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The impact of synthetic musk compounds in biofilms from drinking water bacteria

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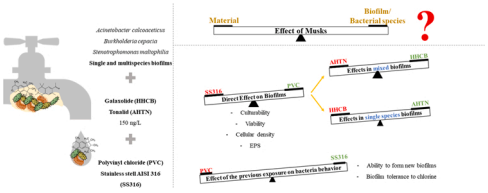
HIGHLIGHTS

- Tonalide increased cellular culturability, viability and density of mixed species biofilms.
- Galaxolide caused significant alterations in single species biofilms.
- Materials where biofilms were formed impact the effects of musk exposure.
- Biofilms on stainless steel were more susceptible to the direct exposure to musks.
- Bacteria exposed to musks in polyvinylchloride have altered ability to form biofilms.

GRAPHICAL ABSTRACT



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ABSTRACT

Musk fragrances have been detected in drinking water (DW) at trace concentrations. However, their impact on the microbial quality of DW has been disregarded. This work provides a pioneer evaluation of the effects of two synthetic musks contaminants, tonalide (AHTN) and galaxolide (HHCB), in microbial biofilms formed on two different surfaces, polyvinyl chloride (PVC) and stainless steel AISI 316 (SS316). Three bacterial species isolated from DW (*Acinetobacter calcoaceticus, Burkholderia cepacia* and *Stenotrophomonas maltophilia*), were used to develop 7-day-old single and mixed species biofilms. The impact of musks was assessed directly on biofilms but also on the bacteria motility, biofilm formation ability and biofilm susceptibility to chlorination. AHTN musk caused the most remarkable effects by increasing the cellular density and viability of mixed biofilms, and the extracellular polysaccharides content of biofilms on SS316. Most of the alterations caused by the direct exposure of biofilms to musks were observed when SS316 was used as an adhesion surface. In contrast, the ability to form biofilms and their susceptibility to chlorine were more affected for bacteria from HHCB-exposed biofilms on PVC. The overall results demonstrate that the presence of musks at residual concentrations influences DW bacterial dynamics, with the potential to impact the DW quality and safety. The type of plumbing material may further impact the effects of musks.

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1. Introduction

Musks fragrances are additives widely used in a multitude of household and personal care products available in the market, such as detergents, shampoos, lotions, perfumes and many other products of indispensable use for the current society. Therefore, the extensive use of musks requires a large production of these compounds. However, some ecological concerns have been pointed out as musks have been detected in water sources worldwide. Their inherent high lipophilicity and low biodegradability hinder their removal in wastewater treatment plants (WWTPs), contributing to the presence and accumulation of musks in the ecosystem, including in drinking water (DW) sources (Romero et al., 2022). Several works have detected musk fragrances in DW at concentrations from 0.06 to 146 ng/L (Wombacher and Hornbuckle, 2009; Pender et al., 2015; Benson et al., 2017; Glassmeyer et al., 2017; Gomes et al., 2020a). Their lipophilicity also explains the frequent detection of musks in human blood, breast milk and even adipose tissues (Taylor et al., 2014). The consequences of these interactions on human health are still inconclusive. However, pieces of evidence are proposing that musks may be endocrine disruptors associated with fertility problems (Taylor et al., 2014). Some studies on carcinogenic and genotoxic effects of musks have been developed in animal models, demonstrating a relationship between musk exposure and tumour development in mice (Mersch-Sundermann et al., 1996; Mersch-Sundermann et al., 2001; Gao et al., 2019).

The ecotoxicological risks of musks have been explored by the scientific community (Parolini et al., 2015; Ehiguese et al., 2021; Jiang et al., 2021). For example, Ehiguese et al. (2021) demonstrated that musks may constitute a significant risk for marine organisms. The microalgae *Phaeodactylum tricornutum* and *Isochrysis galbana* were susceptible to the presence of galaxolide (HHCB) and tonalide (AHTN) at environmentally relevant concentrations (0.005 – 5 μg/L), as well as the larvae development of *Paracentrotus lividus* and *Mytilus gallaprovincialis* was affected by these musks (Ehiguese et al., 2021). Other recent work demonstrated that HHCB negatively affects radish germination, causing severe DNA damage (Jiang et al., 2021). On the other hand, Lyu et al. (2021) evaluated the ecotoxicology of musk fragrances detected in sediments of rivers and lakes and suggested that the sedimentary concentrations of musks pose an extremely low ecological risk to aquatic organisms. Despite the availability of literature on the ecotoxicological risks of musks, their effects on microorganisms remain to be understood. Studies on these contaminants are focused mostly on their impact to animals and plants as well as human health (Maekawa et al., 1990; Eisenhardt et al., 2001; Wan et al., 2007).

Microorganisms are ubiquitously present in drinking water distribution systems (DWDSs), mostly in the form of biofilms adhered to pipe walls. It is estimated that approximately 95% of existing microorganisms in DWDSs are attached to the pipe walls in the form of biofilms, although continuous disinfection processes are applied (Simões and Simões, 2013). Biofilms in DWDSs could represent a hazard for the overall public health since they harbour a diverse microbial community, including opportunistic pathogens. These microorganisms are inevitably exposed to a wide range of emerging contaminants (ECs), making it imperative to evaluate the effect of these contaminants on the microbial cells. Still, there is little to no information about the effect of these contaminants on DW biofilms. Attending the high adaptability of bacterial cells to different stressors as well as the evidence found in the literature that bacteria suffer behavioural and genetic alterations due to the exposure to non-pharmaceutical ECs, even at trace concentrations, it is crucial to understand the impact of the presence of musks in the behaviour of microorganisms in DW (Gomes et al., 2020a). The presence of musk compounds in DW is typically disregarded. Moreover, studies regarding the effects of synthetic musks in DW biofilms are scarce. To the best of our knowledge, there are only two studies evaluating the effects of musks on DW bacteria (Gomes et al., 2018, 2019a).

The main objective of this work was to evaluate whether the presence of polycyclic musks in DWDSs affected the development of bacterial biofilms and their susceptibility to disinfection. The impact of the material used for biofilm formation was further assessed on the musk-induced changes in bacterial dynamics. *Acinetobacter calcoaceticus, Burkholderia cepacia* and *Stenotrophomonas maltophilia* isolated from DW (Simões et al., 2007) were used for the formation of single and mixed species biofilms. These bacterial species have been found in DW and are considered emerging pathogens (Jones et al., 2001; Narciso-da-Rocha et al., 2013; Bae et al., 2019). Two polycyclic musks (AHTN and HHCB) were selected for this study as emerging contaminants. AHTN and HHCB are two of the most widely used synthetic musks (Australian Government, 2016, 2021).

2. Materials and Methods

2.1. Emerging contaminants

Tonalide (AHTN) and galaxolide (HHCB), purchased from Sigma-Aldrich (Germany) were the selected musk frangrances. Stock solutions of each synthetic musk were prepared using dimethyl sulfoxide (DMSO) (Fisher Scientific, UK). Musks were tested at 150 ng/L, which corresponds to approximately the maximum concentration of musk compounds detected in DW and reported in the literature (Gomes et al., 2020a). The final concentration of DMSO applied in all the experiments was 0.003% (v/v). Successive dilutions of the stock solutions of the two musks (HHCB at 500 mg/L and 1000 mg/L) were performed in sterile synthetic tap water (STW) until the desired final concentration was obtained (150 ng/L). STW was prepared as described by Gomes et al. (2018).

2.2. Microorganisms and culture conditions

Acinetobacter calcoaceticus, Burkholderia cepacia and *Stenotrophomonas maltophilia* were used as DW models. These bacteria were isolated from the same DW biofilm (Simões et al., 2007). The co-existence of these three environmental emerging pathogens in the same biofilm as well as their recurrent detection in DW were the main reasons for their use as DW bacterial models (Simões et al., 2007). Moreover, *A. calcoaceticus* can act as a bridging bacteria that will promote the aggregation of other species in DW biofilms (Simões et al., 2008). Each bacterial species was grown overnight in batch cultures in R2A broth, prepared as described by Gomes et al. (2018). The cultures were incubated overnight at 25 °C with agitation (160 rpm). Microorganisms were harvested by centrifugation (Eppendorf centrifuge 5810 R) at 3700g for 15 min and the cells were resuspended in adequate growth media: R2A broth, Luria Bertani broth (LBB, from Sigma-Aldrich, Germany) or in STW, depending on the assay to be performed. The cell density was then adjusted to 10^8 CFU/mL.

2.3. Biofilm formation and contaminant exposure

2.3.1. Substratum for biofilm formation

Polyvinyl chloride (PVC) and stainless steel AISI 316 (SS316) coupons (1 × 1 cm), purchased from Neves & Neves (Trofa, Portugal) were used as surface materials for biofilm formation. PVC coupons were cleaned and sterilized by immersion in a solution of commercial detergent (Continente, Porto, Portugal) for 30 min. The coupons were then rinsed in distilled water and, after that, were immersed in ethanol at 70%, for 30 min. The PVC coupons were then thoroughly rinsed with distilled sterile water and dried overnight. SS316 coupons were autoclaved at 121 °C, for 15 min. Coupons of both surface materials were then placed in a 48-well microtiter plate and exposed to ultraviolet (UV) light for 30 min to ensure sterility, before being used for biofilm formation.

2.3.2. Colonization of PVC and SS316 coupons and exposure to musks

Fig. 1 presents a scheme of the experimental set-up for biofilm formation, exposure to musks contaminants and the assessment of the impact of musks on biofilm and bacteria.

Bacterial suspensions at 10^8 CFU/mL were prepared in R2A broth as described in Section 2.2. A volume of 50 µL of each of the contaminants (HHCB and AHTN, prepared as described in Section 2.1) was added to 4.95 mL of the bacterial suspension. Negative controls correspond to unexposed bacteria. Bacteria exposed to DMSO was also used as control, to ensure that the alterations observed were caused by musks and not by the solvent. Therefore, controls were prepared by adding 50 µL of R2A or 50 µL of DMSO (0.3% v/v) to bacterial suspensions instead of contaminants in solution. Then, 1 mL of bacterial suspension was added to each well of a 48-well microtiter plate containing sterile PVC coupons or sterile SS316 coupons (Fig. 1. A). For mixed species biofilms formed by *A. calcoaceticus, B. cepacia* and *S. maltophilia*, 333 µL of each bacterial suspension (prepared as described in Section 2.2) were added to each well. Afterwards, microtiter plates were incubated for 24 h at 25 ºC and under a constant agitation of 160 rpm, to ensure initial biofilm formation, *i.e.* colonization of coupons.

After 24 h, the colonized coupons were carefully removed from the wells and placed in new sterile wells containing 1 mL of contaminant solution, prepared in STW as described in Section 2.1 – Fig. 1.B. The biofilms were exposed to the musks for 7 days at 25 ºC and 160 rpm. After this exposure period, the coupons were washed in sterile STW to remove the non-attached/weakly adhered bacterial cells, for that colonized coupons were removed from the corresponding wells and placed in new and sterile wells containing STW.

Coupons with biofilms were inserted in 15 mL falcon tubes (VWR, Portugal) containing 5 mL of saline solution (8.5 g/L NaCl) and were vigorously stirred in a vortex (VV3 model, VWR) for 2 min, to ensure the complete biofilm removal from the surface of coupons (Fig. 1. C). Then, biofilms were characterized to assess the direct impact of musks (Fig. 1. D). Moreover, 100 µL of the suspension was used to inoculate 5 mL of fresh R2A broth for cryopreservation (Fig. 1. E) and further characterization of bacteria from musk-exposed biofilms (Fig. 1. F). The inocula prepared for cryopreservation were incubated overnight at 25 ºC under 160 rpm. After overnight growth, a volume of 700 µL of the bacterial cultures was added to sterile cryovials with 300 µL of glycerol, which was homogenized and stored at − 80 ºC, for future experiments.

2.3.3. Biofilm characterization

Exposed and unexposed single and mixed species biofilms were characterized in terms of cellular culturability, viability and density (Section 2.3.3.1). Moreover, the mixed species biofilms, as more representative of DW biofilms for being composed of diverse species, were also characterized in terms of extracellular polymeric substances (EPS) content (Section 2.3.3.2).

2.3.3.1. Cell density, viability and culturability. The number of total and viable cells was determined through the Live/Dead BacLight bacterial viability kit (Invitrogen Life Technologies, Alfagene, Portugal) assay according to Gomes et al. (2019b). This kit is composed of two nucleic acid-binding stains: SYTO9™ (stains all cells in green) and propidium iodide (stains cells with the damaged membrane in red). The biofilm samples prepared as described previously were filtered using a Nucleopore (Whatman, Middlesex) black polycarbonate membrane with 0.22 mm pore size. Then, samples were stained with 250 µL of SYTO 9™ and 50 µL of PI and left in the dark for 10 min. The samples were analyzed using a LEICA DM LB2 epifluorescence microscope attached to a Leica DFC300 FX camera. A minimum of 20 images were collected per sample, corresponding to different view fields. The combination of optical filters used consisted of a 515–560 nm excitation filter combined with a dichromatic mirror at 580 nm and a suppression filter at 590 nm (Gomes et al., 2019b). Bacterial enumeration was done using the free available cell-counting software tool BioFilmAnalyzer (Bogachev et al., 2018). The detection limit of the method was 4.8 log cells/cm^2. This assay was performed in three independent experiments, with duplicates.

To assess the culturable cells, biofilm suspensions were homogenized in a vortex and then serial dilutions were made in saline solution (8.5 g/L NaCl). Dilutions were plated in R2A agar plates and incubated at 25 ºC for 48 h. Then, the number of colony forming units (CFUs) was enumerated. Both single and mixed species biofilms were plated. The detection limit of this method was 1.79 log CFU/cm^2. This assay was performed in three independent experiments, with duplicates.

2.3.3.2. Extracellular polymeric substances (EPS) characterization. The effect of the exposure to AHTN on the EPS matrix content of mixed species biofilms was assessed. AHTN was selected as it was the musk that caused most alterations in these biofilms. Mixed species biofilms were formed on 48-well microtiter plates as described in Section 2.3.2. Negative controls were obtained by exposing biofilms to 0.003% of

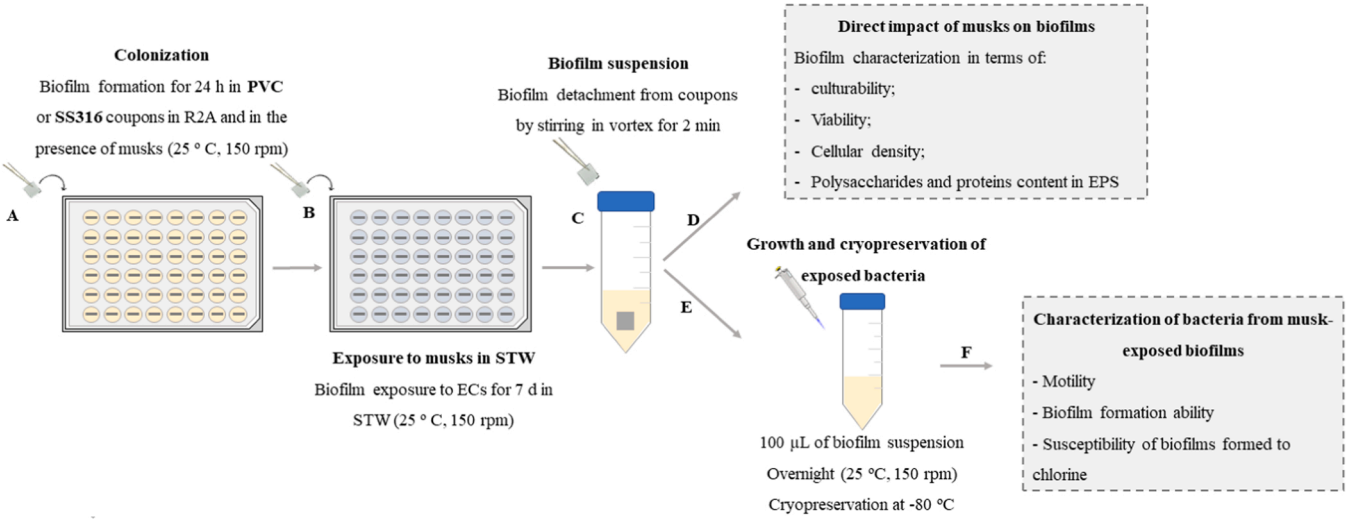

Fig. 1. Experimental set-up for biofilm formation, exposure to musk contaminants and evaluation of the effects of musks contaminants on biofilms and bacteria. A – Biofilm formation for 24 h in R2A broth on PVC and SS316 coupons; B - Biofilm exposure to the selected musk contaminants prepared in STW for 7 days; C - Detachment of biofilm from coupons; D - Characterization of exposed and unexposed biofilms; E – Inoculation of fresh R2A medium with bacteria from musk-exposed biofilms; F - Characterization of bacteria from musk-exposed biofilms.

DMSO (v/v), instead of AHTN at 150 ng/L.

After 7 days of incubation, the coupons containing the biofilms were carefully placed in new wells with sterile STW to wash off the weakly and non-adhered bacterial cells. Then, a total of 8 coupons (per condition) were placed in 50 mL falcon tubes with 7 mL of extraction buffer, prepared according to Frølund et al. (1996). The biofilm was removed from the coupons with vortex agitation for 2 min. To extract the EPS, the biofilm suspension was added to a beaker containing 0.7 g of Dowex® Marathon© resin (Naþ form, strongly acidic, 20–50 mesh, Sigma-Aldrich, Germany). This resin, in the acidic form, is used to remove cations from the matrix of biofilms, breaking the aggregates and leading to the release of EPS (Gomes et al., 2020b). The suspension was then exposed to constant agitation (400 rpm) for 4 h and at a temperature of 4 ºC. After extraction, the suspension was centrifuged at 3700g for 5 min to collect the supernatant, which contained extracellular polymeric substances. The resulting pellet was resuspended in 7 mL of extraction buffer, and both the pellet solution and the supernatant were stored at − 20 ºC for further analysis of the total amount of proteins and polysaccharides. Polysaccharides were quantified by the phenol-sulphuric method ((Dubois et al., 1951) using glucose as standard. The detection limit of the method was 7.08 mg/L. Proteins were quantified according to Sedmak and Grossberg (1977), using bovine serum albumin as standard. The detection limit of the method was 0.12 mg/L.

This experiment was performed in three independent assays, with duplicates.

2.4. Characterization of bacteria from musk-exposed biofilms

2.4.1. Biofilm formation ability of bacteria from musk-exposed biofilms

Musk-exposed bacteria (from cryopreservation) were grown overnight planktonically in R2A broth, at 25 ºC and 160 rpm. Then, the cell density was adjusted to 10^8 CFU/mL in R2A broth. Bacterial suspensions were used to form 24-h-old biofilms in 96 wells polystyrene flat-bottom microtiter plates (VWR, Portugal). For single species biofilms, each well of the microtiter plate was filled with 200 µL of the exposed bacterial suspension. In the case of mixed species biofilms, 66.7 µL of each of the bacterial suspensions was added to each well. Each condition was tested using a total of 10 wells, ensuring five replicates to assess biofilm formation ability and five replicates to assess biofilm susceptibility to disinfection. Microtiter plates were incubated at 25 ºC under 160 rpm of agitation for 24 h.

After incubation, biofilms were treated with sodium hypochlorite (Sigma-Aldrich, Germany). Firstly, the bacterial suspension was removed, and each well was washed with a volume of 200 µL of STW to remove weakly adhered and non-adhered cells. Afterwards, biofilms were exposed to STW (control conditions) or sodium hypochlorite solution at 10 mg/L of free chlorine (treated biofilm) for 30 min. The chlorine solution was prepared accordingly to Gomes et al. (2020b), adjusting the free chlorine concentration through the N, N-diethylp-phenylenediamine (DPD) method (test kit from Hanna Instruments, Woonsocket, USA). After an incubation time of 30 min, the STW and the chlorine solution were discarded, and 200 µL of sodium thiosulphate (0.5% w/v) was added to the wells to neutralize the activity of chlorine (Gomes et al., 2016). After 5 min of incubation, the solution was discarded and the biofilm biomass and activity were evaluated by crystal violet and Alamar blue staining, respectively.

To assess the biomass formed and/or removed, biofilms in each well were fixed with 250 µL of 100% ethanol. After incubating for 15 min at room temperature, the ethanol was discarded. Then, 200 µL of crystal violet (CV) solution (Merck, Germany) at 1% (v/v) was added to the wells and the microtiter plates were left to incubate for 5 min at room temperature. The liquid was again discarded, and 200 µL of acetic acid at 33% was added to each well. Finally, the absorbance was measured at 570 nm. This method serves as a quantitative indicator of the total amount of biofilm since the dye binds to negatively charged molecules in the biofilm and the measured absorbance is proportional to the biofilm mass (Burmølle et al., 2006). The assay was repeated three times with five replicates.

To assess the viability of the bacterial cells in the biofilm, the Alamar blue assay was performed. This method is based on the reduction of a blue dye, Alamar blue, by metabolically active cells, which gradually turns into pink resorufin, a fluorescent compound (Peeters et al., 2008). The Alamar blue (Sigma-Aldrich, Germany) solution at 0.1 mg/mL was prepared in sterile ultrapure water, in the dark. This assay is frequently used to assess bacterial viability, including in biofilms, as the fluorescence of resorufin is proportional to the metabolic activity of the cells within the biofilms (Alonso et al., 2017). After biofilm formation, treatment with chlorine and neutralization, as described previously, 190 µL of R2A liquid broth was added to the wells along with 10 µL of the Alamar blue solution. The microtiter plates were incubated for 20 min in the dark at room temperature, and after this period, fluorescence was measured in a microtiter plate reader (Spectramax M2e, Molecular Devices, Inc.) with an exciting wavelength of 570 nm and an emission wavelength of 590 nm. Three independent assays with four replicates were performed.

2.4.2. Motility of bacteria from musk-exposed biofilms

Bacteria from musk-exposed biofilms were inoculated in LB broth and incubated overnight at 25 ºC under agitation (160 rpm). Afterwards, bacterial cell density was adjusted to 10^8 CFU/mL and a volume of 15 µL was applied in the centre of Petri dishes containing adequate media (1% tryptone from Merck, Germany, 0.25% NaCl (VWR, Belgium) and agar (VWR, Belgium) at 0.3%, 0.7% or 1.5% for swimming motility, swarming motility or twitching motility, respectively) (Borges et al., 2012). Then, the plates were incubated at 25 °C and the motility halos were measured after 72 h of incubation. Each condition was tested in triplicate, in three independent assays.

2.5. Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using the statistical software PAST in its latest version, 4.03. Statistical calculations were based on a confidence level $\geq$ 95%, *i.e.*, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of musk contaminants on bacteria culturability of 7-days old biofilms

The effect of the exposure to musks on bacteria culturability was assessed for biofilms formed on PVC and SS316. Fig. 2. A presents the log CFU/cm^2 of single and mixed species biofilms grown for 7 days in PVC coupons in the presence of the synthetic musks, while Fig. 2. A presents the results obtained for single and mixed species biofilms formed for 7 days on SS316 coupons. In general, it was observed that biofilms formed on SS316 (Fig. 2. A) had more culturable cells than biofilms formed on PVC (Fig. 2. A), although statistically significant differences were not detected ($p > 0.05$). The musk exposure to *A. calcoaceticus* biofilms did not cause significant alterations in bacteria culturability, regardless of the surface material used ($p > 0.05$). *B. cepacia* biofilms displayed an analogous behaviour, *i.e.* a more evident biofilm production on SS316. However, musk exposure did not produce significant effects on *B. cepacia* culturability in comparison to the solvent control ($p > 0.05$). For *S. maltophilia* biofilms, the number of culturable cells tends to be slightly more pronounced on SS316 ($p > 0.05$). Moreover, it is possible to observe that the exposure to AHTN caused a decrease in the number of *S. maltophilia* culturable cells for biofilms formed on PVC and SS316 coupons in comparison to the solvent control ($p < 0.05$, Fig. 2). While the overall results suggest that the presence of musks led to similar responses in all single species biofilms, it seems that the impact of musks

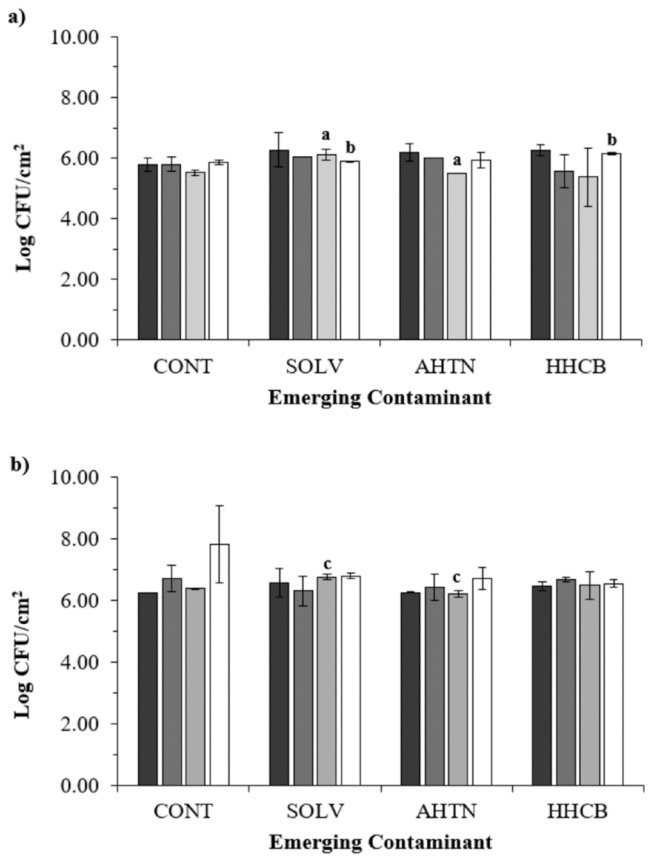

Fig. 2. Bacterial culturability of *A. calcoaceticus* ■, *B. cepacia* ■, *S. maltophilia* ■, or mixed (□) species biofilms formed on PVC (A) and SS316 (B) surfaces for 7 days.
CONT – unexposed bacteria; SOLV – bacteria exposed to DMSO at 0.003% (v/v); AHTN – bacteria exposed to AHTN at 150 ng/L; HHCB – bacteria exposed to HHCB at 150 ng/L. [a-c] - corresponds to conditions that have statistically significant differences from each other ($p < 0.05$).

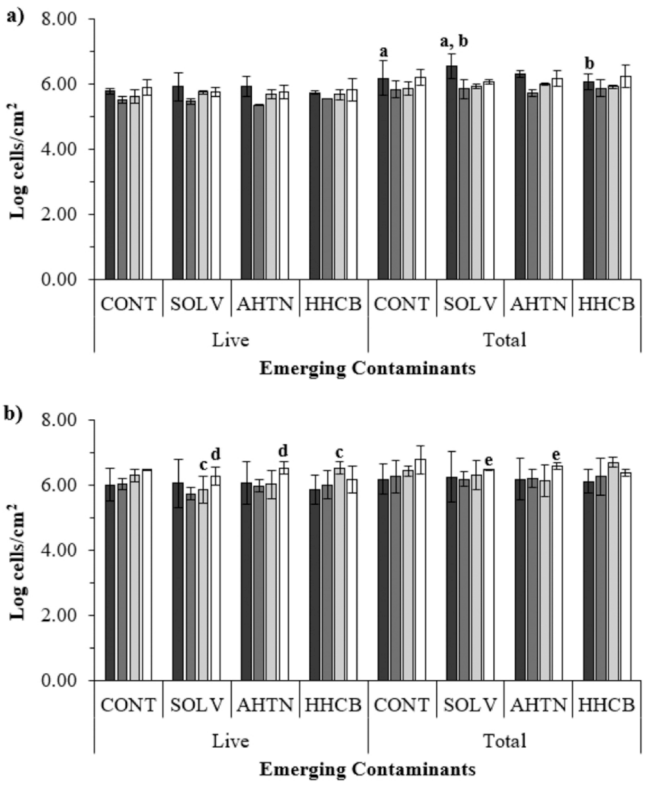

Fig. 3. Cellular density (Live – non-damaged cells; Total – total cells) of *A. calcoaceticus* ■, *B. cepacia* ■, *S. maltophilia* ■, or mixed (□) species biofilms formed on PVC (A) or SS316 (B) surfaces. CONT – unexposed bacteria; SOLV – bacteria exposed to DMSO at 0.003% (v/v); AHTN – bacteria exposed to AHTN at 150 ng/L; HHCB – bacteria exposed to HHCB at 150 ng/L. [a-e] - corresponds to conditions that have statistically significant differences from each other ($p < 0.05$).

on mixed species biofilms did not follow the same trend. Specifically, the exposure to musks seems to boost biofilm production on PVC surfaces (Fig. 2. A). The exposure to HHCB particularly caused an increase in the number of CFU/cm^2 in mixed species biofilms formed on PVC ($p < 0.05$), even though no significant differences were found for biofilms adhered on SS316 surfaces ($p > 0.05$, Fig. 2. B).

3.2. Effect of musk contaminants on the cellular density and viability of 7-day-old biofilms

Fig. 3 presents the cellular density and viability (in log cells/cm^2) of single and mixed 7-d-old biofilms grown on PVC (Fig. 3. A) and SS316 (Fig. 3. B) in the presence of musks (AHTN and HHCB). Overall, bacterial adhesion was slightly favoured on SS316 surfaces than on PVC surfaces, although the differences are not statistically significant ($p > 0.05$). The number of total cells of *A. calcoaceticus* in biofilms formed on PVC was higher in the solvent control ($p < 0.05$) when compared to the unexposed biofilm (CONT). Exposure to HHCB caused a slight decrease in the cellular density ($p < 0.05$) of *A. calcoaceticus* biofilms on PVC. However, their viability was not significantly affected by HHCB ($p > 0.05$). Moreover, musk exposure did not produce any significant effects on *A. calcoaceticus* biofilms formed on SS316 coupons in comparison to the solvent control ($p > 0.05$). Regarding *B. cepacia* biofilms, musk exposure did not produce significant effects on the cellular density and viability in biofilms adhered on SS316 and PVC surfaces ($p > 0.05$). *S. maltophilia* had a higher ability to adhere on SS316 surfaces than on PVC. For SS316 surfaces, the effects from musk exposure were more noticeable. In particular, HHCB exposure appears to be associated with an increase in *S. maltophilia* viability in comparison to the solvent control ($p < 0.05$).

Mixed species biofilms adhered on PVC surfaces were not altered by musk exposure ($p > 0.05$). However, mixed species biofilms formed on SS316 and exposed to AHTN had a higher number of viable and total cells than those exposed to the solvent ($p < 0.05$).

3.3. EPS matrix content of mixed species biofilms after exposure to AHTN

Mixed species biofilms were characterized in terms of EPS content after exposure to AHTN, which was the musk that caused the most significant changes in the cell density (both numbers of total and viable cells). The biofilms formed on the different surface materials were characterized by the content of extracellular proteins and polysaccharides (Table 1). That content was found to be very similar among the biofilms developed, regardless of the surface material used for adhesion. For bacteria exposed to AHTN, the content of proteins was

Table 1
Characterization of the extracellular matrix of mixed biofilms formed with bacteria previously exposed to DMSO at 0.003% (solvent control) and AHTN at 150 ng/L, adhered on PVC and SS316 surfaces.

	PVC		SS316	
EPS content (μg/cm^2)	SOLV	AHTN	SOLV	AHTN
Proteins	11.08 ± 3.66	11.91 ± 4.60	12.68 ± 2.87	12.40 ± 3.71
Polysaccharides	2.75 ± 1.45	3.00 ± 2.63	3.26 ± 2.43	7.28 ± 3.01

similar for biofilms formed on both surfaces ($p > 0.05$). For the polysaccharides content (Table 1), it was possible to observe that biofilms adhered to SS316 and formed in the presence of AHTN presented a slightly higher content than for the solvent control.

3.4. Biofilm formation ability of bacteria from musk-exposed biofilms

Fig. 4 shows that bacteria from biofilms adhered on PVC surfaces displayed, in general, a higher ability to form new biofilms (*i.e.* with higher biomass) than bacteria from biofilms formed on SS316. The exposure of biofilms formed on PVC to musks altered the ability of bacteria to form new biofilms, mainly for *A. calcoaceticus* and *S. maltophilia*. *A. calcoaceticus* presented an increased ability to form biofilms after being exposed to HHCB on PVC surfaces ($p < 0.05$). Regarding *S. maltophilia* from biofilms formed on PVC, it was possible to observe a decrease in their biofilm formation ability when exposed to any of the contaminants tested ($p < 0.05$). Moreover, the exposure of each bacterial strain adhered on PVC to AHTN caused a slight increase in their ability to form new mixed species biofilms ($p > 0.05$). For SS316 surfaces, musk influence on the ability to form biofilms was not so noticeable. The exception was the mixed species biofilms formed by bacteria from AHTN-exposed biofilms, which had more biomass than the respective solvent control ($p < 0.05$).

Chlorine treatment (10 mg/L of free chlorine for 30 min) did not produce significant removal of biofilms formed by bacteria from musk-exposed biofilms on PVC and SS316 ($p > 0.05$) (supplementary file - Table S.1). However, it caused significant biofilm inactivation (Table 2). Nevertheless, complete inactivation was not achieved (maximum inactivation observed was 48.9%). An increase in susceptibility to chlorine for single species biofilms formed by *A. calcoaceticus, B. cepacia* and *S. maltophilia* from HHCB-exposed biofilms formed on PVC surfaces was observed in comparison to the non-exposed counterparts (SOLV) ($p < 0.05$). Similar observations were found for *B. cepacia* and mixed species biofilms formed by bacteria from AHTN-exposed biofilms formed on PVC surfaces ($p < 0.05$). In general, biofilms formed by bacteria from HHCB-exposed biofilms formed on SS316 were more tolerant to chlorine disinfection than non-exposed bacteria ($p < 0.05$). However, a similar trend was observed for mixed species biofilms formed by bacteria from HHCB-exposed biofilms formed on PVC ($p < 0.05$). The exposure of bacteria to AHTN on SS316 leads to the formation of mixed species biofilms more tolerant to chlorine than those formed by non-exposed bacteria (SOLV) ($p < 0.05$).

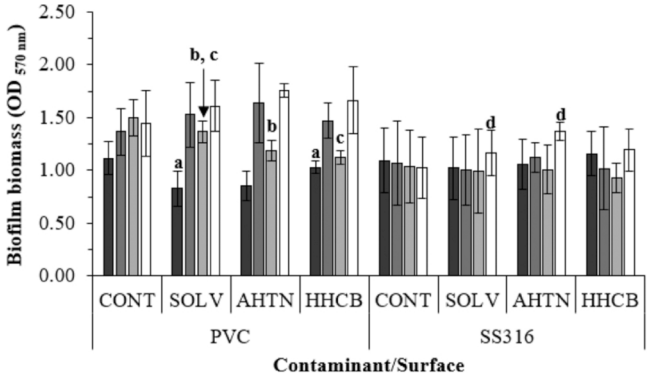

Fig. 4. The ability of exposed and unexposed bacteria from PVC and SS316 surfaces to form new single and mixed species biofilms. Biomass of *A. calcoaceticus* ■, *B. cepacia* ■, *S. maltophilia* ■, or mixed (□) biofilms. $^{a\text{-}d}$ - corresponds to conditions that have statistically significant differences from each other ($p < 0.05$).

3.5. Motility of bacteria from musk-exposed biofilms

Swimming, swarming and twitching motility of bacteria from musk-exposed biofilms were tested for 72 h (Fig. 5). The exposure of *A. calcoaceticus* in biofilms formed on PVC and SS316 to both musk contaminants did not alter the swarming motility in comparison to the solvent control ($p > 0.05$). On the other hand, the exposure of *B. cepacia* to AHTN (regardless of the material surface) increased the swarming motility, mainly in bacteria from biofilms formed on SS316 ($p < 0.05$). Overall, swarming motility was more pronounced in *S. maltophilia* from biofilms formed on SS316 surfaces than on PVC surfaces ($p < 0.05$). However, the exposure to musk contaminants did not alter significantly the swarming motility of *S. maltophilia* in comparison to the solvent control ($p > 0.05$).

Exposure to DMSO caused alterations in the bacterial motility when exposure took place in biofilms formed on PVC surfaces (Fig. 5. C). In particular, an increase in swimming motility was observed for *B. cepacia* ($p < 0.05$). Also, an increase in *A. calcoaceticus* swimming motility was observed for bacteria from musk (AHTN and HHCB)-exposed biofilms in SS316 (in comparison to the solvent control – $p < 0.05$). *B. cepacia* swimming motility was not affected by the exposure of biofilms to musk contaminants, regardless of the surface used ($p > 0.05$). However, there was an increase in swimming motility for *S. maltophilia* from HHCB-exposed biofilms in SS316 ($p < 0.05$). Twitching motility was higher for bacteria from biofilms formed on PVC surfaces (Fig. 5. E) than those on SS316 (Fig. 5. F), however, it was not significantly affected by the exposure to AHTN and HHCB.

4. Discussion

Water should be managed as a whole due to its circularity and the interconnectivity between water in natural and anthropogenic systems, such as water sources (groundwater and surface waters), wastewater, treated wastewater and drinking water, as defended by the One Water Approach (Fitzmorris-Brisolara et al., 2022). A management system based on this concept will ensure wider safety for public health (Fitzmorris-Brisolara et al., 2022). So, this concept supports the need for the study of ECs consequences on water microorganisms. The consequences of ECs on microbial communities may have significant repercussions along the water cycle and on public health. However, the impact of musks in microbial communities has been significantly disregarded. Therefore, it is of utmost importance to understand the impact of these contaminants in DW microbial communities to anticipate potential public health risks and improve the water management to guarantee water quality and safety. In the present work, bacteria were selected as the main representative organisms of DW microbial communities, since most of the microbial cells found in DW biofilms are bacteria (Fish and Boxall, 2018). The mixed species biofilms were used as a more realistic model of DW biofilms since in nature biofilms are not formed by only one bacterial species but by different microorganisms and bacterial species (Fish and Boxall, 2018). So far, only two works have evaluated the effects of musks in DW biofilm models (Gomes et al., 2018, 2019a). This study is the pioneer in the assessment of the impact of musk contaminants on DW biofilms and bacteria, considering the influence of the materials used as biofilm substrate. For that purpose, two materials often found in DWDS (PVC and SS316) were used. The previous works (Gomes et al., 2018, 2019a) did not report a significant impact of musks (AHTN and HHCB) on the biofilms, which is not corroborated by the present study. These differences may result from the differences in the experimental conditions and parameters evaluated (Gomes et al., 2018, 2019a). In the present work, single and mixed species biofilms were formed on SS316 and PVC coupons for 7 days, under exposure to musk contaminants and the direct impact on biofilm was assessed in terms of cellular culturability, viability and density. However, in the previous works, the effects of musk contaminants were assessed only in terms of culturability in 24-h-old *B. cepacia* biofilms formed in 96-wells

Table 2
Inactivation (%) of biofilms formed by exposed and unexposed bacteria from PVC and SS316 surfaces after chlorine treatment (10 mg/L for 30 min). Results are presented as mean ± SD of at least three independent assays; * corresponds to results with statistically significant differences in comparison to SOLV ($p < 0.05$).

	Biofilm inactivation (%)							
	PVC				SS316			
	CONT	SOLV	AHTN	HHCB	CONT	SOLV	AHTN	HHCB
A. calcoaceticus	21.8 ± 8.0	9.9 ± 6.3	25.8 ± 9.0	23.8 ± 1.7 *	27.6 ± 13.7	26.8 ± 12.8	29.4 ± 4.7	13.2 ± 0.2 *
B. cepacia	30.9 ± 0.1	22.8 ± 7.2	48.9 ± 3.4 *	40.8 ± 4.4 *	21.7 ± 4.3	31.3 ± 1.0	42.4 ± 13.1	26.7 ± 6
S. maltophilia	36.3 ± 3.3	19.5 ± 5.0	30.1 ± 9.4	38.0 ± 3.5 *	38.0 ± 32.6	37.1 ± 18.0	25.3 ± 4.5	10.4 ± 6.6 *
Mixed biofilms	24.4 ± 18.8	29.9 ± 8.4	38.5 ± 0.0 *	0.0 ± 0.0 *	33.1 ± 18.7	40.4 ± 12.4	24.4 ± 8.2 *	20.1 ± 2.8 *

polystyrene microtiter plates (Gomes et al., 2019a) or in 26-d-old *S. maltophilia* biofilms on PVC coupons (Gomes et al., 2018).

The present results demonstrated that AHTN and HHCB altered significantly biofilms formed on PVC and SS316 as well as the bacterial motility and ability to form biofilms. It was evident that the impact of musk contaminants was dependent on the bacterial species but also on the materials where biofilms were formed. For example, an increase in the number of culturable cells in mixed species biofilms formed on PVC was found after exposure to HHCB, and the same tendency was not observed for its counterparts formed on SS316. On the other hand, HHCB caused a reduction in the total number of *A. calcoaceticus* cells on single species biofilms formed on PVC, but increased the number of viable cells of *S. maltophilia* biofilms formed on SS316. These results demonstrated that the exposure to trace concentrations of musk contaminants may alter the characteristics of biofilms developed in DWDS. However, the impact of musk was also found to be influenced by the substrate material, bacterial strains and biofilm composition (single and mixed species biofilms). For example, higher numbers of culturable, viable and total cells were observed in biofilms formed on SS316 than on PVC. So, the different characteristics among biofilms formed on different substrata may influence the interaction between musks and biofilm, as reviewed by Gomes et al. (2020a). Biofilms may interact with environmental contaminants in multiple ways: bacteria may uptake ECs from water; ECs may be adsorbed in the bacterial surface by binding to specific sites; bacteria may secret outer membrane vesicles which may play an important role in trapping some contaminants; ECs may interact with the matrix through adsorption to EPS or by chemical interactions with EPS-associated functional groups (Gomes et al., 2020a). So, these interactions will be highly dependent on the characteristics of biofilms, which in its turn are highly influenced by the surface where biofilms are formed (Teughels et al., 2006). The higher impact of musks in biofilms formed on SS316 may be attributed to the higher cellular density in these biofilms. It is well known that AHTN and HHCB have a high potential for adsorption on organic matter resulting in a high propensity for bioaccumulation (Romero et al., 2022). Furthermore, musk effects may also be influenced directly by the material used, since it is known that adsorption mechanisms can occur between musks and plastic materials (Dong et al., 2019). Therefore, for PVC, the concentration of musks available to interact with biofilms and bacteria may be different from these present in SS316 experiments, due to the adsorption of musks in the plastic materials. This may help to explain the higher variability in the characteristics of biofilms formed on SS316 after exposure to musks. Moreover, it is also known that the presence of biofilms on plastic surfaces may influence the adsorption of ECs (Zhang et al., 2022). A recent study evaluated the impact of biofilm formation in microplastics on the chemical adsorption of nine different ECs and varying effects were observed depending on the type of EC and the presence of biofilms (Zhang et al., 2022). ECs adsorption in microplastics with biofilms decreased for miconazole and estrone and increased for carbendazim, carbamazepine, and diclofenac (Zhang et al., 2022). Other recent studies also highlighted that the presence of biofilms or organic matter in plastic surfaces favoured the adsorption of perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), lead, atrazine, 4-acetaminophenol and hexafluoropropylene oxid dimer acid (Ateia et al., 2020; Bhagwat et al., 2021). Attending to these studies, it is not possible to conclude about the impact of biofilms on musk adsorption to PVC coupons. However, the presence of biofilms in plastics has a significant impact on ECs adsorption to these polymeric surfaces.

Biofilms commonly found in nature or anthropogenic environments are characterized by a complex and heterogeneous composition, exhibiting interactions between multiple species, which not only facilitates their survival in various environmental niches but also results in increased resistance to antimicrobial agents (Galié et al., 2018; Arunasri and Mohan, 2019). For that reason, besides studying the formation of single species biofilms, this work includes the study of mixed biofilms, which better represents the scenario found in a DWDS. Mixed species biofilms formed under exposure to AHTN showed a higher content of extracellular polysaccharides than the non-exposed biofilms. Several studies have reported EPS production influenced by the presence of contaminants, such as cytostatic drugs, 2,6-dichlorophenol and sulfonamides (Avella et al., 2010; Li et al., 2016; Xu and Sheng, 2020), usually in the form of increased production as a stress response and protection mechanism against toxic contaminants. On the other hand, the composition of EPS varies for different bacterial strains as well as with the surface material where bacterial attachment takes place (Nuryastuti et al., 2011; Petrova and Sauer, 2012). One particular study by Nuryastuti et al. (2011) concluded that EPS production and biofilm formation by three *Staphylococcus epidermidis* isolates exposed to gentamicin varied with the surface material. While biofilm production was more pronounced on stainless steel surfaces, EPS production suffered an increase when using polyethylene surfaces.

Behind the direct impact of musks on the biofilms formed, the results also describe the alterations in the characteristics of bacteria from musk-exposed biofilms, such as their ability to form biofilms, susceptibility to free chlorine and also their motility. This work demonstrates that biofilm exposure to musks on PVC surfaces resulted in a significant reduction of *S. maltophilia* ability to form new biofilms. It means that these bacteria from musk-exposed biofilms, formed on PVC, will be able to colonize other surfaces to a lower extent than the non-exposed bacteria, which did not seems to be a critical situation in terms of DWDS management. On the other hand, *A. calcoaceticus* from HHCB-exposed biofilms formed on PVC had a higher ability to form single species biofilms. Moreover, bacteria exposed to AHTN on SS316 were also able to produce mixed species biofilms with a higher biomass content. From a perspective of DWDS management, the presence of AHTN in systems composed of SS316 will be more critical than HHCB. AHTN-exposed bacteria demonstrated an increased ability to colonize new surfaces.

After the evaluation of the impact of exposure to musks on the ability of bacteria to form a biofilm, the resulting sessile community was also treated with 10 mg/L of free chlorine. The susceptibility of biofilms to free chlorine was assessed by two methods: one aimed to determine the total biomass providing information on the biofilm removal caused by free chlorine; the other aimed at the evaluation of the inactivation of the metabolic activity of bacteria that was forming the biofilm. Analysing the results from these two tests, it is evident that free chlorine at 10 mg/L is not able to remove biofilms from the surface. However, chlorine was responsible for significant metabolic inactivation in biofilms formed by *A. calcoaceticus, B. cepacia* and/or *S. maltophilia.* The results highlighted

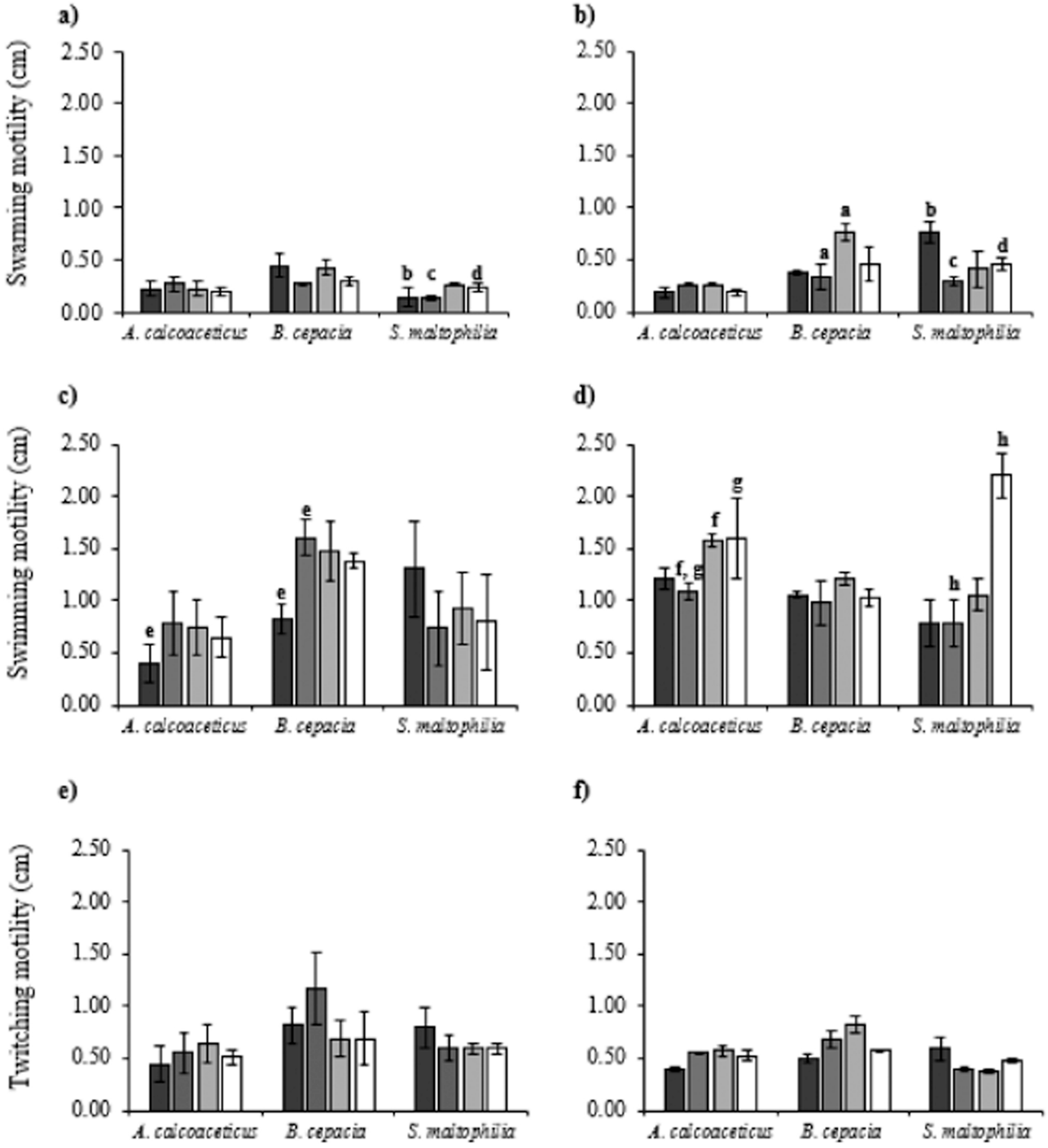

Fig. 5. Swarming (A, B), swimming (C, D) and twitching motility (E, F) of *A. calcoaceticus*, *B. cepacia*, and *S. maltophilia* previously exposed to musk contaminants on PVC (a, c, e) and SS316 (b, d, f) surfaces. Results are presented as mean ± SD of at least three independent assays; CONT ■ – unexposed; SOLV ■ – bacteria exposed to DMSO at 0.003% - solvent control; AHTN ■ – bacteria exposed to AHTN at 150 ng/L; HHCB (□) – bacteria exposed to HHCB at 150 ng/L. $^{a\text{-}h}$ - corresponds to conditions that have statistically significant differences from each other ($p < 0.05$.

that HHCB significantly altered the susceptibility of these biofilms to chlorine. Bacteria from HHCB-exposed biofilms in PVC were able to form biofilms more susceptible to chlorine, which did not represent a scenario of concern for DWDS. It means that the new biofilms formed will be more susceptible to chlorine than these biofilms formed by non-exposed bacteria. It is important to highlight that the exposure of single species biofilms to HHCB caused different effects on the ability of bacteria to form single and mixed species biofilms. Single species biofilms formed after exposure to HHCB on PVC were more susceptible to chlorine. However, the mixed species biofilms formed after bacteria exposure to HHCB in PVC coupons were significantly more tolerant to chlorine action. This last situation is the most critical since it suggests that the previous exposure of bacteria to HHCB in biofilms formed on PVC may affect the effectiveness of DW disinfection and potentially compromise DW quality and safety. HHCB did not alter significantly the total amount of biomass in the exposed biofilms. Therefore, the alterations observed in biofilm susceptibility to free chlorine may be caused by changes in the biofilm structure, affecting the penetration of chlorine.

The results obtained in this study further showed that musk exposure has an impact on bacterial motility. The present study focused on three types of bacterial motility already described for the three bacteria evaluated: swimming, swarming and twitching (Gomes et al., 2016, 2019a; Malešević et al., 2017). Swimming motility requires a functional flagellum and is associated with bacterial cell movement in liquid or low viscosity environments (Ha et al., 2014). Swarming motility is a flagellum driven movement regulated by quorum sensing which allows for biofilm dispersion and colonization of new niches (Daniels et al., 2004). This type of movement is also valuable when environmental characteristics, such as nutrient availability, are not favourable and bacteria need to find new sites with more auspicious conditions (Daniels et al., 2004). Finally, twitching motility is a type of movement dependent on type IV pilli that allows bacterial movement over surfaces (Wall and Kaiser, 1999). Specifically, the results demonstrated that the swarming motility of *B. cepacia* from AHTN-exposed biofilms formed on SS316 and PVC biofilms suffered an increase in comparison to the non-exposed bacteria. Gomes et al. (2019a) also demonstrated that *B. cepacia* swarming motility was increased by 12% in the presence of HHCB. Considering that the exposure to HHCB caused a slight increase in the culturable population of *B. cepacia* biofilms formed on SS316 but did not promote swarming motility for the same conditions, it can be proposed that the changes in biofilm formation were not promoted by the alterations in the swarming motility. Moreover, the increase in the swarming motility of *B. cepacia* after exposure to AHTN in SS316 may be related to an increased ability of bacteria to form mixed species biofilms after exposure to the same conditions. The swarming motility is characterized by requiring intercellular interactions essential for the recruiting of cells. Swarming is a side-by-side movement, which implies the movement of a group of cells (Kearns, 2010). It is known that swarming may be regulated by quorum-sensing, and signal molecules produced by specific bacteria might have a significant impact on the quorum sensing–regulated swarming in other bacteria (Daniels et al., 2004). Therefore, the increase in the formation of mixed species biofilms may result from the increase of swarming expression by *B. cepacia* which may act by recruiting other cells to the biofilm. Regarding swimming motility, the results demonstrate that musk exposure promotes this type of motility, specifically for *A. calcoaceticus* exposed in biofilms adhered onto SS316 surfaces. This means that the exposure to musks could be related to a positive effect in flagellum synthesis and/or activity (Kondakova et al., 2016). So, exposure to AHTN and HHCB may stimulate bacterial movement in liquid environments. A previous work also demonstrated that the presence of ECs in DW, such as erythromycin, may affect the motility of microorganisms (Shunmugam et al., 2021). Most of the alterations observed in bacteria motility resulted from exposure that occurred in SS316, which may be a consequence of the increased number of cells on that surface material.

5. Conclusions

5.1. The musk contaminant of concern

AHTN was the musk contaminant that caused a higher impact in mixed species biofilms. Specifically, AHTN exposure leads to the formation of mixed species biofilms with the highest cellular density and number of viable cells in SS316. AHTN also promoted the production of polysaccharides in mixed species biofilms formed on SS316, as well as increased the ability of bacteria to form new mixed species biofilms.

5.2. The role of biofilm models

The alterations observed in mixed species biofilms after AHTN exposure were not predictable through the results observed in from the experiments with single species biofilms. Therefore, the presence of AHTN in DW has the potential to impact mixed biofilms as higher cellular proliferation and a slight increase in EPS production were observed in comparison to the single species experiments. On the other hand, most of the alterations caused by the exposure to HHCB were observed in single species biofilms. The impact of HHCB in biofilms was found to be dependent on the type of biofilm studied and the most critical situations were the increase in the cellular culturability of mixed species biofilms formed on PVC when exposed to HHCB and the increase of *S. maltophilia* viability in single species biofilms formed on SS316. In general, biofilms formed by bacteria from HHCB-exposed biofilms were able to form new biofilms with higher tolerance to chlorine disinfection. Therefore, the presence of HHCB in DW may also have negative repercussions for the DW quality and safety, as bacteria become more tolerant to disinfection when recolonizing new surfaces of the DWDS.

5.3. The impact of DWDS materials

Most of the alterations caused by the direct exposure of biofilms to musks were related to SS316, specifically the increase in biofilm cellular density and viability, as well as an increase in the polysaccharides content after exposure to AHTN. On the other hand, most of the alterations observed in the ability of bacteria to form new biofilms and their susceptibility to chlorine treatment were mainly observed when the exposure to musks took place on PVC surfaces.

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CRediT authorship contribution statement

Vitória Arruda: Methodology, Investigation, Data curation, Writing – original draft preparation. **Inês Gomes:** Conceptualization, Methodology, Investigation, Writing – review & editing, Writing – review & editing, Supervision. **Manuel Simões:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, funding acquisition.

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.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2022.129185.

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