Article

eDNA inactivation and biofilm inhibition by the polymeric biocide polyhexamethylene guanidine hydrochloride (PHMG-Cl)

Olena V. Moshynets [1,*]**, Taras P. Baranovskyi** [2,6]**, Olga S. Iungin** [1,3]**, Nadiia P. Kysil** [4]**, Larysa O. Metelytsia** [5]**, Ianina Pokholenko** [1]**, Viktoria V Potochilova** [6]**, Geert Potters** [7,8]**, Kateryna L. Rudnieva** [6]**, Svitlana Y. Rymar** [1]**, Ivan V. Semenyuta** [5]**, Andrew J. Spiers** [9]**, Oksana P. Tarasyuk** [5]**, and Sergiy P. Rogalsky** [5,*]

1 Institute of Molecular Biology and Genetics of National Academy of Sciences of Ukraine, 150 Zabolotnoho Str., 03680 Kiev, Ukraine
2 Shupyk National Healthcare University of Ukraine, 9 Dorohozhytska Str., Kiev 04112, Ukraine
3 Kyiv National University of Technologies and Design, Nemyrovycha-Danchenka Street, 2, Kiev 01011, Ukraine.
4 National Children's Specialized Hospital "Okhmatdyt", 28/1 Chornovola Str., 01135 Kiev, Ukraine
5 V. P. Kukhar Institute of Bioorganic Chemistry and Petrochemistry of National Academy of Science of Ukraine, 50, Kharkivske Schose, 02160 Kiev, Ukraine
6 Kyiv Regional Clinical Hospital, 1 Baggovutivska Street, Kiev 04107, Ukraine
7 Antwerp Maritime Academy, Noordkasteel Oost 6, 2030 Antwerp, Belgium
8 Department of Bioscience Engineering, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium
9 School of Applied Sciences, Abertay University, Bell Street, Dundee DD1 1HG, United Kingdom.
* Correspondence: moshynets@gmail.com (OVM); sergey.rogalsky@gmail.com (SPR)

Abstract: The choice of effective biocides used for routine hospital practice should consider the role of disinfectants in the maintenance and development of local resistome and how they might affect antibiotic resistance gene transfer within the hospital microbial population. Currently, there is little understanding of how different biocides contribute to eDNA release that may contribute to gene transfer and subsequent environmental retention. Here we investigated how different biocides affected the release of eDNA from mature biofilms of two opportunistic model strains *Pseudomonas aeruginosa* ATCC 27853 (PA) and *Staphylococcus aureus* ATCC 25923 (SA) and contribute to the hospital resistome in the form of surface and water contaminants and dust particles. The effect of four groups of biocides including alcohols, hydrogen peroxide, quaternary ammonium compounds, and polymeric guanidines were evaluated using PA and SA biofilms. Most biocides, except for PHMG-Cl and 70% ethanol, caused substantial eDNA release and PHMG-Cl was found to block biofilm development when used at concentrations of 0.5% and 0.1%. This might be associated with the formation of DNA-PHMG-Cl complexes as PHMG-Cl is predicted to bind to AT base pairs by molecular docking assays. PHMG-Cl was found to bind high molecular DNA and plasmid DNA and continued to inactivate DNA on surfaces even after four weeks. PHMG-Cl also effectively inactivated biofilm-associated antibiotic resistance gene eDNA released by a pan-drug-resistant *Klebsiella* strain which demonstrates the potential of PHMG-Cl as a new surface-active agent to combat the spread of antibiotic resistance in hospital settings.

Keywords: eDNA, antibiotic resistance, biofilms, biocides, disinfectant, alcohols, hydrogen peroxide, quaternary ammonium compounds, PHMG-Cl

1. Introduction

The risk of multi-drug and pan-drug-resistant bacterial infections during or after a hospital stay increases worldwide. According to the WHO (2009), in general health care-associated infections in Europe lead to death in at least 2.7% of cases and accounted for 135,000 deaths per year. According to the National Expert Group on Infection Control (https://negic.ua) there are up to 1 million cases of hospital-acquired infections and 50,000

deaths occurring in Ukraine each year. Each drug-resistant bacterium can contribute antibiotic resistance genes (ARGs) to the hospital environment or resistome. This includes ARGs located within bacterial genomes (intracellular DNA) as well as cell-free ARGs in extracellular DNA (eARGs).

Microbial eDNA is ubiquitous and can be found everywhere where microbial life is present, as the polymer is involved in microbial survival, often contributes to the structure of biofilms as a matrix component [1-7] and acts to preserve a dynamic pool of genes via horizontal gene transfer (HGT) within the biofilm community. ARGs provide an important source for naturally occurring HGT [8-10] via transduction and conjugation. In contrast, the spread of eARGs by transformation and outer membrane vesicle-associated transport seems to be underestimated [11-12].

Over the last few years, investigations of eDNA distributions and its role in various resistomes has increased considerably. However, research mainly focuses on the wastewater environmental resistome, while the source and fate of eARGs in other environments including hospital-associated niches remain largely overlooked. For instance, while PubMed Central (https://www.ncbi.nlm.nih.gov/pmc) contains over 7,000 references for "eDNA + water" and "eDNA + hospital", less than 2,000 references can be found for "eDNA + antibiotic resistant gene" and "eDNA + antibiotic resistant gene + hospital". Nevertheless, an analysis of eARGs in aquatic sediments and water can potentially provide insights into the role of these eARGs as part of the environmental antibiotic resistome. eARGs persist more easily in sediments which makes them more available for transformation [13]. HGT of such clinically significant eARGs (containing for example extended spectrum beta-lactamases and carbapenem-resistant genes) can then transform environmentally harmless bacteria into pathogens that may then pose a threat to human health. A good demonstration of the importance of HGT in the evolution of human pathogens can be found in water-borne *Vibrio cholera* where the ability to take up eDNA (competence) is induced by chitin which is abundant in aquatic habitats [14].

In contrast, little is known about the impact of eDNA on the hospital resistome [13]. Hospital sewage systems are the source of persistent and recombinant *bla*KPC plasmids which cause hospital-acquired infections in patients [15]. Hospital wastewater sludge is a prime source of eARGs with a higher abundance than normal wastewater sludge, pharmaceutical factory waste, lake sediments or swine manure [16]. Hospital materials [17-19], furniture [20], and small items such as stethoscopes [21], mobile phones [22-25] and keyboards [26] are also known to be the source of hospital-associated infections, and even dust plays an important part in the deposition and transmission of ARGs [27] in the hospital environment.

Bacterial biofilm-forming communities producing eDNA containing ARGs as well as eARGs no longer associated with biofilms therefore pose a significant problem in modern healthcare environments. ARGs can spread throughout a hospital econiche by plasmid transfer, in the genomes of bacteriophages and as naked linear DNA [9-10]. There are only a limited number of studies in which the susceptibility of bacterial communities and eDNA to different biocides have been evaluated, and HGT may still be possible when cell-damaging biocides have been deployed but have failed to kill all bacteria in a biofilm [28-29]. The aim of our research was to evaluate the effect of different biocides on eDNA in biofilms produced by model hospital opportunistic pathogens, with a specific focus on the use of the polymeric biocide polyhexamethylene guanidine hydrochloride (PHMG-Cl) as a potential DNA-deactivating biocide.

2. Results

2.1. Effects of different biocides on eDNA release from PA and SA biofilms

We wondered how much eDNA could potentially be released from biofilm contamination likely to be found in hospital environments following treatment with biocides. To investigate this, we used dehydrated 5-day old *Pseudomonas aeruginosa* ATCC 27853 (PA) and *Staphylococcus aureus* ATCC 25923 (SA) biofilms to mimic dried biofilm material likely to found on a variety of surfaces [30]. We tested these with a number of biocides used frequently in the National Children's Specialized Hospital Okhmatdyt (Kiev, Ukraine), including hydrogen peroxide, ethanol, isopropanol-based Desmanol, 0.05% chlorhexidine and commercially available mixtures of quaternary ammonium compounds such as Maxisan, Arquades-plus and Sanikon, plus the polymeric cationic biocide polyhexamethylene guanidine hydrochloride (PHMG-Cl) which we previously used in the synthesis of a new thermally stable polymeric biocide polyhexamethylene guanidine 2-naphtalenesulfonate to modify polyamide 11 [31]. After 1 h exposure to biocide we isolated eDNA from the biofilms and measured eDNA yields compared to a water-only control treatment (Figure 1). This demonstrated that eDNA release from biofilms differed depending on both the biofilm and biocide.
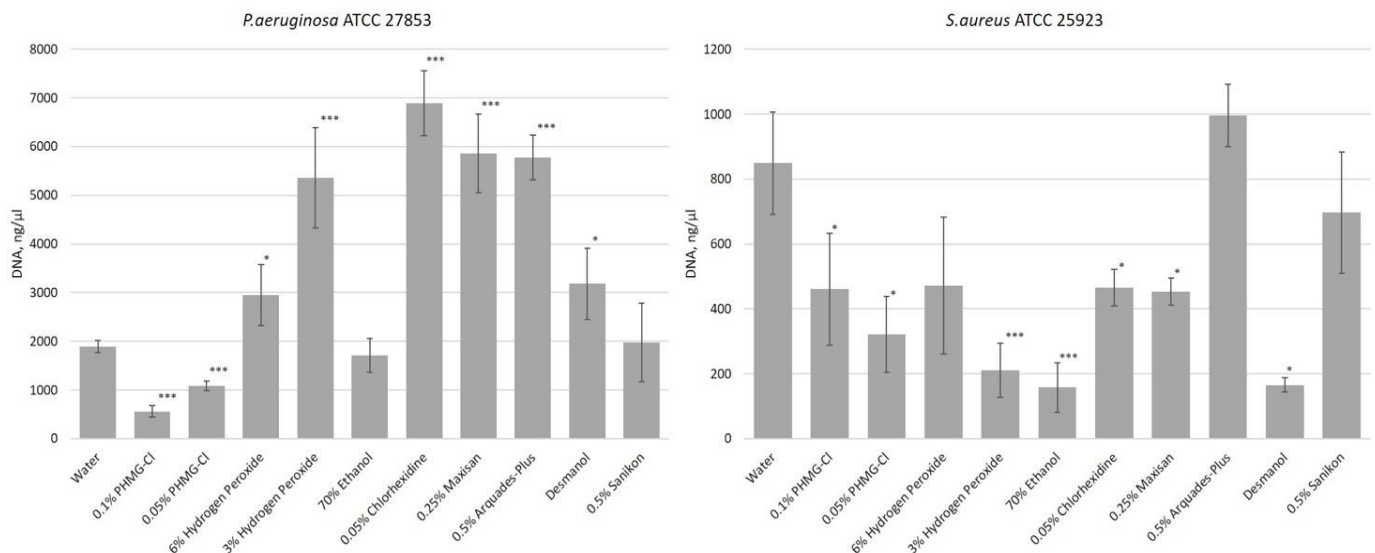

Figure 1. eDNA released from dehydrated *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 biofilms after treatment with biocides. Shown here are eDNA yields (ng/μL) recovered from 5-day-old dehydrated biofilms after 1 hr exposure to biocides (n=3). Statistical significance compared to water exposure (control) when $*p < 0.05$, $**p < 0.01$, $***p < 0.005$.

We also visualised the released eDNA by gel electrophoresis which confirmed that PHMG-Cl reduced eDNA yield from PA biofilms compared to the water-only control treatment. The eDNA was a mix of high molecular weight with substantial amounts remaining in the wells after electrophoresis and highly degraded DNA. In contrast, PHMG-Cl, alcohols, quaternary ammonium compounds and 3% hydrogen peroxide were able to reduce the eDNA yield from SA biofilms (Figure 2) in agreement with our direct measurement of eDNA concentrations.
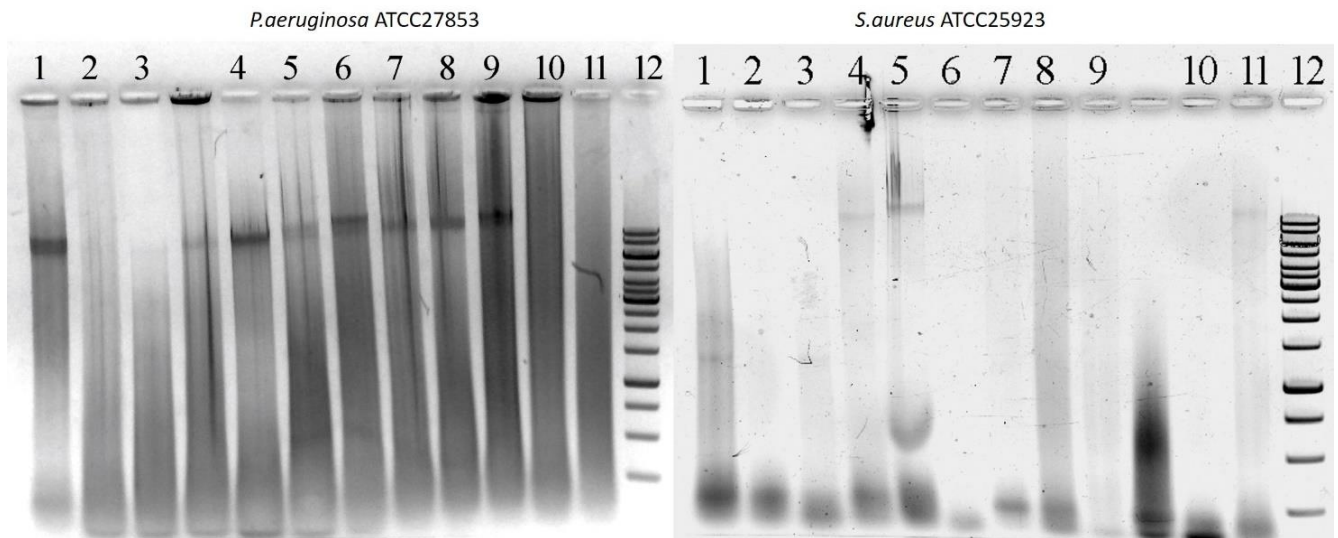
Figure 2. High molecular weight and degraded eDNA released from 5-day-old dehydrated *Pseudomonas aeruginosa* ATCC 27853 (PA) and *Staphylococcus aureus* ATCC 25923 (SA) biofilms after treatment with biocides. Shown here are 1.2% agarose-TA gels stained with EtBr to visualise eDNA released from PA (left) and SA (right) biofilms after 1 hr exposure to biocides. Treatments: (1) Water-only control; (2) 0.1% PHMG-Cl; (3) 0.05% PHMG-Cl; (4) 6% Hydrogen peroxide; (5) 3% Hydrogen peroxide; (6) 70% Ethanol; (7) 0.05% Chlorhexidine; (8) 0.25% Maxisan; (9) Arquades-plus; (10) Desmanol; and (11) Sanikon. A sample of GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, Lithuania) is shown in the rightmost lane of each gel.

Surprisingly, the eDNA released from dehydrated biofilms after exposure to biofilms was largely undamaged as assessed by PCR of 16s rDNA (Figure 3). However, PCR amplification of the PHMG-Cl samples failed, suggesting that, in addition to reducing eDNA yield, this biocide was complexing with the eDNA to prevent amplification of the target sequence.
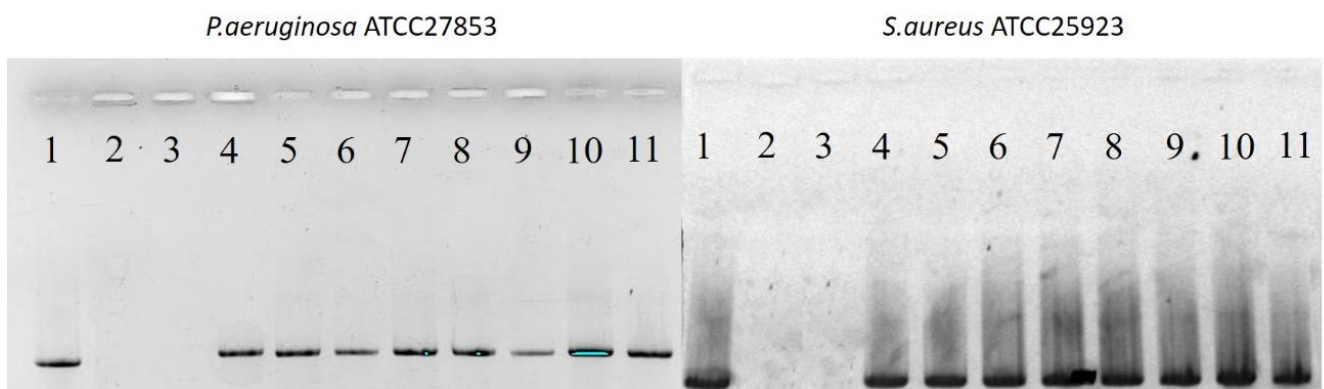

Figure 3. PHMG-Cl inhibits PCR amplification of 16S rDNA target sequences from eDNA released from 5-day-old dehydrated *Pseudomonas aeruginosa* ATCC 27853 (PA) and *Staphylococcus aureus* ATCC 25923 (SA) biofilms after treatment with biocides. Shown here are 1.2% agarose-TA gels stained with EtBr to visualise PCR products from PA (left) and SA (right) biofilms after 1 hr exposure to biocides. Treatments: (1) Water-only control; (2) 0.1% PHMG-Cl; (3) 0.05% PHMG-Cl; (4) 6% Hydrogen peroxide; (5) 3% Hydrogen peroxide; (6) 70% Ethanol; (7) 0.05% Chlorhexidine; (8) 0.25% Maxisan; (9) Arquades-plus; (10) Desmanol; and (11) Sanikon.

2.2. Anti-biofilm activity of PHMG-Cl against PA and SA biofilms.

Given the effectiveness of PHMG-Cl in inactivating eDNA in dehydrated biofilms, we tested PHMG-Cl to see if it could also supress or inhibit the growth of PA and SA biofilms in situ. We treated 24-hour old biofilms with different concentrations of PHMG-Cl and then determined metabolic activity using MTT (Figure 4). In this assay, the lowest concentration of PHMG-Cl which was not significantly different from the EtOH-treated

(dead) negative control biofilm but significantly lower than the water-treated (live) posi-tive control was biocidal. For the PA biofilm, the biocidal concentration was 0.1% and for the SA biofilm it was 0.5% PHMG-Cl. Increasing concentrations of PHMG-Cl also reduced eDNA yield from biofilms (Figure 5). At 0.5% PHMG-Cl, eDNA yield was reduced to less than a quarter of the water-treated positive PA control biofilm and to around one third of the SA control biofilm.
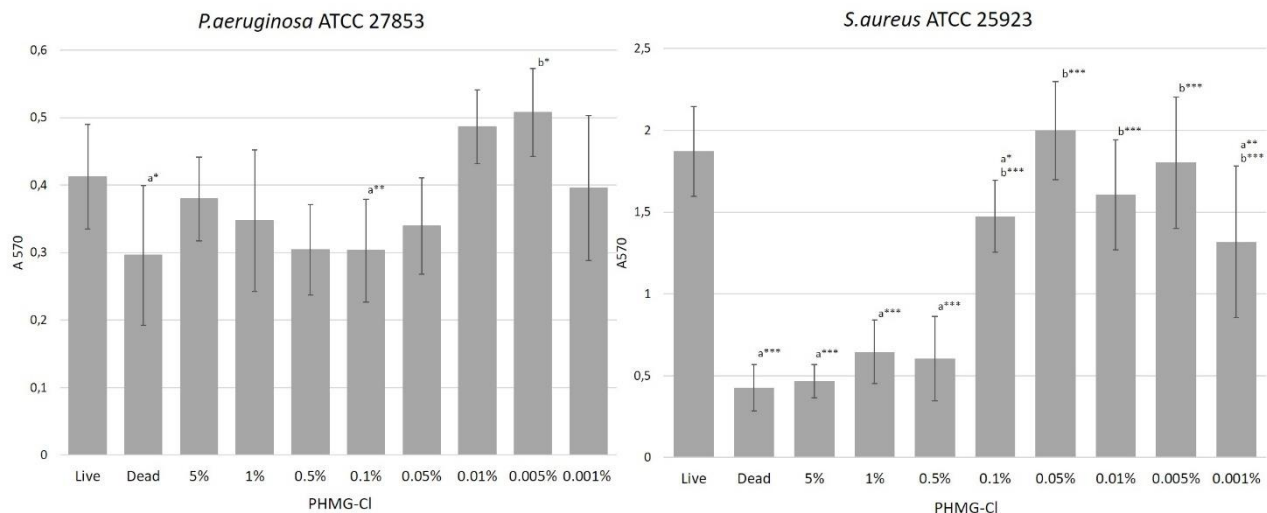

Figure 4. Metabolic assays show biocidal activity of PHMG-Cl on *Pseudomonas aeruginosa* ATCC 27853 (PA) and *Staphylo-coccus aureus* ATCC 25923 (SA) biofilms growing in situ. Shown here are the metabolic activity of 24-hour PA (left) and SA (right) biofilms after treatment with PHMG-Cl for 1 hour (n=8). Statistical differences are determined between treatments and the water-treated (live) positive controls and the EtOH-treated (dead) negative controls, *$p < 0.05$, ** $p < 0.01$, ***$p < 0.005$.
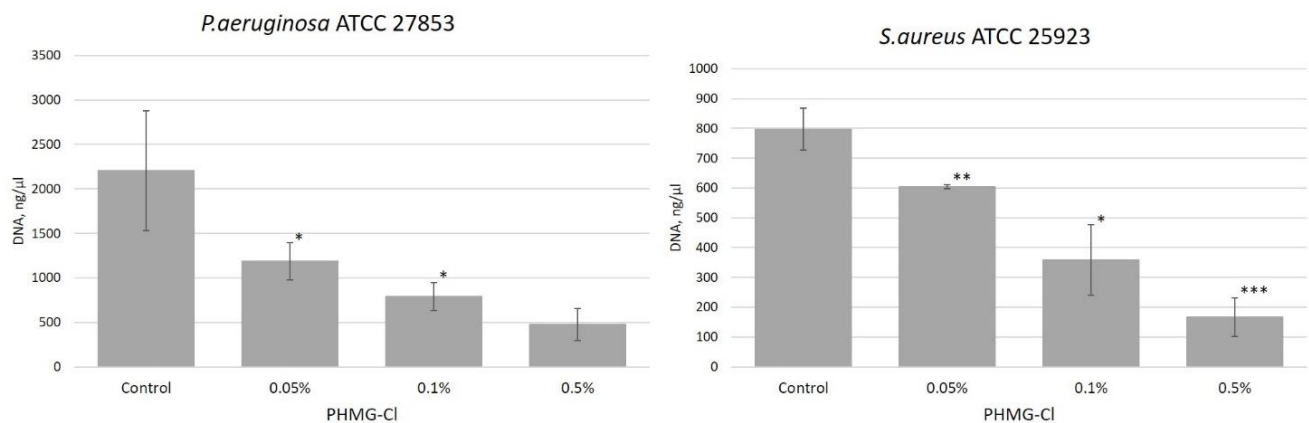

Figure 5. Increasing concentrations of PHMG-Cl reduce eDNA yield from *Pseudomonas aeruginosa* ATCC 27853 (PA) and *Staphylococcus aureus* ATCC 25923 (SA) biofilms. Shown here is the eDNA yield from 3-day old PA (left) and SA (right) biofilms after treatment with PHMG-Cl (n=3). Statistical differences are determined between treatments and the water-treated positive controls for each of the PA and SA biofilms, *$p < 0.05$, ** $p < 0.01$, ***$p < 0.005$.

The spatial distribution of eDNA in 0.5% PHMG-Cl treated PA and SA biofilms was also investigated by CLSM with PI used to visualise eDNA and dead cells and SYBR Green to visualise intracellular DNA in living cells with undamaged membranes [32] (Figure 6). As was expected, eDNA could be visualised in 3 and 5-day old PA and SA biofilms. How-ever, PHMG-Cl had different effects on biofilms that may reflect differences between PA and SA biofilm structure and cell wall organization. PHMG-Cl appeared to crumble bio-film structures and deformed cell shapes, and in older biofilms, PA cells showed substan-tial autolysis and SA biofilms becoming thicker with regions with damaged cells appear-ing less compact than in other areas. In some of the 5-day old PA biofilm samples which

were torn during sampling PHMG-Cl seemed to form large DNA filaments and the bio-film fragmented into smaller section pieces instead of the slimy and amorphous sections seen in younger treated biofilms. The opposite was observed when PHMG-Cl was applied onto older SA biofilms where most of the eDNA was removed and the cells heavily damaged.
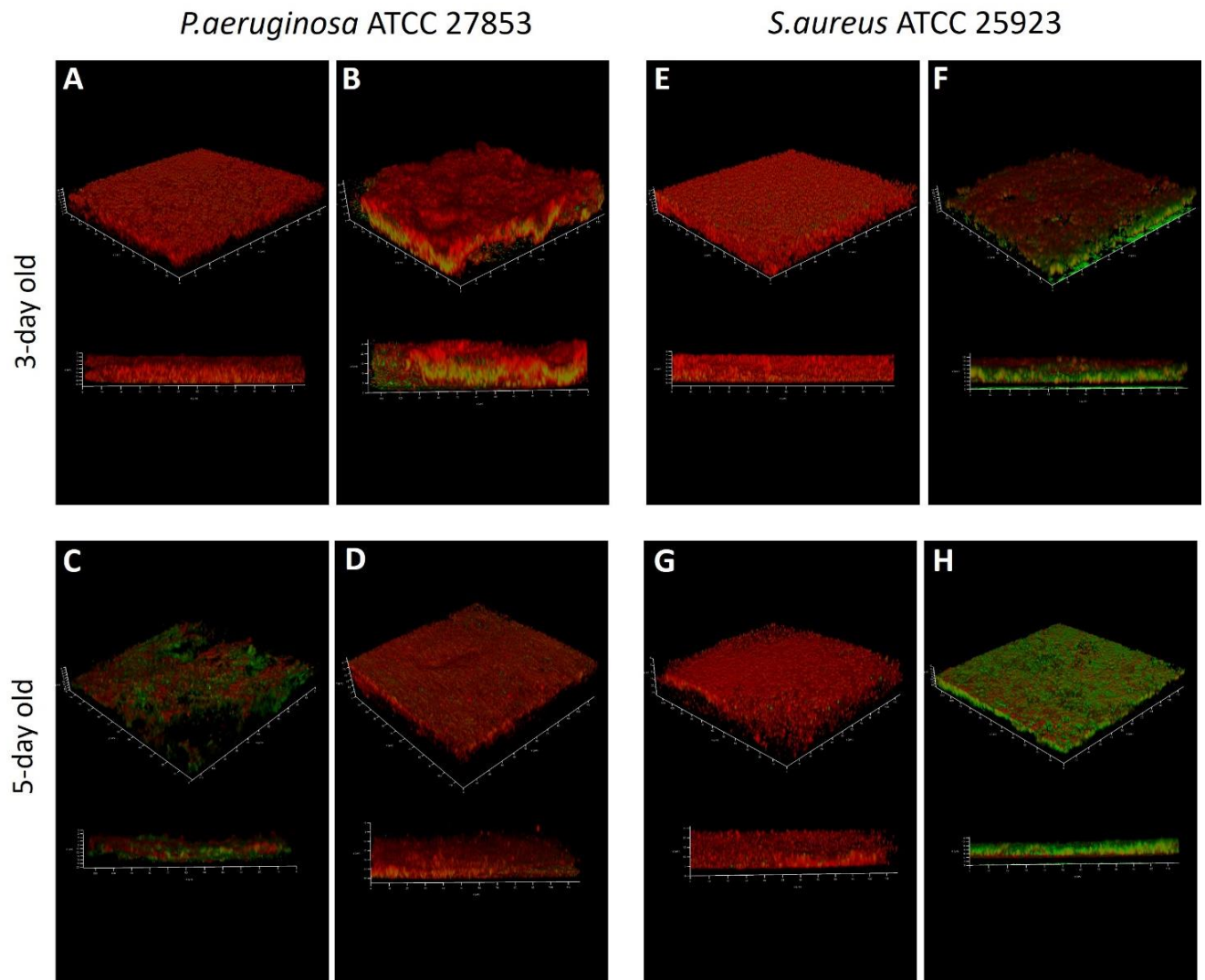

Figure 6. CLSM imaging of *Pseudomonas aeruginosa* ATCC 27853 (PA) and *Staphylococcus aureus* ATCC 25923 (SA) biofilms in situ after exposure to PHMG-Cl. Shown here are images of 3-day old (top row) and 5-day old (bottom row) PA and SA biofilms after treatment with water (A, C, E, G) or 0.5% PHMG-Cl (B, D, F, H). PI (red signal) and SYBR Green (green signal) were used to visualise eDNA, and SYBR Green to visualise the intracellular DNA of living cells with undamaged membranes.

2.3. Inactivation of DNA by PHMG-Cl

PHMG-Cl was the only biocide found to prevent the PCR amplification of 16S rDNA target sequences from eDNA samples (Figure 3). We therefore wondered how eDNA in-activation occurred, given that measurable quantities of eDNA could still be recovered from PHMG-Cl treated biofilms, and used an electrophoretic mobility assay to determine whether this biocide formed DNA-complexes which might prevent PCR amplification. PA eDNA treated with 0.001 – 0.01% PHMG-Cl did not affect the intrinsic charge of DNA molecules but concentrations of 0.05 – 0.5% PHMG-Cl effectively neutralised the DNA which was then lost during gel electrophoresis (Figure 7). However, it is not clear from this assay whether PHMG-Cl binding was co-operative or not, but we assume that with 0.05% PHMG-Cl was sufficiently saturating to bind all surface sites. Dialysis or deprotein-

ization of the eDNA-PHMG-Cl reaction mixture had little impact on electrophoresis suggesting that these complexes were stable and not dependent on the presence of DNA-binding proteins that may have co-isolated with the eDNA (the presence of eDNA was confirmed by NanoDrop spectrophotometry demonstrating that DNA was not lost during processing). PHMG-Cl also complexed with covalently closed circular (CCC) and linear forms of pC1-L plasmid DNA suggesting that this process may not be too sensitive to DNA structure. Treatment of CCC and linear pC1-L also prevented PCR amplification of the LIF gene target sequence (Figure 8). We also included a mixture of PHMG-Cl-treated pC1-L DNA with untreated pC1-L DNA in this PCR assay which demonstrated that once complexed with DNA, PHMG-Cl could not bind new DNA (suggesting a low dissociation constant for the DNA – PHGM-Cl complex) or directly interact with the Taq polymerase to prevent PCR amplification. These assays confirmed that treatment with 0.05% PHMG-Cl effectively inactivated a range of DNA, including both eDNA and CCC and linear plasmid DNA.
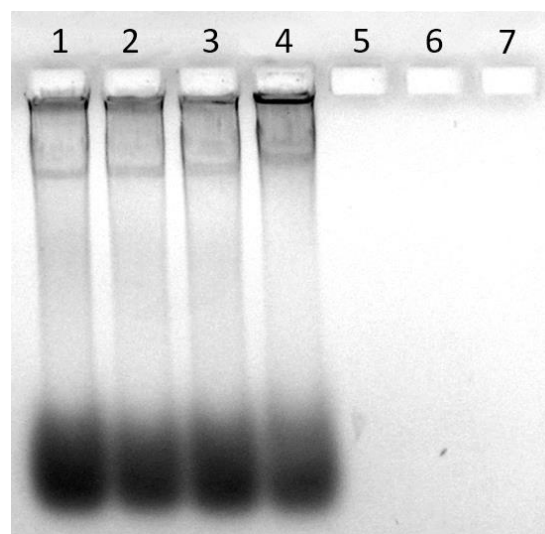

Figure 7. PHMG-1 complexes with eDNA and neutralises the intrinsic charge of DNA molecules. Shown here is a 1.2% agarose-TA gel stained with EtBr to visualise *Pseudomonas aeruginosa* ATCC 27853 (PA) eDNA that had treated with PHMG-Cl. Treatments: (1) Water-only control; (2) 0.001%; (3) 0.005%; (4) 0.01%; (5) 0.05%; (6) 0.1%; and (7) 0.5% PHMG-Cl.
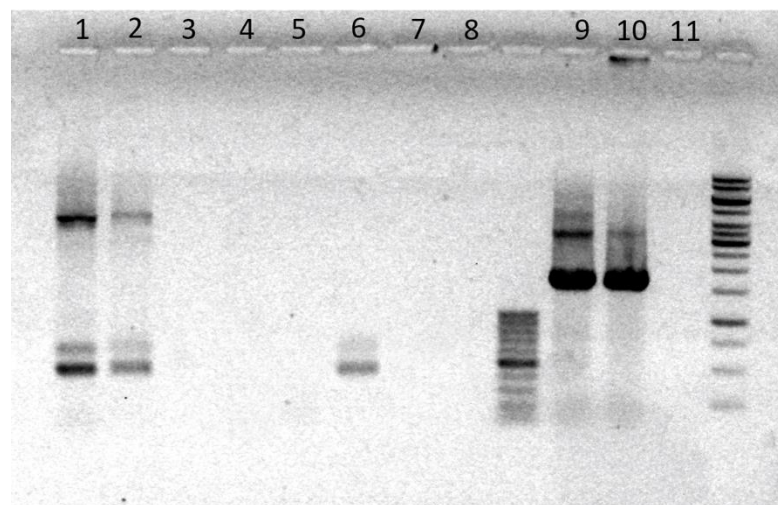

Figure 8. PHMG-Cl – DNA complexes prevent PCR amplification of target sequences in pC1-L plasmid DNA and eDNA. Shown here is a 1.2% agarose-TA gel stained with EtBr to visualise PCR products after amplification with pL1-C LIF gene or 16S-specific primers. CCC pC1-L DNA treatments: (1) Water-only control; (2) Control sample plus an equal volume 0.01% PHMG-Cl sample; (3) Control sample plus an equal volume of 0.05% PHMG-Cl sample; (4) 0.01% PHMG-Cl; (5)

0.05% PHMG-Cl; Linear pC1-L DNA treatments: (6) Water-only control; (7) 0.01% PHMG-Cl; (8) 0.05% PHMG-Cl; eDNA treatments: (9) Water-only control; (10) 0.01% PHMG-Cl; (11) 0.05% PHMG-Cl. A sample of GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, Lithuania) is shown in the rightmost lane of the gel.

2.4. Inactivation of eARG by PHMG-Cl

In order to confirm the direct inactivation of a model ARG in eDNA by PHMG-Cl, we isolated eDNA from the biofilm of a pan-drug-resistant *Klebsiella pneumoniae* hospital isolate (UHI KP 1633). We confirmed that UHI KP 1633 was a pan-drug resistant strain and was resistant to carbapenems and colistin and used Real-time PCR to amplify the carbapenemase (KPC) resistance gene from eDNA treated with PHMG-Cl (Figure 9). Real-time PCR was only able to detect KPC in the eDNA water-only control and samples treated with 0.001 – 0.005% PHMG-Cl but not in samples treated with higher concentrations of PHMG-Cl, in agreement with our earlier tests of PA eDNA and pC1-L plasmid DNA.
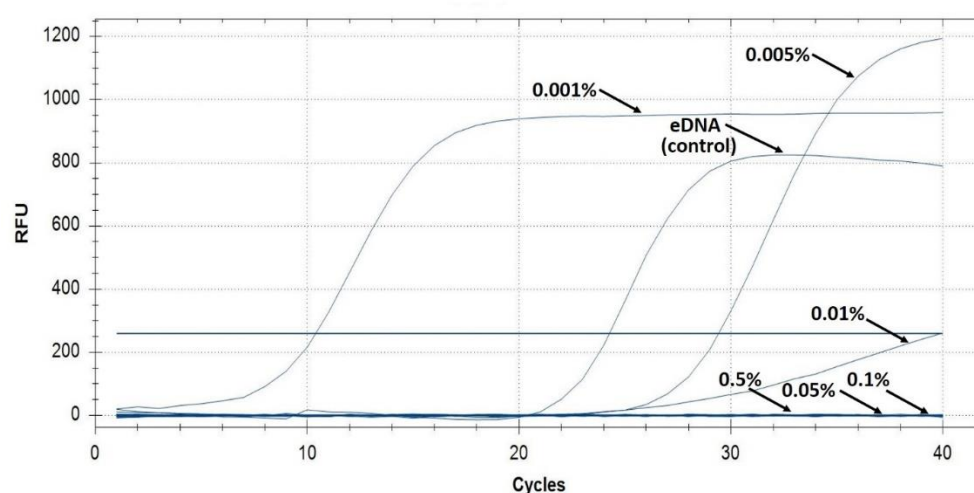

Figure 9. Real-time PCR of a model eARG is inhibited by PHMG-Cl treatment of eDNA. Shown here are the real-time PCR amplification curves of the KPC antibiotic resistance gene from UHI KP 1633 eDNA samples after PHMG-Cl treatment. The water-only control and 0.001 – 0.5% PHMG-Cl treatments are indicated. Note that RFU is not normalized as quantitative results were not needed and these curves are only used to indicate positive PCR amplification. The blue horizontal line is the fluorescent threshold and Cq (not marked) is where the PCR amplification curves cross the threshold.

2.5. DNA-inactivating activity of PHMG-Cl adsorbed onto plastic surfaces

PHMG-Cl is known to have strong absorptive properties, and slow-release from surfaces can provide long-lasting antibacterial defence [33, 34]. We wondered whether the inactivation of DNA we had observed with PHMG-Cl added to dehydrated and growing biofilms and eDNA also occurred when eDNA was allowed to dry onto plastic surfaces treated with PHMG-Cl. 96-well polystyrene plates were treated with PHMG-Cl and PA eDNA added before storage at room temperature for up to 28 days before recovery of DNA and gel electrophoresis (Figure 10). Although the total amount of DNA recovered decreased with age, sufficient DNA remained after 28 days to demonstrate that 0.5 – 1.0% PHMG-Cl treated surfaces could still inactivate eDNA. 0.05 – 0.1% PHMG-Cl could also protect surfaces and inactivate eDNA for 4 – 8 days.
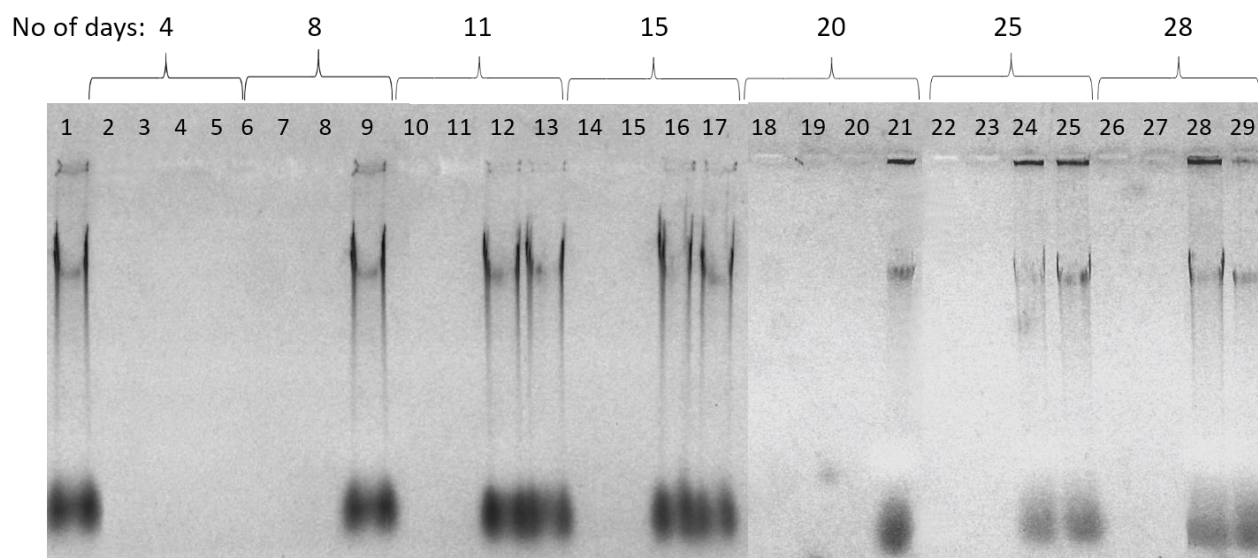
Figure 10. PHMG-Cl surface-treated plastic inactivates eDNA. Shown here is a 1.2% agarose-TA gel stained with EtBr to visualise eDNA recovered from plastic treated with PHMG-Cl for up to 28 days. PHMG-Cl treatments (1%, 0.5%, 0.1% and 0.05% are grouped according to the length of exposure (days). Treatments: (1) Clean-surface control; (2, 6, 10, 14, 18, 22 & 26) 1% PHMG-Cl; (3, 7, 11, 15, 19, 23 & 27) 0.5% PHMG-Cl; (4, 8, 12, 16, 20, 24 & 28) 0.1% PHMG-Cl; (5, 9, 13, 17, 21, 25 & 29) 0.05% PHMG-Cl.

2.6. DNA docking assay

We undertook molecular docking using the DNA oligonucleotide 1DNE to investigate the mechanism which might allow the formation of DNA – PHMG-Cl complexes. 1DNE has been previously used in several studies to investigate the interaction of various ligands with DNA [35-37]. As we were interested in identifying PHMG-Cl interactions in both AT and CG-rich regions, we modelled PHMG-Cl as a dimer following the approach used in studying DNA – PHMB dimer interactions [38]. We obtained a 1DNE DNA – PHMG dimer complex that was stabilized by six hydrogen bonds (1.97 – 3.20 Å) and five electrostatic bonds (1.97 – 3.20 Å), with five additional longer-range electrostatic interactions (4.42 – 5.13Å) occurring via the PHMG amino groups (Figure 11). The DA7, DT6, DT8, DC9, DT18, DA19, and DC21 nucleotide bases in 1DNE playing a key role in stabilizing the complex. Our docking results showed the formation of the DNA – PHGM dimer complex with estimated binding energies of −7.8 kcal/mol predominantly at the AT base pair region of 1DNE.

We validated our approach by redocking the DNA intercalator Netropsin after ligand randomization [39-40] to 1DNA with an estimated binding energy of −7.7 kcal/mol after ligand randomization with a RMSD value for all atoms of 0.2 Å. The similarity between the two estimated binding energies suggests that we selected the correct docking strategy for the PHMG dimer.
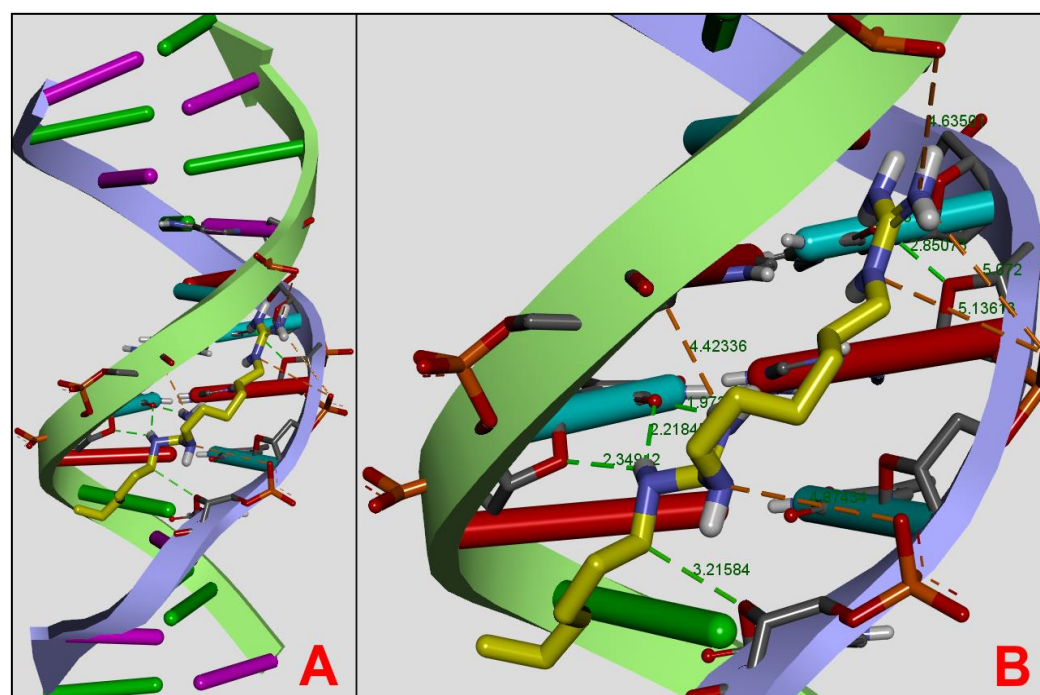
Figure 11. PHMG can dock to DNA in the minor grove and is stabilised by hydrogen and electro-static bonds. Shown here are two views of a DNA – PHMG dimer complex produced by molecular docking studies using the model DNA oligonucleotide 1DNE. (A) A general view of the PHMG dimer (yellow) docking in the minor grove of DNA. (B) Detailed docking position including the hydrogen (green) and electrostatic (orange) bonds with bond lengths (Ấ) indicated.

3. Discussion

3.1. Impact of biocides on eDNA

eDNA is an extracellular matrix polymer found in the biofilms of many important opportunists such as *Acinetobacter baumannii*, *Enterococcus faecalis*, *Helicobacter pylori*, *P. aeruginosa*, *Staphylococcus* spp., etc. [1-7]. It is an important structural element and is stabilized by functional amyloids and cross-linked with polysaccharides [7, 41-42]. eDNA is also a source of nutrients and provides antibacterial, antibiotic-resistant, and regulatory activities [43-45] and has good adhesive properties in *P. aeruginosa* and *Bacillus cereus* biofilms in particular [46-47]. It has been traditionally considered as an inevitable attribute of microbial contamination and in recent years more attention has been paid to eDNA as a source of ARGs contributing to hospital resistomes. eDNA has finally been recognized as a contaminant which should be controlled to reduce HGT intensity and resistomes [6, 48-49]. Like other resistance factors, eDNA in biofilms can be enhanced by sublethal doses of antimicrobials [50-52] and biocides [8, 53] which result in hospital contamination [16]. It is therefore likely that the use of some biocides in hospitals may be counter-productive in controlling biofilms, eDNA, HGT and resistomes.

A better understanding of how biocides affect the release of eDNA from biofilm and contribute to surface and water contaminants and dust particles is required. In this research, the effect of four groups of biocides on eDNA release from dehydrated biofilms produced by two model hospital pathogens, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923. Interestingly, almost all the biocides tested, except for PHMG-Cl and 70% ethanol, cause substantial eDNA release. The amount of eDNA released from these biofilms might be explained structural differences between biofilms. PA biofilms are very rich in eDNA which plays not only a structural role but also an important physiological role providing phenotypic resistance [41, 43-44].

3.2. PHMG blocks biofilm development

Polymeric cationic biocides incorporating guanidinium cations in polymer backbone such as polyhexamethylene biguanide hydrochloride (PHMB-Cl) and polyhexamethylene guanidine hydrochloride (PHMG-Cl) are often considered as cost-effective alternatives to common inorganic antimicrobial agents as they have high efficacy in killing antibiotic-resistant bacteria and fungi and low cytotoxicity [54-58]. Cationic polymers are widely used as effective disinfectants in cooling systems, swimming pools and hospitals, in personal hygiene products, as well as in the food industry [54, 56, 58-59]. Although bacterial biofilm-forming communities pose a significant problem in modern healthcare environments, there are a limited number of studies investigating susceptibility to existing biocides and polymeric agents like PHMG-Cl. PHMG-Cl has been tested on *S. aureus* in liquid cultures (planktonic state) as well as in biofilms [60] and demonstrated an anti-biofilm activity that was significantly higher compared to common antimicrobial agents including benzalkonium chloride, cetrimide, chlorhexidine and hexadecylpyridinium chloride [60]. PHMG-Cl also demonstrated bactericidal advantages over chlorhexidine digluconate against ESKAPE bacteria [61].

In order to evaluate the biocidal effectiveness of PHMG-Cl we measured the total metabolic activity of PA and SA biofilms that confirmed that it is an effective antibacterial compound. Our testing again demonstrated a difference between PA and SA biofilms, with SA biofilms more resistant to PHMG-Cl with a minimal effective anti-biofilm concentration of 0.5% which was 5x lower than that needed for PA biofilms. However, the mechanism behind the effectivity of PHMG-Cl against biofilms or biofilm-resident cells is not yet understood for either gram-negative or gram-positive opportunistic pathogens. We note with interest that PHMG-Cl can effectively release eDNA from our dehydrated biofilms that we used as a model surface contamination as well as from fully hydrated and growing biofilms, suggesting that this biocide might work well with both developing biofilms as well as biofilm residue.

3.3. PHMG binds to DNA and inhibits its functionality

Our molecular docking analysis of a PHMG dimer with a model DNA oligonucleotide suggest that the high antibiofilm activity of PHMG-Cl may be associated with specific DNA binding in high-AT regions. DNA binding is also the likely explanation of why PHMG-Cl treated eDNA and pC1-L plasmid did not enter agarose gels during electrophoresis and the failure of PCR to amplify target sequences. PHMG-Cl was able to inactivate genes including ARGs in when used at 0.01 – 0.05% and plastic surfaces treated with PHMG-Cl continued to show DNA deactivation for up to 28 days after application. For high molecular weight and degraded eDNA, there was a direct correlation between eDNA and PHMG-Cl concentrations needed to inactivate DNA, and 0.01% PHMG-Cl also inactivated CCC and linear plasmid DNA. A similar DNA-inactivation effect has been observed before for polyamide films containing 7 – 10% PHMG-NS [30] where only the PHMG at the surface of the film is actively in contact with biofilm eDNA. There are some reports that polyhexamethylene biguanide (PHMB) might bind selectively and condenses bacterial chromosomes [38, 62].

Selective binding and condensation of intracellular DNA might be one more mechanism inhibiting bacterial growth [62]. To investigate this effect and the spatial distribution of both eDNA and intracellular DNA in PA and SA biofilms, we applied the classical live/dead staining routinely used in microbiology [32] with PI used to visualize eDNA and the DNA of cells with damaged membranes and the membrane permeable SYBR Green to stain all DNA. As both dyes bind in the minor groove of DNA, fluorescence resonance energy transfer (FRET) results in eDNA and dead cells fluorescing only with a red emission [63]. We observed this phenomenon with mature and physically damaged PA biofilms when treated with PHMG-Cl but not with younger or untreated PA biofilms that showed a normal PI signal. PHMG-Cl treatment also resulted in a substantial increase in thickness and robustness of the biofilm material due to strong eDNA-incorporated fibril formation and we suggest that these fibrils might be compact eDNA-PHMG-Cl complexes. The distribution of eDNA in mature and damaged SA biofilms was like that seen

in PA biofilms and confirms the role of eDNA role in the biofilm matrix for this bacterium. However, PHMG-Cl treatment had a different effect on SA biofilms where matrix protein denaturation and the degradation of the gram-positive staphylococcal cell wall preventing PI from penetrating damaged cells. This may favor the intercalation of SYBR Green into intracellular DNA while exposed eDNA – PHMG complexes could no longer bind PI. Finally, we note that the effects of PHMG were not confined to eDNA only. Like others [64] we noticed microscopic evidence of cell wall damage after PHMG-Cl treatment (Figure 12) confirming the generally accepted model of PHMG-Cl activity in which cell walls and membranes are destroyed.
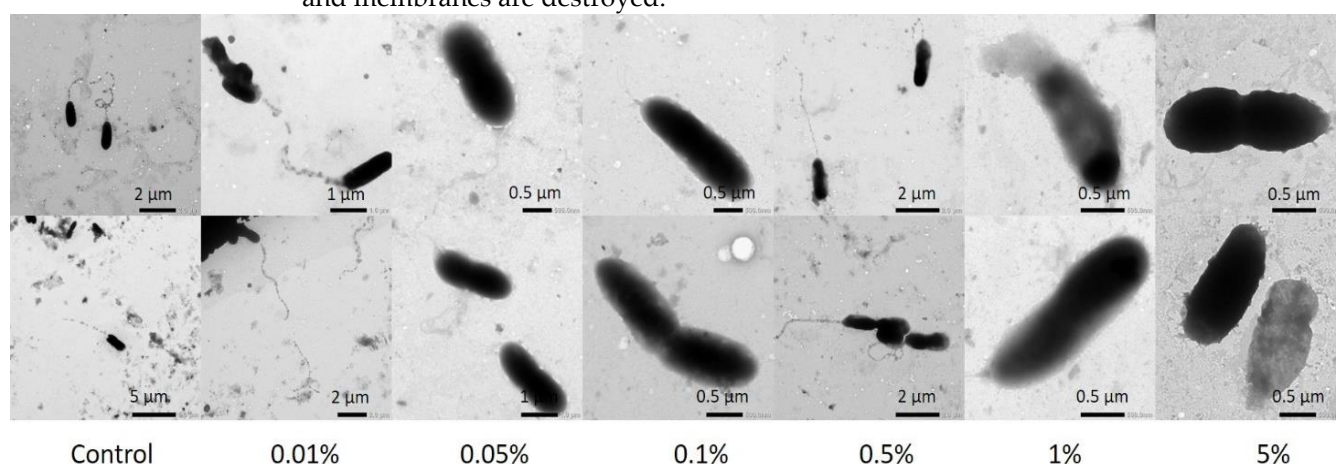

Figure 12. PHMG-Cl effects cell shape causing damage to the cell wall and membrane. Shown here are TEM images of PA cells subjected to different PHMG-Cl concentrations.

4. Materials and Methods

4.1. Synthesis of polymeric biocide polyhexamethylene guanidine hydrochloride (PHMG-Cl)

A mixture of guanidine hydrochloride (20 g, 0.21 mol) and hexamethylenediamine (23.1 g, 0.2 mol) was placed into a round-bottomed flask (500 mL) equipped with a mechanical stirrer (Scheme 1). This mixture was heated to 100 ℃ and stirred for 4 h, then at 130-140 ℃ for 4 h, and finally at 180 ℃ for 4 h to obtain a highly viscous melt of PHMG-Cl. A vitreous solid was obtained after cooling to room temperature which was then dissolved in water (200 mL), filtered, and precipitated by the addition of a saturated aqueous solution of sodium chloride (100 mL). PHMG-Cl was isolated by decantation of the water solution and dried at 140 ℃ for 24 h before powdering in a porcelain mortar. The final product had a melting point of 134-136 ℃ and an intrinsic viscosity of 0.09 dl/g in 0.1 N NaCl at 25 ℃. NMR spectra of PHMG-Cl were recorded in DMSO-d6 on a Varian Gemini-2000 (400 MHz) spectrometer with the following results: 1H NMR (400 MHz, DMSO-D6): $\delta = 1.32$ (m, 4H, CH_2), 1.47 (m, 4H, $N-CH_2CH_2$), 3.16 (m, 4H, $N-CH_2$), 7.1-8.1 (br s, 4H, NH); 13C NMR: 25.3 (CH_2), 28.1 ($N-CH_2CH_2$), 40.5 ($N-CH_2$), 156.6 (NH).
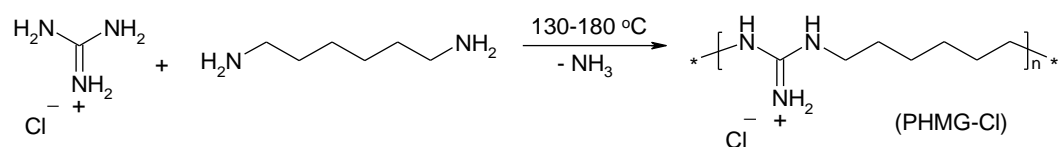
Scheme 1. Synthesis of PHMG-Cl

2.2. Biocides used in the assays.

Seven biocides were used in addition to PHMG-Cl and include 35% hydrogen peroxide (Khimpostachannia, Ukraine), 96% ethanol (UkrSpirt Trade, Ukraine), 0.05% Chlorhexidine (Zdorovie, Ukraine), Maxisan (Interdez, Ukraine) which contains not less than 50% of a mix of four quaternary ammonium compounds, Arquades-plus (O. L. KAR,

Ukraine) containing 10% dimethyldialkylammonium chloride, 5% didecyldime-thylammonium chloride, 2.5% tetrasodium salt, Desmanol (Schülke & Mayr GmbH, Germany) containing 75% isopropanol, and Sanikon (Interdez, Ukraine) which contains not less than 5.5% of a mix of four quaternary ammonium compounds.

2.3. Microorganisms and culturing conditions.

Two model hospital opportunistic pathogens, *Pseudomonas aeruginosa* ATCC 27853 (PA) and *Staphylococcus aureus* ATCC 25923 (SA), were used to study the effects of biocides on established biofilms and eDNA yield. The Ukrainian hospital isolate *Klebsiella pneumoniae* 1633 (KP) was recovered from a patient and identified as a pan-drug-resistant (PDR) strain using antibiotic disc diffusion assays and EUCAST 2021 v.11.0 breakpoints. An AST-N332 card was used to confirm the PDR strain phenotype with the VITEK 2 Advanced Expert System. Antimicrobial susceptibility testing of CMS was performed by SensiTest Colistin (Liofilchem, Italy) broth microdilution assay and results interpreted according to EUCAST breakpoints. Bacterial strains were cultured aerobically at 37°C with shaking in Luria-Bertani (LB) medium to provide inoculum [65]. For the biofilm inactivation assay, LB cultures were diluted to 10% and 200 µL aliquots transferred to the wells of a 96-well plate which was incubated at 37 °C for 24 hours before the biocide inactivation assay. For CLSM and the eDNA yield assay, 10% dilutions were used to inoculate 30 mL glass microcosms containing 2 – 5 mL LB that were incubated statically at 37 °C for 3 – 5 days to produce biofilms. Biofilms were air-dried at room temperature for 14 days before eDNA yield analysis. For CLSM biofilm samples were placed onto a microscopic glass.

2.4. Plasmids used in the study.

The pC1-L plasmid containing the human LIF gene [66] was used to investigate the effect of biocides on covalently closed circular and linear plasmid DNA.

2.5. Biofilm metabolic assay.

Aliquots of an aqueous PHMG-Cl solution were added the wells of a 96-well plate with 24-hour PA and SA biofilm cultures at final concentrations of 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, and 0.001%, with eight replicates per treatment. These were incubated for 1 h at room temperature. A negative control sample was produced using 50% ethanol and a positive (live) control produced by adding sterile distilled water. MTT solution (Sigma-Aldrich, UK) was then added to each well to a final concentration of 0.05% and incubated at 37 °C for 3 h. Biofilms were removed from each well and placed in 1.5 mL plastic tubes that were then centrifuged at 13,000 g for 15 minutes in an Eppendorf 5424 Microcentrifuge (Eppendorf, Germany). The supernatant was discarded, and the pellet dissolved in 500 µL DMSO. Metabolic activity was evaluated using absorbance measurements at 570 nm in BioTek ELx800 microplate spectrophotometer (BioTek Instruments, USA). Net biofilm metabolic activity was calculated by subtracting the negative control values.

2.6. eDNA yield assay.

Biocides were tested at different concentrations to determine eDNA yields. 1 mL of 0.5%, 0.1% and 0.05% PHMG-Cl, 6% and 3% hydrogen peroxide, 70% ethanol, 0.05% chlorhexidine, 0.25% Maxisan, 0.5% Arquadez-plus, 0.5% Desmanol, and 0.5% Sanikon were added to dehydrated PA and SA biofilms at room temperature for 1 h with three replicates per treatment. eDNA was extracted from the liquid phase of each sample. Proteins were first removed with an equal volume of chloroform and DNA precipitated with 70% ethanol and 0.3M sodium acetate pH 8.0. DNA was then dissolved in TE buffer. Nucleic acid concentration was measured by NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, USA). DNA samples were then visualized by 1.2% agarose-TA gel electrophoresis and EtBr staining. GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, Lithuania) was used as a size marker.

2.7. Biofilm eDNA isolation.

PA and KP biofilms were grown in 5 mL LB static microcosm at 37 °C for 5 days. Biofilms were then disintegrated by agitation for 5 minutes. 5 mL of biofilm suspension was centrifuged at 13,000 g for 15 minutes using a microcentrifuge and the supernatant recovered. eDNA in the supernatant was precipitated with 70% ethanol and 0.3M sodium acetate pH 8.0 and then dissolved in TE buffer. Nucleic acid concentration was measured by NanoDrop.

2.8. PHMG-Cl effects on eDNA and pC1-L plasmid DNA.

eDNA isolated from PA 27853 and pC1-L DNA were treated with different PHMG-Cl concentrations. 7 µL (6.7 µg) of eDNA and 7 µL (2.8 µg) of liner or covalently closed circular (CCC) pC1-L DNA were mixed with 3 µL of DNA loading buffer (ThermoFisher Scientific, USA) and final concentrations of 0.01% and 0.05% PHMG-Cl. Samples were placed in D-0530 dialysis tubing (Sigma, USA) and the samples dialysed together to reduce the PHMG-Cl concentration by 10-7x in TE buffer. The DNA samples were visualized by gel electrophoresis.

2.9. PCR of 16S rDNA, LIF and KPC sequences.

PCR was used to determine the effect of biocide-treatment on DNA by amplifying 16S rDNA, LIF and *Klebsiella pneumoniae* Carbapenemase (KPC) target sequences. 25 µL PCR reaction mixtures (Taq PCR Kit, New England Biolabs) with 27F (5'-AGAGTTT-GATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') primers were used to amplify 16S sequences from eDNA samples [67]. Forward (5'-ATGAAGGTCTTGGCGGCAGG-3') and reverse (5'-ACCTCCTGCTAGAAGGCCTG-3') primers were used to amplify the LIF gene from pC1-L samples. PCR conditions involved an initial stage of 5 min at 95°C, followed by 30 cycles at 95°C for 30s, 57°C for 30s and 72°C for 30s, and a final stage at 72°C for 5min. Purified genomic DNA was used as a positive control and the PCR products visualized by gel electrophoresis. KPC sequences were amplified using the AmpliSense MDR KPC/OXA-48-FL reagent kit (AmpliSense, Russia) and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA).

2.10. Effect of PHMG-Cl treated plastic surface on eDNA.

10 µL of 1%, 0.5%, 0.1%, and 0.05% PHMG-Cl were placed into the wells of 96-well polystyrene plates with three replicates per treatment. The plates were allowed to dry at room temperature and left for 4, 8, 11, 15, 20, 25 and 28 days under the same conditions. 10 µL of 880 ng/µL eDNA from PA biofilms was added to the wells and incubated at room temperature for 1 h before nucleic acid concentrations were measured by NanoDrop and visualized by gel electrophoresis.

2.11. Molecular docking assay.

Molecular docking was performed using the model DNA dodecamer (CGCGA-TATCGCG) used to study DNA-binding compounds (RCSB Protein Data Bank 1DNE) [68]. Chains A and B were used for docking and the water molecules and ligand were removed from the crystal structure using Accelrys DS 4.0 [69]. AutoDock Tools (ADT) 1.5.6 [70] was used to make the PHMG dimer ligand and add polar hydrogens to the DNA. The noBondOrder method was used to renumber all atoms including the new hydrogen atoms and the Gasteiger method was used calculate charges. ChemAxon Marvin Sketch 5.3.735 [71] was used to optimize the PHMG dimer structure. The energy minimization and optimization of the PHMG dimer ligand was performed by MOPAC2016 [72] using the Auto Optimization Tool (MMFF94s force field) [73]. Partial charges and torsion angles of the ligand were changed using ADT. The DNA structure and PHMG dimer were used for molecular docking by AutoDock Vina 1.1.2 [74]. A grid box of 30x30x30 points was used with a spacing of 1 Å. The analysis and visualization of interactions were performed by Accelrys DS.

2.12. Confocal laser scanning microscopy (CLSM).

Biofilm samples were stained with 5 µL of a mixture of 100× SYBR Green (Invitrogen) and 1 mM propidium iodide (PI) (Sigma). CLSM analysis was undertaken using a Leica

TCS SPE Confocal system with coded DMi8 inverted microscope (Leica, Germany) and Leica Application Suite X (LAS X) Version 3.4.1. Images were acquired using excitation at 488 nm and emission collected at 490-580 nm for SYBR Green, and excitation at 532 nm and emission collected at 537-670 for PI.

2.13. Transmission electron microscopy (TEM).

An overnight PA culture was diluted to 20% and PHMG-Cl added to a final concentration of 5%, 1%, 0.5%, 0.1%, 0.05%, and 0.01%. Samples were incubated at room temperature for 20 minutes before a 10 μL aliquot was placed onto a formvar covered grid and dried at room temperature. 10 μL of 1% uranyl acetate (Sigma-Aldrich, UK) was dropped onto each grid and dried with filter paper. TEM was performed with JEM-1400 transmission electron microscope (JEOL, Japan).

2.14. Statistical analysis

Replicate data were processed using the statistical software package OriginPro 7.0 and MS Excel for Windows. All results are presented as the mean ± standard deviation. A value of $p < 0.05$ was considered statistically significant.

5. Conclusions

Our investigations clearly demonstrate that biocides commonly used in hospitals can have a significant impact on the release of eDNA from bacterial biofilms. Some biocides, in particular PHMG-C1, were found to block biofilm development and was able to complex with DNA in a manner predicted by molecular docking assays. PHMG-C1 binding to DNA altered the electrophoretic mobility of both high molecular weight and plasmid DNA and prevented the amplification of a target ARG gene from eDNA isolated from a Klebsiella biofilm. PHMG-C1 was also found inactivate DNA when used to treat plastic surfaces up to 28 days after application. These findings demonstrate the potential of PHG-C1 as a surface-active agent that can be used in hospital settings to help reduce the spread of antibiotic resistance through inactivating eDNA commonly found in bacterial biofilms and by limiting the development of biofilms themselves.

References

1. Allesen-Holm, M.; Barken, K.B.; Yang, L.; Klausen, M.; Webb, J.S.; Kjelleberg, S.; Mollin, S.; Givskov. M.; Tolker-Nielsen, T. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Mol. Microbiol.* **2006**; 59, 1114-1128. DOI: 10.1111/j.1365-2958.2005.05008.x.

2. Thomas, V.C.; Hiromasa, Y.; Harms, N.; Thurlow, L.; Tomich, J.; hancock, L.E. A fratricidal mechanism is responsible for eDNA release and contributes to biofilm development of *Enterococcus faecalis*. *Mol. Microbiol.* **2009**, 72, 1022-1036. DOI:10.1111/j.1365-2958.2009.06703.x

3. Vilain, S.; Pretorius, J.M.; Theron, J.; Brozer, V.S. DNA as an adhesion: bacillus cereus requires extracellular DNA to form bio-film. *Appl. Environ. Microbiol.* **2009**, 75, 2861-2868. DOI: 10.1128/AEM.01317-08.

4. Christner, M.; Heinze, C.; Busch, M.; Franke, G.; Hentschke, M.; Buhring, S.B.; Buttner, H.; Kotasinska, M.; Wischnewski, V.; Kroll, G.; Buck, F.; Molin, S.; Otto, M.; Rohde, H. SarA negatively regylates *Staphylococcus epidermidis* biofilm formation by modulation exprtession of 1 MDa extracellular matrix binding protein and autolysis-dependent release of eDNA. *Mol. Microbiol.* **2012**, 86, 394-410.

5. Sahu, P.K.; Iyer, P.S.; Oak, A.M.; Pardesi, K.R.; Chopade, B.A. Characterization of eDNA from the clinical strain *Acinetobacter baumannii* AIIMS7 and its role in biofilm formation. *The Scientific World Journal* **2012**, 973436. DOI:10.1100/2012/973436

6. Grande, R.; Di Marcantonio, M.C.; Robuffo, I.; Pompilio, A.; Celia, C.; Di Marzio, L.; Paolino, D.; Codagnone, M.; Muraro, R.; Stoodley, P.; hall-Stoodley, L.; Mincione, G. Helicobacter pylori ATCC 43629/NCTC 11639 outer membrane vesicles (OMVs) from biofilm and planktonic phase associatied with extracellular DNA (eDNA). *Front. Microbiol.* **2015**, 6, 1569. https://doi.org/10.3389/fmicb.2015.01369

7. Schwartz, K.; Ganesan, M.; Payne, D.E.; Solomon, M.J.; Boles, B.R. Extracellular DNA facilitates the formation of functional amyloids in *Staphylococcus aureus* biofilms. *Mol. Microbiol.* **2016**, 99, 123-134. DOI: 10.1111/mmi.13219

8. Itzek, A.; Zheng, L.; Chen, Z.; Merritt, J.; Kreth, J. Hydrogen peroxide-dependent DNA release and transfer of antibiotic re-sistance genes in *Streptococcus gordonii*. *J. Bacteriol.* **2011**, 193, 6912-6922.

9. Hannan, S.; Ready, D.; Jasni, A.S.; Rogers, M.; Pratten, J.; Roberts, A.P. Transfer of antibiotic resistance by transformation with eDNA within oral biofilms. *FEMS Immunol. Med. Microbiol.* **2010**, 59, 345-349. DOI: 10.1111/j.1574-695X.2010.00661.x.

10. Nolan, L.M.; Turnbull, L.; Katrib, M.; Osvath, S.R.; Losa, D.; Lazenby, J.J.; Whitchurch, C.B. *Pseudomonas aeruginosa* is capable of natural transformation in biofilms. *Microbiology* **2020**, 166, 995-1003. DOI: 10.1099/mic.0.000956

11. Thomas, C.M.; Nielsen, K.M. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nat. Rev. Genet.* **2005**, 3, 711–721.

12. Domingues, S.; Nielsen, K.M. Membrane vesicles and horizontal gene transfer in prokaryotes. *Curr. Opin. Microbiol.* **2017**, 38, 16-21.

13. Sivalingam, P.; Poté, J.; Prabakar, K. Extracellular DNA (eDNA): neglected and potential sources of antibiotic resistant genes (ARGs) in the aquatic environments. *Pathogens* **2020**, 9, 874.

14. Meibom, K. L.; Blokesch, M.; Dolganov, N.A.; Wu, C.Y.; Schoolnik, G.K. Chitin induces natural competence in Vibrio cholerae. *Science* **2005**, 310, 1824–1827.

15. Mathers, A.J.; Crook, D.; Vaughan, A.; Barry, K.E.; Vegesana, K.; Stroesser, N.; Parikh, H.I.; Sebra, R.; Kotay, S.; Walker, A.S.; Sheppard, A.E. *Klebsiella quasipneumoniae* provides a window into carbapenemase gene transfer, plasmid rearrangements, and patient interactions with the hospital environment. *Antimicrob. Agents Chemother.* **2019**, 63, e02513-18. DOI: 10.1128/AAC.02513-18.

16. Dong, P.; Wang, H.; Fang, T.; Wang, Y.; Ye, Q. Assessment of extracellular antibiotic resistance genes (eARGs) in typical envi-ronmental samples and the transforming ability of eARG, *Environment International* **2019**, 125, 90-96.

17. Wiener-Well, Y.; Galuty, M.; Rudensky, B.; Schlesinger, Y.; Attias, D.; Yinnon, A.M. Nursing and physician attire as possible source of nosocomial infections. *Am. J. Infect. Control.* **2011**, 39, 555–559. DOI: 10.1016/j.ajic.2010.12.016.

18. Lopez, P.-J.; Ron, O.; Parthasarathy, P.; Soothill, J.; Spitz, L. Bacterial counts from hospital doctors' ties are higher than those from shirts. *Am. J. Infect. Control* **2009**, 37, 79–80. DOI: 10.1016/j.ajic.2008.09.018.

19. Treakle, A.M.; Thom, K.A.; Furuno, J.P.; Strauss, S.M.; Harris, A.D.; Perencevich, E.N. Bacterial contamination of health care workers' white coats. *Am. J. Infect. Control* **2009**, 37, 101–105, DOI: 10.1016/j.ajic.2008.03.009.

20. Lax, S.; Smith, D.; Sangwan, N.; Handley, K.; Larsen, P.; Richardson, M.; Taylor, S.; Landon, E.; Alverdy, J.; Siegel, J.; Stephens, B.; Knight, R.; Gilbert, J.A. Colonization and succession of hospital-associated microbiota. *Sci. Transl. Med.* **2017**, 9, eaah6500. DOI: 10.1126/scitranslmed.aah6500

21. Marinella, M.A.; Pierson, C.; Chenoweth, C.; The stethoscope. A potential source of nosocomial infection? *Arch. Intern. Med.* **1997**, 157, 786–790.

22. Brady, R.R.W.; Wasson, A.; Stirling, I.; McAllister, C.; Damani, N.N. Is your phone bugged? The incidence of bacteria known to cause nosocomial infection on healthcare workers' mobile phones. *J. Hosp. Infect.* **2006**, 62, 123–125, DOI: 10.1016/j.jhin.2005.05.005.

23. Brady, R.R.W.; Verran, J.; Damani, N.N.; Gibb, A.P. Review of mobile communication devices as potential reservoirs of noso-comial pathogens. *J. Hosp. Infect.* **2009**, 71, 295–300. DOI: 10.1016/j.jhin.2008.12.009.

24. Ulger, F.; Esen, S.; Dilek, A.; Yanik, K.; Gunaydin, M.; Leblebicioglu, H. Are we aware how contaminated our mobile phones with nosocomial pathogens? *Ann. Clin. Microbiol. Antimicrob.* **2009**, 8. https://doi.org/10.1186/1476-0711-8-7.

25. Akinyemi, K.O.; Atapu, A.D.; Adetona, O.O.; Coker, A.O. The potential role of mobile phones in the spread of bacterial infections. *J. Infect. Dev. Ctries.* **2009**, 3, 628–632.

26. Bures, S.;   Fishbain, J.T.; Uyehara, C.F.; Parker, J.M.; Berg, B.W. Computer keyboards and faucet handles as reservoirs of nosocomial pathogens in the intensive care unit. *Am. J. Infect. Control.* **2000**, 28, 465–471. DOI: 10.1067/mic.2000.107267

27. Li, X.; Wu, Z.; Dang, C.; Zhang, M.; Zhao, B.; Cheng, Z.; Chen, L.; Zhong, Z.; Yuhui., Y., Xia Y. A metagenomic-based method to study hospital air dust resistome. *Chemical Engineering Journal* **2021**, 406, 126854

28. Pearce, H.; Messager, S.; Maillard, J.Y. Effect of biocides commonly used in the hospital environment on the transfer of antibiotic-resistance genes in *Staphylococcus aureus*. *J. Hosp. Infect.* **1999**, 43, 101–107. DOI: 10.1053/jhin.1999.0250

29. Jutkina, J.; Marathe N.P.; Flach C.F.; Larsson D.G.J. Antibiotics and common antibacterial biocides stimulate horizontal transfer of resistance at low concentrations. *Sci Total Environ.* **2018**, 616-617:172-178. DOI: 10.1016/j.scitotenv.2017.10.312.

30. Moshynets, O.; Chernii, S.; Chernii, V.; Losytskyy, M.Y.; Karakhim, S.; Czerwieniec, R.; Pekhnyo, V.; Yarmoluk, S.; Kovalska, V. Fluorescent β-ketoenole AmyGreen dye for visualization of amyloid components of bacterial biofilms. *Methods Appl. Fluoresc.* **2020**, 8, 035006. DOI: 10.1088/2050-6120/ab90e0

31. Moshynets, O.; Bardeau, J.-F.; Tarasyuk, O.; Makhno, S.; Cherniavska, T.; Dzhuzha, O.; Potters, G.; Rogalsky, S. Anti-biofilm activity of polyamide 11 modified with thermally stable polymeric biocide polyhexamethylene guanidine 2-naphtalenesulfonate. *Int. J. Mol. Sci.* **2019**, 20, 348. DOI: 10.3390/ijms20020348.

32. Feng, J.; Yee, R.; Zhang, S.; Tian, L.; Shi, W.; Zhang, W.-H.; Zhang, Y. A Rapid growth-independent antibiotic resistance detection test by SYBR Green/Propidium Iodide viability assay. *Front. Med.* **2018**, 5, 127.

33. Chen, S.; Li, C.; Hou, T.; Cai, Y.; Liang, L.; Chen, L.; Li, M. Polyhexamethylene guanidine functionalized chitosan nanofiber membrane with superior adsorption and antibacterial performances, *React. Funct. Polym.* **2019**, 145, 104379. DOI: 10.1016/j.reactfunctpolym.2019.104379

34. Wu, F.; Xu, J.; Yan, R.; Hu, B.; Li, G.; Jin, M.; Jiang, X.; Li, J.; Tang, P.; Zhu, J.; Yan, S. In vitro and in vivo evaluation of antibacterial activity of polyhexamethylene guanidine (PHMG)-loaded TiO₂ nanotubes.   *Biomed. Mater.* **2020**, 15, 045016   DOI: 10.1088/1748-605X/ab7e79

35. Ricci, G. C.; Paulo, N. Docking studies on DNA-ligand interactions: building and application of a protocol to identify the binding mode. *J. Chem. Inf. Model.* **2009**, 49, 1925-35. DOI: 10.1021/ci9001537.

36. Guo, Y.; Yue, Q.; Gao, B. Molecular docking study investigating the possible mode of binding of C.I. Acid Red 73 with DNA. *Int. J. Biol. Macromol.* **2011**, 49, 55-61. DOI: 10.1016/j.ijbiomac.2011.03.009

37. Khosravifar, F.; Dehghan, G.; Bidoki, S.K.; Mahdavi, M. DNA-binding activity and cytotoxic and cell-cycle arrest properties of some new coumarin derivatives: a multispectral and computational investigation. *Luminescence* **2020**, 35, 98-106. DOI: 10.1002/bio.370.

38. Sowlati-Hashjin, S.; Carbone, P.; Karttunen, M. Insights into the polyhexamethylene biguanide (PHMB) mechanism of action on bacterial membrane and DNA: a molecular dynamics study. *J. Phys. Chem. B.* **2020**, 124, 4487-4497.

39. Kopka, M.L.; Yoon, C.; Goodcell, D.; Pjura, P.; Dickerson, R.E. Binding of an antitumor drug to DNA netropsin and C-G-C-G-A-A-T-T-BrC-G-C-G. *J. Mol. Biol.* **1985**, 183, 553-563.

40. Ricci, C.G.; Netz, P.A. Docking studies on DNA-ligand interactions: building and application of a protocol. *J. Chem. Inf. Model.* **2009**, 49, 1925–1935.   DOI: 10.1021/ci9001537

41. Jennings, L.K.; Storek, K.M.; Ledvina, H.E.; Coulon, C.; Marmont, L.S.; Sadovskaya, I.; Secor, P.R.;Tseng, B.S.; Scian, M,; Filloux, A.; Wozniak, D.J.; Howell, P.L.; Parsek, M.R.. Biofilm matrix composition and localization. *PNAS* **2015**, 112, 11353-11358.

42. Wang, S.; Liu, X.; Liu, H.; Zhang, L.; Guo, Y.; Yu, S.; Wozniak, D.J., Ma LZ. The exopolysaccharide Psl-eDNA interaction enables the formation of a biofilm skeleton in *Pseudomonas aeruginosa*. *Environ. Microbiol. Rep.* **2015**, 7, 330-340.

43. Mulcahy, H.; Charron-Mazenod, L.; Lewenza, S. Extracellular DNA chelates cations and induces antibiotic resistance in Pseudomonas aeruginosa biofilms. *PLoS Pathog.* **2008**, 4, e1000213. DOI: 10.1371/journal.ppat.1000213

44. Wilton, M.; Charron-Mazenod, L.; Moore, R.; Lewenza, S. Extracellular DNA acidifies biofilms and induces aminoglycosaside resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **2016**, 60, 544-553.

45. Sharma, D.; Misba, L.; Khan, A.U. Antibiotics versus biofilms: an emerging battleground in microbial communities. *Antimicrob. Resist. Infect. Control.* **2019,** 8, 76. DOI; 10.1186/s13756-019-0533-3.

46. Vilain, S.; Pretorius, J.M.; Theron, J.; Brözel, V.S. DNA as an adhesion: Bacillus cereus requires extracellular DNA to form biofilms. *Appl. Environ. Microbiol.* **2009**, 75, 2861-2868. DOI: 10.1128/AEM.01317-08.

47. van Schaik, E.J.; Giltner, C.L.; Audette, G.F.; Keizer, D.W.; Bautista, D.L.; Slupsky, C.M.; Sykes, B.D.; Irvin, R.T. DNA binding: a novel function of *Pseudomonas aeruginosa* Type IV Pili. *J. Bacteriol.* **2005**, 187, 1455–1464. DOI: 10.1128/JB.187.4.1455-1464.2005

48. Sanderson, H.; Fricker, C.; Brown, R.S.; Majury, A.; Liss, S.N. Antibiotic resistance genes as an emerging environmental contaminant. *Environmental Reviews* **2016**, 24, 205-218.

49. Slipko, K.; Reif, D.; Wögerbauer, M.; Hufnagl, O.P.; Krampe, J.; Kreuzinger, N. Removal of extracellular free DNA and antibiotic resistance genes from water and wastewater by membranes ranging from microfiltration to reverse osmosis. *Water Research* **2019**, 164, 114916. DOI: 10.1016/j.watres.2019.114916

50. Turnbull, L.; Toyofuku, M.; Hynen, A.L.; Kurosawa, M.; Pessi, G.; Petty, N.K.; Osvath, S.R.; Ca´rcamo-Oyarce, G.; Gloag, E.S.; Shimoni, R.; Omasits, U.; Ito, S.; Yap, X.; Monahan, L.G.; Cavaliere, R.; Ahrens, C.H.; Charles, I.G.; Nomura, N.; Eberl, L.; Whitchurch, C. Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nat. Commun.* **2016**, 7, 11220.   DOI: 10.1038/ncomms11220

51. Yu, W.; Hallinen, K.M.; Wood, K.B. Interplay between antibiotic efficacy and druginduced lysis underlies enhanced biofilm formation at subinhibitory drug concentrations. *Antimicrob. Agents Chemother.* **2018**, 62, e01603-17. DOI: 10.1128/AAC.01603-17

52. Tahrioui, A.; Duchesne, R.; Bouffartigues, E.; Rodrigues, S.; Maillot, O.; Tortuel, D.; Hardouin, J.; Taupin, L.; Groleau, M.C.; Dufour, A.; Deziel, E.; Brenner-Weiss, G.; Feuilloley, M.; Orange, N.; lesouhaitier, O.; Cornelis, P.; Chevalier, S. Extracellular DNA release, quorum sensing, and PrrF1/F2 small RNAs are key players in *Pseudomonas aeruginosa* tobramycin-enhanced biofilm formation. *npj Biofilms Microbiomes* **2019**, 5, 15.

53. Liu, S.-S.; Qu, H.-M.; Yang, D.; Hu, H.; Liu, W.-L.; Qiu, Z.-G.; Hou, A.-M.; Guo, J.; Li, J.-W.; Shen, Z.-Q.; Jin, M. Chlorine disinfection increases both intracellular and extracellular antibiotic resistance genes in a full-scale wastewater treatment plant. *Water Research* **2018**, 136, 131-136. DOI: 10.1016/j.watres.2018.02.036.

54. Moore, K.; Gray, D. Using PHMB antimicrobial to prevent wound infection. *Wounds UK* **2007**, 3, 96-102.

55. Kaehn, K. Polyhexadine: a safe and highly effective biocide. *Skin Pharmacol. Physiol.* **2010**, 23, 7-16. DOI: 10.1159/000318237.

56. Oulè, M.K.; Azinwi, R.; Bernier, A.M.; Kablan, T.; Maupertuis, A.M.; Mauler, S.; Koffi-Nevry, R.; Dembèlè, K.; Forbes, L.; Diop, L. Polyhexamethylene guanidine hydrochloride-based disinfectant: a novel tool to fight meticillin-resistant *Staphylococcus aureus* and nosocomial infections. *J. Med. Microbiol.* **2008**, 57, 1523–1528. DOI: 10.1099/jmm.0.2008/003350-0.

57. Zhou, Z.; Wei, D.; Guan, Y.; Zheng, A.; Zhong, J-J. Extensive in vitro activity of guanidine hydrochloride polymer analogs against antibiotics-resistant clinically isolated strains. *Mater. Sci. Eng.* **2011**, 31, 1836–1843. DOI: 10.1016/j.msec.2011.08.015.

58. Choi, H.; Kim, K.-J.; Lee, D.J. Antifungal activity of the cationic antimicrobial polymer-polyhexamethylene guanidine hydrochloride and its mode of action. *Fungal Biol.* **2017**, 121, 53-60. DOI: 10.1016/j.funbio.2016.09.001

59. Zhang, Y.; Jiang, J.; Chen, Y. Synthesis and antimicrobial activity of polymeric guanidine and biguanide salts. *Polymer* **1999**, 40, 6189-6198. DOI: 10.1016/S0032-3861(98)00828-3

60. Caballero Gómez, N.; Abriouel, H.; Grande, J.; Pulido, R.P.; Gálvez, A. Combined treatments of enterocin AS-48 with biocides to improve the inactivation of methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* planktonic and sessile cells. *Int. J. Food Microbiol.* **2013**, 163, 96–100. DOI: 10.1016/j.ijfoodmicro.2013.02.018

61. Zhou, Z.; Wei, D.; Lu, Y. Polyhexamethylene guanidine hydrochloride shows bactericidal advantages against ESKAPE bacteria. *Biotechnol. Appl. Biochem.* **2015**, 62, 268-274. DOI: 10.1002/bab.1255

62. Chindera, K.; Mahato, M.; Sharma, A.K.; Horsley H.; Kloc-Muniak, K.; Kamaruzzaman, N.F.; Kumar, S.; McFarlane, A.; Stach, J.; Bentin, T.; Good, L. The antimicrobial polymer PHMB enters cells and selectively condenses bacterial chromosomes. *Sci. Rep.* **2016**, 6, 23121.

63. Habtewold, T.; Duchateau, L.; Christophides, G.K. Flow cytometry analysis of the microbiota associated with the midguts of vector mosquitoes. *Parasites Vectors* **2016**, 9, 167

64. Zhou, Z.; Wei, D.; Guan, Y.; Zheng, A.; Zhong, J-J. Damage of Escherichia coli membrane by bactericidal agent polyhexamethylene guanidine hydrochloride: micrographic evidences. *J. Appl. Microbiol.* **2010**, 108, 898–907. DOI: 10.1111/j.1365-2672.2009.04482.x.

65. Sambrook, J.; Fritsch; E.R.; Maniatis, T. Molecular Cloning: A Laboratory Manual (2nd ed.). Cold Spring Harbor, NY. 1989. Cold Spring Harbor Laboratory Press.

66. Rymar, S.Yu.; Ruban, T.A.; Irodov, D.M.; Kordium, V.A. Expression and secretion of human recombinant LIF by genetically modified mammalian cells. *Biopolym. Cell*. **2011**, 27, 53-58. DOI: 10.7124/bc.000082

67. DeLong, E.F. Archaea in coastal marine environments. *Proc. Natl. Acad. Sci., U. S. A.* **1992**, 89, 5685 – 5689.

68. Coll, M.; Aymami, J.; Van Der Marel, G.A.; Van Boom, J.H.; Rich, A.; Wang, A.H.-J. Molecular structure of the netropsin-D(CGCGATATCGCG) complex: DNA confirmation in an alternating AT segment; conformation 2. DOI: 10.2210/pdb1dne/pdb

69. Dassault Systèmes BIOVIA, Discovery Studio Visualizer, v4.0.100.13345, San Diego: Dassault Systèmes, 2021

70. Sanner, M. F. Python: A programming language for software integration and development, *J. Mol. Graph. Model.* **1999**, 17, 57-61.

71. Marvin Sketch 5.3.735, 2017, ChemAxon. Available from: http://www.chemaxon.com

72. MOPAC2016, James J. P. Stewart, Stewart Computational Chemistry, Colorado Springs, CO, USA, HTTP://OpenMOPAC.net (2016).

73. Halgren, T.A. MMFF VI. MMFF94s option for energy minimization studies, *J. Comput. Chem.* **1999**, 20, 720-729.

74. Trott, O.; Olson, A.J. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* **2010**, 31, 455-61.