Studii şi Cercetări Ştiinţifice 2016, **17** (2), pp. 199 – 210
Chimie şi Inginerie Chimică, Biotehnologii, Industrie Alimentară

Scientific Study & Research
Chemistry & Chemical Engineering, Biotechnology, Food Industry *ISSN 1582-540X*

ORIGINAL RESEARCH PAPER

EVALUATION OF THE ANTIMICROBIAL EFFECTS OF NEW HETEROCYCLIC BIS-QUATERNARY AMMONIUM COMPOUNDS ON BIOFILMS

Oana E. Constantin[1], Bianca Furdui[2*], Gabriela Bahrim[1], Rodica M. Dinică[2]

[1] *Dunărea de Jos University of Galati, Faculty of Food Science and Engineering, 111 Domnească Street, 800201, Galati, Romania*
[2] *Dunărea de Jos University of Galati, Faculty* of *Sciences and Environment, 111 Domnească Street, 800201, Galati, Romania*

*Corresponding author: bfurdui@ugal.ro

Received: May, 09, 2016
Accepted: June, 24, 2016

Abstract: Considering the well-known mechanism of adaptable resistance of microorganisms to chemical compounds through biofilms formation and the widespread use of N-heterocyclic quaternary ammonium salts (QAC) as disinfectants, in this study we have evaluate the effect of 8 newly synthesized symmetrical and unsymmetrical diquaternary ammonium salts of 1,2-bis-(4-pyridil)-ethane on bacterial biofilms produced by three different bacterial strains. The effect of the exposure to quaternary ammonium salts on biofilm communities was investigated within biofilms obtained in a conventional testing system, on stainless steel and glass surfaces. Differential plate counts were used to characterize the developed communities and the effects of QAC exposure and the results were correlated with epifluorescence microphotographs. The data obtained revealed a significant reduction of bacterial cells in the biofilms tested with 4-7 log CFU for all the QAC.

Keywords: *B. cereus, B. subtilis, biofilm, bis-pyridinium salts, epifluorescence microscopy, P. fluorescens, quaternary ammonium salts*

INTRODUCTION

The use of the commercial disinfectants is limited by the continuing increase of bacterial resistance to many antibacterial agents and that conduct to the necessity of a permanent identification of new compounds with microbiostatic and/or microbicide effect.

An adaptive mechanism of microorganisms' resistance to disinfectants is the formation of protective materials – biofilm [1]. A biofilm can be defined as a functional consortia of microbial cells, attach to each other and/or a surface, linked to and growing at an interface (solid-liquid, solid-air, liquid-liquid, liquid-air), with production of extracellular polymeric substances (EPS). These surface-associated microbial cells are enclosed in hydrated extracellular polymeric substances [2]. The main components of extracellular polymeric matrix are polysaccharides, proteins, phospholipids and even nucleic acids [3]. Mature biofilms are highly organized ecosystems in which water channels are dispersed and can provide passages for the exchange of nutrients, metabolites and waste products [4].

It is well known that bacteria adhere to many surfaces from food processing and storage; the time needed for bacteria to attach and form biofilms on the surface being very short. Formation of biofilms on the surfaces of processing or storage of food is a hygiene problem because of the risk of microbial contamination of food by transferring microorganisms from biofilms on the surface of food, after contact between the biofilm and food [5].

Efficacy of destruction processes is influenced by the organization of bacteria. Thus, numerous reports show that biofilms attached bacteria are more resistant to biocides comparatively with microorganisms in suspension [1, 5 – 7]. When such organisms are returned to their planktonic phase, the cells regain their original sensitivity. Thus, it may be that certain architectural features of biofilms are responsible for increasing tolerance to disinfectants, by preventing penetration of disinfectant agent, mainly due to exopolymeric matrix, slower microbial growth rate or resistance phenotype expression [7].

Therefore recent researches in the field of disinfectants are focused on the destruction and removal of bacteria attached to surfaces, organized as biofilms [5]. Currently there is an unprecedented demand for registration of various disinfectant products claiming to be "antiobiofilm-effective" [8].

Quaternary ammonium salts (QAC) is one of the most used classes of disinfectants, with wide application in hospital environments, water treatment, textiles, paints and food, due to their relatively low toxicity to humans and animals and their very wide antimicrobial spectrum [9, 10]. Numerous studies have been conducted in recent years to synthesis of new quaternary ammonium compounds and to study their antimicrobial properties. Between the synthesized structures, quaternary pyridinium and bis-pyridinium salts is an important group of substances used as biocides [11, 12], drugs [13, 14] and herbicides [15, 16] due to their strong antimicrobial effect even at very low concentrations, on a broad range of Gram-positive and Gram-negative bacteria (eg. *Bacillus subtilis*, *Bacillus cereus, Sarcina lutea*, *Streptococcus pneumoniae, Staphylococcus aureus, Micrococcus luteus, Escherichia coli* etc.), moulds (*Aspergillus niger, Aspergillus glaucus, Geotrichum candidum, Fusarium graminearum* etc.) and yeasts (*Saccharomyces cerevisiae, Rhodoturula glutinis* etc.) [17, 18].

Having regard to recent results on the low cytotoxic effect of some antimicrobial from bis-quaternary pyridinium salts group on human cells [19] may expected that pyridinium and bis-pyridinium salts are potential agents recommended for use in composition of hygiene products in food industry (technological places, equipments and surfaces), and in catering units, hospitals etc.
In our previous studies we have been interested in the development of new nitrogen quaternary salts in particular derived from 4,4'-bipyridil and 1,2-bis-(4-pyridil)-ethane with antimicrobial properties [20 – 22].
In order to identify new and more efficient antimicrobial compounds applicable as antibacterial agents with biofilm destruction and removal efficiency, some symmetrical and unsymmetrical diquaternary ammonium salts of 1,2-bis(4-pyridil)-ethane recently synthesized by us [23 – 25] were used to investigate their microbiostatic and microbicide effect on biofilm with food industry and health impact.

MATERIALS AND METHODS

Chemical compounds tested

The antibacterial potential on biofilms of eight symmetrical and unsymmetrical diquaternary salts derived from 1,2-bis-(4-pyridinium)-ethane, recently synthesized in our laboratory from the reaction of 1,2-bis-(4-pyridil)-ethane with reactive halides [23 –25], were investigated. The structures of investigated compounds are presented in Figure 1.

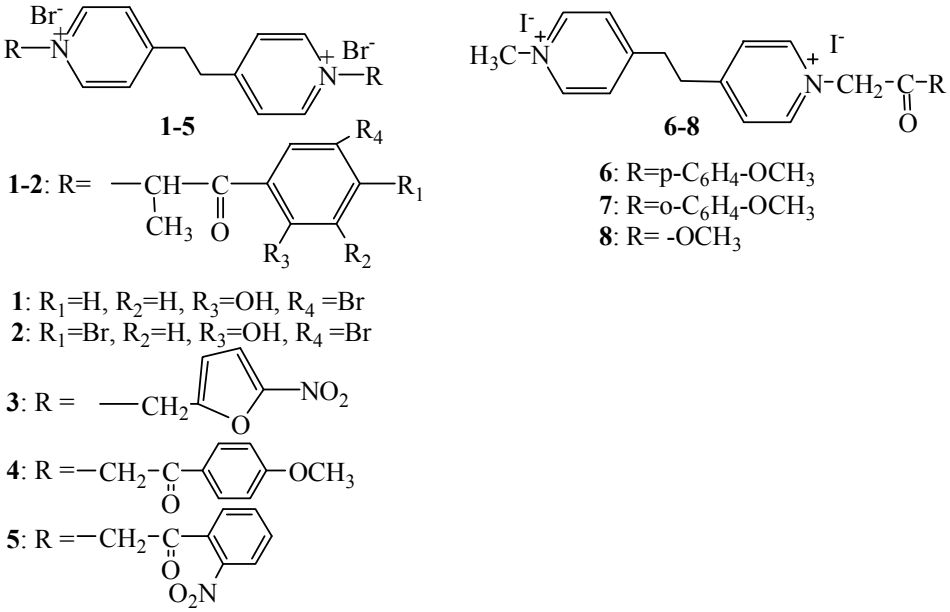


Figure 1. *Structure of tested diquaternary salts*

Test microorganisms and culture preservation

Strains used in this study were *Bacillus subtilis ATCC 19659*, *Bacillus cereus ATCC 10876* and *Pseudomonas fluorescens ATCC 13525*, purchased from American Type

Culture Collection (Microbiologics). Commercial cultures lyophilized were reactivated as specified by the Microbiologics Protocol, being inoculated on agar medium (casein peptone 5.0 $g \cdot L^{-1}$, yeast extract 2.5 $g \cdot L^{-1}$, dextrose 1.0 $g \cdot L^{-1}$, agar 15 $g \cdot L^{-1}$) and incubated at 37 °C for 24-48 h for *Bacillus subtilis* and *Bacillus cereus* and at 25 °C for 24-48 h for *Pseudomonas fluorescens*. After reactivation the pure cultures were preserved at 4 °C.

Antimicrobial activity test

The agar diffusion test was used for the qualitative evaluation of the inhibitory potential of the investigated compounds against the tested bacterial strains. A volume of 50 µL of aqueous solutions (5 $mg \cdot mL^{-1}$) of chemicals tested was impregnated into the sterile filter paper disk (Φ = 19mm) and placed on Tryptone Soya Agar (TSA) plates (Oxoid, UK), which were previously inoculated with 200 µL of 10^6 $CFU \cdot mL^{-1}$ suspension for each tested bacterium. Distilled water was used as a control. Plates were incubated at optimal conditions for culture growth (37 °C, 48 hour). The evaluation of the antimicrobial effect of studied chemical compounds was realized by measuring at every 24 hours, the inhibition zone diameter (D_{IZ}, mm). All of the experiments were performed in triplicate.

Slides preparation for biofilm formation

The slides for biofilm forming were glass and stainless steel plates (4 × 4 cm), treated by immersion in acetone, dried in air flow under, rinse with ultrapure water and then treated with 2N HCl for 2 hours, rinse with distilled water and dried in air flow. Finally, the slides were autoclaved at 121 °C for 15 min.

Attachment of microorganisms and biofilm development in model system

Biomass of bacteria obtained by cultivation on broth medium (beef extract $3g \cdot L^{-1}$, peptone 5 $g \cdot L^{-1}$, *p*H = 7±0.2), in submerged condition, for 48 h, at 30 °C, was separated by centrifugation at 10000 rpm for 15 min., followed by suspension in distilled water (1 g biomass/5 mL distilled water).
The *Bacillus spp.* obtained suspension was heated to 70-80 °C, for 20 min. and then immediately cooled in ice to inactivate vegetative cells. Spores were preserved as dense suspension in sterile distilled water at 4 °C until use. A volume of 5 mL inoculums of *Bacillus cereus* or *Bacillus subtilis* spore suspensions was collected and inoculated in a volume of 500 mL culture medium with composition ($g \cdot L^{-1}$): casein peptone 5.0, yeast extract 2.5, dextrose 1.0, *p*H = 7±0.2, in two separate beakers. For *Pseudomonas fluorescens* strain, biofilm preparation was achieved using a 5 mL overnight culture, grown on liquid medium (beef extract 3 $g \cdot L^{-1}$, peptone 5 $g \cdot L^{-1}$, *p*H = 7±0.2), by cultivation on the shaker at 100 rpm and inoculation to a volume of 500 mL of the same culture medium used as well as for *Bacillus spp*.
The glass and stainless steel slides were clamped vertically and immersed in those culture mediums. Attachment of microorganisms was initiated, and biofilms allowed forming, incubated in a shaking incubator (37 °C), by continuous stirring at 30 rpm, for 6 days. Biofilm development was monitored by optical density measuring at wavelength of 580 nm in cell suspension using a spectrophotometer (UV-Vis Beam PC 8 Scanning

auto cell UVD-3200, Labomed), maintaining the value of 0.5 for OD by adding fresh medium, every two days for 6 days. Once the incubation period was over, the slides were removed from medium containers and rinsed twice with sterile water.

Antibacterial activity assay on biofilm

The obtained biofilms were treated with aqueous solutions (5 $mg \cdot mL^{-1}$) of the tested chemicals, that have shown a stronger inhibitory potential (D_{IZ}) >30mm. Each test biofilm area was rinsed with a volume of 10 mL sterile saline serum (0.9 % NaCl) and treated with a 2 mL of tested chemical solutions. Biofilm slides were than incubated at room temperature (20 °C) for 20 min. After the contact time passed biofilm slides were rinsed with sterile distilled water.

Determination of viable count in biofilm

The biofilms were removed from the culture media by a sterile cotton swab [26]. Recovered biofilm was transferred to 50 cm^3 sterile saline and after intense agitation of the sample were performed immediately decimal dilutions with sterile saline. From the obtained dilutions the inoculation were made in nutrient agar and incubated at 37 °C for 48 hours. After the incubation, the developed colonies were counted.

Evaluation of biofilm removal by epifluorescence microscopy

Initial biofilm formation and biofilm removal potential of tested substances were rapidly assessed by staining with fluorochromes and studied by epifluorescence microscopy. Acridine orange (AO) at concentration of 10 µg $fluorochrome \cdot mL^{-1}$ was used for staining. The slides where biofilms developed were treated with an appropriate quantity of AO solution to cover all slide surface, for 15 min at room temperature in the dark. After 15 minutes of action of the AO, the slides were rinsed with sterile distilled water and dried in air flow. Epifluorescence microscopy images for AO stained biofilms were recorded with the phase-contrast and epifluorescence microscope Olympus BX 41, using fluorochrome specific WB filter, at 460-490 nm excitation wavelength and 520 nm emission wavelength.

Image analysis

Twenty images for each sample were collected from the 6 day-old biofilms treated and untreated with chemical compound measuring the obtained images area (88.69 x 66.5µm). The area of colonization with and without adding the chemical compound was determined using the Digital Image Analysis by using the software, Image J (v. 1.45, NIH, downloadable from the site http://rsb.info.nih.gov/ij). The scanned color image is first converted to grey scale. After adjusting the threshold, biofilm areas as the fraction of the total area were determined by using this software. Binary images are threshold to only two values, typically 0 and 1, but often – as with Image J – 0 and 255, that represent black and white on an 8–bit scale.

RESULTS AND DISCUSSIONS

The tested chemical compounds were chosen from a wide range of symmetrical and unsymmetrical diquaternary salts derived from 1,2-bis-(4-pyridil)ethane recently synthesized during several original studies [23 – 25]. Even though the antimicrobial activity of some of the studied compounds has been previously evaluated [22, 24, 25], all the compounds have been retested for establishing their inhibitory potential against the bacterial strains used for biofilm formation. In Table 1 are presented the results obtained through the diffusion method, after 24 h of cultivation. The highest antimicrobial inhibition spectrum has the following compounds: **1** (37.1±1.01 mm) for *Bacillus subtilis*; **2** (62.1±0.22 mm), **3** (42.33±0.21 mm) and **1** (39.6±0.17 mm) for *Bacillus cereus*; **6** (78.3±0.34 mm), **2** (68.3±1.02 mm) and **8** (43.66±2.76 mm) for *Pseudomonas fluorescens*.
For biofilm removal were selected only those compounds with an inhibition spectrum (D_{IZ}) >30mm, for each tested strain.

Table 1. *The qualitative (D_{IZ}) antibacterial effect of tested compounds*

Tested compound	Bacterial strains/ D_{IZ} [mm]		
	Bacillus subtilis	***Bacillus cereus***	***Pseudomonas fluorescens***
1	37.11±1.01	39.62±0.17	37.63±0.14
2	31.35±1.12	62.10±0.22	68.31±1.02
3	34.02±1.12	42.33±0.21	24.21±0.1
4	34.66±2.16	19.16 ± 0.16	29.33±1.15
5	34.00±1.60	35.33±2.51	21.66±0.57
6	31.33±0.17	19.33 ± 0.33	78.3±0.34
7	19.16 ± 0.16	19.50 ± 0.28	19.33 ± 0.33
8	19.33 ± 0.33	19.16 ± 0.16	43.66±2.76

D_{IZ} – diameter of the inhibition zone. Values are presented as the mean ± SD. The diameter of the test paper discs is 19 mm

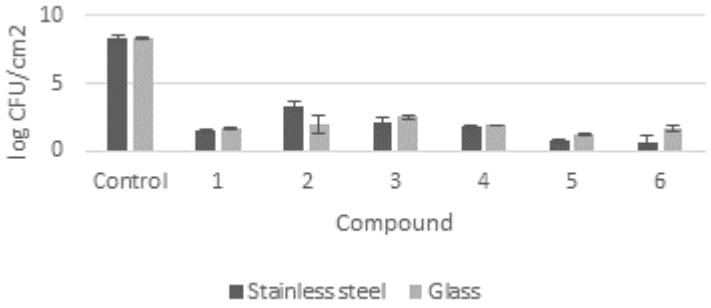
Investigation of bis-pyridinium diquaternary salts antimicrobial efficacy was performed on biofilm testing systems produced by three different bacteria (*B. subtilis*, *B. cereus* and *P. fluorescens*) in a dynamic model system conditions, under continuous stirring [27]. The choice of these strains was motivated by their ability to form biofilms in various field of food industry [28 –30]. Biofilms were induced to develop on glass and stainless steel slides. The material selection was influenced by their properties to support the formation of biofilm, their use in food sector and the property of not adversely affect the viability of the microorganism and allows biofilm detachment after cleaning and sanitizing treatments.
The compounds were applied to 6-day aged biofilms and their effects were assessed on inactivation. Figure 2 shows the number of viable cells attached to glass and stainless steel slides after treatment with bis-pyridinium diquaternary salts. A maximum concentration (5 mg·mL^{-1}) for aqueous solution of the tested compounds has been used.
The initial number of *B. subtilis* cells on stainless steel and glass slides was 8.37 ± 0.19 log CFU·cm^{-2} and 8.34 ± 0.10 log CFU·cm^{-2}, respectively (Figure 2a). For the biofilm formed on stainless steel slides treated with selected salts, the number of cells decreased to below the detection limit for compound **6** and **5** (0.68 ± 0.45 log CFU·cm^{-2} and 0.79 ± 0.06 log CFU·cm^{-2}, respectively) within 20 min. For the biofilm on glass slides,

the number of cells decreased to below the detection limit only for compound **5** (1.20 ± 0.06 log CFU·cm^{-2}) within 20 min.

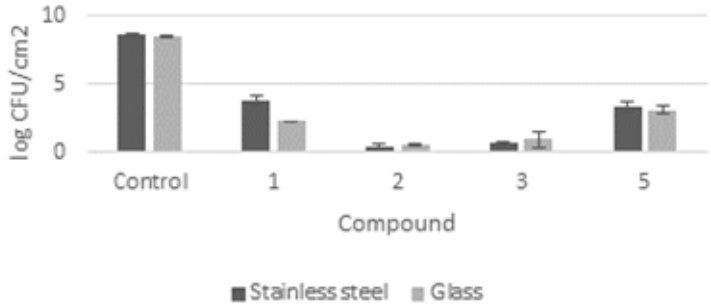
The initial number of *B. cereus* cells on stainless steel and glass slides was 8.64 ± 0.07 log CFU·cm^{-2} and 8.43 ± 0.15 log CFU·cm^{-2}, respectively (Figure 2b). For the biofilm on treated stainless steel slides the number of cells decreased to below the detection limit for compound **2** and **3** (0.47 ± 0.15 log CFU·cm^{-2} and 0.68 ± 0.07 log CFU·cm^{-2}, respectively) within 20 min. For glass slides, the number of cells decreased significant for compounds **2** and **3** (0.53 ± 0.04 log CFU·cm^{-2} and 0.98 ± 0.57 log CFU·cm^{-2}, respectively) within 20 min.

For *Pseudomonas fluorescens* biofilms the initial cell number on stainless steel and glass slides was 8.29 ± 0.07 log CFU·cm^{-2} and 8.40 ± 0.14 log CFU·cm^{-2}, respectively (Figure 2c). The number of cells decreased to below the detection limit for compounds **2** and **6** for both types of slides.

a



b



c

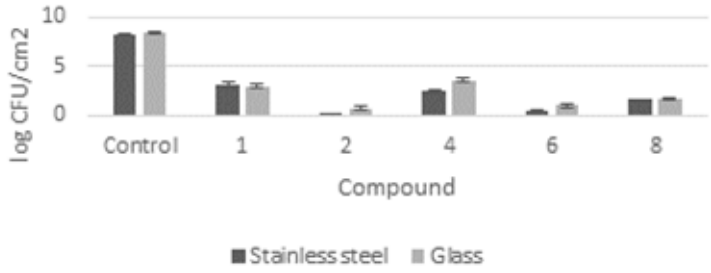


Figure 2. *Effect of heterocyclic bis-quaternary ammonium compounds on biofilms: a) Bacillus subtilis, b) Bacillus cereus, c) Pseudomonas fluorescens*

The antimicrobial activity of the tested compounds was reduced in some cases (ex. salts **1, 4** and **5**), where inhibiting substances may be present (e.g EPS from biofilms matrix). Similar results were obtained by Araújo et al. (2013) [31], showing that the reduction of antimicrobial effects against *B. cereus* and *P. fluorescens* it is due to interaction with biofilm components, for two quaternary ammonium compounds (benzalkonium chloride and cetyltrimethyl ammonium bromide). The effects of diquaternary salts tested on biofilms can be explained by their action on cells: disrupting the cell membranes and cell lysis [32].

The potential for bacterial biofilm removal by tested symmetrical and unsymmetrical bis-pyridinium diquaternary salts was also performed by epifluorescence microscopy, using acridine orange staining and by establishing percentage of biofilm removal after chemical treatment. In figure 3 are presented epifluorescence microscopy images of *Bacillus subtilis*, *Bacillus cereus*, and *Pseudomonas fluorescens* biofilms treated with tested chemical compounds.

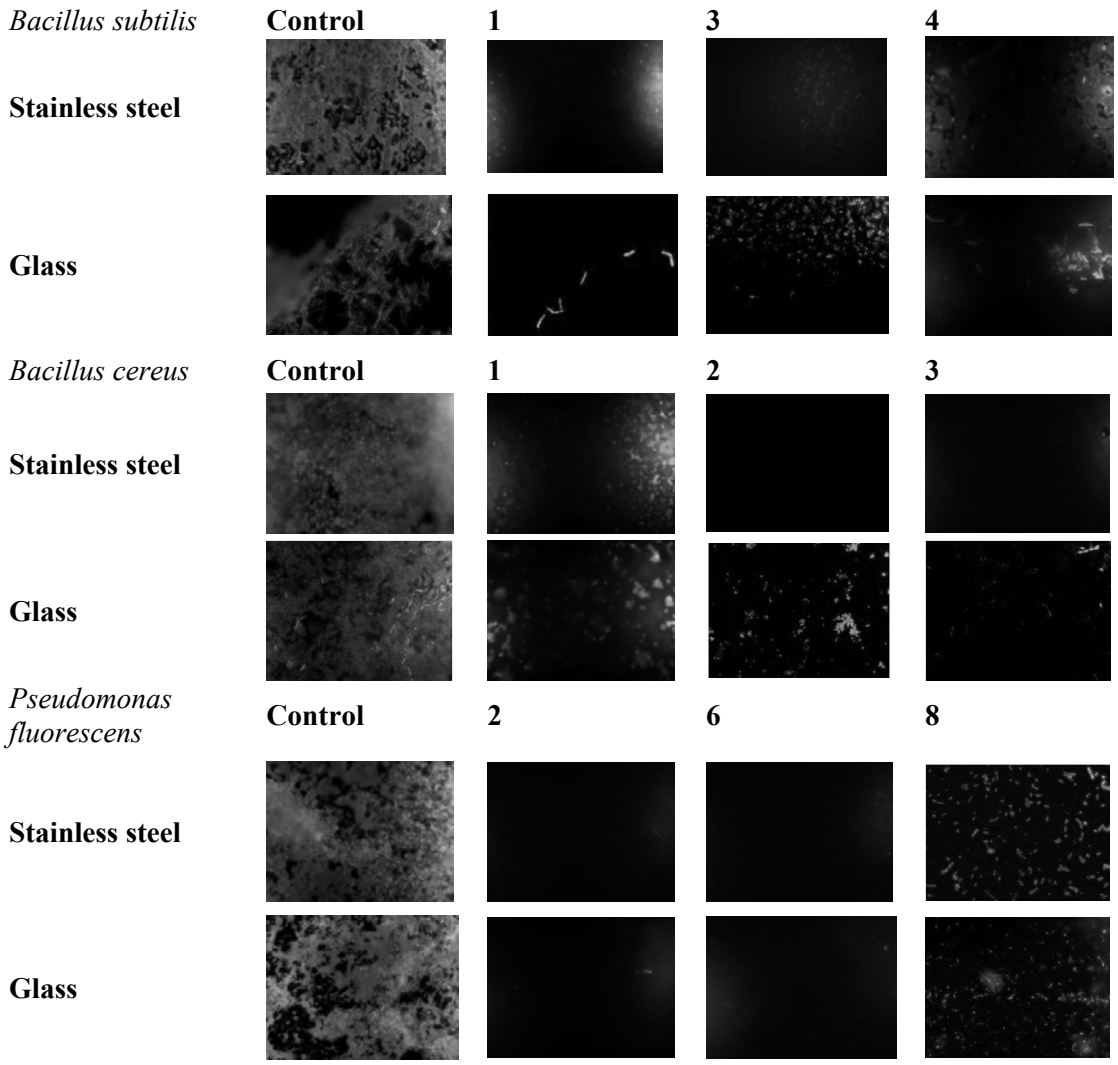


Figure 3. *Epifluorescence microscopy images of Bacillus subtilis, Bacillus cereus, and Pseudomonas fluorescens biofilms before and after applying the chemical compounds*

Fluorochrome staining and epifluorescence microscopy are well known methods to quantify the biomass growth and biofilm formation, for rapid evaluation of the antimicrobial action of disinfectants on microbial cells and for the degree of biofilm's removal [33 – 35]. For this purpose, fluorochromes that are able to interact with nucleic acids present in microbial cells or in the biofilm polymeric extracellular matrix are used. Fluorochrome used in present study was acridine orange (AO) which has the ability to bind to nucleic acids, forming a fluorescent orange red complex for RNA, respectively a green fluorescent complex with DNA [36].
Epifluorescence microscopic images of biofilms after treatment with analysed compounds showed a partial destruction of the studied biofilms, probably due to the reduced ability of chemical compounds to penetrate the biofilm matrix. This matrix can prevent the diffusion of compounds in biofilm, thus giving partial protection against bacterial antimicrobial agent. Besides the physical barrier of diffusion biocides, protective property of EPS matrix may be due to other factors such as absorption or chemical reactions that occur between the chemical agent and biofilm surface.
Table 2 presents the percentage of glass surfaces areas covered with biofilm attached microorganisms.

Table 2. *Quantitative data evaluation on bacterial biofilms removal obtained from fluorescence microscopic image analysis*

Chemical treatment of biofilm	**Percentage of biofilm remaining after chemical treatment, [%]**					
	Bacillus subtilis		*Bacillus cereus*		*Pseudomonas fluorescens*	
	stainless steel	**glass**	**stainless steel**	**glass**	**stainless steel**	**glass**
Control	57.12±0.21	52.92±0.01	55±0.11	69.39±0.21	59±0.41	66.45±0.11
Comp. **1**	26.61±0.12	20.59±0.12	12.21±2.01	13.79±1.01	17.18±0.21	14.51±0.30
Comp. **2**	18.29±0.27	17.12±0.56	3.01±0.45	2.21±0.93	2.99±0.41	3.69±0.01
Comp. **3**	14.98±0.33	13.44±0.03	9.26±1.01	7.14±0.18	-	-
Comp. **4**	17.23±0.31	18.01±0.01	-	-	20±0.65	19±0.01
Comp. **5**	16.01±0.32	16.56±0.56	14.36±0.67	13±0.07	-	-
Comp. **6**	13.13±0.95	15.23±1.47	-	-	3.01±0.18	2.51±0.03
Comp. **8**	-	-	-	-	14.42±0.13	12.1±0.3

Values are presented as the mean ± SD

On *Bacillus subtilis* biofilms bis-pyridinium diquaternary salts caused destruction of both extracellular polymeric substance (EPS) matrix structure and bacterial cells (Figure 2a), highest antimicrobial potential been observed for salt **1**, probably due to its inhibitory potential for *Bacillus subtilis* strain appreciated through agar diffusion method (D_{IZ} = 37.1 ± 1.01mm). Epifluorescence microphotographs from Figure 3 can be correlated with results obtained by analysing the degree of reduction in the area colonized by the biofilm (Table 2). In this case, the action of the chemical compounds reduced the number of adhered cells on stainless steel slides, from 57.12 ± 0.21 % to 13.13 ± 0.95 % for compound **6**. Similar results were obtained for biofilms on glass slides.
For *Bacillus cereus* biofilm the highest degree of destruction was found for compound **2**, which can be explained by his increased antimicrobial activity highlighted by

qualitative effect of tested compound (D_{IZ} = 62.1 ± 0.22 mm). For glass slides the adhered cells were reduced from 69.39 ± 0.21 % (for control) to 2.21 % (for the treatment with the salt **2**), results correlated with the viable cells count (0.53 ± 0.04 log CFU·cm^{-2}). The obtained results also indicate the presence of a bacterial biofilm population susceptible to all the tested compounds at a concentration for which the planktonic cells were completely destroyed.

The highest degree of destruction for *Pseudomonas fluorescens* biofilms has been observed for salts **2** and **6**, which has presented the highest inhibitory potential for this bacterial strain, in planktonic state. The activity of compound **6** led to the destruction of both biofilm bacterial cells and polymer matrix (Figure 3), the colonized biofilm area being only 2.51 ± 0.03 % for glass slides and 3.01 ± 0.18 % (Table 2). Similar results were obtained by treatment with compound **2**, for which only the matrix protein being visible and the number of cells was decreased below the detection limit (Figure 2b). The epifluorescence images obtained for those samples can be explained by Pereira and Vieira study (2001) [37], which have shown that the EPS of biofilms formed by the *Pseudomonas spp*. are composed mainly from proteins, which interact with fluorochrome. Therefore, it is not surprising to observe a polymeric layer fluorescing green, since AO binds to the DNA of the matrix.

The *B. subtilis* biofilms were more susceptible to chemical treatment, followed by *B. cereus* and *P. fluorescens* biofilms. The results support those found by Simões et al. (2009) [38], in which has been shown *P. fluorescens* biofilms on stainless surfaces were more resistant to the chemical treatment with the biocides glutaraldehyde and cetyl trimethyl ammonium bromide, compared with *B. cereus* biofilms. That can be explained by the interactions that can occur between molecules and the biocide in the biofilm matrix, which lead to the decrease of the interactions between biocide and the biofilm cells [39]. Furthermore, Simões et al., 2007 [40] described that the *B. cereus* is a low biofilm EPS producer compared to the *P. fluorescens* biofilms.

CONCLUSIONS

Present study has investigate for the first time in the literature the antibacterial efficacy on biofilms of symmetrical and unsymmetrical bis-pyridinium diquaternary salts derived from 1,2-bis-(4-pyridil) ethane. From the obtained results, it can be concluded that some symmetrical and unsymmetrical bis-pyridinium diquaternary salts can be successfully used as antibacterial agents for the biofilm destruction and removal.

It also been observed that when applying a maximum concentration of chemicals of 5 mg/mL, the antibacterial capability relies especially on compounds inhibitory potential and not necessarily to the antimicrobial activity expressed by minimal inhibitory concentration. Compared to results previously obtained in testing the antimicrobial potential of these diquaternary salts against planktonic cells, in the present study some differences of activity were observed. Thus, bacteria in mature biofilms are more resistant to those antimicrobials (biocides) than planktonic cells.

ACKNOWLEDGMENTS

This work was supported by a grant of the Romanian National Authority for Scientific Research, CNCS – UEFISCDI, project number PN-II-ID-PCE-2011-3-0226

REFERENCES

1. Cloete, T.E.: Resistance mechanisms of bacteria to antimicrobial compounds, *International Biodeterioration & Biodegradation*, **2003**, **51**, 277-282;
2. Shi, X., Zhu, X.: Biofilm formation and food safety in food industries, *Trends in Food Science & Technology,* **2009**, **20**, 407-413;
3. Donlan, R.M.: Biofilms: microbial life on surfaces, *Emerging Infectious Disease,* **2002**, **8** (9), 881-890;
4. Sauer, K., Rickard, A.H., Davies, D.G.: Biofilms and biocomplexity, *American Society for Microbiology*, **2007**, **2**, 347-353;
5. Midelet, G., Carpentier, B.: Impact of cleaning and disinfection agents on biofilm structure and on microbial transfer to a solid model food, *Journal of Applied Microbiology,* **2004**, **97**, 262–270
6. Bremer, P.J., Fillery, S., McQuillan, A.J.: Laboratory scale clean-in-place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms, *International Journal of Food Microbiology*, **2006**, **106**, 254–262;
7. Walker, J.T., Marsh, P.D.: Microbial biofilm formation in DUWS and their control using disinfectants, *Journal of Dentistry,* **2007**, **35**, 721–730;
8. Simoes, M., Simoes, L.C., Vieira, M.J.: A review of current and emergent biofilm control strategies, *Food Science and Technology*, **2010**, **43**, 573–583;
9. Block, S.S.: *Disinfection, Sterilization, and Preservation,* 5th edition, Lippincott, Williams & Wilkins, Philadelphia, U.S.A, **2001**;
10. Liu, S., Ma, J., Zhao, D.: Synthesis and characterization of cationic monoazo dyes incorporating quaternary ammonium salts, *Dyes and Pigments*, **2007**, **75**, 255-262;
11. Kourai, H., Yabuhara, T., Shirai, A., Maeda, T., Nagamune, H.: Syntheses and antimicrobial activities of a series of new bis-quaternary ammonium compounds, *European Journal of Medicinal Chemistry*, **2006**, **41**, 437–444;
12. Chanawanno, K., Chantrapromma, S., Anantapong, T., Kanjana-Opas, A., Fun, H.-K.: Synthesis, structure and in vitro antibacterial activities of new hybrid disinfectants quaternary ammonium compounds: Pyridinium and quinolinium stilbene benzenesulfonates, *European Journal of Medicinal Chemistry*, **2010**, **45**, 4199-4208;
13. Fujimoto, K., Morisaki, D., Yoshida, M., Namba, T., Hye-Sook, K., Wataya, Y., Kurai, H., Kakuta, H., Sasaki, K.: Antimalarial effect of bis-pyridinium salts, N,N'-hexamethylenbis(4-carbamoyl-1-alkylpyridinium bromide), *Bioorganic & Medicinal Chemistry Letters*, **2006**, **16** (10), 2758-2760;
14. Hu, D., Li, X., Sreenivasan, P. K., DeVizio W.: A randomized, double-blind clinical study to assess the antimicrobial effects of a cetylpyridinium chloride mouth rinse on dental plaque bacteria, *Clinical Therapeutics*, **2009**, **31** (11), 2540-2548;
15. Pateiro-Moure, M., Martinez-Carballo, E., Arias-Estevez, M., Simal-Gandara, J.: Determination of quaternary ammonium herbicides in soils - Comparison of digestion, shaking and microwave-assisted extractions, *Journal of Chromatography A*, **2008**, **1196–1197**, 110-116;
16. Pateiro-Moure, M., Nóvoa-Munoza, J.C., Arias-Estévez, M., López-Periago, E., Martínez-Carballo, E., Simal-Gándara, J.: Quaternary herbicides retention by the amendment of acid soils with a bentonite-based waste from wineries, *Journal of Hazardous Materials*, **2009**, **164**, 769-775;
17. Ng, C. L.L., Singhal, V., Widmer F., Wright, L.C., Sorell, T.C., Jolliffe, K.A.: Synthesis, antifungal and haemolytic activity of a series of bis(pyridinium)alkanes, *Bioorganic & Medicinal Chemistry*, **2007**, **15** (10), 3422-3429;
18. Perles, C.E., Matheus, A., Volpe, P.L.O.: The effect of quaternary ammonium surfactants on the inhibition of the aerobic metabolism of *Sacharomyces cerevisiae* – A calorimetric study. *Thermochimica Acta*, **2008**, **479** (1-2), 28-31;
19. Nagamune, H., Maeda, T., Ohkura, K., Yamamoto, K., Nakajima, M., Kourai, H.: Evaluation of the cytotoxic effects of bis-quaternary ammonium antimicrobial reagents on human cells. *Toxicology in Vitro*, **2000**, **14** (2), 139-147;
20. Furdui, B., Bahrim, G., Dinica, R., Druta, I., Demeunynck, M.: In vitro antimicrobial activity of new nitrogen heterocycles, *Roumanian Biotechnological Letters*, **2007**, **12** (1), 3073-3078;
21. Dinica, R., Furdui, B., Bahrim, G., Demeunynck, M.: Precursseurs de Nouveaux Heterocycles avec interet biologiques, *Revue Roumaine de Chimie*, **2008**, **53** (1), 21-24;

22. Furdui, B., Constantin, O., Dinică, R., Bahrim, G. Studies on antimicrobial activities of some new bis-pyridinium derivatives, *Annals of Dunărea de Jos University, Fascicle VI – Food Technology,* **2011**, **35** (2), 66-73;
23. Furdui, B., Dinică, R., Tăbăcaru, A., Pettinari, C.: Synthesis and physico-chemical properties of a novel series of aromatic electron acceptors based on N-heterocycles, *Tetrahedron,* **2012**, **68** (31), 6164-6168;
24. Furdui, B., Constantin, O., Tăbăcaru, A., Dinică, R.: New bis-pyridinium diquaternary salts with antimicrobial properties, *Revista Chimie*, **2012**, **63**, 667-671;
25. Furdui, B., Parfene, G., Ghinea, I. O., Dinică, R.M., Bahrim, G., Demeunynck, M.: Synthesis and in Vitro Antimicrobial Evaluation of New N-Heterocyclic Diquaternary Pyridinium Compounds, *Molecules*, **2014**, **19** (8), 11572-11585;
26. Jeong, D.K., Frank, J.F.: Growth of *Listeria monocytogenes* at 10 ºC in biofilms with microorganisms isolated from meat and dairy processing environments, *Journal of Food Protection*, **1994**, **57**, 576–586;
27. Constantin, O.: *Study on formation and removal of biofilms on food surfaces*, PhD thesis, Dunarea de Jos University of Galati, Romania, **2009**;
28. Sharma, M., Anand, S.K.: Biofilms evaluation as an essential component of HACCP for food/dairy processing industry - a case, *Food Control*, **2002**, **13** (6-7), 469-477;
29. Bagge-Ravn, D., Ng, Y., Hjelm, M., Christiansen, J.N., Johansen, C., Gram, L.: The microbial ecology of processing equipment in different fish industries – analysis of the microflora during processing and following cleaning and disinfection, *International Journal of Food Microbiology*, **2003**, **87** (3), 239-250;
30. Guobjornsdottir, B., Einarsson, H., Thorkelsson, G.: Microbial adhesion to processing lines for fish fillets and cooked shrimp: influence of stainless steel surface finish and presence of Gram negative bacteria on the attachment of *Listeria monocytogenes*, *Food Technology and Biotechnology*, **2005**, **43** (1), 55-61;
31. Araújo, P.A., Lemos, M., Mergulhão, F., Melo, L., Simões, M.: The influence of interfering substances on the antimicrobial activity of selected quaternary ammonium compounds, *International Journal of Food Science*, **2013**, 1-9;
32. Chapman, J.S.: Biocide resistance mechanisms, *International Biodeterioration & Biodegradation*, **200**3, **51** (2), 133–138;
33. Honraet, K., Nelis, H.J.: Use of the modified robbins device and fluorescent staining to screen plant extracts for the inhibition of *S. mutans* biofilm formation, *Journal of Microbiological Methods*, **2006**, **64** (2), 217-224;
34. Hadjiev, D., Dimitrov, D., Martinov, M., Sire, O.: Enhancement of the biofilm formation on polymeric supports by surface conditioning, *Enzyme and Microbial Technology*, **2007**, **40** (4), 840-848;
35. Pascaud, A., Amellal, S., Soulas, M.-L., Soulas, G.: A fluorescence-based assay for measuring the viable cell concentration of mixed microbial communities in soil, *Journal of Microbiological Methods,* **2009**, **76** (1), 81-87;
36. Nadrigny, F., Li, D., Kemnitz, K., Ropert, N., Koulakoff, A., Rudolph, S., Vitali, M., Giaume, C., Kirchhoff, F., Oheim, M.: Systematic Colocalization Errors between Acridine Orange and EGFP in Astrocyte Vesicular Organelles, *Biophysical Journal,* **2007**, **93**, 969–980;
37. Pereira, M.O., Vieira, M.J.: Effects of the interactions between glutaraldehyde and the polymeric matrix on the efficacy of the biocide against Pseudomonas fluorescens biofilms, *Biofouling*, **2001**, **17**, 93–101;
38. Simões, M., Simões, L.C., Vieira, M.J.: Species association increases biofilm resistance to chemical and mechanical treatments, *Water Res*sources, **2009**, **43** (1), 229–237;
39. Melo, L.F.: Biofilm physical structure, internal diffusivity and tortuosity, *Water Science & Technology*, **2005**, **52** (7), 77-84;
40. Simões, L.C., Simões, M., Oliveira, R., Vieira, M.J.: Potential of the adhesion of bacteria isolated from drinking water to materials, *Journal of basic microbiology*, **2007**, **47** (2), 174-183.