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Coaggregation dynamics in drinking water biofilms and implications for chlorine disinfection

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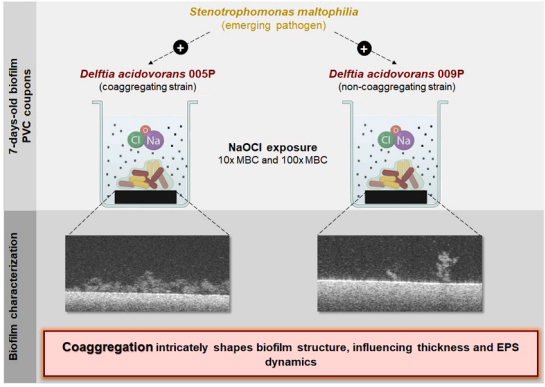
HIGHLIGHTS

- Coaggregation hinders NaOCl, promoting biofilm resilience.
- Biofilm intricate structure challenges NaOCl treatment.
- Membrane damage without culturability loss implies adaptive strategies.
- Coaggregation influences EPS, stability, and structure in biofilm resistance.
- Effective biofilm management requires understanding microbial interactions.

GRAPHICAL ABSTRACT



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ABSTRACT

Biofilms in drinking water (DW) systems persistently challenge traditional disinfection methods due to intricate microbial interactions, with coaggregation playing a crucial role in forming multispecies biofilms. This study examined the implications of coaggregation on tolerance towards sodium hypochlorite (NaOCl) disinfection. Dual-species biofilms were formed for seven days on polyvinyl chloride coupons, comprising a strain of the emerging pathogen *Stenotrophomonas maltophilia* and the coaggregating strain *Delftia acidovorans* 005 P. For comparison, dual-species biofilms were also formed with a non-coaggregation strain (*D. acidovorans* 009 P). The minimum bactericidal concentration (MBC) for each planktonic strain varied (*D. acidovorans*: 1 mg/L, *S. maltophilia*: 1.5 mg/L) below the safe DW treatment limits. However, high NaOCl doses (10 ×MBC and 100 ×MBC,) showed low efficacy against dual-species biofilms, indicating significant biofilm tolerance to disinfection. Membrane damage occurred at sub-MBC without culturability loss, underscoring biofilm resilience. The biofilm analysis revealed a complex interplay between the composition of extracellular polymeric substances and

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the architecture, which was influenced by the presence of the coaggregating strain. Overall, coaggregation significantly influenced biofilm formation and resilience, impacting NaOCl disinfection. These findings underscore the challenges of microbial interactions in biofilms, emphasizing the need for improved disinfection strategies to control biofilms in drinking water systems.

1. Introduction

In aquatic ecosystems, the coexistence of diverse bacterial species is a complex interplay that influences the dynamics of the ecosystem [1,2]. Among these interactions, bacterial coaggregation has emerged as a significant aspect of microbial ecology [3,4]. Coaggregation is a process where genetically distinct cells recognize and adhere to each other through specific molecular interactions, forming complex, multi-species clusters [4]. This phenomenon is not random, being often mediated by adhesins and receptors on the bacterial cell surfaces, enabling specific and stable interactions between different cells [5]. Studying bacterial coaggregation in aquatic systems can help understand the intricate phenomena promoting biofilm formation. Coaggregation has been extensively studied in the oral environment. It is now recognized that coaggregation plays a key role in forming dental plaque, where different microorganisms coaggregate to form complex communities [6,7].

Similarly, in aquatic systems, coaggregation seems to facilitate the formation of multi-species biofilms [3,4]. For instance, a study on *Delftia acidovorans* isolated from drinking water (DW) found that this bacterium selectively coaggregated with various partner bacteria forming stable biofilms. Notably, *D. acidovorans* 005 P (a coaggregating strain) enhanced biofilm formation by *Citrobacter freundii* and *Pseudomonas putida* likely through the production of extracellular molecules that promoted microbial cooperation [5]. In showerhead biofilms, coaggregation was observed among diverse bacterial isolates, including *Brevundimonas*, *Micrococcus*, and *Lysobacter* species, indicating that coaggregation might be a common feature that supports biofilm development in such environments [8]. Additionally, research on DW biofilms identified an *Acinetobacter calcoaceticus* strain as a key bridging bacterium, facilitating the association of other species and enhancing the overall resilience and colonization potential of multi-species biofilms [9]. Moreover, bacterial coaggregation due to the presence of bridging bacteria creates complex communities, which protect individual microorganisms from the effects of disinfectants [10]. Simões et al. [10] evaluated the susceptibility of single and multi-species biofilms composed of six bacterial isolates from DW to sodium hypochlorite (NaOCl). The study revealed that multi-species biofilms demonstrated significantly higher resistance to NaOCl in comparison to single-species biofilms. Specifically, biofilms containing A. calcoaceticus were notably more resilient to disinfection. Nevertheless, that study presented experimental limitations, particularly the use of 96-well microtiter plates, which may not reflect any relevant surface material used in DW distribution systems, and analyzed 72-hour-old biofilms, potentially overlooking structural development and maturation. Additionally, the biofilm structural characteristics, which are crucial for understanding the resistance to disinfection, were not evaluated. Bacterial coaggregation also impacts the composition and structure of biofilms in aquatic environments [11]. The incorporation of pathogenic bacteria within these multi-species biofilms raises concerns about the persistence and potential for disease transmission [8,12]. Thus, the phenomenon of coaggregation has substantial implications not only for the efficiency of water treatment processes but also for DW quality and safety.

The propensity of opportunistic bacteria to become incorporated into multi-species biofilms introduces a challenging dimension to understanding aquatic microbial ecology [13,14]. *Stenotrophomonas maltophilia* has emerged as a pathogen that can be encountered in water systems, posing challenges to public health [15]. This emerging bacterium, once considered an environmental saprophyte, has demonstrated its adaptability to various aquatic environments, including hospital water systems, plumbing networks, and natural water sources [15] and has served as a model in studies with DW biofilms [16–18]. Its increasing prevalence in these settings is particularly critical due to its inherent resistance to a broad spectrum of antibiotics [19,20]. Moreover, the ability of *S. maltophilia* to form biofilms further complicates eradication efforts [15,20]. In this study, an *S. maltophilia* strain isolated from DW [18] was studied in terms of its tolerance to NaOCl, both in planktonic and sessile states and compared when in co-culture with the coaggregating strain *Delftia acidovorans* 005 P. This strain was previously identified as coaggregating with a significant role in biofilm formation and microbial community dynamics [5]. For comparison, a *D. acidovorans* strain without coaggregation ability (*D. acidovorans* 009 P) was tested [5]. For that, single and dual-species biofilms of *S. maltophilia* and *D. acidovorans* 005 P/009 P were formed for seven days on polyvinyl chloride (PVC), a polymeric material commonly used in plumbing systems [18]. Biofilm culturability, bacterial membrane damage, biofilm structure and the content of extracellular polymeric substances (EPS) were evaluated after exposure to NaOCl at concentrations 10 and 100 times the minimum bactericidal concentration (MBC). The results contribute to increasing the understanding of coaggregation dynamics in the dynamics of microbial communities in aquatic systems and its impact on the effectiveness of water disinfection practices.

2. Material and methods

2.1. Bacteria and culture conditions

Three Gram-negative strains isolated from DW were used: *D. acidovorans* 005 P, *D. acidovorans* 009 P and *S. maltophilia* [5,18]. *D. acidovorans* strains were selected considering their coaggregation abilities: *D. acidovorans* 005 P with a high ability to coaggregate and *D. acidovorans* 009 P without the ability to coaggregate [5]. Both strains are deposited in the publicly accessible culture collection MUM (Micoteca da Universidade do Minho, Braga, Portugal) with accession codes MUM 24.11 (*D. acidovorans* 005 P) and MUM 24.12 (*D. acidovorans* 009 P). Regarding *S. maltophilia*, it naturally resides in DW but has been identified as an emerging opportunistic pathogen [15]. All strains were cryopreserved at −80 °C (aliquots of BHI [Brain Heart Infusion] medium [Oxoid, UK] with 15 % (v/v) of glycerol – *D. acidovorans*; aliquots of R2A medium growth with 30 % (v/v) of glycerol – *S. maltophilia*) and were grown overnight in R2A broth prepared according to Gomes et al. [18], at room temperature (23 °C ± 2), under agitation (160 rpm). The number of cells in suspension was adjusted to 1×10^8 CFU/mL for all bacteria. For that, bacteria were harvested from overnight culture by centrifugation (Eppendorf centrifuge 5810 R) at 3772g for 10 min and resuspended in sterile phosphate-buffered saline (PBS, pH 7.4), for the determination of the MBC (Section 2.3) or in fresh R2A broth, for biofilm formation assays (Section 2.4).

2.2. Experimental set-up

The experimental set-up involved two main tasks: (i) evaluating the susceptibility of planktonic cells to NaOCl, and (ii) assessing the tolerance to NaOCl of seven-days-old biofilms formed on PVC. Detailed procedures are provided as Supplementary Information.

2.3. Planktonic assays

2.3.1. Minimum bactericidal concentrations

The MBC of NaOCl (Sigma-Aldrich) against planktonic cells was determined based on the European Standard EN 1276 [21]. Briefly, 1 mL of bacterial suspension was mixed with 1 mL of sterile distilled water and 8 mL of NaOCl solution at 1.25 times the target concentration (0.1 to 1.5 mg/L free chlorine). Free chlorine concentrations were adjusted using a photometer from Hanna Instruments through the N, N-diethyl-phenylenediamine (DPD) method (test kit from Hanna Instruments) [22]. After 30 min at room temperature (23 ± 2 °C), the disinfectant was neutralized with sodium thiosulphate (0.50 % w/v) for 10 min [23]. Serial dilutions were then plated on R2A agar, and CFUs were counted after 24 h. The MBC was defined as the lowest concentration reducing bacterial culturability by ≥ 99.9 % (3-log reduction) [22].

2.3.2. Assessment of membrane integrity

Membrane integrity was assessed using the Live/Dead BacLight kit (Invitrogen Life Technologies, Alfagene, Portugal) [22]. SYTO 9 stains all cells green, while propidium iodide (PI) stains cells with damaged membranes red [24]. After filtering 600 µL of the neutralized test mixture through a 0.22 µm membrane, the sample was stained with 250 µL of SYTO 9 and 50 µL of PI for 7 min in the dark. The membrane was then mounted in BacLight oil and visualized with a LEICA DMLB2 epi-fluorescence microscope (LEICA Microsystems) coupled with a LEICA DFC300 FX camera and a 100 × oil immersion fluorescence objective. Cells were counted across a minimum of 10 fields of view, and membrane integrity was determined from three independent experiments.

2.4. Biofilm assays

2.4.1. Biofilm formation and exposure to NaOCl

Single-species (*D. acidovorans* 005 P, *D. acidovorans* 009 P, *S. maltophilia*) and dual-species biofilms (*D. acidovorans* 005 P/*S. maltophilia*, *D. acidovorans* 009 P/*S. maltophilia*) were grown in 24-well plates with PVC coupons for 7 days at 25 °C with agitation. A volume of 1 mL of bacterial suspension (1×10^8 CFU/mL - for single-species or 0.5 mL each for dual-species biofilms) was added to each well. The growth medium was refreshed every two days. After seven days, biofilms were exposed to NaOCl at 10 × and 100 × MBC for 30 min, at room temperature. Post-exposure, biofilms were neutralized with sodium thiosulphate and assessed for culturability, membrane damage (Section 2.4.2), EPS content (Section 2.4.3), and structural characteristics (Section 2.4.4).

2.4.2. Assessment of biofilm culturability and membrane integrity

After exposure and neutralization, biofilms were vortexed in saline solution to promote cell detachment [17]. Culturability was determined by plating on R2A agar and CFU counting (Section 2.4.2). The number of total cells and the number of cells with a damaged membrane were assessed through the Live/Dead BacLight bacterial viability kit (Invitrogen Life Technologies, Alfagene, Portugal) as described before (Section 2.3.2).

2.4.3. Analysis of biofilm structure

Biofilm structure was analyzed using optical coherence tomography (OCT) to determine the thickness and roughness [17]. For this, seven-days-old single and dual-species biofilms, after NaOCl exposure, were imaged using a Thorlabs Ganymede spectral domain OCT system (Thorlabs GmbH, Dachau, Germany) with a core wavelength of 930 nm [25]. The system provided a 3.66 × 2.98 mm^2 imaging field at a resolution of 1024 × 1024 pixels. The refractive index was adjusted to 1.4 to match the biofilm water content [26]. Biofilms were imaged in three distinct fields to ensure accuracy. The Biofilm Imaging and Structure Classification Automatic Processor (BISCAP) was used to analyze OCT images. Images were converted to binary format to isolate the biofilm from the substrate. The BISCAP software identified three boundaries: a lower bound for the biofilm base, a substratum bound below this, and an upper bound above the biofilm [25]. Thickness was calculated as the distance between the top and bottom interfaces of the biofilm. The roughness coefficient was computed to reflect variations in thickness and biofilm heterogeneity, normalized by the average biofilm thickness [25]. Higher roughness values indicate more robust biofilms. Measurements were replicated across three independent assays, with at least ten OCT images per condition.

2.4.4. Assessment of extracellular polymeric substances

EPS content was quantified to evaluate biofilm matrix composition. Following seven days of biofilm formation and NaOCl exposure, six coupons per condition were vortexed with 5 mL of extraction buffer to dislodge EPS [27]. Therefore, EPS were extracted using Dowex® Marathon® resin (0.1 g/mL of suspension) (NA+ form, strongly acidic, 20–50 mesh, Sigma-Aldrich, Germany), incubated for four hours at 400 rpm and 4 °C, then separated by centrifugation (3700g, 5 min) and stored at −20 °C [28]. EPS protein content was measured using the Total Protein Kit (Micro Lowry, Peterson's Modification, Sigma) with bovine serum albumin (BSA) as a standard. Polysaccharides were quantified using the phenol-sulfuric acid method with glucose as a standard [28–30]. The detection limits were 10.4 µg/cm^2 for polysaccharides and 12.2 µg/cm^2 for proteins. All experiments were performed in triplicate with duplicates.

2.5. Statistical analysis

Data were analyzed using the statistical program SPSS version 27.0 (Statistical Package for the Social Sciences, USA). Descriptive statistics was used to calculate the mean and standard deviation (SD) of three independent assays with duplicates. Data were evaluated in terms of normality (Gaussian distribution) using the D'Agostino-Pearson test. Differences between data were analyzed using the *t*-test for paired samples based on a confidence level of ≥ 95 % ($P < 0.05$ was considered statistically significant).

3. Results and discussion

3.1. Antimicrobial activity against planktonic bacteria

In water treatment, effective disinfection is crucial for public health, with free chlorine being a key method due to its antimicrobial potency and cost-effectiveness [31,32]. However, maintaining secure concentrations of free chlorine within designated limits is imperative to balance its effectiveness in inactivating pathogens with the need to minimize the formation of disinfection byproducts [33]. According to the Centers for Disease Control and Prevention (CDC), doses up to 4 mg/L of free chlorine provide effective disinfection of water distribution systems and are considered safe for DW consumption [34]. Here, planktonic cells of *S. maltophilia* and *D. acidovoran*s were exposed to concentrations

Table 1
Culturability of planktonic cells (Log CFU/mL) of *D. acidovorans* 005 P, *D. acidovorans* 009 P and *S. maltophilia*, non-treated and treated with free chlorine from NaOCl for 30 min. The means ± SD of three independent experiments with three replicates are illustrated. The detection limit (DL) is 1 log CFU/cm^2.

	Control	0.1 mg/L	0.5 mg/L	1 mg/L	1.5 mg/L
***D. acidovorans* 005 P**	6.39 ± 0.04	6.24 ± 0.05	3.40 ± 0.23	0.00 ± $0.00^{<DL}$	0.00 ± $0.00^{<DL}$
***D. acidovorans* 009 P**	6.23 ± 0.07	6.13 ± 0.08	3.43 ± 0.18	0.00 ± $0.00^{<DL}$	0.00 ± $0.00^{<DL}$
S. maltophilia	6.72 ± 0,07	7.02 ± 0.15	4.36 ± 0.03	3.78 ± 0.15	0.00 ± $0.00^{<DL}$

between 0.1 mg/L and 1.5 mg/L of free chlorine for MBC determination. These results are presented in terms of cell culturability (Table 1). The increase in chlorine concentration resulted in higher antimicrobial activity until a complete reduction of culturability was reached. *S. maltophilia* was more tolerant to free chlorine (MBC at 1.5 mg/L) than both *D. acidovorans* strains (MBC equal to 1 mg/L). Therefore, as the MBC values obtained are below the recommended concentration of chlorine in DW, it is considered that this would be an effective treatment against the planktonic cells of the strains tested.

Fig. 1 presents the number of total and membrane-damaged cells in treated and non-treated suspensions. In general, the number of cells with membrane damage increased with the concentration. At the concentration just below the MBC, all cells from each strain tested exhibited membrane damage. Despite significant membrane damage at the lowest concentration tested ($P < 0.05$) in both *D. acidovorans* strains and at 0.5 mg/L for *S. maltophilia* ($P < 0.05$), culturable cells were only undetectable at the MBC. This result corroborates previous findings showing that membrane damage does not inherently lead to cell death [35], reinforcing that culturability loss and membrane damage can be distinct phenomena. The ability of bacterial cells to maintain culturability despite membrane damage suggests the presence of resilient subpopulations or survival strategies within the microbial community [36]. These cells may employ diverse mechanisms, such as the ability to repair membrane damage [35] and/or enter a dormant state [36,37] to withstand the adverse effects of disinfectants. In the present study, exposure to free chlorine altered cell membrane permeability, allowing PI entry. Oliveira et al. [38] observed significant permeability changes in *S. maltophilia* exposed to sodium dichloroisocyanurate (NaDCC), trichloroisocyanuric acid (TCCA), and potassium salt of peroxymonosulfate (OXONE) even at sub-lethal concentrations, aligning with Haas and Engelbrecht [39], who demonstrated that chlorine-based disinfectants cause membrane destabilization due to physiological changes resulting from chlorination.

3.2. Biofilm disinfection

3.2.1. Effect of free chlorine on the culturability and membrane integrity of biofilms

In drinking water distribution systems (DWDS), bacteria predominantly exist in biofilms rather than in a planktonic state, facilitated by the availability of surfaces for adhesion [38]. While biofilms support bacterial survival, their presence challenges water quality management by serving as reservoirs for pathogens and impacting water quality through metabolic activities and interactions with the infrastructure [40,41]. Chlorine disinfection is crucial for managing biofilm proliferation in DWDS [42], but maintaining concentrations below specified thresholds is essential to meet water quality standards [34].

The seven-days-old biofilms formed on PVC were exposed for 30 min to free chlorine at 10 × MBC and 100 × MBC. Specifically, single-species biofilms of *D. acidovorans* were exposed to 10 μg/mL and 100 μg/mL of free chlorine; those of *S. maltophilia* to 15 μg/mL and 150 μg/mL; and dual-species biofilms to 15 μg/mL and 150 μg/mL, considering the highest MBC value, which was 1.5 μg/mL for *S. maltophilia*. Then, the effects on biofilms were evaluated in terms of biofilm culturability, membrane integrity, EPS content and biofilm structure. For cellular culturability (Fig. 2), there is an evident decrease in the number of cells in the treated biofilms formed by all the strains tested, especially at 100 × MBC ($P < 0.05$). However, these high doses of free chlorine were not able to kill completely bacteria within 30 min of exposure. Previous studies observed similar events, in which bacteria from biofilms exposed to concentrations of 10 mg/L of free chlorine persisted [10,43] as well as for higher concentrations of NaOCl [16,42]. In Gomes et al. [42], the use of NaOCl at 125 mg/L and 175 mg/L led to a modest removal of *A. calcoaceticus* (maximum removal of 0.89 log) and *S. maltophilia* (maximum removal of 0.69 log) biofilms from a polyvinyl chloride surface. In the present study, it was found that at 100 × MBC the bacteria persisted (4.54 log CFU/cm^2). In dual-species biofilms (Fig. 2), a significant decrease ($P < 0.05$) in the number of *S. maltophilia* cells, when in co-culture with *D. acidovorans* 009 P, was observed when exposed to both treatments. The number of *S. maltophilia* cells after treatment with 150 μg/mL of free chlorine was slightly higher ($P > 0.05$) in *D. acidovorans* 009 P/*S. maltophilia* dual-species biofilms than in single-species biofilms of *S. maltophilia*. Also, Gomes et al. [16] observed that treatment with a higher concentration of NaOCl does not necessarily result in increased removal of dual-species biofilms of *S. maltophilia*/*A. calcoaceticus*. Looking at the dual-species *D. acidovorans* 005 P/*S. maltophilia*, there was no significant decrease in the number of *S. maltophilia* cells after any treatment, compared to the control, nor in relation to single-species biofilms ($P > 0.05$). These opposite effects on *S. maltophilia* cells when co-cultured with *D. acidovorans* 009 P or *D. acidovorans* 005 P after treatment, suggest a protection of *S. maltophilia* in biofilms containing the coaggregating strain. Simões et al. [10] observed similar behavior for multi-species biofilms that included a coaggregating strain of *A. calcoaceticus*. These authors formed biofilms using a strain exclusion process and it was found that those that did not contain *A. calcoaceticus* were those that suffered higher inactivation after NaOCl exposure.

Regarding membrane integrity (Fig. 3) after exposure to 10 × MBC, a high number of cells with membrane damage was observed in all cases ($P < 0.05$). At 100 × MBC all cells had membrane damage ($P < 0.05$). Looking at the results of dual-species biofilms, and contrary to what was observed for cell culturability, the cellular damage of *S. maltophilia* was similar regardless of whether in co-culture with *D. acidovorans* 005 P or 009 P. However, this damage appears to have a modest impact on biofilm control, as the number of culturable cells was never less than 4 log (Fig. 2). This result is in agreement with those obtained with planktonic cells, where it was observed that exposure to NaOCl caused changes in membrane permeability, which may not been translated into cell death. Furthermore, it was already known that biofilms are extremely tolerant to concentrations that are effective against the same cells in a planktonic

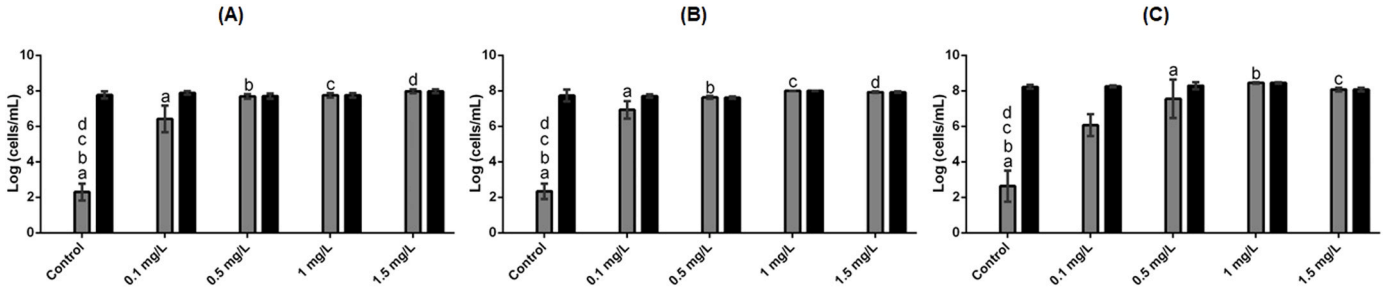

Fig. 1. Cellular density (damaged ■ and total cells ■) of *D. acidovorans* 005 P (A), *D. acidovorans* 009 P (B) and *S. maltophilia* (C), non-treated and treated with free chlorine from NaOCl for 30 min. The means ± SD of three independent experiments with three replicates are illustrated. a, b, c – samples were statistically different from the respective controls (t-test $P < 0.05$).

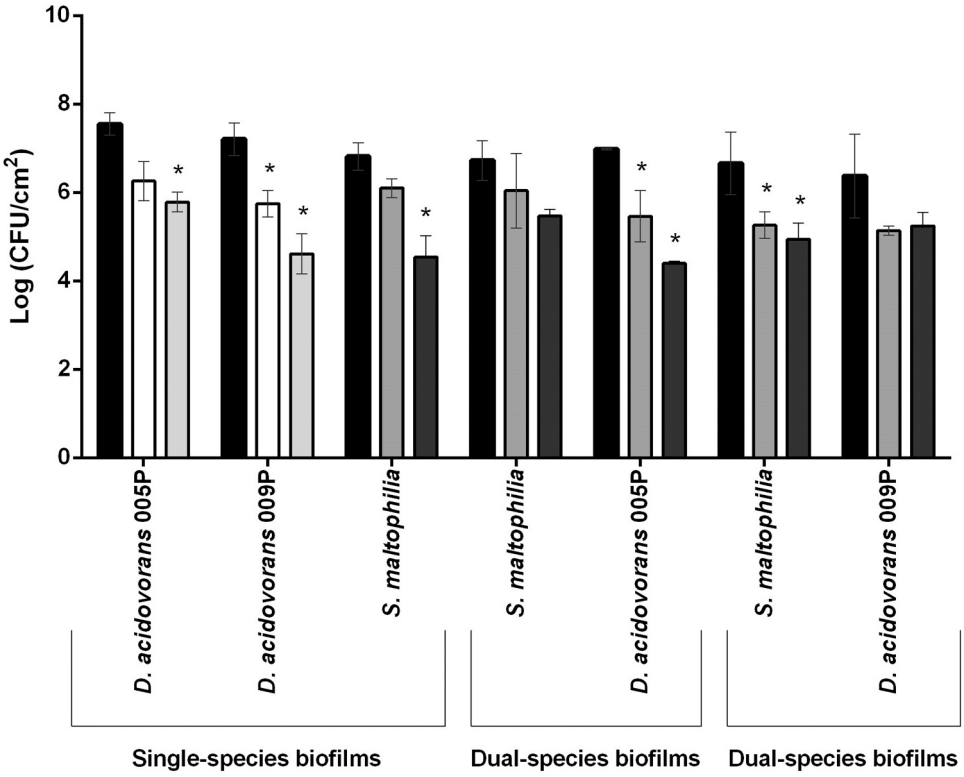

Fig. 2. Cellular culturability (Log CFU/cm^2) of seven-days-old single and dual-species biofilms formed on PVC and non-treated and treated with free chlorine from NaOCl for 30 min (control ■, 10 μg/mL □, 15 μg/mL ■, 100 μg/mL ■ and 150 μg/mL ■). The means ± SD of three independent experiments with three replicates are illustrated. *samples were statistically different from the respective controls (*t*-test, $P < 0.05$).

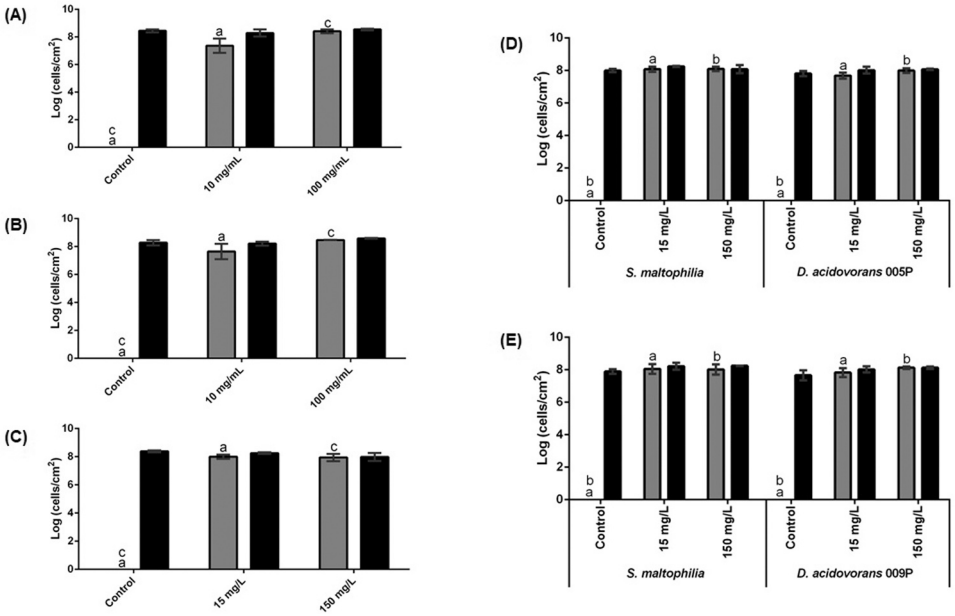

Fig. 3. Cellular density (Log cells/cm^2) of seven-days-old single (A – *D. acidovorans* 005 P, B - *D. acidovorans* 009 P and C – *S. maltophilia*) and dual-species biofilms (D and E) formed on PVC and non-treated and treated with free chlorine from NaOCl for 30 min (damaged ■ and total cells ■). The means ± SD of three independent experiments with three replicates are illustrated. a, b, c – samples were statistically different from the respective controls (t-test $P < 0.05$).

state [44].

3.2.2. *Effect of free chlorine on the biofilm structure*

The biofilm structure was analyzed by OCT and the values of the thickness and roughness coefficient are presented in Table 2. *D. acidovorans* 005 P formed the thickest biofilms (27.21 ± 3.73 μm), followed by *S. maltophilia* biofilms (19.16 ± 8.42 μm), dual-species *D. acidovorans* 005 P/*S. maltophilia* biofilms (15.78 ± 8.43 μm), *D. acidovorans* 009 P biofilms (14.53 ± 1.81 μm) and dual-species *D. acidovorans* 009 P/*S. maltophilia* biofilms (11.56 ± 4.51 μm). For

Table 2
Biofilm thickness (μm) and roughness coefficient obtained for seven-days-old single and dual-species biofilms formed on PVC coupons, non-treated and treated with free chlorine from NaOCl at 10 × MBC and 100 × MBC for 30 min. The means ± SD of three independent experiments with three replicates are illustrated. *samples were statistically different from the respective controls (*t*-test, $P < 0.05$); [a,b] statistically significant difference between mixed biofilms of different strains of *D. acidovorans* but under the same experimental condition.

		Biofilm thickness (μm)	Roughness coefficient
***D. acidovorans* 005 P**	**Control**	27.21 ± 3.73	0.76 ± 0.04
	10 mg/L	18.85 ± 7.77 *	0.74 ± 0.16
	100 mg/L	14.02 ± 5.49 *	0.70 ± 0.16
***D. acidovorans* 009 P**	**Control**	14.53 ± 1.81	0.69 ± 0.31
	10 mg/L	10.60 ± 2.99 *	0.68 ± 0.37
	100 mg/L	8.30 ± 3.89 *	0.57 ± 0.28
S. maltophilia	**Control**	19.16 ± 8.42	0.81 ± 0.36
	15 mg/L	11.69 ± 3.03 *	0.61 ± 0.15
	150 mg/L	10.22 ± 3.57 *	0.50 ± 0.23
D. acidovorans* 005 P/*S. maltophilia	**Control**	15.78 ± 8.43	0.66 ± 0.20
	15 mg/L	13.17 ± 2.91[a]	0.50 ± 0.16
	150 mg/L	12.83 ± 3.76[b]	0.55 ± 0.09
D. acidovorans* 009 P/*S. maltophilia	**Control**	11.56 ± 4.51	0.52 ± 0.04
	15 mg/L	10.29 ± 1.50[a]	0.52 ± 0.19
	150 mg/L	9.49 ± 2.12[b]	0.43 ± 0.09 *

single-species biofilms, a significant decrease ($P < 0.05$) in the biofilm thickness was observed with both NaOCl concentrations and in comparison to these untreated biofilms. In the case of dual-species biofilms, NaOCl exposure only led to a slight decrease in the thickness ($P > 0.05$). This observation shows that, although dual-species biofilms are thinner, they are more tolerant to NaOCl exposure. If one compares the thickness between the dual-species biofilms after treatment, *D. acidovorans* 005 P/*S. maltophilia* biofilms remain thicker than those of *D. acidovorans* 009 P/*S. maltophilia* ($P < 0.05$). Regarding the roughness coefficient, no differences were observed with the application of NaOCl ($P > 0.05$), except 10 × MBC against *D. acidovorans* 009 P/*S. maltophilia* biofilms, whose roughness decreased after chlorination ($P < 0.05$). In general, OCT analysis revealed a significant impact of NaOCl exposure in single-species biofilms but not for dual-species biofilms. Despite the higher thickness of *D. acidovorans* 005 P/*S. maltophilia* than that of *D. acidovorans* 009 P/*S. maltophilia* ($P < 0.05$), no clear evidence exists to highlight the role of the coaggregating strain in providing a stable structure. This is underscored by the lack of statistically significant reductions in dual-species biofilm thickness with NaOCl at the concentrations tested. To validate this hypothesis, further investigation involving the use of a higher concentration of free chlorine should be conducted.

Additionally, to further understand the impact of chlorination on the biofilm structure, it is crucial to consider the broader effects of chlorine disinfection. Research has shown that chlorine can alter biofilm architecture by redistributing cell clusters and inducing bacteria into a viable but non-culturable (VBNC) state, complicating biofilm assessment in terms of viability [45]. Chlorine disinfection also leads to heterogeneous disintegration of biofilms, which supports our finding that NaOCl had a more pronounced effect on single-species biofilms than against these dual-species [46]. The tolerance of dual-species biofilms to NaOCl exposure may be due to their structural complexity and potentially enhanced protection mechanisms. Synergistic interactions between species in dual-species biofilms can contribute to increased tolerance against disinfectants [10]. Previous mechanistic studies on the coaggregation of *D. acidovorans* 005 P have demonstrated that this strain expresses extracellular or surface proteins when co-cultured or near other cells [5,47].

3.2.3. *Effect of free chlorine on extracellular polymeric substances*

The complex structure of the biofilm EPS matrix poses a disinfection challenge by entrapping and shielding microorganisms from disinfectants [44]. This matrix is primarily composed of a complex blend of polysaccharides, proteins, nucleic acids, and lipids [48]. In this study, the EPS content was characterized in terms of extracellular polysaccharides and proteins (Table 3). In general, higher concentrations of both polysaccharides and proteins were observed for untreated biofilms, with a gradual decrease of its content with increasing concentrations of free chlorine. Looking at the results obtained for single-species biofilms, *D. acidovorans* 005 P presented a higher percentage of polysaccharides and proteins, followed by *D. acidovorans* 009 P biofilms and finally those of *S. maltophilia*. For dual-species biofilms, the content of polysaccharides and proteins was higher in those harboring the coaggregating strain (005 P) ($P < 0.05$). The protein-to-polysaccharide ratio (Table 3) consistently exceeded 1, indicating that protein concentrations were always higher than those of polysaccharides. Overall, the ratio increased with exposure to free chlorine, except in single-species biofilms of *S. maltophilia*, where a decrease was observed. This might reflect the dynamic biofilm behavior, in which the active production of proteins seems to be involved in the response to stress conditions [49,50]. Looking at the dual-species biofilm ratio values, these were highest for *D. acidovorans* 005 P/*S. maltophilia*. This suggests that biofilms produced by these species seem to contain a higher protein content relative to polysaccharides, leading to stronger aggregation and increased hydrophobicity, similar to what has been observed in other studies with elevated EPS-protein/polysaccharide ratios being reported [51–53]. These findings are consistent with previous research, which shows that

Table 3
EPS content (μg/cm^2) from single and dual-species biofilms formed on PVC, non-treated and treated with free chlorine from NaOCl at 10 × MBC and 100 × MBC for 30 min. The means ± SD of three independent experiments with three replicates are illustrated. The protein/polysaccharides ratio is also presented. *samples were statistically different from the respective controls (*t*-test, $P < 0.05$); [a,b] statistically significant difference between mixed biofilms of different strains of *D. acidovorans* but under the same experimental condition. Detection limits (DL): 10.4 μg/cm^2 (polysaccharides); 12.2 μg/cm^2 (proteins).

		Polysaccharides (μg/cm^2)	Proteins (μg/cm^2)	Ratio
***D. acidovorans* 005 P**	**Control**	25.25 ± 2.23	40.65 ± 3.85	1.59
	10 mg/L	13.15 ± 2.11 *	35.53 ± 4.58	2.71
	100 mg/L	7.18 ± 0.68 *, <DL	24.98 ± 4.84 *	3.48
***D. acidovorans* 009 P**	**Control**	13.04 ± 1.95	31.94 ± 4.25	2.45
	10 mg/L	5.23 ± 1.82 *, <DL	29.44 ± 3.067	5.63
	100 mg/L	4.61 ± 0.54 *, <DL	16.77 ± 4.99 *	3.63
S. maltophilia	**Control**	8.98 ± 0.68<DL	20.81 ± 2.87	2.32
	15 mg/L	6.01 ± 1.95<DL	14.07 ± 3.89 *	2.34
	150 mg/L	4.37 ± 0.82 *, <DL	5.17 ± 2.66 *, <DL	1.18
D. acidovorans* 005 P/*S. maltophilia	**Control**	11.32 ± 2.35[a]	25.57 ± 4.20[a]	2.26
	15 mg/L	7.57 ± 0.98<DL	22.72 ± 2.65[b]	3.00
	150 mg/L	4.68 ± 0.84 *,b, <DL	14.54 ± 4.09 *,c	3.10
D. acidovorans* 009 P/*S. maltophilia	**Control**	9.13 ± 1.83[a], <DL	17.59 ± 1.77[a]	1.93
	15 mg/L	6.01 ± 2.18<DL	14.04 ± 3.37 *,b	2.34
	150 mg/L	2.50 ± 0.98 *,b, <DL	6.21 ± 3.61 *,c, <DL	2.49

high protein/polysaccharide ratios correlate with enhanced aggregation and sedimentation efficiency, as well as higher hydrophobicity and reduced surface charge, potentially influencing the biofilm structural conformation and its interaction with surfaces [51–53]. These findings are in line with the results observed for culturability and biofilm structure. In practical terms, when biofilms underwent disinfection, EPS in biofilms, particularly proteins, reacted rapidly with chlorine, potentially modifying the structure of the EPS matrix and influencing its diffusional properties [43]. These changes, occurring within a 30-minute disinfection period, allowed biofilm cells to survive, helping to explain the persistence of viable cells even after exposure to high disinfectant concentrations [43]. Notably, the biofilm thickness is expected to correlate with EPS content, although exceptions were observed in *D. acidovorans* 009 P and *S. maltophilia* single-species biofilms. However, it is crucial to acknowledge that, while the influence of EPS content on the biofilm structure is well-established, this association can exhibit variations dependent on the microbial species, environmental conditions, and other factors shaping the process of biofilm formation and development [48]. It is noteworthy that protein-saccharide interactions play a role in auto and coaggregation [3], and *D. acidovorans* 005 P biofilms exhibited a higher content of polysaccharides and proteins. Furthermore, differences in these components were also observed within dual-species biofilms ($P < 0.05$). Bacterial coaggregation intricately shapes the EPS matrix composition and architecture [11]. In Hede et al. [11], the aggregation ability of freshwater, estuarine, and marine biofilm bacteria was characterized by their cell-bound EPS (CB-EPS). Estuarine and freshwater biofilm bacteria demonstrated higher biofilm-forming potential, attributed to distinct sugar types within CB-EPS, in contrast to marine bacteria. The study underscored the crucial role of CB-EPS in coaggregation, evidenced by the loss of coaggregation ability post-CB-EPS removal. Specific bacterial pairs from estuarine and freshwater environments retained their aggregation capability through the expression of lipids and proteins, indicating a potential adaptive response of CB-EPS to stressful conditions. Therefore, these authors demonstrated that coaggregation fosters a dynamic interplay, shaping the diversity and structure of EPS components, with coaggregated bacterial cells actively contributing with distinct extracellular materials such as proteins and polysaccharides, thereby enhancing the EPS matrix with a diverse and heterogeneous array of constituents [11].

In short, while dual-species biofilms generally exhibit smaller thickness and lower EPS content, the reduction of these parameters after treatment is not as pronounced as in single-species biofilms. For the *D. acidovorans* 005 P/*S. maltophilia* dual-species biofilms, both thickness values and polysaccharide and protein content, after exposure to 100 × MBC, consistently surpass those of single-species *S. maltophilia* biofilms under the same condition. In contrast, the dual-species biofilms *D. acidovorans* 009 P/*S. maltophilia* were close to or lower than those of single-species *S. maltophilia* biofilms. Despite cell culturability results suggest that coaggregation may protect *S. maltophilia* in dual-species biofilms, results regarding membrane damage, thickness, and EPS content are less conclusive for this hypothesis. However, OCT images reveal that the presence of the coaggregating strain influences the biofilm structure (Figs. 4 and 5). Analyzing single-species biofilms (Fig. 4), all strains form heterogeneous structures, with the primary distinction being the size/thickness of these structures (higher for *D. acidovorans* 005 P biofilms). These structures are less frequent in *D. acidovorans* 009 P and *S. maltophilia* strains. The same trend was observed for dual-species biofilms of *D. acidovorans* 009 P/*S. maltophilia* (Fig. 5B). In dual-species biofilms of *D. acidovorans* 005 P/*S. maltophilia* (Fig. 5), a less scattered structure was observed, resulting in a more compact and homogeneous biofilm. Hence, it is unsurprising that coaggregation promotes the synthesis of new EPS elements and alters the three-dimensional arrangement of the biofilm structure.

4. Conclusions

Although chlorine disinfection is effective in combating bacteria in planktonic state, its impact tends to be limited against biofilms. This reduced efficacy is due to the biofilm complex structure, the presence of EPS, and interactions among microorganisms that create protective microenvironments. This study highlights the role of coaggregation in enhancing biofilm tolerance to disinfection by providing physical protection, stabilizing the structure, and promoting EPS production. Coaggregation with *D. acidovorans* 005 P enhanced the protective effect in dual-species biofilms by creating a more compact and uniform biofilm structure. This change indicates that the presence of *D. acidovorans*

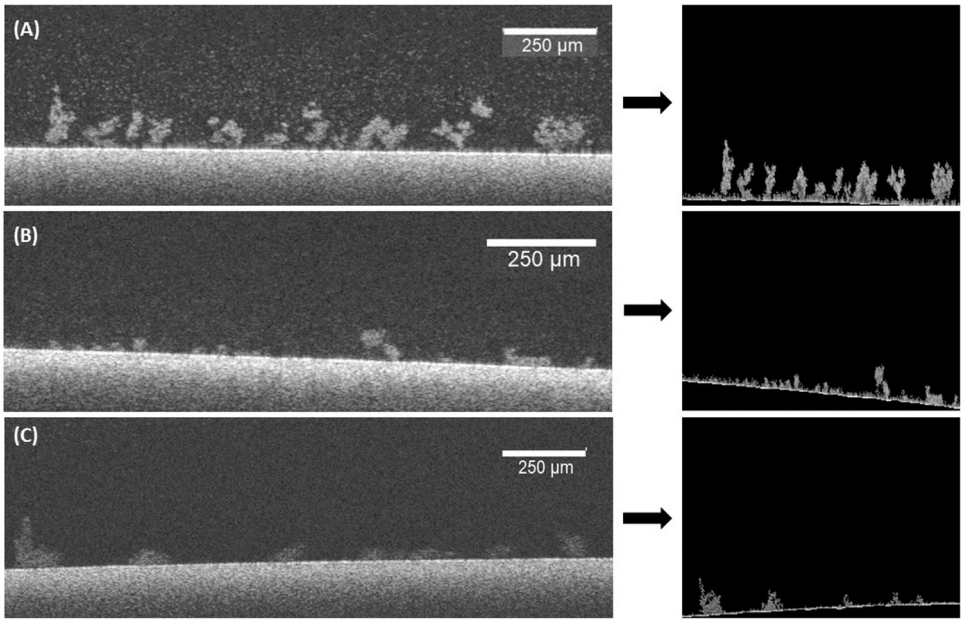

Fig. 4. Biofilm structure obtained for seven-days-old single-species biofilms of (A) *D. acidovorans* 005 P, (B) *D. acidovorans* 009 P and (C) *S. maltophilia* formed on PVC without exposure to any treatment. The panels on the right display the results of post-processing with BISCAP software. The average thicknesses of the biofilms represented are 34.23 µm, 13.88 µm and 9.17 µm, respectively for *D. acidovorans* 005 P, *S. maltophilia* and *D. acidovorans* 009 P. The visualization field was 3.66 × 2.98 mm^3 at the X–Z section corresponding to 1024 pixels × 1024 pixels.

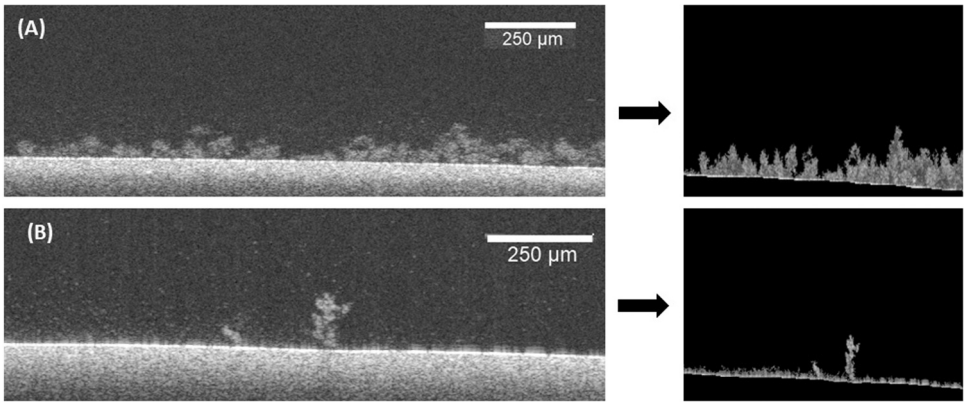

Fig. 5. Biofilm structure obtained for seven-days-old dual-species biofilms of (A) *D. acidovorans* 005 P/*S. maltophilia* and (B) *D.* acidovorans 009 P/*S. maltophilia* formed on PVC without exposure to any treatment. The panels on the right display the results of post-processing with BISCAP software. The average thicknesses of the biofilms represented are 31.02 µm and 19.43 µm, respectively for *D. acidovorans* 005 P/*S. maltophilia* and *D. acidovorans* 009 P/*S. maltophilia.* The visualization field was 3.66 × 2.98 mm^3 at the X–Z section corresponding to 1024 pixels × 1024 pixels.

005 P promotes higher EPS production and reconfigures the biofilm three-dimensional arrangement, improving its resistance to chlorine exposure. The membrane damage caused by NaOCl exposure occurred without significant culturability loss, suggesting the presence of resilient subpopulations within the biofilm. The overall results highlight the role of coaggregation in biofilm resilience and propose that understanding the interactions between microorganisms, the EPS content, and biofilm architecture is relevant for developing efficient water treatment strategies to manage biofilm-related issues in distribution systems, ensuring microbiological safe water.

Environmental implication

Complex microbial interactions within drinking water (DW) biofilms, particularly the phenomenon of coaggregation, pose significant challenges to traditional disinfection methods. The study underscores the limited effectiveness of chlorine disinfection against biofilms formed on polyvinyl chloride (PVC). Despite high chlorine concentrations, biofilms persist due to specific microbial interactions, when in the presence of the DW coaggregating strain *Delftia acidovorans* 005 P. Understanding the complex dynamics between chlorine disinfection, PVC surfaces, and microbial interactions is crucial for developing efficient water treatment approaches to mitigate environmental and public health risks associated with biofilm formation in DW distribution systems.

CRediT authorship contribution statement

Ana Afonso: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Lúcia Chaves Simões:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Francisca Massano:** Writing – review & editing, Methodology, Investigation. **Mª Saavedra:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Methodology, Funding acquisition, Conceptualization. **Inês Gomes:** Writing – review & editing, Visualization, Validation, Software, Methodology, Investigation, 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.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.135948.

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