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# Neutral natural deep eutectic solvents as anti-biofilm agents

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ABSTRACT

Natural deep eutectic solvents (NADES) are a class of liquids with promising properties as components in pharmaceutical formulations, such as a low toxicity profile, biodegradability and versatility. Recently, their potential use as anti-biofilm agents has been proposed, due to their ability to solubilize and stabilize biological macromolecules. In the current work, the ability to break down biofilm matrix and the biofilm killing activity of three NADES of neutral pH were investigated against *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 9027 biofilms. The tested NADES were choline chloride:xylitol (ChX), choline chloride:glycerol (ChG) and betaine:sucrose (BS). Two of the NADES (ChX and ChG) significantly reduced the number of remaining viable cells of both bacterial species in pre-formed biofilm by 4–6 orders of magnitude, while the average biofilm biomass removal for all NADES was 27–67% (*S. aureus*) and 34–49% (*P. aeruginosa*). The tested NADES also inhibited biofilm formation of both bacterial species at concentrations at or below 0.5 x the minimal inhibitory concentration (MIC), possibly in part due to observed restrictions imposed by NADES on planktonic growth. These results demonstrate the potential value of neutral NADES as anti-biofilm agents in future anti-microbial preparations.

## 1. Introduction

Natural deep eutectic solvents (NADES) have rapidly become a focus in the search for new "green" media as alternatives to conventional organic solvents. Their applications as extraction and reaction media in fields such as the chemical industry have already been widely studied [1–5]. Recently, attention has grown for the potential use of NADES as solvents or excipients for pharmaceutical purposes due to their special properties [6,7]. NADES consist of supramolecular networks of two or more natural components, such as organic acids and bases, sugars or amino acids [8,9]. The deep eutectic mixture is formed when the components are mixed in a specific molar ratio, resulting in a liquid with significantly lower melting point than its individual constituents [10]. Some of the advantages of NADES include the low production cost, their adjustable physicochemical properties through changing the composition or water content, as well as their solubilizing properties [11–14]. As the components are naturally abundant in living biological systems, the solvents have promising properties in pharmaceutical formulation, such as being biodegradable, environmentally friendly and presumably non-toxic [15]. Several studies have reported how NADES are able to extract, solubilize and stabilize biopolymers and other macromolecules, exemplified by bovine serum albumin, various enzymes and phenolic metabolites [16–19]. In addition, a recent study found that certain NADES had biofilm matrix destruction properties [20].

Biofilms are three-dimensional matrices made up of microbial cells immersed in a self-generated extracellular polymeric substance (EPS). Biofilm formation poses a substantial challenge for efficient treatment of a range of skin and soft tissue infections in the human body, and is a well-known contributor to the development of chronic wounds [21]. The EPS is commonly composed of polysaccharides, proteins, and/or extracellular DNA, and provides protection against toxic substances, immune cells during infection, and inflammatory responses, as well as unexpected changes in the growth environment [22–24]. Therefore, the biofilm matrix can enhance the ability of an organism to survive exposure to antibiotics, potentially resulting in tolerance development and treatment failure [25]. Treatment strategies targeting the EPS, either by dissolving or disrupting the matrix, would be of potential great value in healing skin and soft tissue infections. Thus, a possible biofilm dissolving effect of NADES could lead to promising new antimicrobial formulations

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**Abbreviations**

| | |
|---|---|
| BS | betaine:sucrose (2:1) |
| CFU | colony forming units |
| ChG | choline chloride:glycerol (1:1) |
| ChX | choline chloride:xylitol (5:2) |
| EPS | extracellular polymeric substance |
| MBEC | minimal biofilm eradication concentration |
| MHB + gluc. | Mueller Hinton broth with 0.5% added glucose |
| MIC | minimum inhibitory concentration |
| NADES | natural deep eutectic solvents |
| PBS | phosphate buffered saline; |
| SEM | Scanning electron microscopy |
| TSB | Tryptic soy broth |

involving these solvents.

A previous study of biofilm matrix destruction properties of NADES did not include investigations in a biological system [20]. Therefore, the effects of NADES on bacterial biofilm should be expanded to *in vitro* biofilm models, in order to increase knowledge about novel potential applications of this group of solvents. The application of NADES in antimicrobial preparations is further actualized by studies reporting that these solvents could function synergistically, improving the antimicrobial activity of other compounds, e.g. catechins or porphyrins [26,27]. There are also indications that some NADES, in particular those with acidic properties, exhibit intrinsic antibacterial effects [28,29]. NADES with neutral pH are considered less toxic than their acidic counterparts, but are still able to dissolve biofilm components [20]. It would, therefore, be of interest to study the anti-biofilm effects of neutral NADES in particular.

In the present study, three NADES with neutral pH were investigated for their ability to break biofilm matrix structure and as biofilm formation inhibitors in different *in vitro* biofilm assays. Furthermore, their antibacterial effect in pre-formed biofilm was investigated. Neutral NADES were selected to avoid potential antimicrobial effects due to acid/base properties [30]. *Pseudomonas aeruginosa* and *Staphylococcus aureus* were chosen as model organisms, representative of Gram-negative and Gram-positive species, as they are both common biofilm-forming pathogens linked to topical infections. The selected model strains were sensitive to most common antibiotics.

## 2. Materials and methods

### 2.1. Materials

All materials were of analytical grade and were purchased from commercial sources.

### 2.2. Bacterial strains and growth medium

*S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 9027 were cultivated at 37 °C in Tryptic Soy Broth (TSB) or on tryptic soy agar (TSA) plates unless otherwise stated. Original stocks of each strain were stored at −80 °C, and used to streak out bacterial cultures on master plates, which were stored at 4 °C for a maximum of two weeks before being discarded.

### 2.3. Neutral natural deep eutectic solvents

The NADES applied in this study were prepared as explained in a previous study [31]. In short, components were mixed and dissolved in warm water (50 °C), followed by evaporation of the water for 20 min in a rotary evaporator, resulting in a clear, viscous liquid. Table 1 gives an overview of the selected NADES, their constituents, and their measured water content. Unless otherwise stated, samples of NADES were diluted in the relevant growth media for the test organism. Concentrations are presented as % (v/v) due to the liquid nature of NADES.

**Table 1**
Components of the natural deep eutectic solvents (NADES) included in the study, their molar ratios and measured water content when undiluted (from Ref. [31]).

| NADES | Molar ratio | Component 1 | Component 2 | Water content (% v/v) |
|---|---|---|---|---|
| **ChG** | 1:1 | Choline chloride | Glycerol | 15 |
| **ChX** | 5:2 | Choline chloride | Xylitol | 12 |
| **BS** | 2:1 | Betaine | Sucrose | 23 |

### 2.4. Minimum inhibitory concentration measurements and bacterial growth curves during exposure to NADES

The minimum inhibitory concentration (MIC) of each selected NADES at concentrations ranging from 5 to 35% was determined for both bacterial strains as a basis for further experiments. Over-night cultures of the strains in LB or TSB were prepared and incubated at 37 °C with shaking at 150 rpm prior to sample preparation. Inoculum (100 μl) was added to all samples in a 96-well round-bottom polystyrene microtiter plate, to a final volume of 200 μl and a starting $OD_{600}$ of 0.01, in each well (Costar, Corning Inc., Kennebunk, USA). This was repeated for different starting inocula to investigate a possible inoculum effect of NADES. Plates were incubated at 37 °C for 18 h without shaking, followed by visual inspection to determine the MIC. Negative control (blank) wells (no bacteria added) and positive growth control wells were included in each plate, and the bacterial inoculum was controlled by plating of serial dilutions and colony counts.

Planktonic growth curves in growth medium with added NADES were obtained at 37 °C with shaking at 150 rpm over a 24 h period, with absorbance measurements ($OD_{600}$) every 10 min using a plate reader (Clariostar, BMG Labtech, Ortenberg, Germany), and were compared to untreated controls. In this assay, each NADES was added in two different sub-MIC concentrations (diluted in TSB growth medium) to sterile non-treated flat bottom polystyrene 96-well microplates (Tissue culture plates, VWR, USA) containing over-night cultures diluted in fresh growth medium (TSB) to a final starting optical density of $OD_{600} = 0.01$. Final concentrations of NADES were: ChX: 5% and 10%; ChG: 2.5% and 5%, BS: 10% and 20% (respectively). The plates were covered with sealing foil (Breathe-Easy membrane, Merck KGaA, Darmstadt, Germany) prior to measurements. Four biological replicates were performed for the growth curve experiments, each with four technical replicates for each growth condition for each bacterium.

### 2.5. Effects of NADES on preformed biofilm

#### 2.5.1. Biofilm formation

*P. aeruginosa* and *S. aureus* biofilms were formed in a peg lid system (Calgary device) (Innovotech, Inc., Edmonton, Canada). Over-night cultures were prepared and incubated as described in section 2.4, and were then diluted hundred-fold in fresh growth medium and incubated for 3 h at 37 °C with shaking at 150 rpm. The precultures were then adjusted to a starting $OD_{600} = 0.01$, and 160 μl was added to each well of a 96-well microtiter plate (Innovotech, Inc., Edmonton, Canada). *P. aeruginosa* biofilm was grown in TSB over 24 h at 25 °C and shaking at 110 rpm. The *S. aureus* biofilm was formed in Mueller-Hinton broth with 0.5% added glucose (MHB + gluc.) over 72 h at 37 °C and 110 rpm shaking. In both cases, the incubation was performed in a humid environment by placing the plates in sealed-off containers with moist tissue paper.

#### 2.5.2. Minimal biofilm eradication concentration (MBEC) and biofilm killing determination

Established biofilm (on pegs) was challenged with concentrations of NADES ranging from 10 to 30% in 96-well round bottom polystyrene microplates (Costar, Corning Inc., Kennebunk, USA), followed by incubation for 24 h under similar conditions as for biofilm formation (2.5.1). Untreated controls were included. In order to measure total biomass the remaining biofilm was stained with 0.3% (w/v) crystal violet for 20 min, then washed in phosphate buffered saline (PBS) and dissolved in 180 μl acetone:ethanol (1:3) over 10 min in an ultrasound bath (SONOREX RK 100, Bandelin electronic GmbH & Co KG, Berlin, Germany). Absorbance measurements at 570 nm were obtained in 96-well flat bottom polystyrene microplates (Costar, Corning Inc., Kennebunk, USA) using an endpoint measuring method in a plate reader (Clariostar, BMG Labtech, Ortenberg, Germany) (the canonical wavelength for crystal violet is 595 nm). Samples from *P. aeruginosa* biofilm were diluted ten-fold prior to measurements, whereas *S. aureus* biofilm samples were measured undiluted. The experiments were performed in three to five biological replicates, each with 16 technical replicates.

To investigate the number of remaining viable bacteria in biofilm following NADES treatment, biofilm was formed and challenged with NADES as described above. Instead of crystal violet staining, the biofilm was gently dislodged from the pegs into wells containing 200 μl 0.9% NaCl by placing the microplates on a metal surface in an ultrasound bath (SONOREX RK 100, Bandelin electronic GmbH & Co KG, Berlin, Germany) for 30 min. Each sample was then serial diluted ($10^1$–$10^7$ fold) in 0.9% NaCl and plated on TSA plates, followed by incubation at 37 °C for 18 h and counting of colony forming units (CFU). The viability was studied using three to four biological replicates, each with eight technical replicates.

### 2.6. Biofilm formation assay

The effects of NADES on biofilm formation were studied in liquid broth cultures in 96-well round bottom polystyrene microplates (Costar, Corning Inc., Kennebunk, USA). NADES were added at sub-MIC concentrations, corresponding to a final high sub-MIC (0.25 x - 0.5 x MIC) and low sub-MIC (0.1 x - 0.25 x MIC) concentration respectively, in the microtiter plate wells (Supplementary Table S1), after addition of an equal volume of preculture, grown as described in 2.5.1 and prediluted to $OD_{600}$ = 0.02 (final starting $OD_{600}$ = 0.01). The microplates were incubated for the same duration and temperature as previously described (2.5.1). Staining of the biofilm formed in the plate wells and the following absorbance measurements were performed as described in section 2.5.2, with the modification that the biofilm was dissolved in 150 μl of acetone:ethanol (1:3) and then diluted two-fold (*S. aureus*) or four-fold (*P. aeruginosa*) in acetone:ethanol (1:3) prior to measurements. The experiment was performed in four biological replicates of each strain, with 16 technical replicates.

### 2.7. Scanning electron microscopy imaging

Biofilm samples for scanning electron microscopy (SEM) imaging were fixed through a double fixation protocol. Biofilm was formed and challenged with NADES as described in section 2.5, then fixed twice with a solution containing 2% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde in 0.1 M HEPES buffer. The first fixation was performed as a single strength fix over 15 min at a temperature of 25 °C or 37 °C for the *P. aeruginosa* and *S. aureus* samples, respectively. The second fixation was done at room temperature, followed by refrigeration of the samples until imaging.

The fixed biofilm was dehydrated by 10 min equilibration in a sequence of ethanol concentrations (70, 80, 90 and 96%), then equilibrated four times for 15 min in 100% ethanol. The samples were critical point dried (BAL-TEC CPD 030, Critical Point Dryer, BAL-TEC AG, Liechtenstein), mounted on aluminum SEM stubs with silver epoxy, then sputter coated with 10 nm platinum (Cressington 308UHR, Ted Pella Incorporated, Redding, CA, USA). The samples were examined with a Hitachi scanning electron microscope (Hitachi S-4800 FEG, Tokyo, Japan).

### 2.8. Statistical analysis

Microsoft Excel (Microsoft Office 360) software was used for normalization of data prior to statistical analyses. The experimental data were obtained from at least three independent experiments with technical replicates, and were presented as mean ± standard deviation (SD). Statistical analyses were performed by using one-way or two-way ANOVA followed by the Tukey's multiple comparison test using GraphPad Prism version 9.1.0 for Windows (GraphPad Software, La Jolla, CA, USA; www.graphpad.com).

## 3. Results

### 3.1. Minimal inhibitory concentration (MIC) determination of NADES towards P. aeruginosa and S. aureus

The MICs of the three selected NADES were determined against planktonic cultures of *P. aeruginosa* ATCC 9027 and *S. aureus* ATCC 6538 (Table 2), both at a starting $OD_{600}$ equal to 0.01 (equivalent to approximately $4*10^6$ CFU/ml for *P. aeruginosa* and $1*10^7$ CFU/ml for *S. aureus*, respectively). Note that the values are reported as % (v/v) due to the liquid nature of NADES. The lowest MIC was found for choline chloride:glycerol (ChG), with 20% against *P. aeruginosa* ATCC 9027 and 20–25% against *S. aureus* ATCC 6538, while betaine:sucrose (BS) was the apparently least effective, with an MIC of 30% against *P. aeruginosa* and no apparent inhibition (>35%) of *S. aureus* growth in the tested concentration range (5–35%). Interestingly, an inoculum effect was observed for all three NADES tested (Supplementary Table S2; no data available for BS with *S. aureus*, as no growth inhibition was observed in the tested concentration range, 5–35%).

### 3.2. Effect on biofilm biomass and biofilm killing effect of NADES

Pre-established biofilms were challenged with the selected NADES, and Figs. 1 and 2 portray the effects on biofilm biomass of the tested NADES against *P. aeruginosa* ATCC 9027 and *S. aureus* ATCC 6538, respectively. The biofilm was stained with crystal violet prior to absorbance measurements, and a low absorbance value would correspond to a higher degree of biofilm biomass removal (lower residual biofilm). Statistically significant ($p < 0.05$–$0.001$) disruption of the biofilm relative to untreated control was found for choline chloride:xylitol (ChX) (all concentrations), betaine:sucrose (BS) (20% and 30%), and choline chloride:glycerol (ChG) (30%) against both bacterial strains (Figs. 1 and 2), as well as for ChG (10% and 20%) and BS (10%) against *S. aureus* (Fig. 2). However, none of the NADES were able to eradicate all biofilm biomass at the tested concentrations. To control for the possible effects of a reduced volume of growth medium in the samples containing 20–30% NADES, samples added identical concentrations (v/v) of PBS were included. PBS samples did not affect the preformed biofilm significantly (Figs. 1 and 2). For *S. aureus* the various NADES treatments lead to an on average 27–67% removal of biofilm biomass compared to

**Table 2**
Minimum inhibitory concentration (% v/v) of the NADES ChX, ChG and BS against *Pseudomonas aeruginosa* ATCC 9027 and *Staphylococcus aureus* ATCC 6538, both at starting $OD_{600}$ = 0.01. ChX: choline chloride:xylitol; ChG: choline chloride:glycerol; BS: betaine:sucrose.

| | ChX | ChG | BS |
|---|---|---|---|
| *P. aeruginosa* ATCC 9027 | 20% | 20% | 30% |
| *S. aureus* ATCC 6538 | 25% | 20–25% | >35% |

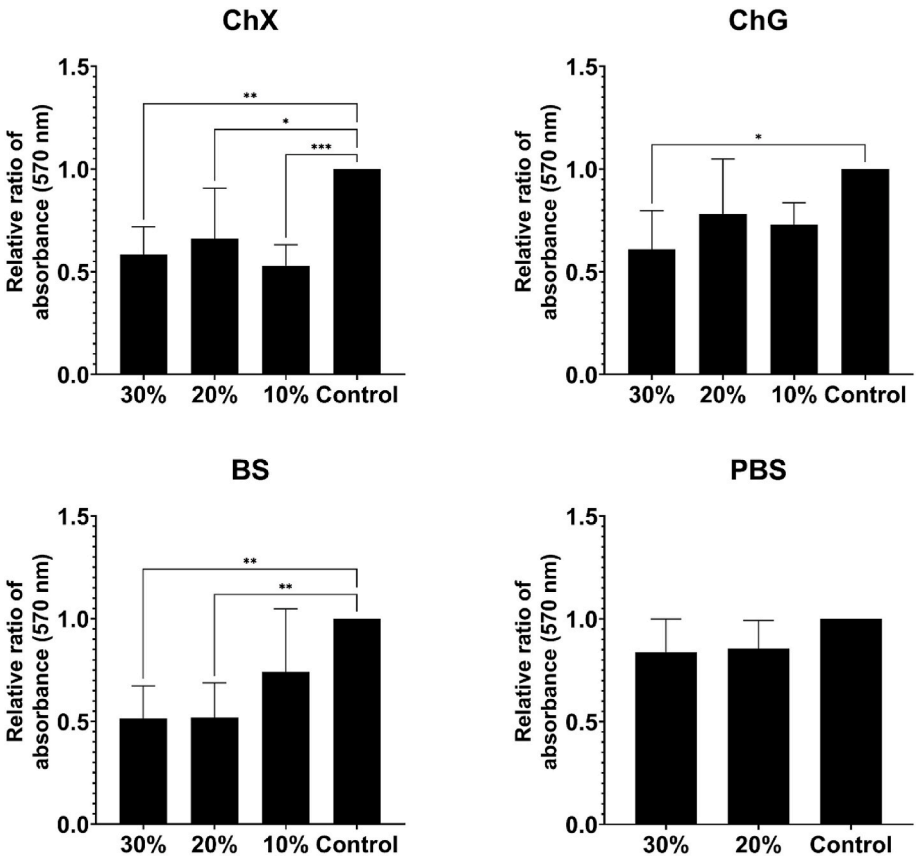


**Fig. 1.** Biofilm biomass of *Pseudomonas aeruginosa* ATCC 9027 after 24 h challenge with NADES ChX, ChG and BS on pre-established biofilm, evaluated through staining the biofilm with crystal violet and measuring absorbance intensity (570 nm) (n = 3). Control samples challenged with 30% and 20% PBS (v/v, bottom right) were included to exclude effects on viability from a reduced volume of growth media in the samples containing 20–30% NADES. The indicated control was sample not added neither NADES nor PBS. Samples were diluted ten-fold in acetone: ethanol (1:3) prior to measurement. Results were normalized to control prior to statistical analyses, which were performed using one-way ANOVA. Error bars represent the standard deviation (SD) of normalized data from five independent experiments. Asterisks (*) denote significant differences, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

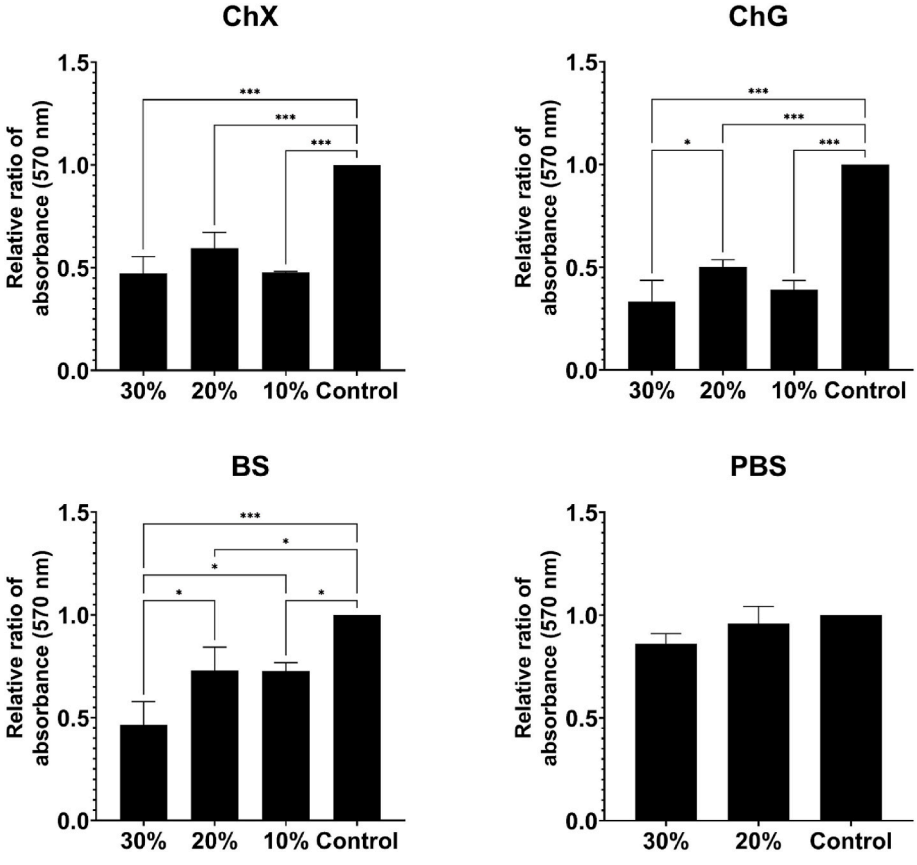


**Fig. 2.** Biofilm biomass of *Staphylococcus aureus* ATCC 6538 after 24 h challenge with NADES ChX, ChG and BS on pre-established biofilm, evaluated through staining the biofilm with crystal violet and measuring absorbance intensity (570 nm) (n = 5). PBS samples at 30% and 20% (v/v) were included to exclude effects on viability from a reduced volume of growth media in the samples containing 20–30% NADES. The indicated control sample was not added neither NADES nor PBS. Results were normalized to control prior to statistical analyses, which were performed using one-way ANOVA. Error bars represent the standard deviation (SD) of normalized data from five independent experiments. Asterisks (*) denote significant differences, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

control (Fig. 2), while for *P. aeruginosa* NADES treatment resulted in an average biofilm disruption of 34–49% (Fig. 1).

Although crystal violet staining provides insight into the amount of biomass eradicated by NADES treatment, it does not shed light on the effects of NADES on bacterial viability. Therefore, cell viability within the biofilm was investigated post treatment with NADES. The number of viable (CFU/ml) *P. aeruginosa* ATCC 9027 and *S. aureus* ATCC 6538 cells after 24 h challenge of the biofilm with the various NADES is presented in Figs. 3 and 4, respectively. Except for 10% ChG with *P. aeruginosa*, all other tested concentrations of ChX and ChG significantly affected the number of viable cells of both species ($p < 0.05$–$0.001$) compared to control, with an average $\log_{10}$ reduction of 5–6 observed in the case of ChG (30%) against *P. aeruginosa* (Fig. 3), and average $\log_{10}$ reduction of 4–5 for ChG (20% and 30%) against *S. aureus* (Fig. 4). None of the organisms were susceptible to BS (Figs. 3 and 4), with no significant change ($p < 0.05$) in viability following exposure (Fig. 3). Again, no significant change in number of viable cells was observed in the samples added PBS to 20% or 30% (Figs. 3 and 4). Note the log-scale change in value for the vertical axis between the experiments with different NADES.

### 3.3. Effects of NADES on biofilm formation

The effects of NADES ChX, ChG and BS on *P. aeruginosa* and *S. aureus* biofilm formation are shown in Fig. 5, with applied concentrations of NADES at or below 0.5 x the determined MIC values at starting $OD_{600}$ = 0.01 (Table 2). Two concentrations were used for each NADES, a high sub-MIC concentration (0.25 x – 0.5 x MIC) and a low sub-MIC concentration (0.1–0.25 x MIC) (Supplementary Table S1; Supplementary Table S2). The high sub-MIC concentrations of ChX and ChG significantly reduced biofilm formation for both strains relative to control (Fig. 5a and b), whereas the BS high sub-MIC sample only affected *P. aeruginosa* biofilm formation significantly ($p < 0.001$) (Fig. 5a). None of the low sub-MIC samples (0.1 x - 0.25 x MIC) were able to inhibit biofilm formation of *P. aeruginosa* (Fig. 5a), whereas ChX and ChG at low sub-MIC concentrations significantly ($p < 0.05$–$0.001$) affected *S. aureus* biofilm formation relative to control (Fig. 5b). For all three NADES, increasing the concentration from low sub-MIC to high sub-MIC levels had a significant inhibitory effect on *P. aeruginosa* biofilm formation (Supplementary Table S3). This effect was not observed for *S. aureus*.

In order to determine if NADES ChX, ChG and BS had an impact on planktonic bacterial growth rates, which may potentially also affect initial phases of biofilm formation, growth curve experiments were performed at two different sub-MIC concentrations of each compound (Fig. 6). The slope value for each growth curve was calculated during the exponential growth phase (as identified by log-transformation of the growth curve) for each individual culture condition, and was used as a measure for the bacterial growth rate (Supplementary Table S4). Also, delay of growth for the various NADES treatments relative to control was quantified, by determining the time to reach $OD_{600}$ = 0.20 (corresponding to mid-exponential growth for the untreated control samples), and the time to reach $OD_{600}$ = 0.50, respectively (Supplementary Table S4). All NADES samples inhibited the growth of the bacteria to a significant degree at the highest sub-MIC concentration tested (relative to control) both for *P. aeruginosa* and *S. aureus*, and for *P. aeruginosa* also at the lower sub-MIC concentrations, leading to significant changes in exponential phase growth rate (Supplementary Tables S4 and S5). In addition, experiments investigating the time to reach exponential growth phase (time to reach $OD_{600}$ = 0.20 and 0.50, respectively) for each culture condition showed that NADES ChX and BS at the highest tested sub-MIC concentrations conferred a significant growth delay for *P. aeruginosa* (both at $OD_{600}$ = 0.20 and $OD_{600}$ = 0.50), while NADES ChX and ChG at the highest tested sub-MIC concentrations conferred a significant growth delay for *S. aureus* at $OD_{600}$ = 0.50 (Supplementary Tables S4 and S6).

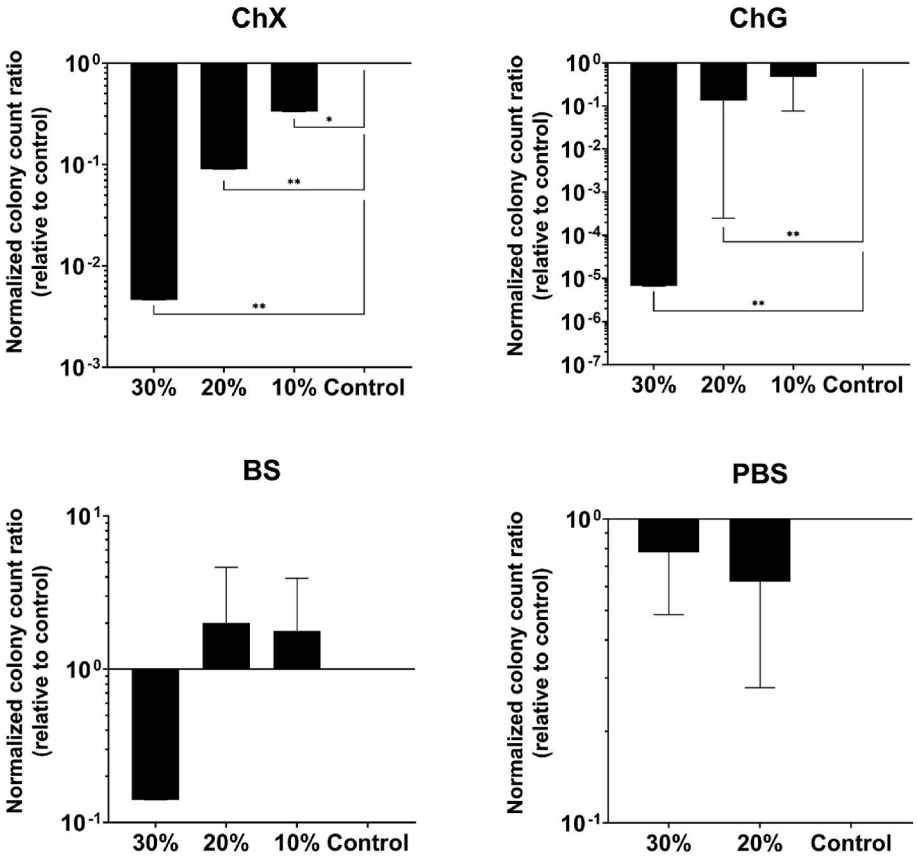


**Fig. 3.** Viable *Pseudomonas aeruginosa* bacteria (CFU/ml) in biofilm after challenge with NADES ChX, ChG and BS for 24 h (n = 3), relative to control. PBS samples at 30% and 20% (v/v) were included to exclude effects on viability from a reduced volume of growth media in the samples containing 20–30% NADES. In the indicated control sample, neither NADES nor PBS was added. Results were normalized to the control prior to statistical analyses, which were performed using one-way ANOVA. Error bars represent the standard deviation (SD) of normalized data from four independent experiments. Asterisks (*) denote significant differences, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

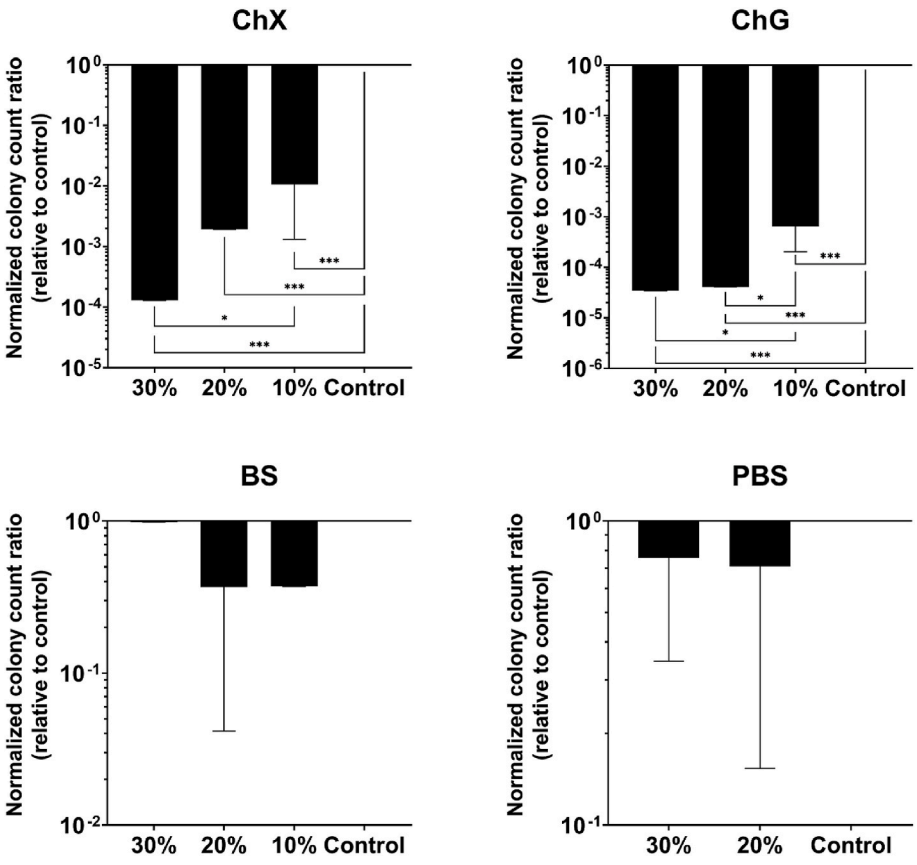


**Fig. 4.** Viable *Staphylococcus aureus* bacteria (CFU/ml) in biofilm after challenge with NADES ChX, ChG and BS for 24 h (n = 4), relative to control. PBS samples at 30% and 20% (v/v) were included to exclude effects on viability from a reduced volume of growth media in the samples containing 20–30% NADES. In the indicated control sample, neither NADES nor PBS was added. Results were normalized to the control prior to statistical analyses, which were performed using one-way ANOVA. Error bars represent the standard deviation (SD) of normalized data from four independent experiments. Asterisks (*) denote significant differences, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

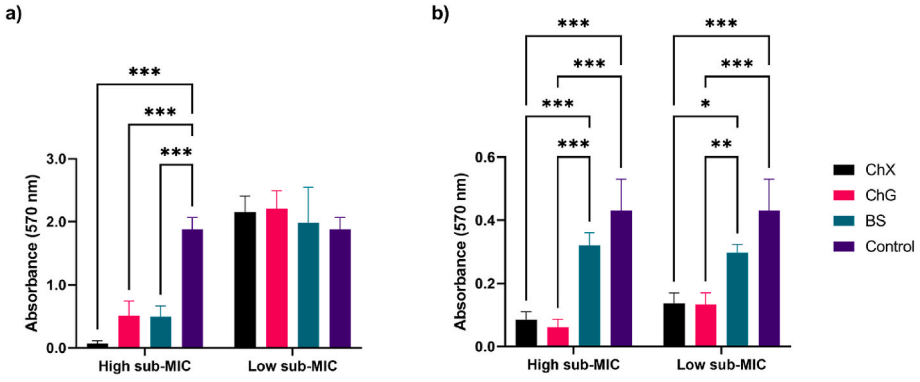


**Fig. 5.** Effect of NADES ChX, ChG and BS in sub-MIC concentrations corresponding to high sub-MIC (0.25 x - 0.5 x) and low sub-MIC (0.1 x - 0.25 x) concentrations, respectively, on *P. aeruginosa* (a) and *S. aureus* (b) biofilm formation (n = 4). Absorbance (570 nm) was measured after staining the biofilm with crystal violet. *S. aureus* samples were diluted two-fold, whereas *P. aeruginosa* samples were diluted four-fold in acetone:ethanol (1:3) prior to measurements. Statistics were performed using two-way ANOVA. Error bars represent the standard deviation (SD) of normalized data from four independent experiments. Asterisks (*) denote significant reduction in formed biofilm, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. Statistics for comparisons between samples across the two different NADES concentrations are shown in Supplementary Table S3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

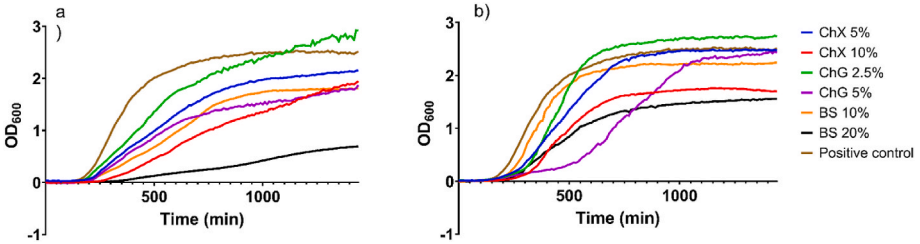


**Fig. 6.** Growth curves ($OD_{600}$) of *P. aeruginosa* ATCC 9027 (a) and *S. aureus* ATCC 6538 (b), in the presence of NADES ChX, ChG and BS at different concentrations. $OD_{600}$ of each culture was measured every 10 min over 24 h (n = 4). Values for slope and delay of growth relative to control are shown in Supplementary Table S4, and statistical analysis using two-way ANOVA is provided in Supplementary Tables S5 and S6.

### 3.4. Scanning electron microscopy (SEM) imaging of biofilms challenged with NADES

*P. aeruginosa* ATCC 9027 and *S. aureus* ATCC 6538 biofilms challenged with NADES were analysed by scanning electron microscopy (SEM), and representative images are shown in Figs. 7 and 8, respectively. The experiment was performed twice and at least nineteen images were analysed for each treatment for each strain. Challenge with NADES was performed identically to the procedure used for the MBEC assay, employing the Calgary device model (3.2), and imaging was performed directly on the plastic pegs where biofilm was formed, at the section corresponding to the liquid-air interface. Images of the samples with 30% concentration of NADES were chosen, to clearly observe potential differences between samples and control (Fig. 7a and e and Fig. 8a and e shows untreated *P. aeruginosa* and *S. aureus* biofilm, respectively).

The images of *P. aeruginosa* biofilm exposed to 30% BS (Fig. 7b and f) and 30% ChG (Fig. 7c and g) indicated damages to the surface of the cells compared to untreated control (Fig. 7a and e). Changes in the morphology (i.e. irregularities on the surface) of the treated cells could be observed for both 30% BS and 30% ChG treatments (Fig. 7f and g). Cell damage was not observed in the ChX samples. Changes in the morphology (i.e. irregularities on the surface) of the treated cells could also be observed (Fig. 7f and g). However, the biofilms were clearly not fully eradicated following NADES treatment (Fig. 7b, c and d), with the SEM images showing intact residual biofilm matrix after challenge with all tested NADES at 30%, in line with the MBEC results measuring biofilm biomass using crystal violet staining (Figs. 1 and 2).

Images of *S. aureus* biofilm treated with NADES (Fig. 8a–d) are in line with results from the MBEC experiments (Figs. 1 and 2), which indicate some biofilm removal compared to controls. The morphology of *S. aureus* ATCC 6538 cells was affected by all three different NADES, e.g. as shown for 30% BS (Figs. 8f), 30% ChG (Figs. 8g) and 30% ChX (Fig. 8h) compared to control (Fig. 8a and e). The morphological effect was thereby more pronounced for *S. aureus* ATCC 6538 in the BS samples than for *P. aeruginosa* ATCC 9027.

## 4. Discussion

Biofilms are notoriously more difficult to treat with antimicrobials than planktonic cells, and new treatment strategies for biofilm destruction are needed. NADES have emerged as promising solvents for pharmaceutical formulations and may have a place in novel antimicrobial preparations targeting biofilm. This work investigated the potential value of neutral NADES as anti-biofilm agents in future antimicrobial formulations, employing *S. aureus* and *P. aeruginosa* as representative indicator organisms.

MIC values of the selected NADES toward the indicator bacteria were determined prior to the anti-biofilm assays (Table 2). Both ChX and ChG were found to show anti-bacterial activity, inhibiting growth of the test

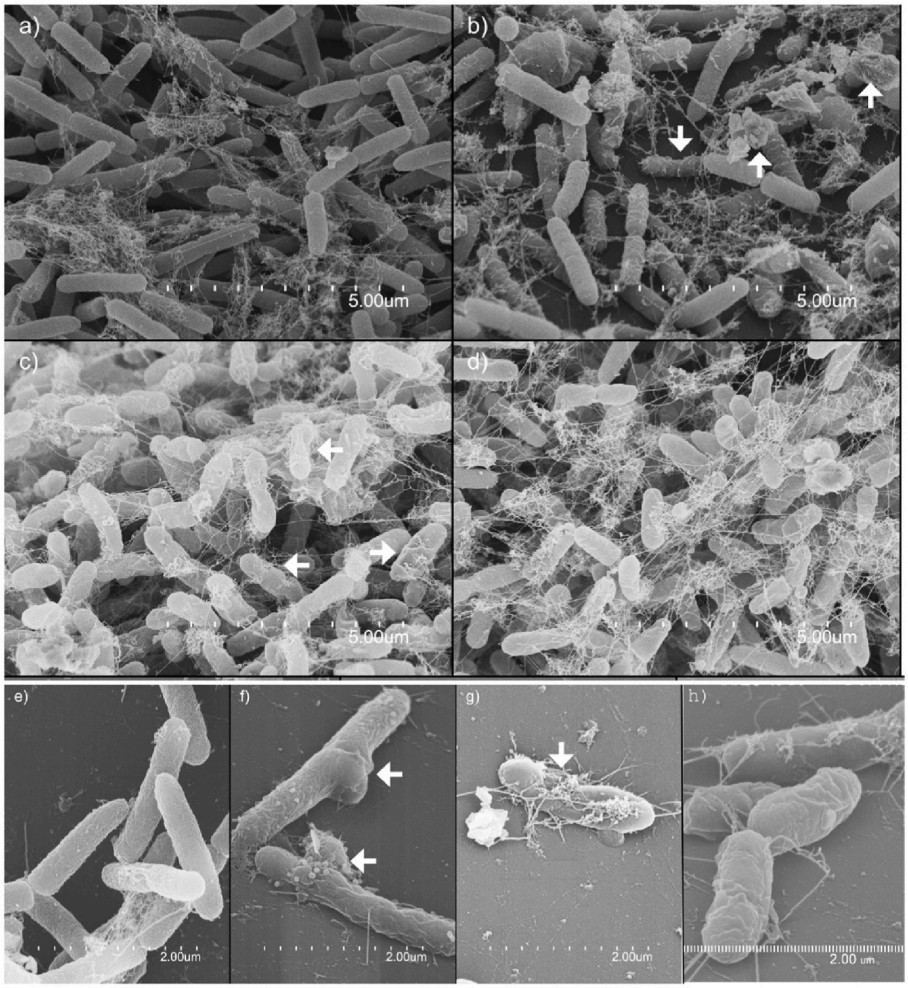


**Fig. 7.** Scanning electron microscopy (SEM) images of *P. aeruginosa* biofilm: a) and e) without treatment, b) and f) after treatment with BS 30%, c) and g) after treatment with ChG 30%, d) and h) after treatment with ChX 30%. Examples of damaged bacteria after treatment with BS 30% and with ChG 30% are indicated by white arrows. White dotted lines are space bars indicating size.

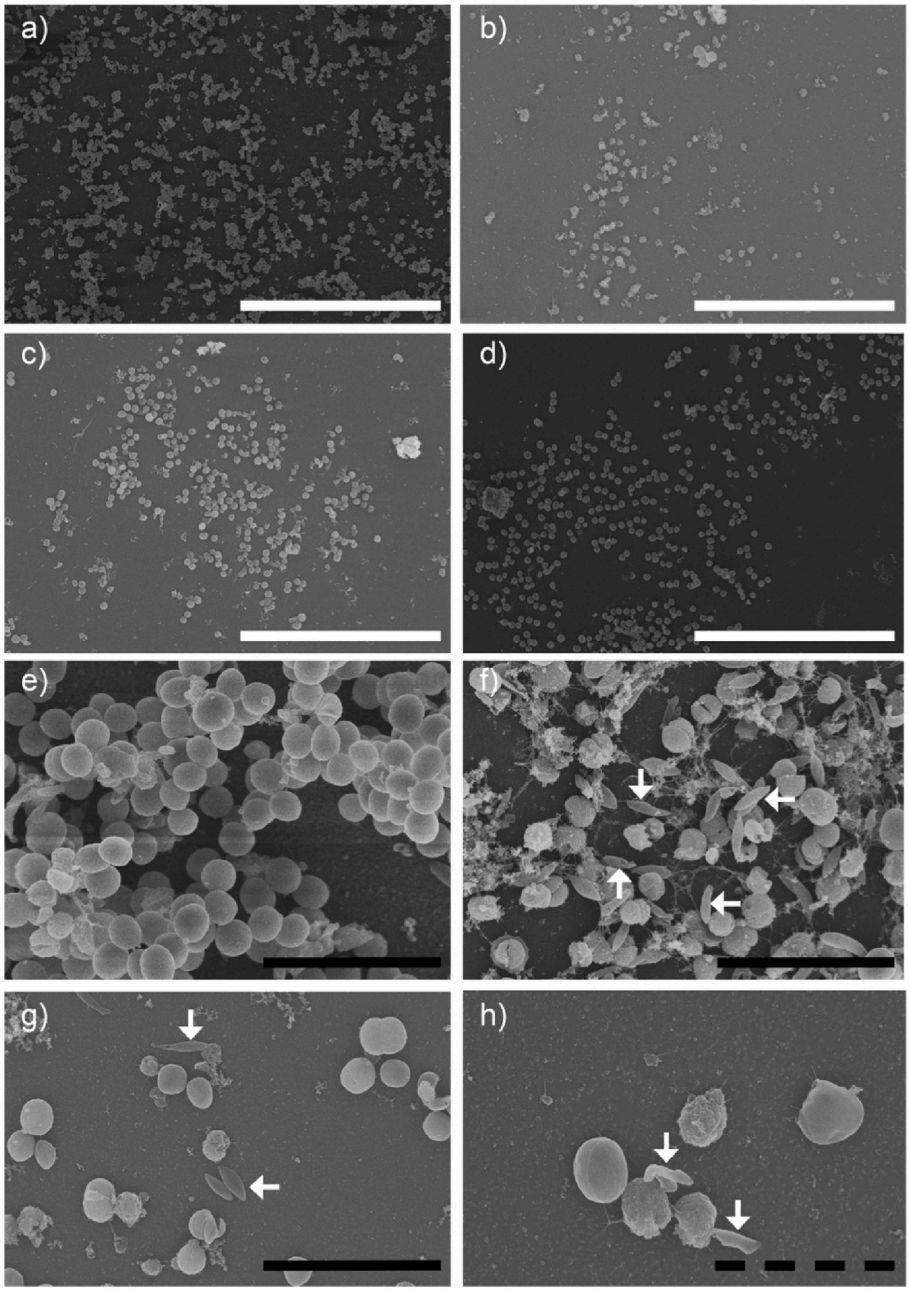


**Fig. 8.** Scanning electron microscopy (SEM) images of *S. aureus* biofilm: a) and e) without treatment, b) and f) after treatment with NADES BS 30%, c) and g) after treatment with ChG 30% and d) and h) after treatment with ChX 30%. White arrows indicate cell morphological changes after NADES treatment. Space bars: white = 20 μM, black = 5 μM and dotted line = 2 μM.

organisms at concentrations 20–25% v/v in growth medium. The antimicrobial activities of ChX and ChG have also been investigated in previous studies. Radosevic et al. (2018) reported no inhibition for ChX against *S. aureus* and *P. aeruginosa* in similar concentrations as those used here [29]. However, that study utilized a disk diffusion method, meaning that the antimicrobial activity is highly dependent on diffusion of the NADES into the surrounding solid medium. The high viscosity of most NADES could hamper diffusion, thus possibly resulting in a lower antimicrobial effect in a disk diffusion method compared to MIC determination employing broth microdilution methods, which also better reflect the use of NADES as antimicrobial and anti-biofilm agents. In a short communication by Ref. [32]; ChG did not exhibit antimicrobial properties [32]. That study did however not specify the tested concentrations of NADES, nor describe the method for MIC determination, and the results are therefore not necessarily comparable. Our experiments therefore to our knowledge constitute the first demonstration of antibacterial activity of neutral NADES.

Growth of both indicator organisms was considerably affected in the presence of the highest sub-MIC concentrations of each NADES used for the growth curve experiments (corresponding to 0.25 x – 0.67 x MIC for the different NADES) relative to control, both with respect to growth rate (all tested NADES, both bacteria) and delay of exponential phase growth (ChX and BS for *P. aeruginosa*, ChX and ChG for *S. aureus*) (Fig. 6; Supplementary Tables S4–S6). The lower sub-MIC NADES concentrations (corresponding to 0.125 x – 0.33 x MIC) had a significant effect on growth rate only for *P. aeruginosa* (all tested NADES), and did not significantly delay entry into exponential growth for any of the two bacteria tested. Although it should be noted that the concentrations used

to assay planktonic growth corresponded to slightly different fold MICs for each of the respective NADES, the results clearly showed that the tested neutral NADES, even at sub-MIC concentrations, could inhibit the growth of the bacteria, which is supported by a previous study on other types of NADES [33].

To our knowledge, the effect of NADES on viability of bacterial cells within a biofilm has never been subject to previous study. Here it was observed that although complete eradication of biofilm biomass was not observed at tested concentrations for any of the three NADES investigated, the number of remaining viable biofilm cells of both test organisms was significantly affected after challenge of established biofilm with ChG and ChX (except for the lowest concentration of ChG with *P. aeruginosa*; Figs. 3 and 4). A 4-6-range $\log_{10}$ reduction in viable cells was seen for the 30% ChG sample against both species, corresponding to a 99.99%–99.9999% reduction in viable bacteria, showing that specific NADES can exhibit high antibacterial efficacy, also against bacteria within a protective biofilm.

An alternative interpretation of this data could be ChG and ChX inducing biofilm dispersal, with biofilm cells becoming planktonic during challenge, and the exact cellular and molecular mechanism(s) responsible for the reduction in viable bacteria within the biofilm remain to be mapped. However, clearly these NADES are able to penetrate into the biofilm, and all tested NADES appeared to have effects on cell morphology (Figs. 7 and 8). Penetration into the biofilm may potentially lead to direct interactions of NADES with bacterial cell surface structures, and/or possibly induce osmotic shock of the cells due to the hyperosmolar NADES environment. The compounds applied in this study would have an expected osmolality from 350 to >3000 mOsm/kg (based on previous measurements of 10–15 fold diluted NADES), depending on the type of NADES and concentration [31]. In line with this, the SEM images (Figs. 7 and 8) showed clear damage to the bacterial membrane and changes in size and morphology of the cells. Also, a previous study in our group identified similar shrinkage to artificial cell membranes in the presence of NADES [31].

It has previously been proposed that Gram-positive bacteria may be more resistant to NADES than Gram-negative species due to the differences in cell wall structure [34]. A hypothesised method of action for NADES is hydrogen bond- or electrostatic interactions of the NADES components with polysaccharides and peptides in the cell wall [9,35]. As the Gram-positive cell wall is composed of a thicker layer of these macromolecules than the Gram-negative wall, resistance towards NADES could potentially be increased in Gram-positive species. The MIC assay showed that all three NADES tested inhibited growth by the Gram-negative bacterium *P. aeruginosa* at somewhat lower concentrations than for *S. aureus* (Gram-positive) (Table 2). This could support the proposed theory, although other factors could also be involved.

Crystal violet staining is a well-known output parameter for total biofilm biomass, staining both bacterial cells and the surrounding EPS. Results in this study indicated that the three tested NADES were able to remove some of the established biofilm, although only in the range 27–67% (Figs. 1 and 2). Nava-Ocampo *et al.* [20] reported that two out of seven tested NADES were able to solubilize EPS and other biomolecules present in biofilms, thus exhibiting biofilm matrix destruction properties in an *in vitro* setting [20]. The study did not investigate the same NADES as the present work, although choline chloride and betaine were components in some of the tested solvents. Among these, only the NADES composed of betaine and urea was reported to have biofilm matrix destruction properties. In the present study however, both the NADES containing betaine (BS) and NADES containing choline chloride (ChG and ChX) showed some degree of biofilm matrix destruction property compared to control. NADES ChG and ChX both affected biofilm cell viability (Figs. 3 and 4) and planktonic bacterial growth rate (Fig. 6; Supplementary Tables S4–S5). BS, however, did not significantly affect the viability of preformed biofilm, thus appearing less effective as an anti-biofilm agent compared to ChX or ChG, as primarily shown by the MBEC cell viability results (Figs. 3 and 4). As mentioned previously, many NADES are of a highly viscous nature, and among the NADES tested in the present work, BS has a considerably higher viscosity than the other solvents (reported to be 874 mPa S undiluted in previous measurements by our group [31]). A high viscosity environment has previously been found to increase the tolerance of *P. aeruginosa* and *S. aureus* to antibiotics [36,37], and it can at present not be excluded that the poor anti-biofilm properties of BS could in part be due to its high viscosity.

In addition, planktonic growth was clearly affected by the presence of NADES, even at concentrations below the MIC, as shown by the growth curve assays (Fig. 6; Supplementary Tables S4–S6). In a biofilm culture, bacteria are in an equilibrium between being in planktonic form and residing in the biofilm [37]. Also, during the initial stages of a biofilm formation assay, the bacterial cells are primarily in planktonic growth. Therefore, the inhibition effect of NADES on planktonic growth could potentially over time affect the biofilm formation capacity, and, although the assays performed for biofilm formation and planktonic growth were different in setup (e.g. different growth media, shaking frequency, NADES concentrations) one can not exclude that the observed reduction in biofilm formation imposed by NADES at least partly could be due to growth restrictions of the planktonic bacteria, in addition to any potential direct inhibition of biofilm formation mechanisms *per se*.

## 5. Conclusions

To the best of our knowledge, this is the first study on anti-biofilm activity of NADES in a biological model system. In this paper, neutral NADES ChX and ChG were demonstrated to have anti-biofilm properties, by exhibiting bactericidal activity against *P. aeruginosa* and *S. aureus* cells in pre-established bacterial biofilm. Furthermore, biofilm formation was inhibited at sub-MIC concentrations of NADES, and the inhibitory effect observed by NADES on bacterial planktonic growth could potentially contribute to this anti-biofilm effect. There was no clear evidence of strong biofilm matrix destruction properties or biofilm eradication by neutral NADES within the present data, and the potential use of NADES in this context needs further investigation.

## Credit author statement

Helene Liepelt Nystedt: Conceptualization, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. Krister Gjestvang Grønlien: Investigation, Methodology, Visualization, Writing – review & editing. Rebekka Rekkedal Rolfsnes: Investigation, Methodology, Writing – review & editing. Hanne Cecilie Winther-Larsen: Supervision, Resources, Writing – review & editing. Ole Andreas Løchen Økstad: Supervision, Resources, Investigation, Methodology, Funding acquisition, Writing – review & editing. Hanne Hjorth Tønnesen: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioflm.2023.100114.

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