

Monomeric and gemini surfactants as antimicrobial agents — influence on environmental and reference strains*

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Quaternary ammonium salts (QAS) belong to surfactant commonly used both, in the household and in different branches of industry, primarily in the process of cleaning and disinfection. They have several positive features inter alia effectively limiting the development of microorganisms on many surfaces. In the present work, two compounds were used as biocides: hexamethylene-1,6-bis-(*N,N*-dimethyl-*N*-dodecylammonium bromide) that belongs to the gemini surfactant (GS), and its single analogue — dodecyl(trimethyl)ammonium bromide (DTAB). Two fold dilution method was used to determine the minimum concentration of compounds (MIC) which inhibit the growth of bacteria: *Staphylococcus aureus* (ATCC 6538 and an environmental strain), *Pseudomonas aeruginosa* (ATCC 85327 and an environmental strain), and yeast *Candida albicans* (ATCC 11509 and an environmental strain). The viability of cells in liquid cultures with addition of these substances at ¼ MIC, ½ MIC and MIC concentrations were also determined. The obtained results show that DTAB inhibits the growth of bacteria at the concentration of 0.126–1.010 µM/ml, and gemini surfactant is active at 0.036–0.029 µM/ml. Therefore, GS is active at more than 17–70-fold lower concentrations than its monomeric analogue. Strains isolated from natural environment are less sensitive upon testing biocides than the references strains. Both compounds at the MIC value reduced the number of cells of all strains. The use of too low concentration of biocides can limit the growth of microorganisms, but often only for a short period of time in case of special environmental strains. Later on, they can adapt to adverse environmental conditions and begin to evolve defence mechanisms.

Key words: gemini surfactants, antimicrobial activity, *Candida*, *Pseudomonas*, *Staphylococcus*

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INTRODUCTION

Surfactants, including quaternary ammonium salts (QAS), are organic compounds composed of a hydrophilic head and a hydrophobic tail. Thanks to this structure, they are very readily soluble in many solvents. Owing to their foaming, wetting, softening and emulsifying abilities, and thermal stability, quaternary ammonium salts are widely used in many industries (such as food, paper and cosmetics). These compounds are also applied as biocides which are highly efficient against lipophilic viruses, bacteria or fungi at very low concentrations. Besides these, they are used for destruction of biofilms

formed by microorganisms in cooling systems (Rucka *et al.*, 1983). It has been found that QASs are more effective against Gram-positive than Gram-negative bacteria. They find application in medicine as antimicrobial agents and even medications (Raghavendra, 2002; Xiao *et al.*, 2008; Hegstad *et al.*, 2010). When applied according to the manufacturer's recommendations, QASs are not toxic to humans, do not corrode surfaces when used for cleaning and are biodegradable.

Gemini surfactants (GS) are a relatively new group of compounds derived from classical surfactants, the use of which has been increasing in recent years. They consist of two monomeric surfactants separated by a spacer. Due to the structure, they lower the surface tension more efficiently, and micelles formed by them are characterized by greater stability and length of life than their monomeric analogues (Hait & Moulik, 2002; Sekhon, 2004; Shukla & Tyagi, 2006). Dimeric surfactants also have a higher antimicrobial activity and a broader spectrum of action compared to monomeric surfactants (Oblak *et al.*, 2014). Their effectiveness depends on the length of the hydrophobic chain and the spacer. Surfactants containing ten or twelve carbon atoms in the hydrophobic part have an optimal activity, and a short spacer reduces the value of the minimum concentrations (MIC) which inhibit the growth of microorganisms.

It should be remembered, however, that biocidal activity is affected by several factors, such as concentration, contact time, temperature, pH, the presence of interfering material, and the types, numbers and conditions of microorganisms (Russell, 2003). Many procedures and standards (CLSI document M07-A9; PN-EN 1276, 1650) describing methods of testing the antimicrobial activity of new biocides recommend using reference strains. They are grown under laboratory conditions on selected microbiological media, which is undoubtedly conducive to their development. However, environmental strains grow under varying conditions, using different carbon or nitrogen sources, often in the presence of organic compounds that may be a protective medium for them. Sometimes, there are microorganisms that have already been exposed to disinfectants, antiseptics or antibiotics. All this means that strains isolated from such environ-

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Abbreviations: QAS, quaternary ammonium salts; GS, gemini surfactant; MIC, minimal inhibitory concentration; DTAB, dodecyl(trimethyl)ammonium bromide; C6, hexamethylene-1,6-bis-(*N,N*-dimethyl-*N*-dodecylammonium bromide); MEB, Malt Extract Broth; TSB, Tryptic Soy Agar; PBS, phosphate-buffered saline; SDS, dodecyl sulfate sodium salt; PN-EN, Polish Norm-English Norm; CLSI, clinical and Laboratory Standards Institute

Table 1. Minimal inhibitory concentration ($\mu\text{M}/\text{ml}$) of surfactants.

Species	Strains	Biocide ($\mu\text{M}/\text{ml}$)					
		Gemini surfactant C6			Monomeric surfactant DTAB		
		MIC	$\frac{1}{2}$ MIC	$\frac{1}{4}$ MIC	MIC	$\frac{1}{2}$ MIC	$\frac{1}{4}$ MIC
<i>Staphylococcus aureus</i>	ATCC 6538	0.0036	0.0018	0.0009	0.252	0.126	0.063
	environmental	0.0073	0.0036	0.0018	0.126	0.063	0.031
<i>Pseudomonas aeruginosa</i>	ATCC 85327	0.0073	0.0036	0.0018	0.126	0.063	0.031
	environmental	0.0145	0.0073	0.0036	1.01	0.5	0.252
<i>Candida albicans</i>	ATCC 10231	0.0145	0.0073	0.0036	0.5	0.252	0.126
	environmental	0.029	0.0145	0.0073	1.01	0.5	0.252

ments may have a different, usually lower sensitivity to biocides. The use of too low concentrations of biocides (i.e. sub-MIC) or antibiotics, may lead to the selection of resistant strains, which in turn contributes to the search for new substances that efficiently overcome pathogenic microorganisms. The aim of this work was to determine the antimicrobial activity of bis-quaternary ammonium salts and their monomeric analogues at MIC and sub-MIC concentrations against pathogenic bacteria and yeast. This study was conducted comparing reference and environmental strains that are often isolated from clinical as well as industrial environments.

MATERIALS AND METHODS

Strains. In the present study, the following reference bacteria and yeast strains were used: *Staphylococcus aureus* ATCC 6538; *Pseudomonas aeruginosa* ATCC 85327; *Candida albicans* ATCC 10231, as well as environmental strains (marked as E) isolated at the hospital in Lodz: *St. aureus* — from the genital tract; *C. albicans* — from urogenital tract, both identified by biochemical tests, and *P. aeruginosa* — from skin surface and identified by molecular methods.

Antimicrobial agents used were cationic gemini surfactant (GS) hexamethylene-1,6-bis-(*N,N*-dimethyl-*N*-dodecylammonium bromide) (C6) and its single analogue (MS, monomeric surfactant) — dodecyl(trimethyl)ammonium bromide (DTAB). Molecular structure and synthesis of GS has been described previously (Brycki *et al.*, 2011).

Minimal inhibitory concentration. The MIC values for bacteria and yeast were determined by a tube standard two fold dilution method (Brycki *et al.*, 2011). 24 - hour culture of bacteria in TSB medium and yeast culture in MEB medium were centrifuge (8000 rpm, for 10 minutes), resuspended in physiological salt solution and diluted 100 folds (10^7 cfu/ml bacteria and 10^6 cfu/ml yeast). One mL of microorganism suspension was mixed with 1 mL of media (TSB for bacteria, MEB for yeast) containing serial dilutions of the tested compounds and incubated at 37°C for 24 h — bacteria, 48 h — yeast. As a growth control, a suspension of microorganisms in TSB/MEB medium without the biocides was used. The MICs were defined as the lowest concentration of the compounds at which there was no visible growth. The test was repeated three times.

Hemolysis. Surfactants were tested for hemolytic activity according to a method describe by Obląk and coworkers (2014). Morphotic elements of sheep blood were obtained by centrifugation of 5 ml blood (2500 rpm, 15 min). Next they were washed in PBS phosphate-buffered saline (pH=7.4) and resuspended in PBS. SDS (1% in PBS) was used as reference for 100% hemolysis. Surfactants in aqueous solution were diluted with PBS. These compounds, at different concentrations (MIC and above), were mixed with 500 μl of red blood cells. All samples were incubated for 1.5 h at 37°C. Absorbance was measured at 540 nm. The test was repeated three times.

Viability. 2 ml of gemini/monomeric surfactant or 2 ml sterile water (non-treated samples, control) and 0.2 ml inoculums were added to Erlenmeyer flask containing TSB medium for bacteria and MEB for yeast (17.8 ml). In every sample, the suspension of bacteria was $1.0\text{--}2.0 \times 10^7$ cfu/ml, yeast $1.0\text{--}2.0 \times 10^6$ cfu/ml; biocide concentration equal to $\frac{1}{4}$ MIC; $\frac{1}{2}$ MIC or MIC were added (Table 1). After 1, 4, 8, 12, 24 and 48 h, viability of microorganisms was determined by a plate count method. Each experiment was repeated three times.

RESULTS AND DISCUSSION

The minimum concentrations of the tested compounds which inhibit the growth of microorganisms are shown in Table 1. As for dimeric cationic surfactant, they are respectively 17–70 folds lower when compared to the monomeric compound. Similar correlations between gemini quaternary ammonium salts and monomeric compounds were observed by Koziróg and coworkers (2015) for *Staphylococcus epidermidis* and *Brochothrix thermosphacta* strains or by Shirai and coworkers (2006) for *Staphylococcus aureus* and *Escherichia coli*. Also, Obląk and coworkers (2014) determined the MIC concentrations of gemini compounds containing a spacer with two or three methylene groups. The results obtained by them for *S. aureus* ATCC 6538 are more than five folds higher when compared to the MIC concentrations presented in this work. This was confirmed by Brycki and coworkers (2011), who reported the lowest MIC values for compounds containing five or six methylene groups in the spacer. The comparison of environmental and reference strains showed a higher susceptibility of reference strains. MIC concentrations that should be used to inhibit the

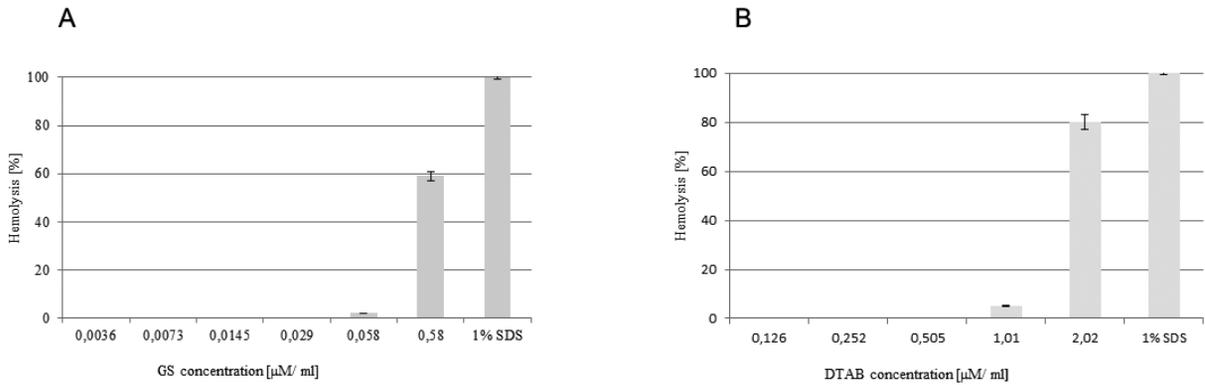


Figure 1. Hemolytic activity of surfactants:(A) gemini surfactant C6 and (B) monomeric surfactant DTAB.

growth of environmental microorganisms are two to eight folds higher. Therefore, it is important to check the sensitivity of strains isolated from the environment in which a particular antibiotic or biocide will be used, which has also been noted by Lambert *et al.* (2001), Wojcicka *et al.* (2006) and Gutarowska *et al.* (2012). The use of too low concentrations may contribute to the emergence of resistant strains.

The tested microorganisms can be ranked in terms of sensitivity to gemini surfactants (starting with the most sensitive) as: *S. aureus* > *P. aeruginosa* > *C. albicans*. This applies to both, reference and environmental strains. This rule is also confirmed by results obtained previously (Koziróg, 2015), where Gram-negative strains *Brochothrix thermosphacta* were less sensitive when comparing to Gram-positive *Staphylococcus aureus*. Differences in the bacterial sensitivity to surfactants may result from the construction of their cell walls, which in Gram-negative

bacteria contain an outer membrane providing an additional barrier (Castillo *et al.*, 2006; Vieira *et al.*, 2014).

Surfactants with MIC values were tested for their hemolytic activity. Gemini surfactant did not exhibit hemolytic activity at the highest MIC (0.029 $\mu\text{M}/\text{ml}$). Two-fold increase of concentration slightly lysed erythrocytes (Fig. 1). The gemini surfactant at a concentration of 0.58 $\mu\text{M}/\text{ml}$ led to a considerable disintegration of the erythrocyte membranes. Monomeric surfactant DTAB, at the highest MIC of 1.01 $\mu\text{M}/\text{ml}$ (for *P. aeruginosa* and *C. albicans* — environmental strains), caused a slight hemolysis. In samples with the addition of DTAB at a concentration of 2.02 $\mu\text{M}/\text{ml}$, a high degree (60%) of hemolysis was observed.

Cellular viability was analysed for all microorganism strains for which MICs were previously determined. Figures 2–4 illustrate variation of cfu/ml relative to time for environmental and reference strains, after gemini and monomeric surfactant treatment. Each environmental

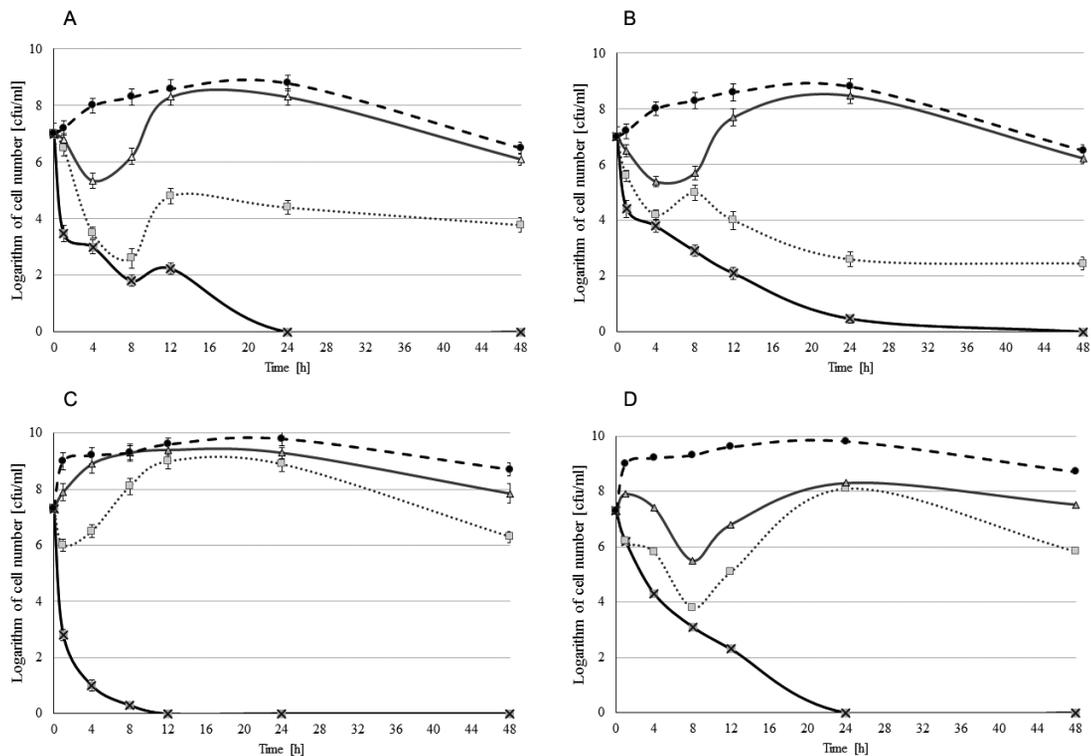


Figure 2. Growth curves of *S. aureus* reference (A, B) and environmental (C, D) strains, measured over time for different concentrations (x, MIC; □, 1/2 MIC; △, 1/4 MIC; ●, control) of gemini C6 (A, C) and monomeric DTAB (B, D) surfactant.

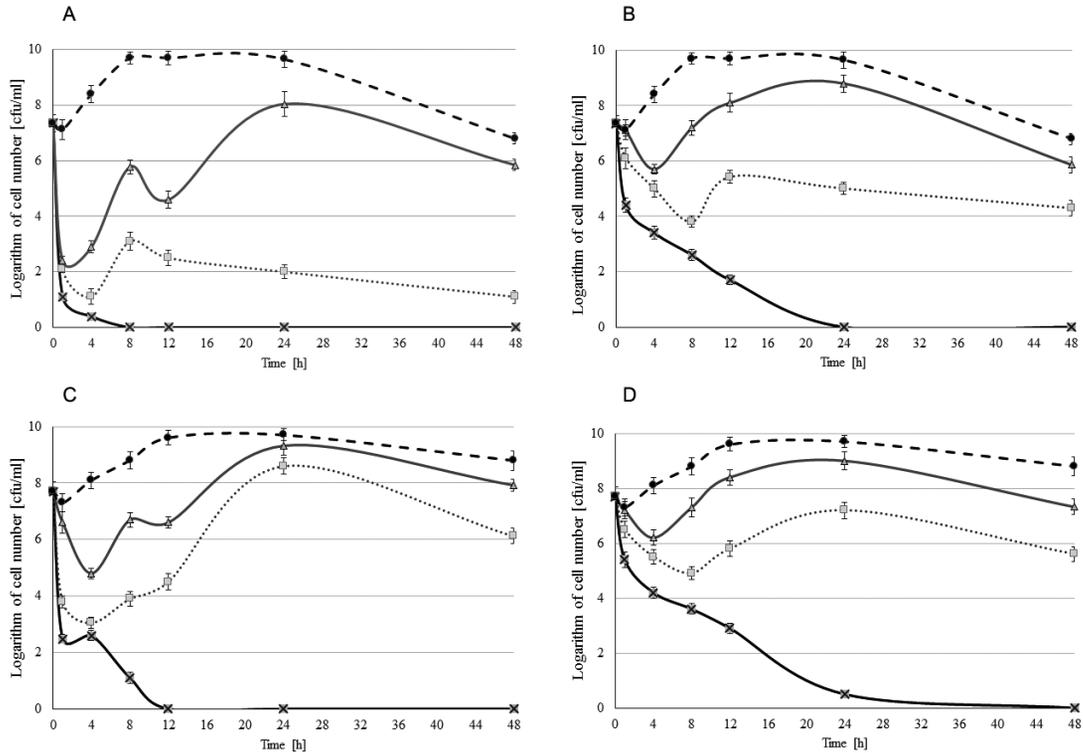


Figure 3. Growth curves of *P. aeruginosa* reference (A, B) and environmental (C, D) strains measured over time for different concentration (x, MIC; □, 1/2 MIC; △, 1/4 MIC; ●, control) of gemini C6 (A, C) and monomeric DTAB (B, D) surfactant.

strain grew faster than the reference strains in the absence of any surfactant.

The results clearly show that regardless of the type of the studied microorganism, in cultures with the addition of the gemini surfactant and its monomeric analogue at the MIC concentration, their growth was inhibited after

24 hours, and the number of viable cells was ≤ 1 log. The same relationships were found for *B. thermosphacta* and *S. epidermidis* (Koziróg, 2015). It should be noted that the growth of almost all strains (except *S. aureus* ATCC — Fig. 2) which were treated with the dimeric surfactant, was inhibited after twelve hours, although

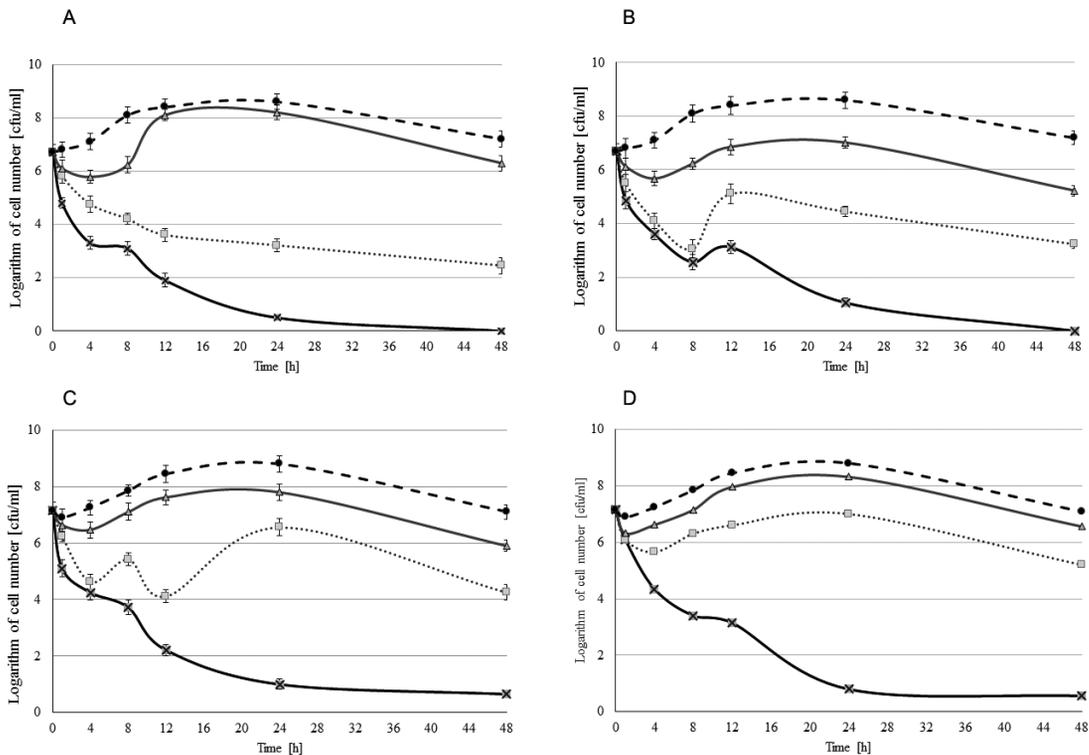


Figure 4. Growth curves of *C. albicans* reference (A, B) and environmental (C, D) strains measured over time for different concentration (x, MIC; □, 1/2 MIC; △, 1/4 MIC; ●, control) of gemini C6 (A, C) and monomeric DTAB (B, D) surfactant.

the concentrations of this compound were much lower when compared to the monomeric analogue.

The application of $\frac{1}{2}$ MIC concentrations in environmental strains initially caused their growth inhibition. The largest reduction in the number of cells (about 4.5 log) was found for the *P. aeruginosa* strain (Fig. 3), after treatment with the C6 compound for four hours. However, after one to twelve hours, depending on the type of microorganism, their cells adapt to unfavourable environmental conditions. After 24 hours, the number of bacteria ranged from 7.2 to 8.9 log, and yeasts from 6.5 to 7 log. As for reference bacterial strains, the amount of cfu/ml decreased in the early hours of culture (0–4 h or 0–8 h). Then, a short-term growth occurred within up to twelve hours, followed by a gradual reduction in cells capable of growth, unlike in environmental strains. Similar growth properties in a culture with the addition of DTAB at $\frac{1}{2}$ MIC have been observed in the case of yeast (Fig. 4). The exception is the reference strain culture with the addition of the gemini surfactant, where the number of cells in the culture gradually decreased despite the use of sub-MIC concentration. Similar results were obtained by Dong *et al.* (2012), who studied the influence of antimicrobial agents, such as chlorhexidine, at $\frac{1}{2}$ MIC concentrations on *Streptococcus mutans*.

Another two-fold reduction in the concentration of the compounds, to $\frac{1}{4}$ MIC, also caused a temporary (1–4 h) inhibitory effect, but this time without a clear distinction between reference and environmental strains. The reduction amounts to as little as 1 log in yeast, and to 2–5 logs in bacteria. Slightly different reactions were observed in the environmental strain of *S. aureus*. After treatment with the monomeric surfactant at a concentration of $\frac{1}{4}$ MIC, the growth inhibition of this strain lasted longer — up to eight hours. But in the culture containing the gemini surfactant at $\frac{1}{4}$ MIC, the growth of this strain was not inhibited at all. After four hours, a dynamic growth of cfu/ml was observed in almost all cultures.

Short-term and one-time exposure to sub-MIC concentrations may contribute to reducing the sensitivity of microorganisms to antibiotics or biocides. However, repeated exposure to sub-MIC concentrations can result in a stable strain resistance, which makes it extremely difficult, and sometimes even impossible, to eliminate it from the environment (Thomas *et al.*, 2000; Aka & Haji, 2015).

The results of our study show that gemini quaternary ammonium salts have a better antimicrobial activity than their monomeric analogues. Reference strains are more sensitive than environmental strains, which should be taken into account when using new biocides. While the use of antimicrobial agents at MIC concentrations is effective in limiting the growth of microorganisms, the use of too low (sub-MIC) concentrations leads to short-term growth inhibition, which may result in the development of resistant strains. This applies particularly to environmental strains which fairly easily adapted to exposures to $\frac{1}{2}$ MIC under the testing conditions.

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