Chemosphere 368 (2024) 143704

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Parabens alter the surface characteristics and antibiotic susceptibility of drinking water bacteria

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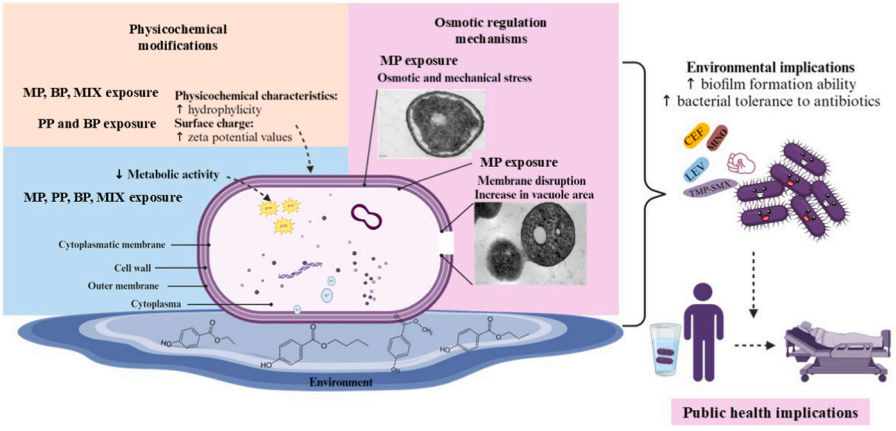
HIGHLIGHTS

- MP, BP, and MIX alter physicochemical bacterial cell surface properties and charge.
- MP, PP, BP, and MIX at 15000 μg/L affect bacterial metabolic activity.
- MP induce osmotic regulation mechanisms in *A. calcoaceticus*.
- MP-exposed bacteria at 15 and 15000 μg/L are higher biofilm producers.
- Long exposure to MP at 15 and 15000 μg/L increases antibiotic tolerance.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling editor: Magali Houde

Keywords:
Antibiotic susceptibility
Bacterial morphology
Biofilms
Cell surface
Mechanism of action
Parabens

ABSTRACT

Parabens are markedly present in products of daily use, considered emerging environmental contaminants that can harm human health and aquatic life, due to their release into aquatic sources. The impact of the exposure of microbial communities to parabens remains unclear. This study investigates aspects of the mode of action of methylparaben (MP), propylparaben (PP), butylparaben (BP), and MIX at environmental (15 μg/L) and in-use (15000 μg/L) concentrations, against two bacterial strains of *Acinetobacter calcoaceticus* and *Stenotrophomonas maltophilia* previously isolated from drinking water (DW). BP showed the strongest antimicrobial activity, while MP exhibited the weakest. The mechanism of action of parabens at the selected concentrations was found to be related to perturbations on physicochemical bacterial cell surface properties and charge, by causing an increase of bacterial cell envelope hydrophilicity and zeta potential values. In addition, parabens may activate osmotic regulation mechanisms as observed by the increase in vacuole area for MP-exposed *A. calcoaceticus*. The bacterial metabolic activity as well as bacterial size was also affected by parabens exposure. MP exposure further enhanced the biofilm formation ability and increased bacterial tolerance to antibiotics. The results raise environmental

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https://doi.org/10.1016/j.chemosphere.2024.143704
Received 23 September 2024; Received in revised form 29 October 2024; Accepted 5 November 2024
Available online 12 November 2024

implications, particularly concerning water quality and public health, as parabens exposure can potentiate the virulence of DW bacteria, increasing the risk of human exposure to harmful microorganisms.

1. Introduction

Parabens are preservatives widely used in many pharmaceutical, food, and cosmetic products due to their low toxicity (Nowak et al., 2021). They are widely used in products commercially available in Europe at levels up to 2 mg/kg in food, 1 mg/L in drinks, 0.4% in cosmetics European Commission EU, 2011, 2014), and up to 1% in pharmaceuticals (Soni et al., 2001) - to prevent bacterial growth and prolong the shelf life of the products. Commonly used parabens include methylparaben (MP), ethylparaben (EP), propylparaben (PP), and butylparaben (BP). They have antimicrobial properties, being stronger against fungi followed by Gram-positive bacteria, and Gram-negative bacteria (Lincho et al., 2021).

Due to their incomplete elimination in wastewater treatment plants (WWTP), parabens can reach aquatic environments contaminating the water cycle and affecting drinking water (DW) (Pereira et al., 2023b). Therefore, bacterial communities living on these aquatic matrices are constantly exposed to these pollutants of emerging concern. While the endocrine-disrupting and carcinogenic effects of parabens on human health and their cytotoxicity against aquatic organisms are well-known, there is a significant gap regarding the impact of these molecules on the exposed microbial communities (Wei et al., 2021).

The mechanism of action of parabens, when used as preservatives, is mainly related to modifications in the bacterial cell envelope, affecting the physicochemical characteristics (Flasiński et al., 2016, 2018). In extreme conditions, parabens may cause total disruption of the bacterial membrane integrity (Bolujoko et al., 2021). Other researchers argue that parabens can also induce changes in bacterial membrane transport processes (Bredin et al., 2005), and disturbances in the transmembrane potential and microbial respiration (Kosová et al., 2015). For example, exposure to PP at 0.5 g/L was found to cause the leakage of intracellular potassium (K^+) in *Escherichia coli* (Bredin et al., 2005) and *Enterobacter gergoviae* (Davin-Regli et al., 2006). Loeffler et al. (2020) recently reported the disruption of the cytoplasmatic membrane and the interference with ATPase by MP at 0.15% (w/v%) of *Listeria innocua* and *Pseudomonas fluorescens*. The combination of PP at 3 mM with UV-A irradiation (at 2015 μW/cm^2 or *D*-value of 4.89 ± 0.66 min) was also found to affect *E. coli* viability by increasing the production of intracellular reactive oxygen species (ROS) after a 10 min exposure (Ding and Tikekar, 2020). An increase in ROS production was also observed under the same conditions but after exposure to PP at 4 mM combined with plasma-activated water (Liu et al., 2021). Moreover, Murata et al. (2019) reported that the leakage of internal substances from *Saccharomyces cerevisiae* occurred when combining sulforaphane (1 mmol/L) and MP (0.125 mg/mL). The inhibition of the bacterial synthesis of DNA and RNA was also considered a mechanism of antimicrobial action of parabens (Halla et al., 2018). However, the mode of action of parabens under low concentrations, such as those encountered in the environment, is not yet fully understood, and it is expected that their interaction with microorganisms may vary with the concentration and parabens chain length (Liu et al., 2023). The number of studies evaluating the effects of parabens at environmental concentrations against aquatic biofilms is modest (Liu et al., 2023, 2024; Pereira et al., 2023a, 2024; Pereira and Gomes, 2024).

Increased ecotoxicity for longer alkyl-chain parabens in water sources is commonly observed (Pereira et al., 2023b). For example, EC_{50} values against *Aliivrio fisheri* range between 2.34 and 16.8 mg/L for BP and MP, respectively, whereas lethal concentration (LC_{50}) values from 11.2 to 73.4 mg/L towards *Daphnia magna* were obtained for BP and MP, respectively (Lee et al., 2018a,b). However, other studies argue that the toxicity profile of parabens is not directly proportional to their chain length but defend that different mechanisms of interaction between parabens and bacteria may occur depending on the length of the alkyl chain (Liu et al., 2023, 2024). Liu et al. (2023) reported that MP tends to remain in the cytoplasm, triggering apoptosis, while longer alkyl-chain parabens like BP enter the cytomembrane, causing necrosis (Liu et al., 2023). In the same study, MP was classified as a reactive-acting toxicant, whereas BP was classified as a baseline toxicant. However, the predicted effective concentration (EC_{50}) for MP and BP were 1.83 mg/L and 0.24 mg/L, respectively (Liu et al., 2023). Another study revealed that exposure to PP caused the strongest impact on freshwater biofilms, revealing high chemical reactivity potential through theoretical calculations and bioinformatic analysis in comparison to other parabens (MP, PP, and BP) (Liu et al., 2024). PP also caused a strong disturbance in the assembly process and quorum sensing system of freshwater biofilms at different environmental concentrations (1 and 10 mg/L) (Liu et al., 2024). Interestingly, the increase of pathogens in these bacterial communities was higher with parabens exposure at a low dosage (1 mg/L) (Liu et al., 2024). Therefore, the mode of action of parabens appears to be different according to their chemical structure and the concentration used.

In a recent study, Pereira et al. (2023a) reported changes in the behaviour and characteristics of aquatic environmental bacterial biofilms exposed to trace concentrations (150 ng/L) of parabens (MP, PP, BP, and a triple combination of all). Some modifications included increased biofilm proliferation (in culturability, density, extracellular polymeric substances - EPS production, and biofilm thickness) and an increase in the number of bacteria with damaged membranes (Pereira et al., 2023a). Increased motility of bacteria from parabens-exposed biofilms as well as increased production of virulence factors (protease and gelatinase activity) was also found in comparison to non-exposed environmental biofilms. Modifications on the biofilm architecture were observed after exposure to MP (Pereira et al., 2024). More concerning was the fact that MP exposure compromised DW disinfection by increasing the tolerance of DW biofilms to free chlorine (with the lower logarithmic reduction values of culturability of MP-exposed bacteria in comparison to the non-exposed counterparts) (Pereira and Gomes, 2024). Indeed, the changes induced in bacterial behaviour by long-term environmental exposure to parabens may also contribute to antimicrobial tolerance (Alampanos and Samanidou, 2021). This phenomenon was reported by Davin-Regli et al. (2006), who found a co-selection of antimicrobial resistance by parabens against *E. gergoviae*. Overall, the lack of information about the impact of parabens as environmental contaminants allied to their environmental persistence and their effects on microorganisms as preservatives highlights the need to understand the microbial changes induced by the exposure to parabens at the residual concentrations encountered in the environment.

The main goal of this work was to study the mode of action of parabens (MP, PP, BP), individually and in combination (MIX), which is a triple formulation composed of MP, PP, and BP on two strains of *Acinetobacter calcoaceticus* and *Stenotrophomonas maltophilia* (both Gram-negative bacteria) isolated from DW. The effects of parabens on biofilm formation and bacterial tolerance to antibiotics were also studied. The combination of parabens was planned to mimic the chemical heterogeneity encountered in real conditions and to provide novel insights into how diverse parabens affect bacterial characteristics and behaviour.

The impact of each paraben formulation at an environmental concentration, representative of water sources/systems contamination (15 μg/L), and a concentration 1000 times higher (15000 μg/L), representative of in-use concentrations (among the maximum limits allowed in food and drink products in European Union) was evaluated on different bacterial physiological indexes such as bacterial surface hydrophobicity

and charge, bacterial size distribution, and metabolic activity. The deregulation of membrane potential and ROS production were also assessed before and after parabens exposure at the highest concentration (15000 μg/L) tested. The potential increase of bacterial virulence induced by MP exposure at 15000 μg/L was evaluated in terms of biofilm formation ability. In addition, bacterial tolerance against a broad spectrum and clinically different relevant antibiotics (ceftazidime - CEF; levofloxacin - LEV; minocycline - MINO; and trimethoprim-sulfamethoxazole - TMP-SMX) was evaluated after long-term exposure to MP at both concentrations, to understand the selective pressure induced by these environmental contaminants. This information will help to clarify why bacterial communities change their characteristics and behaviour when exposed to parabens and will facilitate the prediction of the environmental and human health challenges posed by these parabens-exposed bacteria.

2. Materials and methods

2.1. Parabens solutions

MP, PP, and BP were purchased from Sigma-Aldrich (Germany). These parabens are the most commonly detected in aquatic systems worldwide (Bolujoko et al., 2021). In the present study, these selected parabens were tested individually and in combination (MIX formulation), which is a triple formulation composed of MP, BP, and PP (at the same proportion). Sterile ultrapure water (UPW) was used as the solvent for MP, whereas acetone at 0.005 % (v/v) was used as the solvent for BP and PP. The selected parabens were tested at subinhibitory concentrations: 15 μg/L, which is representative of environmental concentrations of parabens detected in water sources/systems, such as wastewater treatment plants (WWTP) and surface waters (Pereira et al., 2023b), and a concentration 1000 times higher (15000 μg/L). This last selected concentration of parabens is at the same level of concentrations found in approved European food and drinks products (2 mg/kg in food; 1 mg/L in drinks) but lower than those acceptable in European cosmetics (4000 mg/L) (European Commission EU, 2011, 2014) and pharmaceuticals products (10000 mg/L) (Soni et al., 2001). Therefore, a concentration of parabens equal to 15000 μg/L is defined as in-use concentration.

2.2. Bacteria and culture conditions

Acinetobacter calcoaceticus and *Stenotrophomonas maltophilia* were isolated from a drinking water distribution system (DWDS) and identified by 16 rRNA gene sequencing as described previously by Simões et al. (2007). Both selected bacteria are considered opportunistic pathogens frequently found in DWDS and associated with hospital outbreaks, presenting increased antimicrobial resistance (Alawi et al., 2024; Tsvetanova et al., 2022). *S. maltophilia* has been associated with infections in hospital water systems (Auld et al., 2023), while *A. calcoaceticus* plays a key role in biofilm formation due to its coaggregation abilities (Simões et al., 2008a,b). These characteristics make them important environmental models for studying their potential public health risks.

Both bacteria were grown overnight in R2A broth medium prepared as described by Gomes et al. (2018) at 25 °C and under agitation (160 rpm) in an orbital incubator (New Brunswick Scientific, I26, USA). Afterwards, bacterial cells were harvested by centrifugation (MegaStar 600R, VWR, Germany) at 3772×*g* for 10 min, washed and resuspended in the appropriate medium, and adjusted to 1×10^8 colony forming units (CFU/mL) for further experiments.

2.3. Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of each paraben (MP, PP and BP) against *A. calcoaceticus* and *S. maltophilia* was determined by the broth microdilution method according to McBain et al. (2004). For each bacterium, 16 wells of 96-well microtiter plates were filled with cells in R2A (180 μL) adjusted to an optical density (OD) at 600 nm of 0.132 and parabens (20 μL) at different concentrations (100, 150, 200, 250, 300, 350, 400 and 450 mg/L). Cell suspensions with UPW or acetone at 0.005% (v/v) and without parabens were used as controls. The OD was measured at 600 nm using a microplate reader before and after 24 h of incubation at 25 °C and 160 rpm. The MIC was determined as the lowest concentration of parabens at which complete inhibition growth was detected.

2.4. Effects of parabens exposure on bacterial cell envelope

2.4.1. Bacterial surface physicochemical properties

The surface physicochemical properties of parabens non-exposed (control) and exposed bacteria were measured by the sessile drop contact angle technique described by Busscher et al. (1984). A volume of 30 mL of bacterial suspension (adjusted to 1×10^8 CFU/mL in sterile tap water - STW prepared as described by Gomes et al. (2019a) was exposed to parabens solutions (MP, PP, BP and MIX) at 15 and 15000 μg/L. After 24 h of exposure, the suspension was filtered using a 0.45 μm cellulose nitrate filter (Sartorius Stedim Biotech GmbH, Germany) forming uniform lawns of bacteria. Afterwards, contact angle data were obtained from at least 15 determinations for each liquid and each experiment, at room temperature using a model OCA 15 Plus (DATAPHYSICS, Germany) that allowed image acquisition and data analysis, as described by Simões et al., 2008a,b. The surface tension components were obtained by measuring the contact angles with three pure liquids as reference: two polar liquids (water and formamide - Sigma, Portugal), and one apolar (α-bromo-naphthalene - Sigma, Portugal). The surface tension parameters of the reference liquids were taken from the literature (Janczuk et al., 1993). Then, the total surface hydrophobicity was evaluated based on contact angle data as proposed by van Oss (1986, 1993). According to the extended Young equation (Equation (1)), the contact angles (θ) of a liquid (L) on a surface are related to the total surface tension $\left(\gamma, \mathrm{mJ}/m^2\right)$, which can be separated into two components: apolar $\left(\gamma^{LW}\right)$ and polar $\left(\gamma^{AB}\right)$, with $\gamma_L = \gamma^{LW} + \gamma^{AB}$ and $\gamma^{AB} = 2\sqrt{\gamma^+\gamma^-}$. Where γ^+ and γ^- are the electron-acceptor and electron-donor parameters, respectively.

$$(1+\cos\theta)\gamma_L = 2\left(\sqrt{\gamma^{LW}\gamma_L^{LW}} + \sqrt{\gamma_L^+\gamma^-} + \sqrt{\gamma_L^-\gamma^+}\right) \quad (1)$$

The degree of hydrophobicity $\left(\Delta G_{iwi}, \mathrm{mJ}/m^2\right)$ of a given material was expressed in Equation (2), as the free energy of interaction between two identical entities (i) when immersed in water (w), as the sum of Liftshitz - van der Waals (LW) and Lewis acid-base (AB) interaction free energies. *LW* component includes London dispersion forces, Debye induction (dipole-induced dipole) and Keesom orientation (dipole-dipole) interactions. *AB* interactions include electron donor (γ^-) and electron acceptor (γ^+) contributions, thus accounting for hydrogen bonding and π-electron interactions too (Janczuk et al., 1993).

$$\Delta G_{iwi} = \Delta G_{iwi}{}^{LW} + \Delta G_{iwi}{}^{AB} = -2\,\gamma_{iw}^{LW} - 2\,\gamma_{iw}^{AB} \quad (2)$$

Where, $\gamma_{iw}^{LW} = \left(\sqrt{\gamma_i^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2$ and $\gamma_{iw}^{AB} = 2\left(\sqrt{\gamma_i^+\gamma_i^-} + \sqrt{\gamma_w^+\gamma_w^-} - \sqrt{\gamma_i^+\gamma_w^-} - \sqrt{\gamma_i^-\gamma_w^+}\right)$.

Thermodynamically, for $\Delta G_{iwi} < 0$ mJ/m^2, the bacterial surface is considered hydrophobic, whereas the bacterial surface is considered as hydrophilic for $\Delta G_{iwi} > 0$ mJ/m^2 (Borges et al., 2012). Non-exposed bacteria (adjusted in STW) and bacteria exposed to acetone at 0.005% were used as negative controls.

2.4.2. Bacterial surface charge and cell size

The surface charge of cells is frequently determined based on their zeta potential, which is calculated from the motility of cells in the

presence of an electrical field under defined pH and salt concentrations (Borges et al., 2013). The bacterial surface charge and the cell size distribution of non-exposed and parabens-exposed bacteria were determined using a Nano Zetasizer (Malvern Instruments, UK). For that, bacterial cell suspensions adjusted to 1×10^8 CFU/mL in STW were exposed to parabens (MP, PP, BP, MIX) at 15 and 15000 μg/L for 24 h (similarly to parabens exposure described in section 2.4.1). Bacterial suspensions in STW, in the absence of parabens and the presence of acetone at 0.005% (v/v) were used as negative controls. After that, bacterial suspensions non-exposed and parabens-exposed were centrifuged (MegaStar 600R, VWR, Germany) at 3772×*g* for 15 min and the pellet was resuspended in sterile UPW (Borges et al., 2013). Bacterial samples were vortexed to avoid the presence of any aggregates or clumps before being analyzed. After that, a small volume of the bacterial cell suspension was carefully loaded into a disposable cuvette that was placed inside the device (Nano Zetasizer). By measuring the electrophoretic mobility of the cells, the zeta potential values of non-exposed and parabens-exposed bacterial suspensions were obtained. These values reflect the bacterial surface charge as the electric potential difference between the bulk solution and the bacterial cell surface (Wilson et al., 2001).

Based on the fluctuations in the intensity of scattered laser light caused by the Brownian motion of particles in the sample, the hydrodynamic diameter of bacterial cells, which is an estimation of their size when suspended in a liquid medium was given by dynamic light scattering (DLS) (Falke and Betzel, 2019). The hydrodynamic diameter is calculated from the translational diffusion coefficient by using the Stokes-Einstein equation (Equation (3)) (Malvern, 2023).

$$D_h = \frac{KT}{3\pi\eta D} \tag{3}$$

Where, D_h (nm) is the hydrodynamic diameter, D (m^2/s) is the translational diffusion coefficient, K (J/K) is the Boltzmann's constant, T (K) is the absolute temperature, and η (Pa·s) the viscosity. These experiments were repeated at least two times with triplicates.

2.4.3. Bacterial membrane potential

The changes in the bacterial cell membrane potential from the exposure to parabens (MP, PP, BP and MIX) at 15000 μg/L were evaluated using a membrane potential sensitive probe bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC4(3); Molecular Probes, Invitrogen, Thermo Fisher Scientific, USA] (Kang et al., 2018). DiBAC4 (3) is a lipophilic fluorescent dye that only enters depolarized cells. Therefore, the fluorescence level of the dye accumulated inside cells can be used to assess the extent of depolarization in their membranes (Kang et al., 2018).

Bacterial suspensions according to section 2.4.1 to achieve a final concentration of 1×10^8 CFU/mL were exposed for 90 min to parabens (MP, PP, BP and MIX) at 15000 μg/L. Afterwards, non-exposed and parabens-exposed suspensions were centrifuged at 13000×*g* for 10 min (Eppendorf centrifuge 5418, VWR, Germany) to remove parabens, and the cell pellet was resuspended in STW (Kang et al., 2018). After that, the suspensions were incubated with DiBAC4(3) in the dark at 25 °C for 90 min, at a final concentration of 5 μM to allow sufficient dye loading into the membranes (Usmani et al., 2021), and subsequently centrifuged again (13000×*g* for 10 min) to remove the excess of dye. Then, a volume of 200 μL of resuspended bacterial suspension in STW was added to each well of a 96-well microtiter plate (VWR, USA). Bacterial suspensions in STW and with acetone at 0.005% (v/v) (solvent of PP, BP, and MIX) were assigned as negative controls and positive controls were performed by substituting the exposure to parabens for heat-shocked at 100 °C for 10 min (Novo et al., 2000). Fluorescence intensity was measured in a Fluostar Omega microplate reader (FLUOstar Omega, BMG Labtech, Spain) at excitation and emission wavelengths of 485/20 nm and 535/20 nm, respectively (Kang et al., 2018).

2.4.4. Evaluation of cell structure and morphology by transmission electron microscopy

Transmission electron microscopy (TEM) was used to evaluate the ultrastructure and morphological changes observed in planktonic *A. calcoaceticus* after exposure to MP at 15 and 15000 μg/L for 24 h in STW. For that, centrifuged and adjusted bacteria in STW as described in section 2.2 were exposed to MP at 15 and 15000 μg/L for 24 h. Then, both exposed and non-exposed bacteria cells were fixed in a solution containing 2.5% glutaraldehyde (Electron Microscopy Sciences - EMS, UK) and 2% paraformaldehyde (EMS, UK) in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 days at room temperature. Following this, the suspended cells were washed with 0.1 M sodium cacodylate buffer and subsequently embedded in HistogelTM (Thermo Fisher Scientific, UK) for en bloc treatment. Post-fixation was carried out with 2% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) solution, followed by another wash with the same buffer. Afterwards, the cells were incubated overnight in 1% uranyl acetate, washed with buffer, dehydrated using a series of graded ethanol, and finally embedded in Epon (EMS, UK). Ultrathin sections, cut at 50 nm thickness, were prepared on an RMC Ultramicrotome (PowerTome, USA) using a diamond knife and placed on 200 mesh copper grids (EMS, UK). A double contrast method with 2% uranyl acetate and saturated lead citrate solution was applied to the sections. The visualization step was performed at 80 kV using a JEM 1400 microscope (JEOL, Japan), and digital images were acquired utilizing a CCD digital camera Orious 1100 W (Gatan, Japan).

2.5. Evaluation of reactive oxygen species formation after parabens exposure

The formation of ROS was evaluated when *A. calcoaceticus* and *S. maltophilia* were in contact with parabens (MP, PP, BP and MIX) at 15000 μg/L. Dichloro-dihydro-fluorescein diacetate, H2DCFDA, (Sigma-Aldrich, Germany) was used to determine intracellular ROS formation. This probe passively diffuses through the cell membrane and is hydrolyzed in the cell to form the dichlorofluorescein (DCFH) carboxylate anion (Kang et al., 2018). DCFH is oxidized by ROS inside the cell membrane resulting in a fluorescent molecule - 2′,7′-dichlorofluorescein (DCF) (Kang et al., 2018). Therefore, the extent of generation of intracellular ROS was determined by increasing the fluorescence values of DCF. After centrifugation, both bacteria were washed once and resuspended in STW to achieve a final concentration of 1×10^8 CFU/mL. Parabens (MP, PP, BP, and MIX) and H2DCFDA (prepared in dimethyl sulfoxide - DMSO) were added to adjusted bacterial suspensions at final concentrations of 15000 μg/L and 50 μM, respectively. After 90 min of incubation at 25 °C in the dark (Vale et al., 2022), both bacterial suspensions were centrifuged at 130000×*g* for 10 min (Eppendorf centrifuge 5418, VWR, Germany) to remove the excess of H2DCFDA, and resuspended in STW (Gomes et al., 2019b). A volume of 200 μL of resuspended bacterial suspension was added to each well of a 96-well microtiter plate (VWR, USA). Two negative controls were performed: only with STW (a negative control without bacterial growth) and with bacteria not marked with H2DCFDA (to evaluate the possible autofluorescence of bacteria unrelated to the formation of ROS) (Gomes et al., 2019b). Positive control was obtained by replacing parabens with hydrogen peroxide (H_2O_2) at 30% (v/v) since H_2O_2 is one of the most common ROS generators within cells, acting as redox signalling molecules, and causing oxidative stress (Szymaszek et al., 2023). Nevertheless, bacteria non-exposed to parabens (adjusted in STW) and exposed to acetone 0.005% (v/v) were used also as a control to evaluate fluorescence intensity differences obtained. The fluorescence intensity was measured in a Fluostar Omega microplate reader (FLUOstar Omega, BMG Labtech, Spain) at a fluorescence excitation wavelength of 485/20 nm and an emission wavelength of 535/20 (Vale et al., 2022). To evaluate the fluorescence intensity per bacterial cell, the total number of cells in resuspended samples was analyzed by 4′,6-diamidino-2-phenylindole (DAPI) staining as described by Pereira et al.

(2023a) (Supplementary Material - B). However, since the total number of cells presented in samples did not differ statistically, the results were presented as fluorescence intensity per sample.

2.6. Assessment of bacterial metabolic activity

The metabolic activity of non-exposed and parabens-exposed bacteria was evaluated through the resazurin assay (Ribeiro et al., 2018). For that, after centrifugation as described in section 2.3, both bacteria were adjusted to 1×10^8 CFU/mL in fresh R2A medium broth and were exposed to MP, PP, BP, and MIX, each one at 15 and 15000 μg/L for 24 h. Then, 180 μL of fresh R2A and 20 μL of resazurin (0.1 mg/mL) were sequentially added to each well of 96-well microtiter plates. After 1 h of incubation in the dark at 25 °C and 160 rpm, the fluorescence intensity was measured in a microplate reader (FLUOstar Omega, BMG Labtech, Spain) at 570 nm excitation wavelength and 590 nm emission wavelength.

2.7. Bacterial biofilm formation after exposure to MP

The ability to form biofilms was evaluated for the conditions that caused always an effect on the cell envelope characteristics and metabolic activity of the bacteria tested. Therefore, biofilm formation ability was evaluated after exposure to MP at 15 and 15000 μg/L for 24 h. For that, planktonic bacteria (*A. calcoaceticus* and *S. maltophilia*) adjusted to 1×10^8 CFU/mL in R2A were exposed to MP at both concentrations for 24 h. After that, parabens-exposed and non-exposed bacteria were characterized in terms of biofilm production as described by Gomes et al. (2018). For that, bacterial biofilms were formed in sterile 96-well polystyrene microtiter plates (VWR, USA), according to Stepanović et al. (2000). Each well was filled with 200 μL of bacterial suspension (1×10^8 CFU/mL in R2A broth). Negative control wells were filled with sterile R2A broth. After 24 h incubation at 25 °C under 160 rpm, biofilms were washed with NaCl at 8.5 g/L to remove all non-adherent and weakly adhered bacteria. Then, biofilm production was assessed in terms of CFU enumeration in R2A agar (Merck, Germany), and by crystal violet (Merck, Germany) staining as described by Gomes et al. (2018).

2.8. Effect of MP on bacterial susceptibility to antibiotics

MP, the paraben that consistently altered bacterial characteristics, was also used to assess the correlation between parabens' mode of action and their potential impact on bacterial susceptibility to antibiotics. For that, both *A. calcoaceticus* and *S. maltophilia* were grown on R2A agar without MP and supplemented with MP at 15 and 15000 μg/L for 10 weeks. During this time, every week both bacteria were passed to a new fresh agar plate. The susceptibility to antibiotics was evaluated in the 5th and 10th weeks by the disk diffusion susceptibility methods according to the Clinical and Laboratory Standards Institute (CLSI, 2015). CEF at 30 μg; LEV at 5 μg; MINO at 30 μg and TMP-SMX at 1.25/23.75 μg were the selected antibiotics, according to the CLSI guidelines (CLSI, 2015) For that, bacteria were adjusted to 0.135 at 600 nm to match 0.5 MacFarland and spread on Mueller-Hinton agar – MHA (Merck, Germany) for all conditions. Then, antibiotic-containing discs (Oxoid, UK) were inserted on the top of the spread MHA plate and further incubated at 37 °C for 24 h before measuring the diameter of growth inhibition. Each condition was tested in duplicate with three independent experiments. Inhibition halos (cm) are presented as average ± standard deviation.

2.9. Statistical analysis

Statistical analysis of the acquired results was performed using the statistical program GraphPad Prism 5.0 (GraphPad Software, USA). The mean and standard deviations (SDs) within samples were calculated for all cases based on a minimum of three independent assays, with duplicates. Data were analyzed using one-way analysis of variance (ANOVA), with Tukey post hoc test (to compare more than two groups) and unpaired *t*-test to analyze the differences between the two groups. Statistical calculations were based on a confidence level ≥95% ($P < 0.05$ were related to statistically significant different results).

3. Results

3.1. Minimum inhibitory concentration

The MIC corresponds to the lowest concentration of selected parabens that causes complete inhibition of microbial growth. Therefore, MIC was used as a measure of the antimicrobial activity of parabens against two bacteria previously isolated from DW: *A. calcoaceticus* and *S. maltophilia*. The MIC values obtained for parabens towards both planktonic bacteria are presented in Table A1 in Supplementary Material. The MIC ranged between 200 and 400 mg/L. Among all parabens tested, MP had the higher MIC value (400 mg/L). For *A. calcoaceticus*, the MIC obtained for PP and BP were 300 and 200 mg/L, respectively. Regarding *S. maltophilia*, PP and BP MIC values were 250 and 200 mg/L, respectively (Table A1). Acetone at 0.005 % (v/v), as solvent control, did not affect the growth of both bacteria.

3.2. Bacterial surface physicochemical properties

The physicochemical characterization of bacterial surface, particularly the total surface hydrophobicity, contributes to the understanding of the mode of action of parabens on bacterial cell envelope. The surface tension parameters and hydrophobicity of non-exposed and exposed bacteria to each selected paraben at 15 and 15000 μg/L are shown in Tables 1 and 2. Based on the free energy of interaction, both bacteria were classified as hydrophilic ($\Delta G_{iwi} > 0$ mJ $/m^2$). Both bacteria had a strong ability to donate electrons (γ^-) and a weak ability to accept electrons (γ^+). *A. calcoaceticus* was not predisposed to changes in total surface hydrophobicity and even to other physicochemical components of the surface tension when exposed to all parabens at 15 μg/L ($P > 0.05$). However, exposure to parabens at 15000 μg/L caused modifications in the bacterial surfaces (Table 1). In particular, MP increased *A. calcoaceticus* total surface hydrophilicity (ΔG_{iwi}) by 68% from 29 ± 0.80 to 48 ± 7.1 mJ/m^2 ($P < 0.05$). This increase in the total hydrophilicity was mainly due to the increase in the polar component of the free energy (ΔG_{iwi}^{AB}) ($P < 0.05$). The same trend for increasing the total surface hydrophilicity was observed for the MIX at 15000 μg/L revealing an increase in ΔG_{iwi}^{AB} by 64% when compared to non-exposed counterparts ($P < 0.05$). PP and BP at the highest concentration tested (15000 μg/L) caused modifications in the polar component of the bacterial surface tension (γ^{AB}), capacity to accept electrons (γ^+), and the polar component of the free energy (ΔG_{iwi}^{AB}) ($P < 0.05$), without affecting the total surface hydrophobicity ($P > 0.05$).

S. maltophilia revealed some changes in physicochemical surface properties after exposure to MP and BP at 15 μg/L ($P < 0.05$) (Table 2). Both, MP and BP, promoted an increase in the total surface hydrophilicity values (ΔG_{iwi}) higher than 20% ($P < 0.05$). This increase in the total hydrophilicity was mainly due to the contribution of the polar component of the free energy with an increase of ΔG_{iwi}^{AB} ($P < 0.05$). The other parabens (PP and MIX at 15 μg/L) did not cause any modification in *S. maltophilia* hydrophobicity ($P > 0.05$). For the highest concentration of parabens tested (15000 μg/L), MP, BP and MIX revealed an increase in ΔG_{iwi} values and consequently, in ΔG_{iwi}^{AB} ($P < 0.05$). In addition, other physicochemical parameters were also affected after parabens exposure. Specifically, the apolar (γ^{LW}) and polar surface tension (γ^{AB}), the capacity to accept electrons (γ^+) and the apolar component of the free energy (ΔG_{iwi}^{LW}) values were significantly altered after exposure to MP at 15 μg/L ($P < 0.05$) (Table 2). These modifications were more pronounced after exposure to MP at 15000 μg/

Table 1
Apolar (γLW) and polar (γAB) components of the surface tension (mJ/m^2), and hydrophobicity ($\Delta Giwi$) (mJ/m^2) of *A. calcoaceticus* non-exposed (control) and exposed to parabens at 15 and 15000 μg/L.

		Surface tension parameters $(\mathbf{mJ}/\boldsymbol{m^2})$			Hydrophobicity $(\mathbf{mJ}/\boldsymbol{m^2})$			
		γ^{LW}	γ^{AB}	γ^{+}	γ^{-}	ΔG_{iwi}	ΔG_{iwi}^{LW}	ΔG_{iwi}^{AB}
	Control (STW)	33.0 ± 2.2*	21.1 ± 3.1*	2.1 ± 0.6*	55.7 ± 5.4	28.5 ± 0.8*	−2.1 ± 0.5*	30.7 ± 0.6*
	Control (Ac)	33.3 ± 1.5	19.5 ± 2.0**	1.8 ± 0.3**	53.3 ± 2.0**	30.9 ± 2.0	−2.4 ± 0.5	33.3 ± 1.5**
15 μg/L	MP	34.1 ± 0.8	20.9 ± 1.1	2.1 ± 0.3	53.0 ± 2.1	29.5 ± 2.6	−2.8 ± 0.3	32.3 ± 2.9
	PP	33.2 ± 1.5	20.8 ± 2.9	2.1 ± 0.6	52.4 ± 0.6	29.3 ± 0.7	−2.4 ± 0.6	31.7 ± 1.3
	BP	33.5 ± 1.4	20.5 ± 2.2	2.0 ± 0.4	53.3 ± 4.9	30.3 ± 5.5	−2.5 ± 0.6	32.8 ± 5.0
	MIX	33.4 ± 1.0	21.1 ± 3.2	2.2 ± 0.7	52.5 ± 3.3	29.0 ± 4.3	−2.5 ± 0.4	31.5 ± 4.4
15000 μg/L	MP	**37.7 ± 1.5***	**5.9 ± 4.1***	**0.2 ± 0.3***	61.4 ± 3.9	**47.8 ± 7.1***	**−4.4 ± 0.7***	**52.1 ± 7.5***
	PP	34.8 ± 0.9	**14.3 ± 2.4****	**1.0 ± 0.3****	54.1 ± 1.4	34.6 ± 2.2	−3.0 ± 0.4	**37.6 ± 1.9****
	BP	36.2 ± 3.1	**13.8 ± 2.5****	**1.0 ± 0.3****	**48.8 ± 0.4****	27.8 ± 0.4	−3.7 ± 1.4	31.5 ± 0.9
	MIX	33.8 ± 2.9	**8.3 ± 4.1****	**0.3 ± 0.3****	**64.1 ± 3.7****	**50.9 ± 6.9****	−2.7 ± 1.1	**53.5 ± 6.5****

If $\Delta G_{iwi} > 0$ mJ/m^2, hydrophilic and if $\Delta G_{iwi} < 0$ mJ/m^2, hydrophobic. γ^{+}- electron-acceptor parameter; γ^{-}- electron-donor parameter; LW - Liftshitz-van der Waals; AB - Lewis acid-base. The values are the means ± SDs of three independent experiments. * – samples were statistically different from non-exposed bacteria (STW) (*t*-test, P < 0.05), ** samples were statistically different from non-exposed bacteria (Ac) (*t*-test, P < 0.05).

Table 2
Apolar (γLW) and polar (γAB) components of the surface tension (mJ/m^2), and hydrophobicity ($\Delta Giwi$) (mJ/m^2) of *S. maltophilia* non-exposed (control) and exposed to parabens at 15 and 15000 μg/L.

		Surface tension parameters $\left(\frac{\mathbf{mJ}}{\boldsymbol{m^2}}\right)$				Hydrophobicity $(\mathbf{mJ}/\boldsymbol{m^2})$		
		γ^{LW}	γ^{AB}	γ^{+}	γ^{-}	ΔG_{iwi}	ΔG_{iwi}^{LW}	ΔG_{iwi}^{AB}
	Control (STW)	29.3 ± 4.6*	27.3 ± 6.4*	3.8 ± 1.9*	51.7 ± 2.0	25.7 ± 4.0*	−1.3 ± 1.0*	27.1 ± 4.9*
	Control (Ac)	36.4 ± 2.6	17.0 ± 3.4	1.4 ± 0.5	53.6 ± 2.1**	31.5 ± 3.3**	−3.8 ± 1.2	35.3 ± 4.5**
15 μg/L	MP	**37.9 ± 2.0***	**16.0 ± 1.5***	**1.2 ± 0.2***	54.4 ± 0.8	**32.4 ± 0.9***	**−4.5 ± 1.0***	**36.9 ± 1.8***
	PP	29.5 ± 6.1	23.9 ± 10.6	3.2 ± 2.8	51.4 ± 2.0	27.2 ± 6.0	−1.5 ± 1.4	28.7 ± 7.3
	BP	34.0 ± 1.5	14.4 ± 1.9	0.9 ± 0.2	**58.6 ± 2.1****	**40.0 ± 2.9****	−2.7 ± 0.6	**42.8 ± 2.3****
	MIX	34.1 ± 1.4	19.4 ± 2.9	1.8 ± 0.6	52.8 ± 2.7	30.2 ± 4.2	−2.8 ± 0.6	32.9 ± 4.2
15000 μg/L	MP	**38.3 ± 3.4***	**13.8 ± 3.7***	**0.9 ± 0.5***	55.1 ± 1.2	**34.4 ± 1.1***	**−4.7 ± 1.6***	**39.1 ± 2.5***
	PP	38.3 ± 2.0	12.4 ± 1.5	0.7 ± 0.1	54.0 ± 2.7	34.0 ± 3.3	−4.6 ± 1.0	38.7 ± 2.4
	BP	37.9 ± 3.7	12.4 ± 3.0	0.7 ± 0.3	**58.4 ± 2.3****	**39.4 ± 2.5****	−4.5 ± 1.8	**43.9 ± 0.7****
	MIX	34.2 ± 0.3	16.0 ± 0.6	1.1 ± 0.1	**59.0 ± 0.3****	**39.4 ± 0.7****	−2.8 ± 0.1	**42.1 ± 0.8****

If $\Delta G_{iwi} > 0$ mJ/m^2, hydrophilic and if $\Delta G_{iwi} < 0$ mJ/m^2, hydrophobic. γ^{+}-; γ^{-}- electron-donor parameter; LW - Liftshitz-van der Waals; AB - Lewis acid-base. The values are the means ± SDs of three independent experiments. * – samples were statistically different from non-exposed bacteria (STW) (*t*-test, P < 0.05), ** samples were statistically different from non-exposed bacteria (Ac) (*t*-test, P < 0.05).

L (P < 0.05) (Table 2). A statistically significant increase in the capacity to donate electrons (γ^{-}) was also found after exposure to BP at both concentrations and after exposure to MIX at 15000 μg/L (P < 0.05).

3.3. Bacterial surface charge and cell size

The bacterial zeta potential was measured to evaluate changes in bacterial surface charge after exposure to the parabens. The results obtained from the zeta potential measurement show that both bacteria had a negative surface charge: 30 mV for *A. calcoaceticus*, and - 32 mV for *S. maltophilia* (Table 3). After exposure to parabens, changes in the surface charge of *A. calcoaceticus* cells to less negative values were verified, particularly after exposure to MP and BP at the highest concentration (15000 μg/L) (P < 0.05). The same trend in zeta potential values occurred for *S. maltophilia* exposed to MP at 15 and 15000 μg/L (P < 0.05). On the other hand, for *S. maltophilia*, exposure to PP at the lowest concentration (15 μg/L) promoted an increase in the negativity of surface charge (P < 0.05) (Table 3).

Regarding the cell size, *A. calcoaceticus* had a hydrodynamic diameter of 2847 ± 365.3 nm and *S. maltophilia* 999 ± 94.9 nm (Table 4).

Table 3
Zeta potential values (mV) of *A. calcoaceticus* and *S. maltophilia* non-exposed and exposed to parabens at 15 and 15000 μg/L for 24 h.

	Zeta potential (mV)									
			15 μg/L				15000 μg/L			
	Control (STW)	**Control (Ac)**	**MP**	**PP**	**BP**	**MIX**	**MP**	**PP**	**BP**	**MIX**
A. calcoaceticus	−30.1 ± 3.6*	−27.6 ± 1.8**	−28.0 ± 0.2	−26.8 ± 0.9	−27.6 ± 1.4	−27.4 ± 2.8	−25.7 ± 1.0*	−26 ± 1.9	−24.2 ± 0.03**	−25.1 ± 3.1
S. maltophilia	−31.8 ± 0.1*	−27.7 ± 0.5**	−30.7 ± 0.05*	−30.3 ± 0.4**	−30.4 ± 1.4	−30.6 ± 2.9	−29.4 ± 0.7*	−28.5 ± 1.0	−28.7 ± 3.6	−24.4 ± 4.5

* – samples were statistically different from non-exposed bacteria (STW) (*t*-test, P < 0.05), ** samples were statistically different from non-exposed bacteria (Ac) (*t*-test, P < 0.05).

Table 4
Bacterial size distribution (nm) of A. calcoaceticus and S. maltophilia non-exposed and exposed to parabens at 15 and 15000 μg/L for 24 h.

	Size (nm)									
			15 μg/L				15000 μg/L			
	Control (STW)	Control (Ac)	MP	PP	BP	MIX	**MP**	**PP**	**BP**	**MIX**
A. calcoaceticus	2847.3 ± 365.3	2425.8 ± 587.6**	3223.5 ± 291.6	3840.5 ± 611.2**	4324.7 ± 1229**	2804.8 ± 176.5	2938.5 ± 1235	4546.3 ± 1195**	3127.2 ± 330.2	4282.5 ± 641.3**
S. maltophilia	998.7 ± 94.9*	887.6 ± 146.7**	914.6 ± 282.9	1096.5 ± 42.5	1203.9 ± 52.0**	1166.8 ± 80.4**	1329.7 ± 217.8*	1267.9 ± 275.0**	1192.3 ± 337.6**	1095.2 ± 127.9

* – samples were statistically different from non-exposed bacteria (STW) (*t*-test, $P < 0.05$), ** samples were statistically different from non-exposed bacteria (Ac) (*t*-test, $P < 0.05$).

Overall, parabens exposure seems to increase the bacterial size (Table 4). Regarding *A. calcoaceticus*, this increase in bacterial size after parabens exposure was more pronounced for PP (58%) and BP (78%) exposure at 15 μg/L ($P < 0.05$). The same trend was verified after exposure to PP and MIX at 15000 μg/L to *A. calcoaceticus*, with increases in bacterial size of 87% and 77%, respectively ($P < 0.05$). Concerning *S. maltophilia*, an increase in bacterial size was verified after exposure to BP (36%) and MIX (31%) at 15 μg/L and for MP (33%), PP (43%) and BP (34%) at 15000 μg/L ($P < 0.05$).

3.4. Bacterial membrane potential

The bacterial membrane potential was determined to assess the impact of parabens exposure on membrane depolarization as an indicator of cell viability and membrane integrity. Only the highest concentration was tested (15000 μg/L), since it caused more impactful modifications on the previously studied bacterial cell envelope characteristics (bacterial surface physicochemical properties and bacterial surface charge). According to the results obtained (Figure C1 in Supplementary Material), parabens exposure at 15000 μg/L did not cause depolarization of both *A. calcoaceticus* and *S. maltophilia* biofilm cells ($P > 0.05$). Therefore, at lower concentrations than 15000 μg/L, parabens are not expected to cause membrane depolarization.

3.5. Bacterial cell structure and morphology

To assess the consequences of parabens exposure on bacterial cell structure and morphology, *A. calcoaceticus* bacterial suspensions exposed to MP at 15 and 15000 μg/L and non-exposed were visualized by TEM (Fig. 1). MP was selected as the representative of parabens compounds since, among other parabens, was the one reporting more impactful and constant perturbations on bacterial cell envelope in the previous assays.

Non-exposed *A. calcoaceticus* has a dense cytosol (dark colour), an

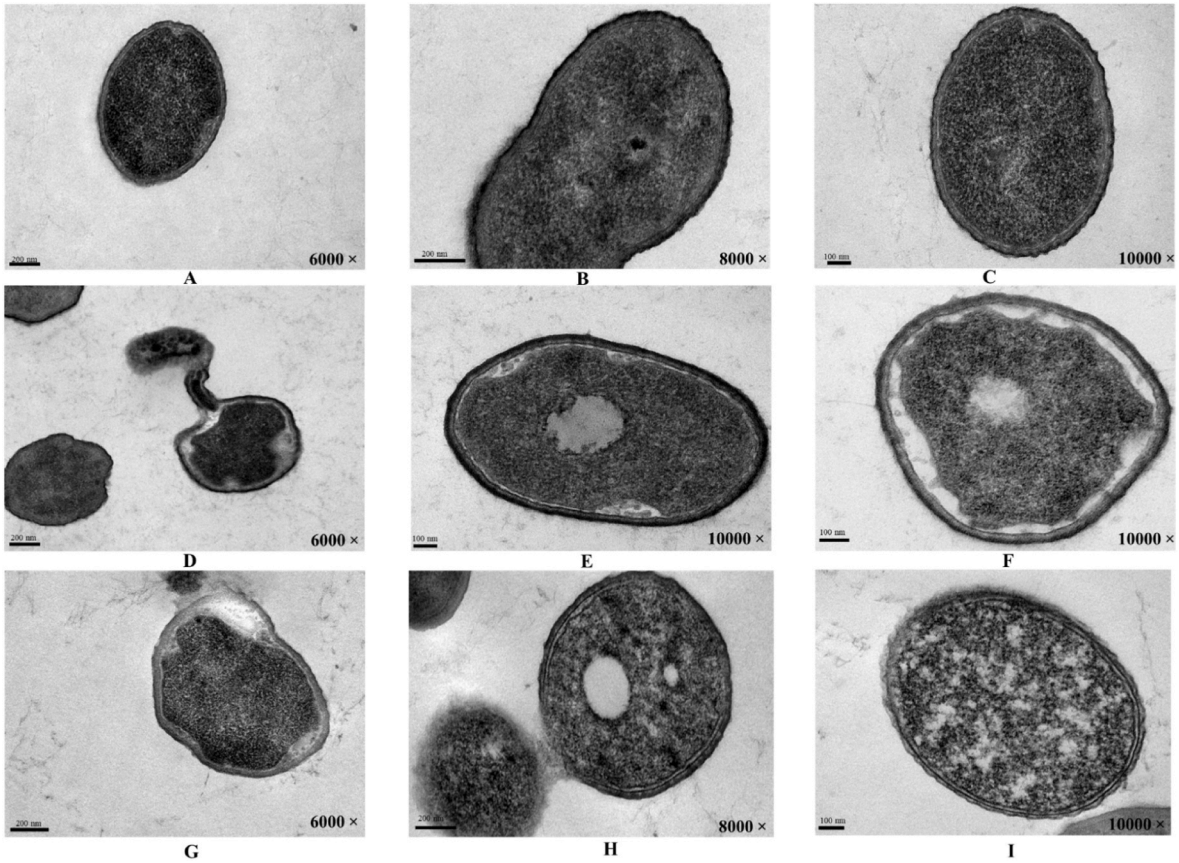

Fig. 1. Transmission electron micrographs of *A. calcoaceticus* non-exposed to MP (A, B, and C) and after 24h of exposure to MP at 15 μg/L (D, E, and F) and exposed to MP at 15000 μg/L (G, H, I).

intact cell envelope and a high and uniform electron density (Fig. 1 – A, B, and C). Well-defined outer and inner membranes are observed in Fig. 1 – A, B, and C). However, MP-exposed *A. calcoaceticus* at both concentrations showed the appearance of vacuoles (white zones of cytoplasm) (Fig. 1 – D, E, F, G, and H). Moreover, Fig. 1 – D to H also suggested that the bacterial cell envelope of *A. calcoaceticus* was greatly affected by MP exposure at 15 and 15000 μg/L, revealing some irregularities. TEM images show that the MP-exposed bacterial cell envelope appears jagged and not smooth. The disruption of the bacterial membrane/cell wall was verified with the release of intracellular components reported by the presence of translucent zones in the cytoplasm and a decrease in intracellular electron density (Fig. 1 – D to H). The results also suggest the occurrence of cytoplasm plasmolyze for MP-exposed *A. calcoaceticus* (Fig. 1 – E and F).

3.6. Reactive oxygen species formation

Both bacteria treated with H_2O_2 at 30% (positive control) showed a greater fluorescent intensity (>30000 fluorescence units - Figure D1) in comparision to both controls (STW and Ac), which allows to validate the implemneted method.

Overall, it was possible to observe a higher formation of ROS in *S. maltophilia* than in *A. calcoaceticus* (Figure D1) ($P < 0.05$). No evident effect from the exposure to parabens at 15000 μg/L on the formation of ROS was observed ($P > 0.05$). However, an increasing trend in ROS production was found for *S. maltophilia* exposed to BP at 15000 μg/L, resulting in an increase in fluorescence units by 44% higher, although not statistically significant.

3.7. Bacterial metabolic activity

The results obtained for the metabolic activity of both planktonic bacteria are presented in Fig. 2. Overall, these results suggest that the exposure to parabens at both concentrations for 24 h caused a reduction in the metabolic activity of planktonic *A. calcoaceticus* ($P < 0.05$). MP at 15 and 15000 μg/L was the more impactful in reducing the metabolic activity of *A. calcoaceticus* by 29% and 42%, respectively ($P < 0.05$). BP at both concentrations reduced the metabolic activity of planktonic *A. calcoaceticus* by 23% (on average) ($P < 0.05$). While PP only affected the metabolic activity of *A. calcoaceticus* for the highest concentration tested (15000 μg/L) reducing by 28%, MIX caused a reduction of 25% at 15 μg/L ($P < 0.05$). Regarding *S. maltophilia*, only MP and MIX at the highest concentration (15000 μg/L) caused a reduction in the metabolic activity of 31% and 34%, respectively. Curiously, PP at 15000 μg/L caused the opposite effect by increasing in 27% the metabolic activity of *S. maltophilia* ($P < 0.05$). The other conditions tested did not affect the metabolic activity of *S. maltophilia*.

3.8. Effect of MP on bacterial biofilm formation

The mode of action of parabens may have a direct impact on bacterial virulence, including the ability to form biofilms. A higher bacterial ability to form biofilms is commonly associated with more virulent bacteria. To understand if exposure to parabens causes changes in bacterial virulence, previously exposed bacteria to MP at 15 and 15000 μg/L for 24 h were selected to evaluate the impact of MP on the bacterial ability for biofilm production.

The exposure to 15 μg/L of MP increased *the ability of S. maltophilia* to form biofilms ($P < 0.05$) (Fig. 3 - A). This resulted in an increase of 24% in the biofilm biomass production of MP-exposed *S. maltophilia* in comparison to non-exposed bacteria. However, *A. calcoaceticus* was not significantly affected by MP exposure at 15 μg/L in terms of biofilm biomass production ($P > 0.05$) (Fig. 3 - A). Besides that, exposure to MP at an in-use concentration (15000 μg/L) resulted in an increase of 59% in the biofilm biomass production of MP-exposed *A. calcoaceticus* ($P < 0.05$) (Fig. 3 - A). Regarding biofilm formation in terms of culturability, only MP exposure at 15000 μg/L was able to notably increase the ability of both bacteria to form biofilms ($P < 0.05$) (Fig. 3 - B). This increase in biofilm formation ability consisted of a four- and two-fold increase in the number of *A. calcoaceticus* and *S. maltophilia* biofilm culturable cells, respectively.

3.9. Effect of MP long exposure on bacterial susceptibility to antibiotics

The effects of MP on bacterial tolerance to antibiotics were evaluated as an environmental implication resulting from long-term exposure to MP. For that, both environmental planktonic bacteria (*A. calcoaceticus* and *S. maltophilia*) were constantly exposed to MP at 15 and 15000 μg/L for 10 weeks and bacterial tolerance to antibiotics (CEF, LEV. MINO, and TMP-SMX) was tested periodically. The diameter of the inhibition halo against the antibiotics tested was determined after 5 and 10 weeks of exposure (Fig. 4). After 5 weeks of MP exposure at both concentrations, *A. calcoaceticus* bacteria were less susceptible to TMP-SMX in comparison to non-exposed bacteria ($P < 0.05$) (Fig. 4 – A). Moreover, MP-exposed *A. calcoaceticus* at 15000 μg/L was also less susceptible to CEF, LEV, and MINO ($P < 0.05$). This resulted in a 19%, 14%, 10%, and 19% decrease in the inhibition halo of MP-exposed *A. calcoaceticus* for CEF, LEV, MINO, and TMP-SMX, respectively. *S. maltophilia* was less susceptible to TMP-SMX after exposure to 15000 μg/L for 5 weeks resulting in a 26% decrease of inhibition halo in comparison to non-exposed counterparts ($P < 0.05$) (Fig. 4 – C). Therefore, it seems that *A. calcoaceticus* was more prone to acquire resistance to antibiotics than

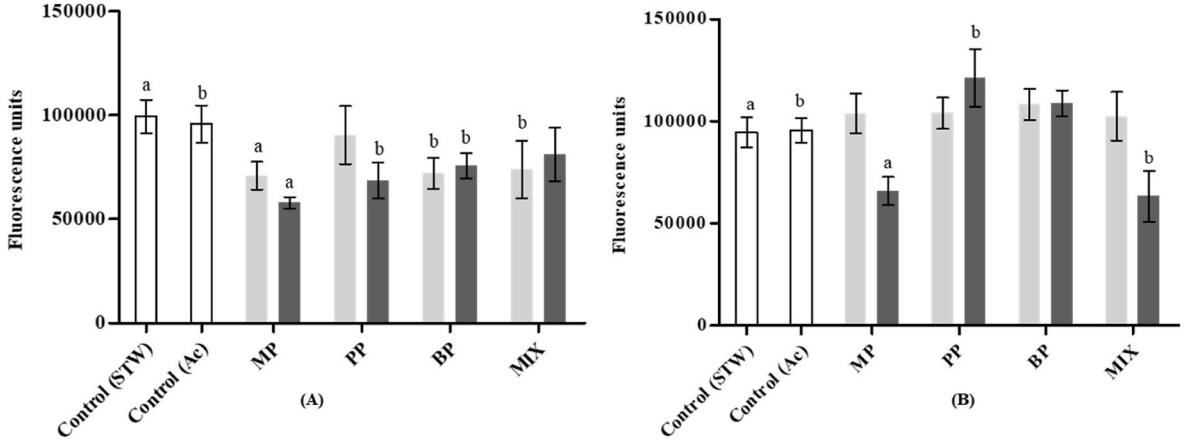

Fig. 2. Metabolic activity of planktonic *A. calcoaceticus* (A) and *S. maltophilia* (B) non-exposed (□) and exposed to MP, PP, BP, and MIX at 15 μg/L (▩) and 15000 μg/L (■) for 24 h. [a, b] - corresponds to conditions that have statistically significant differences from the respective control (Tukey's test, $P < 0.05$).

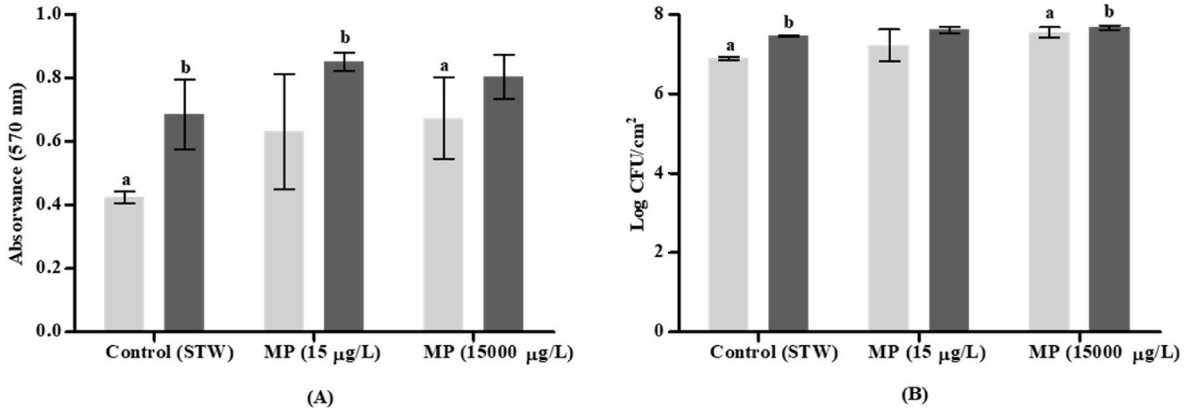

Fig. 3. Biofilm mass (A) and culturable cells - Log CFU/cm2 (B) of MP-exposed and non-exposed *A. calcoaceticus* (■) and *S. maltophilia* (■). [a, b] - corresponds to conditions that have statistically significant differences from the respective control (*t*-test, $P < 0.05$).

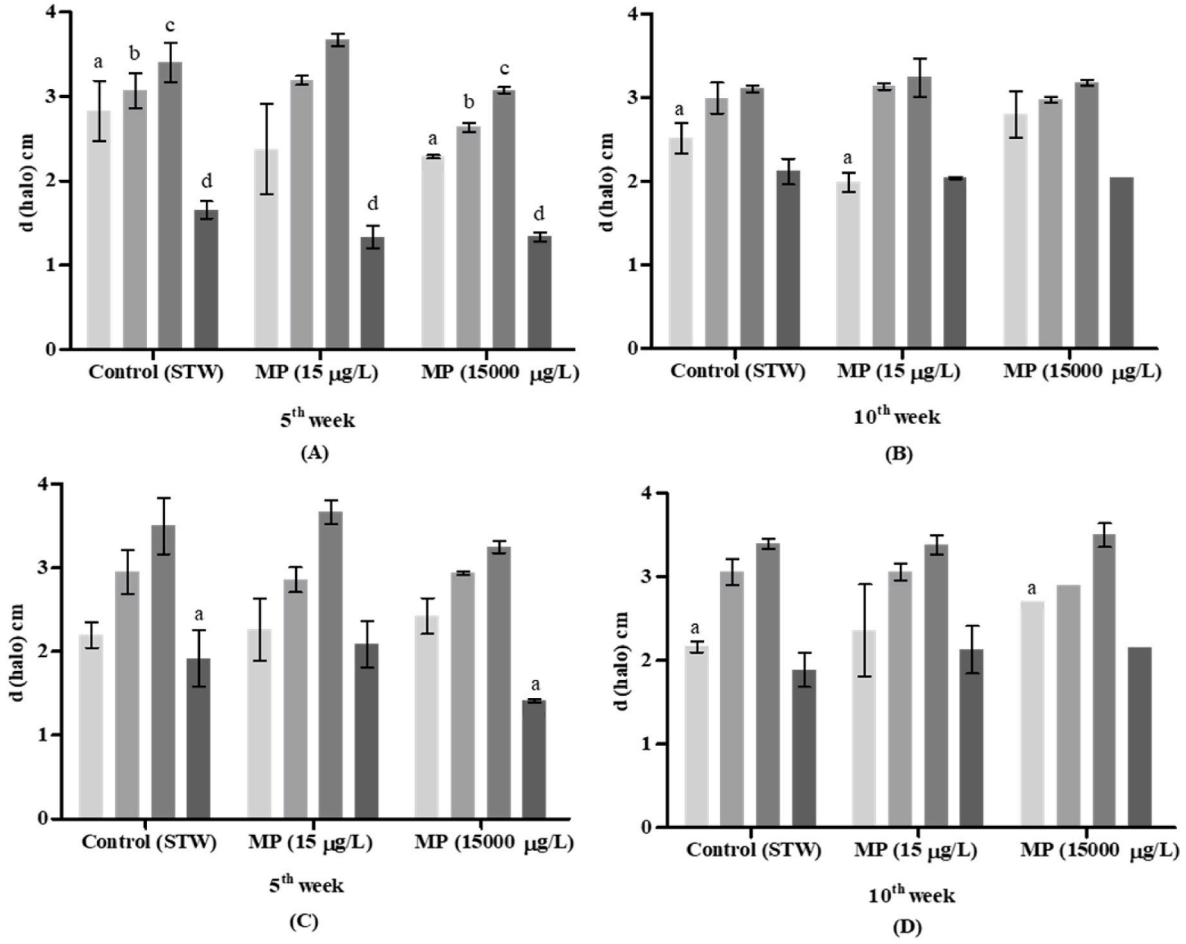

Fig. 4. Diameter (d) of the inhibition halos (cm) of *A. calcoaceticus* (A and B) and *S. maltophilia* (C and D) non-exposed and MP-exposed (15 and 15000 µg/L) for 5 and 10 weeks against CEF ■, LEV ■, MINO ■, and TMP-SMX ■ [a, b, c, d] - corresponds to conditions that have statistically significant differences from the respective control (*t*-test, $P < 0.05$).

S. maltophilia in 5 weeks of exposure to MP. Moreover, for a longer exposure to MP (for 10 weeks) at 15000 µg/L, *S. maltophilia* lost the increased resistance to TMP-SMX (reported before) and became more susceptible to CEF ($P < 0.05$), increasing the inhibition halo against this antibiotic in 23% (Fig. 4 - D). Curiously, after 10 weeks of MP exposure at 15 µg/L, *A. calcoaceticus* became more tolerant to CEF showing a 20% decrease in the inhibition halo diameter ($P < 0.05$) (Fig. 4 – B). However, this increase in antibiotic tolerance after 10 weeks of MP exposure was not observed for the highest concentration tested (15000 µg/L) ($P > 0.05$) (Fig. 4 - B).

4. Discussion

4.1. The impact of parabens on bacteria surface physicochemical properties and morphology may be related to changes in osmotic regulation

The extensive use of parabens followed by their release into the environment and accumulation in aquatic systems have raised concerns regarding their potential impact on ecological systems (Pereira et al., 2023b). Although some researchers have reported some aspects of the mode of action of parabens as preservatives against microorganisms, their effects as environmental contaminants on bacteria, and their potential environmental implications have been disregarded. The present work aimed to extend our current understanding of the mechanisms by which parabens (MP, PP, BP), individually and in combination (MIX), interfere with bacterial cells (*A. calcoaceticus* and *S. maltophilia* isolated from DW) at environmentally relevant (15 μg/L) and in-use concentrations (15000 μg/L).

Parabens are known to possess broad-spectrum antimicrobial activity (Wei et al., 2021). In this study, the MIC values obtained (200–400 mg/L) for parabens against both planktonic bacteria were much higher than the MIC values commonly found for antibiotics and traditional disinfectants (such as chlorine and quaternary ammonium compounds) (Higgins et al., 2001). The results showed that the antimicrobial activity of parabens was higher for the most hydrophobic compounds (MP < PP < BP). These results corroborate the findings of Fransway et al. (2019) who showed that the antimicrobial activity of parabens increases with increasing alkyl chain length. In this study, MP was the paraben with the highest MIC (400 mg/L) obtained against both bacteria. On the other hand, BP was the paraben with higher antimicrobial activity (MIC of 200 mg/L), as previously reported by Zhang et al. (2021). These MICs are higher than the relevant environmental concentrations of parabens (ng/L - μg/L) and in-use concentrations (mg/L) commonly reported in the literature (Pereira et al., 2023b).

Therefore, the use of subinhibitory concentrations (15 and 15000 μg/L) in the present study is particularly relevant, as it allows for a more realistic assessment of how parabens interact with bacteria in environmental settings, where complete bacterial inhibition is unlikely to occur. This will help to deeply assess the structural, and metabolic, modifications that result from their interaction, as well as their environmental implications, such as increased bacterial virulence.

Since the bacterial cell envelope is the main defensive barrier to external factors (such as parabens exposure), assessing its physicochemical properties before and after parabens exposure is crucial to understanding the interaction between parabens and bacteria (Hamadi et al., 2012). In this study, both bacteria showed to be hydrophilic $\left(\Delta G_{iwi} > 0\ mJ/m^2\right)$ and this characteristic was potentiated by the exposure to parabens at both concentrations, suggesting modifications in the physicochemical properties of the bacterial cell envelope. The bacterial cell surface of *S. maltophilia* was shown to be greatly affected by parabens, even at 15 μg/L, and in comparison to *A. calcoaceticus*. An increase in hydrophilicity and polarity of bacterial cell surfaces was found after parabens exposure. This increase in hydrophilicity was detected in *A. calcoaceticus* after MP and MIX exposure at 15000 μg/L, and for *S. maltophilia* when exposed to MP and MIX at 15 μg/L and to MP, BP, and MIX at 15000 μg/L. These data suggest that parabens containing hydroxyl (-OH) and ester functional groups may interact with the components of the bacterial surface (with a negative charge mainly due to the presence of lipopolysaccharides) through electrostatic interactions (Halder et al., 2015). These interactions may alter cell surface permeability and accordingly change the physicochemical characteristics of bacterial cell envelopes as already reported by Flasiński et al. (2016, 2018). These authors found that MP, EP, PP, and BP ($10^{-6} - 10^{-3}$ M) induced bacterial surface film modifications affecting the lipid monolayer characteristics (Flasiński et al., 2016). These concentrations of parabens are in the range of 0.15–190 mg/L, which includes the highest concentration tested in the present study.

The changes in the bacterial surface induced by parabens may also be reflected in changes in the bacterial cell surface charge, highlighting stressful conditions for bacteria (Ferreyra Maillard et al., 2021). Bacterial zeta potential provides information about bacterial surface stability and their interactions with external substances in a liquid medium, reflecting the surface charge and electrical potential at the interface of bacterial cells (Halder et al., 2015). Both bacteria showed a negative surface charge (approximately - 30 mV), which was already expected due to the presence of anionic groups (e.g., carboxyl and phosphate) on the outer cell envelope of Gram-negative bacteria (Borges et al., 2013). A zeta potential above ±30 mV (which is the case of the study) is a strong indication of the stability of the system because it reflects high repulsion of the particles, preventing their aggregation or sedimentation (Ferreira et al., 2021). In general, exposure to parabens resulted in less negative zeta potential values, being this effect more pronounced after exposure to MP and BP at 15000 μg/L (*A. calcoaceticus*) and MP at 15 and 15000 μg/L (*S. maltophilia*). The results corroborate those obtained for physicochemical changes, suggesting the perturbation of the bacterial cell surface. Changes in bacterial surface charge reflected by alterations in zeta potential values may be associated with perturbations of cell surface permeability (Halder et al., 2015). Less negative zeta potential values are commonly associated with a lower bacterial metabolism and decreased viability (Lee et al., 2018a,b). Parabens-exposed *A. calcoaceticus*, and MP- and MIX-exposed *S. maltophilia* were significantly less metabolically active than the non-exposed counterparts.

TEM inspections revealed bacterial surface perturbations induced by MP exposure. Undefined and disrupted membranes and an increase in the area of vacuole structures were observed after *A. calcoaceticus* exposure to MP at both 15 and 15000 μg/L. The structure of vacuoles is intrinsically related to osmotic regulation and surface pressure mediated by mechanosensitive channels of small (MscS) and large conductance (MscL) (Blount and Iscla, 2020). These channels allow bacteria to respond and adapt to osmotic and mechanical stress and membrane tension changes (Blount and Iscla, 2020). Subsequently, the presence of vacuoles regulating the bacterial cell volume and cell surface perturbations suggests that MP may activate bacterial osmotic regulation mechanisms (Hu et al., 2023). Kamaraju and Sukharev (2008) reported increased bacterial cell surface pressure on *E. coli* provoked by EP, PP, and BP at 1 mM and desensitization of *Escherichia coli* MscS, due to parabens integration on the bacterial membrane. Another study reported the ability of EP and PP at 1 mM to intercalate into lipid membranes dysregulating both MscS and MscL (Nguyen et al., 2005).

When increasing parabens concentration an increase in the gap between the bacterial membrane and cytoplasm was observed by TEM, which may also be associated with the bacterial osmotic response (Sleator and Hill, 2002). Other authors argued that the membrane and periplasm adaptation involves an increase in the proportion of anionic phospholipids, which consequently increases the surface charge (Sleator and Hill, 2002). The present study reported an increase in the zeta potential (mV) of *A. calcoaceticus* surface after exposure to MP and BP at 15000 μg/L. Overall, it seems that parabens exposure at environmental and in-use concentrations greatly affects bacterial cell surface physicochemical properties, leading to the disruption of membrane integrity, which may result in the activation of osmotic regulation mechanisms to manage osmotic stress.

Although the present results suggest that the parabens tested at 15000 μg/L were not able to cause bacterial cell membrane depolarization, it is known that MP at much higher concentrations (3.41×10^8 μg/L) caused mitochondrial depolarization and depletion of cellular ATP in humans (Soni et al., 2002). This proposes that the effects of parabens exposure significantly vary according to the concentrations used, and the effects on human cells should not be translated into the effects of parabens on communities of prokaryotic cells. This situation was also observed in terms of ROS production after parabens exposure. Although some authors reported an increase in intracellular ROS

production induced by PP at concentrations from 5.5 to 7.2 × 10^5 μg/L for disinfection purposes (Ding and Tikekar, 2020; Liu et al., 2021), in the present study parabens at 15000 μ/L did not cause a significant change in bacterial ROS production.

Besides the effects of parabens on bacterial cell envelope, an increase in the hydrodynamic diameter of parabens-exposed bacteria at both concentrations tested was also found, which may provide new insights about the possible interaction between parabens and bacteria. This increase in bacterial size was not observed by TEM. However, it may be related to the bacterial swelling from osmotic regulation mechanisms caused by the presence of MP, reflected by the increase in the vacuole area (Wood, 2015). In addition, it is known that some bacteria can degrade parabens and use their metabolites as carbon and energy sources, which may also increase the bacterial cell size (Amin et al., 2010). Future studies aiming to explore the effects of parabens on bacterial biofilms would provide results that mimic the effects encountered in realistic environmental conditions.

4.2. Environmental and in-use concentrations of MP promote biofilm formation and ceftazidime-acquired resistance

Biofilms are the main mode of living of bacteria in nature (Flemming et al., 2016). These complex microbial structures, when associated with pathogenic microorganisms, constitute an important virulence, essential for bacteria survival under stressful conditions. Biofilms can be up to 1000 times harder to eradicate than planktonic bacteria (Mah, 2012). Therefore, it is of utmost relevance to understand how MP exposure may affect the formation of these microbial communities. MP exposure at environmental and in-use concentrations promoted biofilm formation by *A. calcoaceticus* and *S. maltophilia.* Indeed, MP exposure at 15 μg/L increased *S. maltophilia* biofilm biomass production. MP at 15000 μg/L caused more impactful modifications on bacterial biofilm formation ability, reflected by increased biomass production for *A. calcoaceticus* and increased number of culturable cells for *A. calcoaceticus* and *S. maltophilia* biofilms, respectively. Moreover, the increase in biofilm formation ability may be associated with increased bacterial virulence, which may have some outcomes for public health.

The impact of MP exposure on the bacterial ability to form biofilm becomes more critical if associated with increased tolerance to antimicrobials (Caioni et al., 2023). It was found that continuous exposure to environmental and in-use concentrations of the most often used paraben, MP, can reduce the susceptibility of *A. calcoaceticus* and *S. maltophilia* to multiple antibiotics (CEF, LEV, MINO, and TMP-SMX), indicating a potential contribution to antimicrobial tolerance (Caioni et al., 2023). Curiously, the increased tolerance to CEF revealed by both MP-exposed bacteria remains even after the bacterial passages into the growth medium without MP for an additional 10 weeks (Figure E – Supplementary Material). Therefore, MP exposure seems to cause CEF-acquired resistance in exposed bacteria. This could be due to genetic alterations which may result, for example in the potentiation of efflux systems, enzymatic inactivation, or modification of the cellular target sites in paraben-exposed bacteria (Xie et al., 2019). Indeed, Subirats et al. (2018) reported that bacterial communities exposed to MP in combination with other emerging contaminants revealed an increase in the abundance of *sul1* and *intl1* genes, involved in antibiotic (sulfonamides) resistance and the spread of resistance genes among bacteria, respectively. Another study reported that MP was more likely to spread antibiotic resistance genes among microbial communities despite its lower lethality compared to other parabens with longer alkyl chains (Liu et al., 2023).

A. calcoaceticus was the most affected bacteria by MP exposure revealing an increase in their tolerance to all antibiotics selected, particularly for TMP-SMX. Curiously, longer exposure to MP (10 weeks) showed a hormetic response, with an adaptative response to MP at 15 μg/L resulting in higher *A. calcoaceticus* tolerance to CEF, that disappeared at the highest concentration tested (15000 μg/L) (Agathokleous et al., 2021). Yang and Lee (2023) also reported increased tolerance to tetracycline and sulfamethoxazole, for parabens (MP, EP, PP, and BP)-exposed microbial communities at 20 mg/L, being this tolerance increased more pronounced after the 15th week of parabens exposure. This suggests that the continuous addition of parabens may lead to adaptation/selection pressure on the microbiome (Yang and Lee, 2023).

Notably, MP-exposed *S. maltophilia* exhibited increased tolerance to TMP-SMX, which is the most common therapeutic option used to treat *S. maltophilia* infections (Sánchez and Martínez, 2015). This may be potentially linked to the overexpressing of SmeDEF efflux pump (Sánchez and Martínez, 2015). Overall, the non-linear responses observed suggest that environmental concentrations of parabens could induce adaptive bacterial responses. Our findings suggest increased bacterial virulence and tolerance to antibiotics when MP was present, which is of utmost concern since human-acquired resistant infections may come from environmental bacteria, particularly, from water environments (Stanton et al., 2022).

5. Conclusion

This study provides pioneer insights into the mechanism of action of parabens (MP, PP, BP, and MIX) at an environmentally relevant level (15 μg/L) and an in-use concentration (15000 μg/L) - on planktonic bacterial cells isolated from DW, specifically *A. calcoaceticus* and *S. maltophilia*. Most of the selected parabens (MP, BP, and MIX) caused an increase of bacterial cell envelope hydrophilicity and charge values. All tested parabens were able to reduce the metabolic activity of *A. calcoaceticus*, suggesting stressful mechanisms also observed for *S. maltophilia* after exposure to MP, PP and MIX solutions at the highest concentration tested (15000 μg/L). An increase in the bacterial size after exposure to PP, BP, and MIX at 15 μg/L, and for all parabens solutions at 15000 μg/L was also observed, suggesting the existence of bacterial osmotic regulation mechanisms. This osmotic regulation was more pronounced for MP exposure at both concentrations as corroborated by TEM evaluation reflecting the existence of vacuoles in MP-exposed *A. calcoaceticus*. The results further propose that it becomes evident that the presence of parabens, even at environmental concentrations, can have extensive consequences on microbial ecosystems. These physicochemical and metabolic modifications appear to lead to important implications on biofilm formation and antimicrobial resistance since MP-exposed bacteria were found to have increased biofilm formation ability and increased antibiotic resistance. These effects pose environmental and public health risks that require further attention and regulatory consideration on the anthropogenic usage and disposal of parabens.

Funding

This work was supported by Project InnovAntiBiofilm (ref. 101157363) financed by the European Commission (Horizon-Widera 2023-Acess-02/Horizon-CSA) and by national funds through FCT/MCTES (PIDDAC): LEPABE, UIDB/00511/2020 (DOI: 10.54499/UIDB/00511/2020) and UIDP/00511/2020 (DOI: 10.54499/UIDP/00511/2020) and ALiCE, LA/P/0045/2020 (DOI: 10.54499/LA/P/0045/2020); PRESAGE – Aquatic/0007/2020, funded by national funds through FCT/MCTES (PIDDAC); Ana Rita Pereira's PhD scholarship (2021.06226.BD) and Inês B. Gomes contract 2022.06488.CEECIND/CP1733/CT0008 (DOI: 10.54499/2022.06488.CEECIND/CP1733/CT0008) both provided by FCT.

CRediT authorship contribution statement

Ana Rita Pereira: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Inês B. Gomes:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Formal

analysis, Conceptualization. **Manuel Simões:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.143704.

Data availability

Data will be made available on request.

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