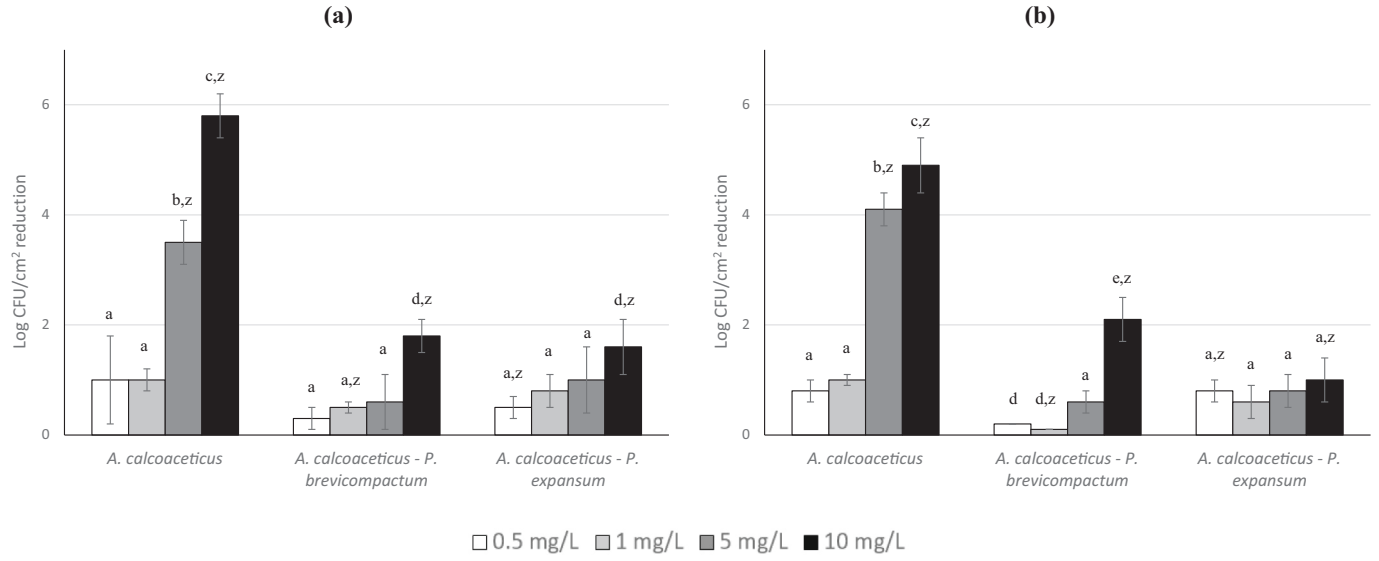

Fig. 7. Log CFU/cm^2 reduction of *A. calcoaceticus* in 48 h-old single and inter-kingdom biofilms formed under 0.05 (a) and 1.6 Pa (b) and exposed to several SHC concentrations (0.1, 0.5, 1 and 10 mg/L). Values are means ± SDs from at least three independent experiments. The mean values with different letters (from a to e) are statistically different for the same shear stress ($P < 0.05$). The letter z shows results statistically different when comparing the same experimental conditions for the two shear stresses.

the spore and the adhesion surface (Fernandes et al., 2019).

At 24 h, both ff had similar biomass ($P > 0.05$) and were both strong biofilm producers under both shear stress conditions. The 48 h-old fungal biofilms were characterized as strong producers, but *P. brevicompactum* seems more fit to grow under the lower shear stress while *P. expansum* thrives under 1.6 Pa ($P < 0.05$). These results demonstrate that ff biofilm productivity was both dependent on the species and on the shear stress under which the biofilms were formed. The biomass of both fungal biofilms was notably higher than that of *A. calcoaceticus* biofilms and increased with the biofilm age, particularly when grown under 1.6 Pa of shear stress ($P < 0.05$). These results propose that after germling formation (Fernandes et al., 2019; Simões et al., 2015), hyphal growth for surface invasion and biofilm maturation is a highly dynamic process, benefiting biomass production.

In terms of inter-kingdom studies, adhesion was observed for the two bacterium-ff associations with comparable biomass production under 0.05 and 1.6 Pa ($P > 0.05$). This fact might mean that the initial steps for adhesion are similar for both consortia, a result apparently related to the strong dominance of the bacterial population, forming a basal layer which will harbour spores and facilitate their adhesion. While spore adhesion is essentially based on physico-chemical interactions (Fernandes et al., 2019), bacterial adhesion benefits from the existence of both physico-chemical and biological interactions (Kimkes and Heinemann, 2020).

The biofilm maturation was beneficial for biomass production by *A. calcoaceticus - P. expansum* under both 0.05 and 1.6 Pa, even if both mature inter-kingdom associations were found to be strong biofilm producers. Comparing the results of inter-kingdom adhesion with those obtained for single *A. calcoaceticus*, it is possible to verify that no significant differences were found for adherent biomass values obtained under both hydrodynamic conditions ($P > 0.05$). However, for 24 and 48 h-old biofilms, the biomass was significantly higher for the inter-

kingdom than for *A. calcoaceticus* single biofilms and increased over time, particularly under 1.6 Pa ($P < 0.05$). One exception was the 24 h-old *A. calcoaceticus – P. brevicompactum* biofilms under 0.05 Pa that had significantly lower biomass than *A. calcoaceticus* single biofilms ($P < 0.05$). A recent study reported inter-kingdom biofilm formation by *A. calcoaceticus – P. brevicompactum* and *Methylobacterium oryzae – P. brevicompactum* under 150 rpm and found an increase in biofilm production when compared to bacterial single biofilms (Afonso et al., 2019). This proposes that no standardization in bacterial - ff biofilm formation can be considered. The different microbial associations and process conditions (*i.e.* shear stress) formed biofilms with distinct biomass production and respiratory activity. The present study further shows that the shear stress under which biofilms were formed has a role in their development, particularly these inter-kingdom biofilms. For 48 h, the inter-kingdom biofilms presented higher values of biomass in comparison to the single species biofilms formed under 1.6 Pa, while the opposite was observed under 0.05 Pa ($P < 0.05$). These results propose that the presence of *A. calcoaceticus* influenced positively and negatively the biofilm production at 1.6 and 0.05 Pa, respectively. Taking into account the results of Afonso et al. (2019) using 150 rpm for biofilm formation and the present data, it appears that inter-kingdom biofilm production benefits from the use of high shear stresses. This is potentially related to an increase in proton translocation, which facilitates and strengthens cell-cell interactions, and further leads to the creation of a highly dense community (Liu and Tay, 2001; Teo et al., 2000). In addition, high shear stresses tend to form more complex and denser biofilms, benefiting from the lower effects of mass transfer limitations (Simões et al., 2008; Krsmanovic et al., 2021).

In terms of specific respiratory activity, the first stages of biofilm development were the most metabolically active. Following microbial adhesion, the formation of a complex extracellular polymeric matrix is known to increase the non-metabolically active biomass (Simões et al., 2005, 2007b), decreasing the specific respiratory activity. However, regardless of the shear stress used, all microorganisms and consortia tested were metabolic active. The single bacterial biofilms (24 and 48 h) were more active than ff single biofilms under both shear stresses ($P < 0.05$). Regarding the specific respiratory activity of the inter-kingdom sessile structures, and for the adhesion times (2 and 4 h), *A. calcoaceticus – P. brevicompactum* (2 h) under 0.05 Pa and *A. calcoaceticus – P. expansum* (4 h) under 1.5 Pa were significantly more active than the counterparts ($P < 0.05$). For the biofilm times (24 and 48 h), *A. calcoaceticus – P. brevicompactum* was found to be the most metabolic active under 0.05 Pa (24 and 48 h) and 1.6 Pa (24 h) ($P < 0.05$). The release of different extracellular molecules by the bacterium and/or ff is probably involved in the different developmental characteristics of the inter-kingdom biofilms. A recent study (Afonso et al., 2020) demonstrated that *A. calcoaceticus* biofilm development beneficiated from *P. expansum* presence whereas *M. oryzae* did not. Such results were due to the presence of molecules involved in quorum sensing inhibition that were produced by the ff (Afonso et al., 2020).

The log CFU/cm^2 of *A. calcoaceticus* from single and inter-kingdom biofilms significantly increased with the culture time ($P < 0.05$), while the respiratory activity sharply decreased after 24 h of culture. It is important to highlight that the respiratory activity results are presented per mass of biofilm ($OD_{570\ nm}$), which means that the increase of biofilm mass over time was followed by an increase in *A. calcoaceticus* cell density, an effect more significant for the inter-kingdom biofilms and shear stress of 1.6 Pa ($P < 0.05$). However, specific respiratory activity is known to be highly affected by the biofilm age. This assay is highly dependent on cell respiratory efficiency, which in turn is related to the growth phase, and the age and thickness of the biofilm (Pantanella et al., 2013). Moreover, as the time of resazurin reduction is species- and strain-related, some experimental conditions are difficult to standardize (Pantanella et al., 2013). The main advantage of the resazurin assay is that being a biological dye, it is non-toxic to cells.

Of all the chemical disinfectants used for DWDS treatment, chlorine is the most commonly used. The disinfection associated with the maintenance of chlorine residual through the distribution systems is a very popular strategy to prevent the regrowth of microorganisms during water transportation (Simões, 2013). Also, chlorination was reported by Kim et al. (2009) as relevant for the control of anaerobic bacteria and biofilms. Disinfected drinking waters have concentrations in the range of 0.2–1 mg/L (Fish and Boxall, 2018). In this study, concentrations between 0.1 and 10 mg/L were tested for biofilm removal and inactivation. Even if, for most of the cases, an increase in chlorine concentration increased biofilm removal and inactivation. Total biofilm mass removal or respiratory inactivation (*i.e.* 100 % biofilm mass removal or biofilm inactivation) was not achieved for any of the conditions tested. Removal was found to be low or moderate for all the biofilms tested. Excellent inactivation was only observed when 10 mg/L of chlorine was applied against *A. calcoaceticus* biofilms formed under both shear stresses and for *P. expansum* biofilms formed under 0.05 Pa. Of particular concern was the behaviour of *P. brevicompactum* biofilms. These were remarkably resistant to removal and inactivation, regardless of the shear stress used for biofilm formation and the chlorine concentration applied. *P. brevicompactum* presence in inter-kingdom biofilms also increased resistance to removal and inactivation, particularly when compared to *A. calcoaceticus* single biofilms. The effect was more significant when *P. brevicompactum* formed single and inter-kingdom biofilms. Even if the biofilm production of *A. calcoaceticus - P. brevicompactum* was lower than that of *A. calcoaceticus - P. expansum*, the interaction of biofilms formed by *A. calcoaceticus - P. brevicompactum* enhanced the bacterial tolerance to disinfection ($P < 0.05$). This result might be related to the distinct structure and composition of these inter-kingdom biofilms (Karygianni et al., 2020). The increased resistance of multispecies biofilms in comparison to their single species counterparts is not a new research topic (Joshi et al., 2021). However, to the best of our knowledge, no previous studies have shown the remarkable resistance to chlorine disinfection of bacteria-ff. The role of inter-kingdom biofilms in *A. calcoaceticus* survival is remarkable, proposing that the presence of ff in DWDS has the potential to compromise the microbiological quality of the water due to poor disinfection.

5. Conclusions

P. expansum and *P. brevicompactum* can adhere and form single and inter-kingdom biofilms under two distinct shear stress conditions. The inter-kingdom associations were found to produce more biofilm than *A. calcoaceticus* alone, a phenomenon potentiated under 1.6 Pa of shear stress. The ff appear to establish in the inter-kingdom biofilm over a basal layer of *A. calcoaceticus*. In general, biofilms formed under 1.6 Pa were more susceptible to removal by SHC exposure than those formed under 0.05 Pa. The opposite was observed in terms of inactivation. *P. brevicompactum* biofilms were highly resistant to removal and inactivation. The presence of this fungus in the inter-kingdom biofilms appears to contribute to the consortium resistance to disinfection, highlighting its potential role in DW microbiological safety and its relevance as a model for DW inter-kingdom biofilm studies. *A. calcoaceticus* was significantly protected from SHC action when in the inter-kingdom biofilms.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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